Semester – II

18BTP203 **ENZYME TECHNOLOGY** **4H-4C**

Instruction Hours / week: L: 3 T: 1 P: 0 **Marks:** Internal: **40**External: **60** Total: **100**
End Semester Exam: 3 Hours

Course Objectives:

- To enable the students to understand in detail about the fundamentals of enzymes and their functions.

Course Outcomes (CO's):

1. This course includes the isolation, purification and characterization of enzymes and their applications.

UNIT – I Definition:

Nomenclature and classification of enzymes, Isozymes, characteristics of enzymes, Enzyme cofactors, Catalytic power, Catalytic strategies, Substrate specificity, Lock and key model, Induced fit hypothesis, Active site- structure, substrate binding, role of catalytic amino acid residues, Catalytic mechanisms of enzymes with representative examples, Types of enzyme inhibition, regulation, kinetics of enzyme-catalyzed reactions, effect of pH and temperature, Thermodynamics, Enzyme pathways and regulatory networks.

UNIT – II Properties of Enzymes:

Thermal stability and catalytic efficiency of enzyme, site directed mutagenesis and enzyme engineering– selected examples, Delivery system for protein pharmaceuticals, structure function relationship in enzymes, structural motifs and enzyme evolution. Methods for analysis of secondary and tertiary structures of enzymes. Protein folding *in vitro* & *in vivo*.

UNIT – III Improvement of enzymes:

Strategies for the discovery of improved and novel enzymes for industrial applications (homology and structure based approaches, screening methods, use of mutants). Optimization of industrial enzymes by mutagenesis; Protein engineering strategies to improve enzyme stability, specificity and activity; Enzyme immobilization - types, advantages, drawbacks and applications; Artificial enzymes; Isolation and purification of industrially important enzymes.

UNIT – IV Enzyme Technology:

Methods for large scale production of enzymes. Immobilized enzyme and their comparison with soluble enzymes, Methods for immobilization of enzymes. Immobilized enzyme reactors. Application of Immobilized and soluble enzyme in health and industry. Application to fundamental studies of biochemistry. Enzyme electrodes.

UNIT – V Applications of enzymes:

Enzymes used in different industries, Enzyme catalysis in organic solvents, enzyme replacement therapy – definition, modes of administration, enzyme deficiency disorders and enzyme therapy; Application of enzymes: Cosmetic benefits, Enzyme-based biosensors; Enzymes in clinical diagnosis: primary and secondary serum enzymes, Intracellular distribution of diagnostic enzymes, Enzyme markers of Xenobiotic toxicity - Pharmacogenomics related to polymorphism of drug metabolizing enzymes, , KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway.

SUGGESTED READINGS

1. Robert Murray, K., David Bender, A., Kathleen Botham, M., Peter Kennelly, J., Victor Rodwell, W., Anthony Weil, P. (2009). *Harper's illustrated Biochemistry* (28th ed.). McGrawHill.

2. Lubert Stryer, (2006). *Biochemistry* (6th ed.). WH Freeman.

3. Donald Voet, & Judith Voet, (1995). *Biochemistry* (2nd ed.). John Wiley andSons.

4. Mary K., & Shawn O.Farrell, (2005). *Biochemistry* (5th ed.). Cenage Learning.

5. Nicholas Price, & Lewis Stevens (1999) *Fundamentals of Enzymology*. Oxford University Press.

KARPAGAM ACADEMY OF HIGHER EDUCATION*(Deemed to be University Established Under Section 3 of UGC Act 1956)* Coimbatore – 641 021.

Enable | Enlighten | Enrich
(Deemed to be University)
(Under Section 3 of UGC Act 1956)

LECTURE PLAN

DEPARTMENT OF BIOTECHNOLOGY

STAFF NAME: Dr. U. USHANI

SUBJECT NAME: Enzyme Technology SUB.CODE:18BTP203

SEMESTER:II CLASS: I M.Sc (BT)

S.No	Lecture Duration (hr)	Topics	Support materials
Unit I			
1	1	Nomenclature and classification of enzymes, Isozymes, characteristics of enzymes	T1: 104-310
2	1	Enzyme cofactors, Catalytic power, Catalytic strategies, Substrate specificity	T1: 304-313
3	1	Lock and key model, Induced fit hypothesis, Active site- structure, substrate binding	T2: 314-317
4	1	Role of catalytic amino acid residues	T2: 325-330
5	1	Catalytic mechanisms of enzymes with representative examples	T2: 359-360
6	1	Types of enzyme inhibition, regulation, kinetics of enzyme-catalyzed reactions	T1: 98-100
7	1	Effect of pH and temperature	T1: 255-258
8	1	Thermodynamics	W1
9	1	Enzyme pathways and regulatory networks	W1
10	1	Revision	

Unit II			
11	1	Thermal stability and catalytic efficiency of enzyme	W2
12	1	Site directed mutagenesis and enzyme engineering– selected examples	W1
13	1	Delivery system for protein pharmaceuticals	W3
14	1	Structure function relationship in enzymes, structural motifs and enzyme evolution	W3
15	1	Methods for analysis of secondary and tertiary structures of enzymes	T2: 648-653
16	1	Protein folding *in vitro* & *in vivo*	W1
17	1	Revision	
Unit III			
18	1	Strategies for the discovery of improved and novel enzymes for industrial applications (homology and structure based approaches, screening methods, use of mutants).	W2
19	1	Optimization of industrial enzymes by mutagenesis;	W2
20	1	Protein engineering strategies to improve enzyme stability, specificity and activity	W1
21	1	Enzyme immobilization - types, advantages, drawbacks and applications	T1: 336-346
22	1	Artificial enzymes	W2
23	1	Isolation and purification of industrially important enzymes	T3: 291-309
Unit IV			
24	1	Methods for large scale production of enzymes	T1: 106-108
25	1	Immobilized enzyme and their comparison with soluble enzymes	W1
26	1	Methods for immobilization of enzymes	T1: 108-109
27	1	Immobilized enzyme reactors	W1
28	1	Application of Immobilized and soluble enzyme in health and industry	W2
29	1	Application to fundamental studies of biochemistry. Enzyme electrodes	W2
30	1	Revision	
Unit V			

31	1	Enzymes used in different industries, Enzyme catalysis in organic solvents,	W3
32	1	Enzyme replacement therapy – definition modes of administration	W2
33	1	Enzyme deficiency disorders and enzyme therapy	W3
34	1	Application of enzymes: Cosmetic benefits,	W3
35	1	Enzyme-based biosensors	W3
36	1	Enzymes in clinical diagnosis: primary and secondary serum enzymes	W1
37	1	Intracellular distribution of diagnostic enzymes, Enzyme markers of Xenobiotic toxicity	W3
38	1	Pharmacogenomics related to polymorphism of drug metabolizing enzymes & KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway	W2
39	1	Revision	
40	1	End semester question paper discussion	

References

1. T1: Dr. U. Satyanarayana. Biochemistry (2007), Books & Allied (P) Ltd.
2. T2: Lubert stryer, (2006), Biochemistry 6th Ed., WH. Freeman
3. W1: WWW.Slideshare.net
4. W2: www.Biology.disscusion.com
5. W3: https://nptel.ac.in/102103017/pdf/lecture.

UNIT – I Definition:

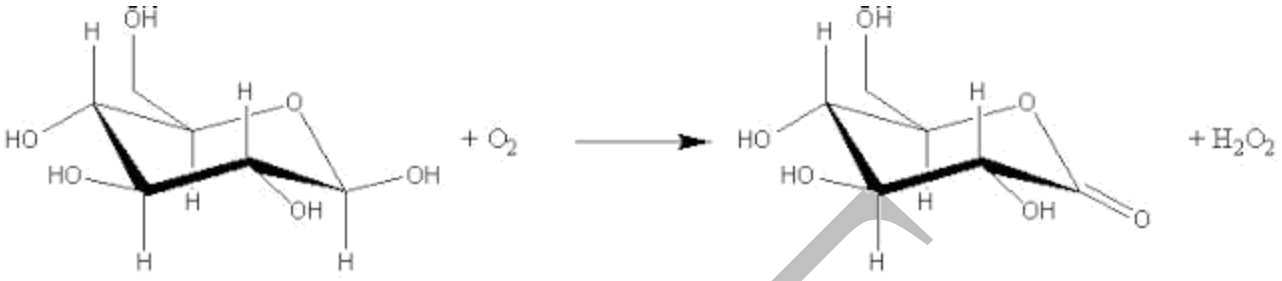
Nomenclature and classification of enzymes, Isozymes, characteristics of enzymes, Enzyme cofactors, Catalytic power, Catalytic strategies, Substrate specificity, Lock and key model, Induced fit hypothesis, Active site- structure, substrate binding, role of catalytic amino acid residues, Catalytic mechanisms of enzymes with representative examples, Types of enzyme inhibition, regulation, kinetics of enzyme-catalyzed reactions, effect of pH and temperature, Thermodynamics, Enzyme pathways and regulatory networks.

Enzyme nomenclature

All enzymes contain a protein backbone. In some enzymes this is the only component in the structure. However there are additional non-protein moieties usually present which may or may not participate in the catalytic activity of the enzyme. Covalently attached carbohydrate groups are commonly encountered structural features which often have no direct bearing on the catalytic activity, although they may well effect an enzyme's stability and solubility. Other factors often found are metal ions (**cofactors**) and low molecular weight organic molecules (**coenzymes**). These may be loosely or tightly bound by noncovalent or covalent forces. They are often important constituents contributing to both the activity and stability of the enzymes. This requirement for cofactors and coenzymes must be recognised if the enzymes are to be used efficiently and is particularly relevant in continuous processes where there may be a tendency for them to become separated from an enzyme's protein moiety.

Enzymes are classified according the report of a Nomenclature Committee appointed by the International Union of Biochemistry (1984). This assigned each enzyme a recommended name and a 4-part distinguishing number. It should be appreciated that some alternative names remain in such common usage that they will be used, where appropriate, in this text. The enzyme commission (**EC**) numbers divide enzymes into six main groups according to the type of reaction catalysed:

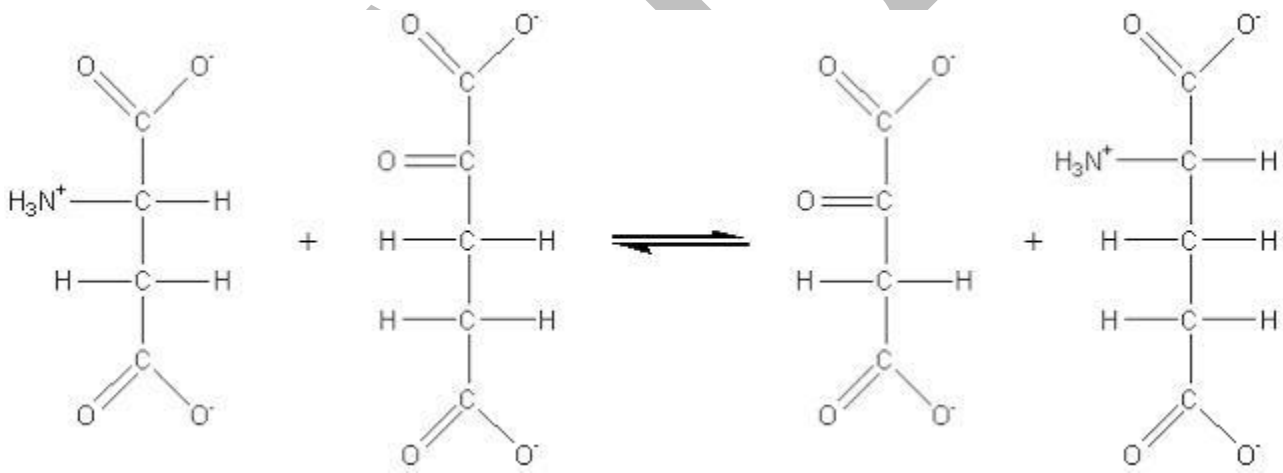
(1) **Oxidoreductases** which involve redox reactions in which hydrogen or oxygen atoms or electrons are transferred between molecules. This extensive class includes the dehydrogenases (hydride transfer), oxidases (electron transfer to molecular oxygen), oxygenases (oxygen transfer from molecular oxygen) and peroxidases (electron transfer to peroxide). For example: glucose oxidase (EC 1.1.3.4, systematic name, b-D-glucose:oxygen 1-oxidoreductase).



[1.1]

b-D-glucose + oxygen ⟶ D-glucono-1,5-lactone + hydrogen peroxide

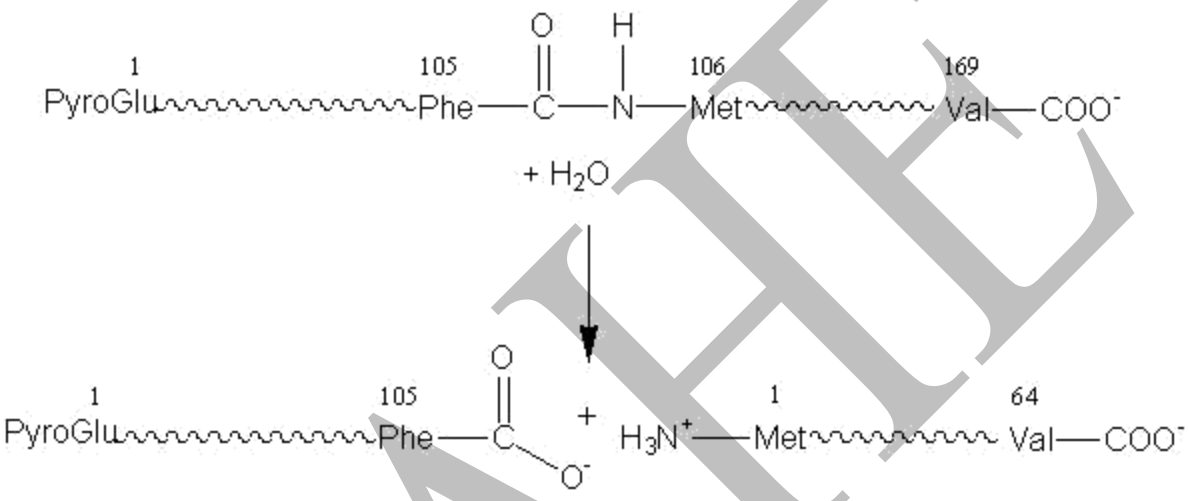
(2) **Transferases** which catalyse the transfer of an atom or group of atoms (e.g., acyl-,alkyl- and glycosyl-), between two molecules, but excluding such transfers as are classified in the other groups (e.g. oxidoreductases and hydrolases). For example: aspartate aminotransferase (EC 2.6.1.1, systematic name, L-aspartate:2-oxoglutarate aminotransferase; also called glutamic-oxaloacetic transaminase or simply GOT).



[1.2]

L-aspartate + 2-oxoglutarate ⇌ oxaloacetate + L-glutamate

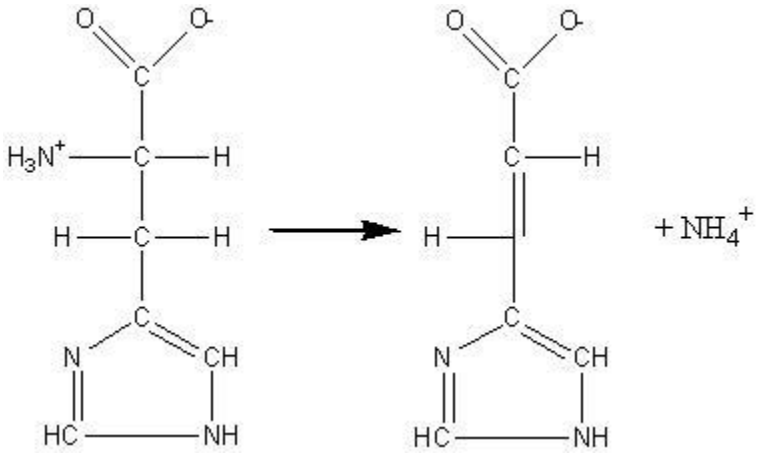
(3) **Hydrolases** which involve hydrolytic reactions and their reversal. This is presentlythe most commonly encountered class of enzymes within the field of enzyme technology and includes the esterases, glycosidases, lipases and proteases. For example: chymosin (EC 3.4.23.4, no systematic name declared; also called rennin).



[1.3]

k-casein + water ⟶ *para*-k-casein + caseino macropeptide

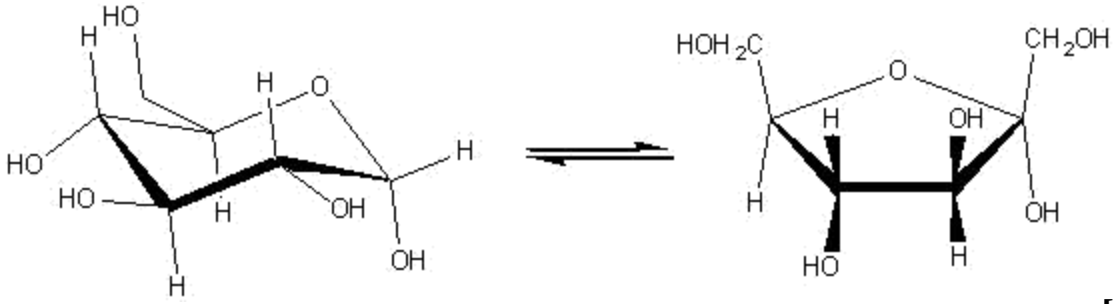
(4) **Lyases** which involve elimination reactions in which a group of atoms is removedfrom the substrate. This includes the aldolases, decarboxylases, dehydratases and some pectinases but does not include hydrolases. For example: histidine ammonia-lyase (EC 4.3.1.3, systematic name, L-histidine ammonia-lyase; also called histidase).



[1.4]

L-histidine ⟶ urocanate + ammonia

(5) **Isomerases** which catalyse molecular isomerisations and includes the epimerases, racemases and intramolecular transferases. For example: xylose isomerase (EC 5.3.1.5, systematic name, D-xylose ketol-isomerase; commonly called glucose isomerase).

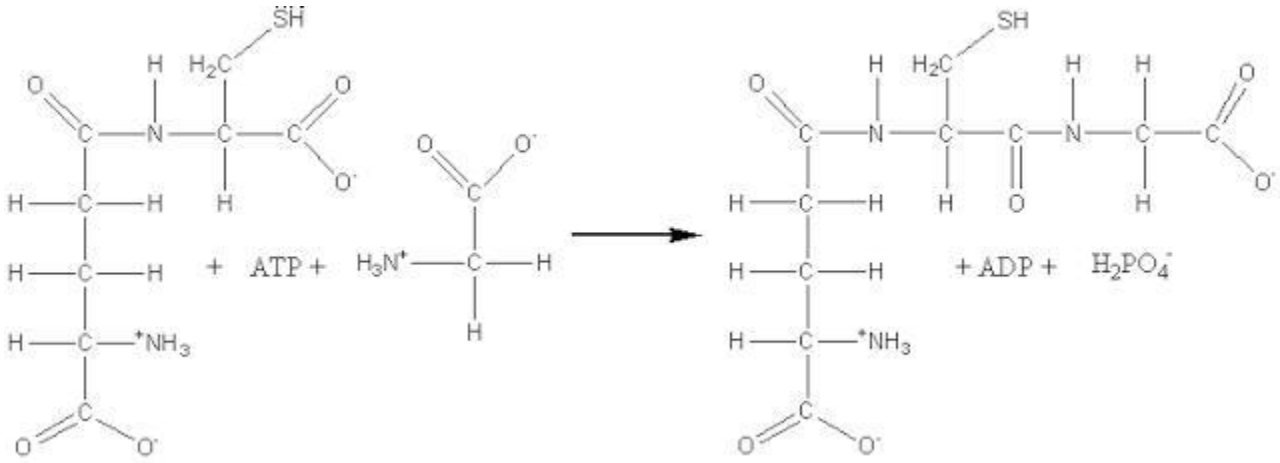


[1.5]

a-D-glucopyranose ⇌ a-D-fructofuranose

(6) **Ligases**, also known as synthetases, form a relatively small group of enzymeswhich involve the formation of a covalent bond joining two molecules together, coupled with the hydrolysis of a nucleoside triphosphate. For example:

glutathione synthase (EC 6.3.2.3, systematic name, g-L-glutamyl-L-cysteine:glycine ligase (ADP-forming); also called glutathione synthetase).



[1.6]

ATP + g-L-glutamyl-L-cysteine + glycine ⟶ ADP + phosphate + glutathione

Classification and Nomenclature of Enzymes by the Reactions they Catalyze

1. General principles

Because of their close interdependence, it is convenient to deal with the classification and nomenclature together.

The *first general principle* of these 'Recommendations' is that names purporting to be names of enzymes, especially those ending in *-ase*, should be used only for single enzymes, *i.e.* single catalytic entities. They should not be applied to systems containing more than one enzyme. When it is desired to name such a system on the basis of the overall reaction catalysed by it, the word *system* should be included in the name. For example, the system catalysing the oxidation of succinate by molecular oxygen, consisting of succinate dehydrogenase, cytochrome oxidase, and several intermediate carriers, should not be named *succinate oxidase*, but it may be called the

succinate oxidase system. Other examples of systems consisting of several structurally and functionally linked enzymes (and cofactors) are the *pyruvatedehydrogenase system*, the similar *2-oxoglutarate dehydrogenase system*, and the *fatty acid synthase system*.

In this context it is appropriate to express disapproval of a loose and misleading practice that is found in the biological literature. It consists in designation of a natural substance (or even of an hypothetical active principle), responsible for a physiological or biophysical phenomenon that cannot be described in terms of a definite chemical reaction, by the name of the phenomenon in conjugation with the suffix *-ase*, which implies an individual enzyme. Some examples of such *phenomenase* nomenclature, which should be discouraged even if there are reasons to suppose that the particular agent may have enzymic properties, are: *permease, translocase, reparase, joinase,replicase, codase, etc.*.

The *second general principle* is that enzymes are principally classified and named according to the reaction they catalyse. The chemical reaction catalysed is the specific property that distinguishes one enzyme from another, and it is logical to use it as the basis for the classification and naming of enzymes.

Several alternative bases for classification and naming had been considered, *e.g.* chemical nature of the enzymes (whether it is a flavoprotein, a hemoprotein, a pyridoxal-phosphate protein, a copper protein, and so on), or chemical nature of the substrate (nucleotides, carbohydrates, proteins, *etc.*). The first cannot serve as a general basis, for only a minority of enzymes have such identifiable prosthetic groups. The chemical nature of the enzyme has, however, been used exceptionally in certain cases where classification based on specificity is difficult, for example, with the peptidases The second basis for classification is hardly practicable, owing to the great variety of substances acted upon and because it is not sufficiently informative unless the type of reaction is also given. It is the overall reaction, as expressed by the formal equation, that should be taken as the basis. Thus, the intimate mechanism of the reaction, and the formation of intermediate complexes of the reactants with the enzyme is not taken into account, but only the observed chemical change produced by the complete enzyme reaction. For example, in those cases in which the enzyme contains a prosthetic group that serves to catalyse transfer from a donor to an acceptor (*e.g.* flavin, biotin, or pyridoxal-phosphate enzymes) the name of the prosthetic group is not normally included in the name of the enzyme. Nevertheless, where alternative names are possible, the mechanism may be taken into account in choosing between them.

A consequence of the adoption of the chemical reaction as the basis for naming enzymes is that a systematic name cannot be given to an enzyme until it is known what chemical reaction it catalyses. This applies, for example, to a few enzymes that have so far not been shown to catalyse any chemical reaction, but only isotopic exchanges; the isotopic exchange gives some idea of one step in the overall chemical reaction, but the reaction as a whole remains unknown.

A second consequence of this concept is that a certain name designates not a single enzyme protein but a group of proteins with the same catalytic property. Enzymes from different sources (various bacterial, plant or animal species) are classified as one entry. The same applies to isoenzymes (see below). However, there are exceptions to this general rule. Some are justified because the mechanism of the reaction or the substrate specificity is so different as to warrant different entries in the enzyme list. This applies, for example, to the two cholinesterases, EC 3.1.1.7 and 3.1.1.8, the two citrate hydro-lyases, EC 4.2.1.3 and 4.2.1.4, and the two amine oxidases, EC 1.4.3.4 and 1.4.3.6. Others are mainly historical, *e.g.* acid and alkaline phosphatases (EC 3.1.3.1 and EC 3.1.3.2).

A *third general principle* adopted is that the enzymes are divided into groups on the basis of the type of reaction catalysed, and this, together with the name(s) of the substrate(s) provides a basis for naming individual enzymes. It is also the basis for classification and code numbers.

Special problems attend the classification and naming of enzymes catalysing complicated transformations that can be resolved into several sequential or coupled intermediary reactions of different types, all catalysed by a single enzyme (not an enzyme system). Some of the steps may be spontaneous non-catalytic reactions, while one or more intermediate steps depend on catalysis by the enzyme. Wherever the nature and sequence of intermediary reactions is known or can be presumed with confidence, classification and naming of the enzyme should be based on the first enzyme-catalysed step that is essential to the subsequent transformations, which can be indicated by a supplementary term in parentheses, *e.g. acetyl-CoA:glyoxylate C-*

acetyltransferase (thioester-hydrolysing, carboxymethyl-forming) (EC2.3.3.9, *cf.*section 3).

To classify an enzyme according to the type of reaction catalysed, it is occasionally necessary to choose between alternative ways of regarding a given reaction. Some considerations of this type are outlined in section 3 of this chapter. In general, that alternative should be selected which fits in best with the general system of classification and reduces the number of exceptions.

One important extension of this principle is the question of the direction in which the reaction is written for the purposes of classification. To simplify the classification, the direction chosen should be the same for all enzymes in a given class, even if this direction has not been demonstrated for all. Thus the *systematic* names, on which the classification and code numbers are based, may be derived from a written reaction, even though only the reverse of this has been actually demonstrated experimentally. In the list in this volume, the reaction is written to illustrate the classification, *i.e.* in the direction described by the systematic name. However, the *common* name may be based on either direction of reaction, and is often based on the presumed physiological direction.

Many examples of this usage are found in section 1 of the list. The reaction for EC 1.1.1.9 is written as an oxidation of xylitol by NAD^+, in parallel with all other oxidoreductases in subgroup EC 1.1.1, and the systematic name is accordingly, *xylitol:*NAD^+*2-oxidoreductase (*D-*xylulose-forming)*. However, the common name, based on the reverse direction of reaction, is D-*xylulose reductase*.

2. Common and Systematic Names

The first Enzyme Commission gave much thought to the question of a systematic and logical nomenclature for enzymes, and finally recommended that there should be two nomenclatures for enzymes, one systematic, and one working or trivial. The systematic name of an enzyme, formed in accordance with definite rules, showed the action of an enzyme as exactly as possible, thus identifying the enzyme precisely. The trivial name was sufficiently short for general use, but not necessarily very systematic; in a great many cases it was a name already in current use. The introduction of (often cumbersome) systematic names was strongly criticised. In many cases the reaction catalysed is not much longer than the systematic name and can serve just as well for identification, especially in conjunction with the code number.

The Commission for Revision of Enzyme Nomenclature discussed this problem at length, and a change in emphasis was made. It was decided to give the trivial names more prominence in the Enzyme List; they now follow immediately after the code number, and are described as Common Name. Also, in the index the common names

are indicated by an asterisk. Nevertheless, it was decided to retain the systematic names as the basis for classification for the following reasons:

(i) the code number alone is only useful for identification of an enzyme when a copy of the Enzyme List is at hand, whereas the systematic name is self-explanatory;

(ii) the systematic name stresses the type of reaction, the reaction equation does not;

(iii) systematic names can be formed for new enzymes by the discoverer, by application of the rules, but code numbers should **not** be assigned by individuals;

(iv) common names for new enzymes are frequently formed as a condensed version of the systematic name; therefore, the systematic names are helpful in finding common names that are in accordance with the general pattern.

It is recommended that for enzymes that are not the main subject of a paper or abstract, the common names should be used, but they should be identified at their first mention by their code numbers and source. Where an enzyme is the main subject of a paper or abstract, its code number, systematic name, or, alternatively, the reaction equation and source should be given at its first mention; thereafter the common name should be used. In the light of the fact that enzyme names and code numbers refer to reactions catalysed rather than to discrete proteins, it is of special importance to give also the source of the enzyme for full identification; in cases where multiple forms are known to exist, knowledge of this should be included where available.

When a paper deals with an enzyme that is not yet in the Enzyme List, the author may introduce a new name and, if desired, a new systematic name, both formed according to the recommended rules. A number should be assigned only by the Nomenclature Committee of IUBMB.

The Enzyme List contains one or more references for each enzyme. It should be stressed that no attempt has been made to provide a complete bibliography, or to refer to the first description of an enzyme. The references are intended to provide sufficient evidence for the existence of an enzyme catalysing the reaction as set out. Where there is a major paper describing the purification and specificity of an enzyme, or a major review article, this has been quoted to the exclusion of earlier and later papers. In some cases separate references are given for animal, plant and bacterial enzymes.

3. Scheme for the classification of enzymes and the generation of EC numbers

The first Enzyme Commission, in its report in 1961, devised a system for classification of enzymes that also serves as a basis for assigning code numbers to them. These code numbers, prefixed by EC, which are now widely in use, contain four elements separated by points, with the following meaning:

(i) the first number shows to which of the six main divisions (classes) the enzyme belongs,

(ii) the second figure indicates the subclass,

(iii) the third figure gives the sub-subclass,

(iv) the fourth figure is the serial number of the enzyme in its sub-subclass.

The subclasses and sub-subclasses are formed according to principles indicated below.

Class 1. Oxidoreductases.

To this class belong all enzymes catalysing oxidoreduction reactions. The substrate that is oxidized is regarded as hydrogen donor. The systematic name is based on *donor:acceptor oxidoreductase*. The common name will be *dehydrogenase*, wherever this is possible; as an alternative, *reductase* can be used. *Oxidase* is only used in cases where O_2 is the acceptor.

The second figure in the code number of the oxidoreductases, unless it is 11, 13, 14 or 15, indicates the group in the hydrogen (or electron) donor that undergoes oxidation: 1 denotes a -CHOH- group, 2 a -CHO or -CO-COOH group or carbon monoxide, and so on, as listed in the key.

The third figure, except in subclasses EC 1.11, EC 1.13, EC 1.14 and EC 1.15, indicates the type of acceptor involved: 1 denotes $NAD(P)^+$, 2 a cytochrome, 3 molecular oxygen, 4 a disulfide, 5 a quinone or similar compound, 6 a nitrogenous group, 7 an iron-sulfur protein and 8 a flavin. In subclasses EC 1.13 and EC 1.14 a different classification scheme is used and sub-subclasses are numbered from 11 onwards.

It should be noted that in reactions with a nicotinamide coenzyme this is always regarded as acceptor, even if this direction of the reaction is not readily demonstrated. The only exception is the subclass EC 1.6, in which NAD(P)H is the donor; some other redox catalyst is the acceptor.

Although not used as a criterion for classification, the two hydrogen atoms at carbon-4 of the dihydropyridine ring of nicotinamide nucleotides are not equivalent in that the hydrogen is transferred stereospecifically.

Class 2. Transferases.

Transferases are enzymes transferring a group, *e.g.* a methyl group or a glycosyl group, from one compound (generally regarded as donor) to another compound (generally regarded as acceptor). The systematic names are formed according to the scheme *donor:acceptor grouptransferase*. The common names are normally formed according to *acceptor grouptransferase* or *donor grouptransferase*. In many cases, the donor is a cofactor (coenzyme) charged with the group to be transferred. A special case is that of the transaminases (see below).

Some transferase reactions can be viewed in different ways. For example, the enzyme-catalysed reaction

X-Y + Z = X + Z-Y

may be regarded either as a transfer of the group Y from X to Z, or as a breaking of the X-Y bond by the introduction of Z. Where Z represents phosphate or arsenate, the process is often spoken of as 'phosphorolysis' or 'arsenolysis', respectively, and a number of enzyme names based on the pattern of *phosphorylase* have come into use. These names are not suitable for a systematic nomenclature, because there is no reason to single out these particular enzymes from the other transferases, and it is better to regard them simply as *Y-transferases*.

In the above reaction, the group transferred is usually exchanged, at least formally, for hydrogen, so that the equation could more strictly be written as:

X-Y + Z-H = X-H + Z-Y.

Another problem is posed in enzyme-catalysed transaminations, where the - NH_2 group and -H are transferred to a compound containing a carbonyl group in exchange for the =O of that group, according to the general equation:

R^1-CH(-NH_2)-R^2 + R^3-CO-R^4 $\rightarrow$ R^1-CO-R^2 + R^3-CH(-NH_2)-R^4.

The reaction can be considered formally as oxidative deamination of the donor (*e.g.* amino acid) linked with reductive amination of the acceptor (*e.g.* oxo acid), and the transaminating enzymes (pyridoxal-phosphate proteins) might be classified as oxidoreductases. However, the unique distinctive feature of the reaction is the transfer

of the amino group (by a well-established mechanism involving covalent substrate-coenzyme intermediates), which justified allocation of these enzymes among the transferases as a special subclass (EC 2.6.1, *transaminases*).

The second figure in the code number of transferases indicates the group transferred; a one-carbon group in EC 2.1, an aldehydic or ketonic group in EC 2.2, an acyl group in EC 2.3 and so on.

The third figure gives further information on the group transferred; *e.g.* subclass EC

2.1 is subdivided into *methyltransferases* (EC 2.1.1), *hydroxymethyl*-and *formyltransferases* (EC 2.1.2) and so on; only in subclass EC 2.7, does the third figure indicate the nature of the acceptor group.

Class 3. Hydrolases.

These enzymes catalyse the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including phosphoric anhydride bonds. Although the systematic name always includes *hydrolase*, the common name is, in many cases, formed by the name of the substrate with the suffix *-ase*. It is understood that the name of the substrate with this suffix means a hydrolytic enzyme.

A number of hydrolases acting on ester, glycosyl, peptide, amide or other bonds are known to catalyse not only hydrolytic removal of a particular group from their substrates, but likewise the transfer of this group to suitable acceptor molecules. In principle, all hydrolytic enzymes might be classified as transferases, since hydrolysis itself can be regarded as transfer of a specific group to water as the acceptor. Yet, in most cases, the reaction with water as the acceptor was discovered earlier and is considered as the main physiological function of the enzyme. This is why such enzymes are classified as hydrolases rather than as transferases.

Some hydrolases (especially some of the esterases and glycosidases) pose problems because they have a very wide specificity and it is not easy to decide if two preparations described by different authors (perhaps from different sources) have the same catalytic properties, or if they should be listed under separate entries. An example is *vitamin A esterase* (formerly EC 3.1.1.12, now believed to be identical with EC 3.1.1.1). To some extent the choice must be arbitrary; however, separate entries should be given only when the specificities are sufficiently different.

Another problem is that proteinases have 'esterolytic' action; they usually hydrolyse ester bonds in appropriate substrates even more rapidly than natural peptide bonds. In

this case, classification among the peptide hydrolases is based on historical priority and presumed physiological function.

The second figure in the code number of the hydrolases indicates the nature of the bond hydrolysed; EC 3.1 are the *esterases*; EC 3.2 the *glycosylases*, and so on.
The third figure normally specifies the nature of the substrate, *e.g.* in the esterases

the *carboxylic ester hydrolases* (EC 3.1.1), *thiolester hydrolases* (EC 3.1.2), *phosphoric monoester hydrolases* (EC 3.1.3); in the glycosylases the *O-glycosidases* (EC 3.2.1), *N-glycosylases* (EC 3.2.2), *etc*. Exceptionally, in the case ofthe peptidyl-peptide hydrolases the third figure is based on the catalytic mechanism as shown by active centre studies or the effect of pH.

Class 4. Lyases.

Lyases are enzymes cleaving C-C, C-O, C-N, and other bonds by elimination, leaving double bonds or rings, or conversely adding groups to double bonds. The systematic name is formed according to the pattern *substrate group-lyase*. The hyphen is an important part of the name, and to avoid confusion should not be omitted, *e.g. hydro-lyase* not 'hydrolyase'. In the common names, expressions like *decarboxylase, aldolase, dehydratase* (in case of elimination of CO_2, aldehyde, or water) are used. Incases where the reverse reaction is much more important, or the only one demonstrated, *synthase* (not synthetase) may be used in the name. Various subclasses of the lyases include pyridoxal-phosphate enzymes that catalyse the elimination of a β- or γ-substituent from an α-amino acid followed by a replacement of this substituent by some other group. In the overall replacement reaction, no unsaturated end-product is formed; therefore, these enzymes might formally be classified as *alkyl-transferases* (EC 2.5.1...). However, there is ample evidence that the replacement is atwo-step reaction involving the transient formation of enzyme-bound α,β(or β,γ)-unsaturated amino acids. According to the rule that the first reaction is indicative for classification, these enzymes are correctly classified as *lyases*. Examples are *tryptophan synthase* (EC 4.2.1.20) and *cystathionine β-synthase* (EC 4.2.1.22).

The second figure in the code number indicates the bond broken: EC 4.1 are carbon-carbon lyases, EC 4.2 carbon-oxygen lyases and so on.

The third figure gives further information on the group eliminated (*e.g.* CO_2 in EC 4.1.1, H_2O in EC 4.2.1).

Class 5. Isomerases.

These enzymes catalyse geometric or structural changes within one molecule. According to the type of isomerism, they may be called *racemases, epimerases, cis-trans-isomerases, isomerases, tautomerases, mutases* or *cycloisomerases*.

In some cases, the interconversion in the substrate is brought about by an intramolecular oxidoreduction (EC 5.3); since hydrogen donor and acceptor are the same molecule, and no oxidized product appears, they are not classified as oxidoreductases, even though they may contain firmly bound $NAD(P)^+$.

The subclasses are formed according to the type of isomerism, the sub-subclasses to the type of substrates.

Class 6. Ligases.

Ligases are enzymes catalysing the joining together of two molecules coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate. The systematic names are formed on the system *X:Y ligase (ADP-forming).* In earlier editions of the list the term *synthetase* has been used for the common names. Many authors have been confused by the use of the terms *synthetase* (used only for Group 6) and *synthase* (used throughout the list when it is desired to emphasis the synthetic nature of the reaction). Consequently NC-IUB decided in 1983 to abandon the use of synthetase for common names, and to replace them with names of the type *X-Y ligase*. In a few cases in Group 6, where the reaction is more complex or there is a common name for the product, a synthase name is used (*e.g.* EC 6.3.2.11 and EC 6.3.5.1).

It is recommended that if the term *synthetase* is used by authors, it should continue to be restricted to the ligase group.

The second figure in the code number indicates the bond formed: EC 6.1 for C-O bonds (enzymes acylating tRNA), EC 6.2 for C-S bonds (acyl-CoA derivatives), *etc.* Sub-subclasses are only in use in the C-N ligases.

In a few cases it is necessary to use the word *other* in the description of subclasses and sub-subclasses. They have been provisionally given the figure 99, in order to leave space for new subdivisions.

From time to time, some enzymes have been deleted from the List, while some others have been renumbered. However, the old numbers have ***not*** been allotted to new enzymes; rather the place has been left vacant and cross-reference is made according to the following scheme:

[**EC 1.2.3.4 Deleted entry:** old name]

or

[**EC 1.2.3.4 Transferred entry:** now EC 5.6.7.8 - common name].

Entries for reclassified enzymes transferred from one position in the List to another are followed, for reference, by a comment indicating the former number.

It is regarded as important that the same policy be followed in future revisions and extensions of the Enzyme List, which may become necessary from time to time.

4. Rules for Classification and Nomenclature

(a) General Rules for Systematic Names and Guidelines for Common Names Rule 1.

(Common Names)

Generally accepted trivial names of substrates may be used in enzyme names. The prefix D- should be omitted for all D-sugars and L- for individual amino acids, unless ambiguity would be caused. In general, it is not necessary to indicate positions of substituents in common names, unless it is necessary to prevent two different enzymes having the same name. The prefix *keto* is no longer used for derivatives of sugars in which -CHOH- has been replaced by -CO-; they are named throughout as dehydro-sugars.

(Systematic Names)

To produce usable systematic names, accepted trivial names of substrates forming part of the enzyme names should be used. Where no accepted and convenient trivial names exist, the official IUPAC rules of nomenclature should be applied to the substrate name. The 1,2,3 system of locating substituents should be used instead of the α,β,γ system, although group names such as β-aspartyl-, γ-glutamyl-, and also β-alanine and γ-lactone are permissible; α,β should normally be used for indicating configuration, as in α-D-glucose. For nucleotide groups, *adenylyl* (not adenyl), *etc*. should be the form used. The name oxo acids (not keto acids) may be used as a class name, and for individual compounds in which $-CH_2-$ has been replaced by -CO-, oxo should be used.

Rule 2.

Where the substrate is normally in the form of an anion, its name should end in *-ate* rather than *-ic; e.g. lactate dehydrogenase,* not 'lactic dehydrogenase' or 'lacticacid dehydrogenase'.

Rule 3.

Commonly used abbreviations for substrates, *e.g.* ATP, may be used in names of enzymes, but the use of new abbreviations (not listed in recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature) should be discouraged. Chemical formulae should not normally be used instead of names of substrates. Abbreviations for names of enzymes, *e.g.* GDH, should not be used.

Rule 4.

Names of substrates composed of two nouns, such as glucose phosphate, which are normally written with a space, should be hyphenated when they form part of the enzyme names, and thus become adjectives, *e.g. glucose-6-phosphate 1-dehydrogenase* (EC 1.1.1.49). This follows standard practice in phrases where twonouns qualify a third; see, for example, *Handbook for Chemical Society Authors*, 2nd edn, p. 14 (The Chemical Society, London, 1961).

Rule 5.

The use as enzyme names of descriptions such as *condensing enzyme*, *acetate-activating enzyme*, *pH 5 enzyme* should be discontinued as soon as the catalysedreaction is known. The word *activating* should not be used in the sense of converting the substrate into a substance that reacts further; all enzymes act by activating their substrates, and the use of the word in this sense may lead to confusion.

Rule 6.

(Common Names)

If it can be avoided, a common name should not be based on a substance that is not a true substrate, *e.g.* enzyme EC 4.2.1.17 should not be called 'crotonase', since it does not act on crotonate.

Rule 7.

(Common Names)

Where a name in common use gives some indication of the reaction and is not incorrect or ambiguous, its continued use is recommended. In other cases a common name is based on the same general principles as the systematic name (see Rule 7 below) but with a minimum of detail, to produce a name short enough for convenient

use. A few names of proteolytic enzymes ending in *-in* are retained; all other enzyme names should end in *-ase*.
(Systematic Names)

Systematic names consist of two parts. The first contains the name of the substrate or, in the case of a bimolecular reaction, of the two substrates separated by a colon. The second part, ending in *-ase*, indicates the nature of the reaction.

Rule 8.

A number of generic words indicating a type of reaction may be used in either common or systematic names: *oxidoreductase, oxygenase, transferase* (with a prefix indicating the nature of the group transferred), *hydrolase, lyase, racemase, epimerase,isomerase, mutase, ligase.*

Rule 9.

(Common Names)

A number of additional generic words indicating reaction types are used in common names, but not in the systematic nomenclature, *e.g. dehydrogenase, reductase,oxidase, peroxidase, kinase, tautomerase, deaminase, dehydratase, etc.*.

Rule 10.

Where additional information is needed to make the reaction clear, a phrase indicating the reaction or a product should be added in parentheses after the second part of the name *e.g. (ADP-forming), (dimerizing), (CoA-acylating).*

Rule 11.

(Common Names)

The direct attachment of *-ase* to the name of the substrate will indicate that the enzyme brings about hydrolysis.

(Systematic Names)

The suffix *-ase* should never be attached directly to the name of the substrate.

Rule 12.

(Common Names)

The name 'dehydrase' which was at one time used for both dehydrogenating and dehydrating enzymes, should not be used. *Dehydrogenase* will be used for the former and *dehydratase* for the latter.

Rule 13.

(Common Names)

Where possible, common names should normally be based on a reaction direction that has been demonstrated, *e.g.* *dehydrogenase* or *reductase*, *decarboxylase* or *carboxylase*.

(Systematic Names)

In the case of reversible reactions, the direction chosen for naming should be the same for all the enzymes in a given class, even if this direction has not been demonstrated for all. Thus, systematic names may be based on a written reaction, even though only the reverse of this has been actually demonstrated experimentally.

Rule 14.

(Systematic Names)

When the overall reaction includes two different changes, *e.g.* an oxidative demethylation, the classification and systematic name should be based, whenever possible, on the one (or the first one) catalysed by the enzyme; the other function(s) should be indicated by adding a suitable participle in parentheses, as in the case of *sarcosine:oxygen oxidoreductase (demethylating)* (EC 1.5.3.1); D-*aspartate:oxygen oxidoreductase (deaminating)* (EC 1.4.3.1); L-*serine hydro-lyase (adding indoleglycerol-phosphate)* (EC 4.2.1.20).

Other examples of such additions are *(decarboxylating), (cyclizing), (acceptor-acylating), (isomerizing).*

Rule 15.

When an enzyme catalyses more than one type of reaction, the name should normally refer to one reaction only. Each case must be considered on its merits, and the choice must be, to some extent, arbitrary. Other important activities of the enzyme may be indicated in the List under 'Reaction' or 'Comments'.

Similarly, when any enzyme acts on more than one substrate (or pair of substrates), the name should normally refer only to one substrate (or pair of substrates), although in certain cases it may be possible to use a term that covers a whole group of substrates, or an alternative substrate may be given in parentheses.

Rule 16.

A group of enzymes with closely similar specificities should normally be described by a single entry. However, when the specificity of two enzymes catalysing the same reactions is sufficiently different (the degree of difference being a matter of arbitrary choice) two separate entries may be made, *e.g.* EC 1.2.1.4 and EC 1.2.1.7. Separate entries are also appropriate for enzymes having similar catalytic functions, but known to differ basically with regard to reaction mechanism or to the nature of the catalytic groups, *e.g. amine oxidase (flavin-containing)* (EC 1.4.3.4) and *amine oxidase(copper-containing)* (EC 1.4.3.6).

(b) Rules and Guidelines for Particular Classes of Enzymes Class 1

Rule 17.

(Common Names)

The terms *dehydrogenase* or *reductase* will be used much as hitherto. The latter term is appropriate when hydrogen transfer from the substance mentioned as donor in the systematic name is not readily demonstrated. *Transhydrogenase* may be retained for a few well-established cases. *Oxidase* is used only for cases there O_2 acts as an acceptor, and *oxygenase* only for those cases where the O_2 molecule (or part of it) is directly incorporated into the substrate. *Peroxidase* is used for enzymes using H_2O_2 as acceptor. *Catalase* must be regarded as exceptional. Where no ambiguity is caused, the second reactant is not usually named; but where required to prevent ambiguity, it may be given in parentheses, *e.g.* EC 1.1.1.1, *alcohol dehydrogenase* and EC 1.1.1.2, *alcohol dehydrogenase* ($NADP^+$).

(Systematic Names)

All enzymes catalysing oxidoreductions should be named *oxidoreductases* in the systematic nomenclature, and the names formed on the pattern *donor:acceptoroxidoreductase.*

Rule 18.
(Systematic Names)

For oxidoreductases using NAD^+ or $NADP^+$, the coenzyme should always be named as the acceptor except for the special case of Section 1.6 (enzymes whose normal physiological function is regarded as reoxidation of the reduced coenzyme). Where the enzyme can use either coenzyme, this should be indicated by writing $NAD(P)^+$.

Rule 19.

Where the true acceptor is unknown and the oxidoreductase has only been shown to react with artificial acceptors, the word *acceptor* should be written in parentheses, as in the case of EC 1.3.99.1, *succinate:(acceptor) oxidoreductase.*

Rule 20.

(Common Names)

Oxidoreductases that bring about the incorporation of molecular oxygen into one donor or into either or both of a pair of donors are named *oxygenase*. If only one atom of oxygen is incorporated the term *monooxygenase* is used; if both atoms of O_2 are incorporated, the term *dioxygenase* is used.

(Systematic Names)

Oxidoreductases bringing about the incorporation of oxygen into one of paired donors should be named on the pattern *donor,donor:oxygen oxidoreductase (hydroxylating).*

Class 2.

Rule 21.

(Common Names)

Only one specific substrate or reaction product is generally indicated in the common names, together with the group donated or accepted.

The forms *transaminase, etc.,* may be replaced if desired by the corresponding forms *aminotransferase, etc.*.

A number of special words are used to indicate reaction types, *e.g. kinase* to indicate a

phosphate transfer from ATP to the named substrate (not 'phosphokinase'), *diphosphokinase* for a similar transfer of diphosphate.
(Systematic Names)

Enzymes catalysing group-transfer reactions should be named *transferase* and the names formed on the pattern *donor:acceptor group-transferred-transferase*, *e.g.ATP:acetate phosphotransferase* (EC 2.7.2.1). A figure may be prefixed to show theposition to which the group is transferred, *e.g. ATP:*D-*fructose 1-phosphotransferase* (EC 2.7.1.3). The spelling 'transphorase' should not be used. Inthe case of the phosphotransferases, ATP should always be named as the donor. In the case of the transaminases involving 2-oxoglutarate, the latter should always be named as the acceptor.

Rule 22.

(Systematic Names)

The prefix denoting the group transferred should, as far as possible, be non-committal with respect to the mechanism of the transfer, *e.g. phospho-*, rather than *phosphate-*.

Class 3.

Rule 23.

(Common Names)

The direct addition of *-ase* to the name of the substrate generally denotes a hydrolase. Where this is difficult, *e.g.* for EC 3.1.2.1, the word *hydrolase* may be used. Enzymes should not normally be given separate names merely on the basis of optimal conditions for activity. The acid and alkaline phosphatases (EC 3.1.3.1-2) should be regarded as special cases and not as examples to be followed. The common name *lysozyme* is also exceptional.

(Systematic Names)

Hydrolysing enzymes should be systematically named on the pattern *substratehydrolase*. Where the enzyme is specific for the removal of a particular group, thegroup may be named as a prefix, *e.g. adenosine aminohydrolase* (EC 3.5.4.4). In a number of cases this group can also be transferred by the enzyme to other molecules, and the hydrolysis itself might be regarded as a transfer of the group to water.

Class 4.

Rule 24.
(Common Names)

The old names *decarboxylase, aldolase, etc*., are retained; and *dehydratase* (not 'dehydrase') is used for the hydro-lyases. 'Synthetase' should not be used for any enzymes in this class. The term *synthase* may be used instead for any enzyme in this class (or any other class) when it is desired to emphasize the synthetic aspect of the reaction.

(Systematic Names)

Enzymes removing groups from substrates non-hydrolytically, leaving double bonds (or adding groups to double bonds) should be called *lyases* in the systematic nomenclature. Prefixes such as *hydro-, ammonia-* should be used to denote the type of reaction, *e.g. (*S*)-malate hydro-lyase* (EC 4.2.1.2). Decarboxylases should be regarded as *carboxy-lyases*. A hyphen should always be written before *lyase* to avoid confusion with hydrolases, carboxylases, *etc*.

Rule 25.

(Common Names)

Where the equilibrium warrants it, or where the enzyme has long been named after a particular substrate, the reverse reaction may be taken as the basis of the name, using *hydratase, carboxylase, etc., e.g. fumarate hydratase* for EC 4.2.1.2 (in preference to 'fumarase', which suggests an enzyme hydrolysing fumarate).

(Systematic Names)

The complete molecule, not either of the parts into which it is separated, should be named as the substrate.

The part indicated as a prefix to *-lyase* is the more characteristic and usually, but not always, the smaller of the two reaction products. This may either be the removed (saturated) fragment of the substrate molecule, as in *ammonia-, hydro-, thiol-lyases,etc*. or the remaining unsaturated fragment, *e.g.* in the case of *carboxy-, aldehyde-*or *oxo-acid-lyases*.

Rule 26.

Various subclasses of the lyases include a number of strictly specific or group-specific pyridoxal-5-phosphate enzymes that catalyse *elimination* reactions of β- or γ-substituted α-amino acids. Some closely related pyridoxal-5-phosphate-containing enzymes, *e.g. tryptophan synthase* (EC 4.2.1.20) and *cystathionine* β-*synthase* (EC 4.2.1.22) catalyse *replacement* reactions in which a β- or γ-substituent is replaced by a second reactant without creating a double bond. Formally, these enzymes appear to be transferases rather than lyases. However, there is evidence that in these cases the elimination of the β- or γ-substituent and the formation of an unsaturated intermediate is the first step in the reaction. Thus, applying rule 14, these enzymes are correctly classified as lyases.

Class 5.

Rule 27.

In this class, the common names are, in general, similar to the systematic names which indicate the basis of classification.

Rule 28.

Isomerase will be used as a general name for enzymes in this class. The types of isomerization will be indicated in systematic names by prefixes, *e.g. maleate cis-trans-isomerase* (EC 5.2.1.1), *phenylpyruvate keto-enol-isomerase* (EC 5.3.2.1), *3-oxosteroid* ^{5}- ^{4}*-isomerase* (EC 5.3.3.1). Enzymes catalysing an aldose-ketose interconversion will be known as *aldose-ketose-isomerases*, *e.g.* L-*arabinose aldose-ketose-isomerase* (EC 5.3.1.4). When the isomerization consists of an intramolecular transfer of a group, the enzyme is named a *mutase*, *e.g.* EC 5.4.1.1, and the *phosphomutases* in sub-subclass 5.4.2; when it consists of an intramolecular lyase-type reaction, *e.g.* EC 5.5.1.1, it is systematically named a *lyase (decyclizing)*.

Rule 29.

Isomerases catalysing inversions at asymmetric centres should be termed *racemases* or *epimerases*, according to whether the substrate contains one, or more than one, centre of asymmetry: compare, for example, EC 5.1.1.5 with EC 5.1.1.7. A numerical prefix to the word *epimerase* should be used to show the position of the inversion.

Class 6.

Rule 30

(Common Names)

Common names for enzymes of this class were previously of the type *XY synthetase*. However, as this use has not always been understood and synthetase has been confused with synthase (see Rule 24), it is now recommended that as far as possible the common names should be similar in form to the systematic names.

(Systematic Names)

The class of enzymes catalysing the linking together of two molecules, coupled with the breaking of a diphosphate link in ATP, *etc*. should be known as *ligases*. These enzymes were often previously known as 'synthetases'; however, this terminology differs from all other systematic enzyme names in that it is based on the product and not on the substrate. For these reasons, a new systematic class name was necessary.

Rule 31

(Common Names)

The common names should be formed on the pattern *X-Y ligase*, where X-Y is the substance formed by linking X and Y. In certain cases, where a trivial name is commonly used for XY, a name of the type *XY synthase* may be recommended (*e.g.* EC 6.3.2.11, *carnosine synthase*).

(Systematic Names)

The systematic names should be formed on the pattern *X:Y ligase (ADP-forming)*, where X and Y are the two molecules to be joined together. The phrase shown in parentheses indicates both that ATP is the triphosphate involved, and also that the terminal diphosphate link in broken. Thus, the reaction is X + Y + ATP = X-Y + ADP + P_i.

Rule 32.

(Common Names)

In the special case where glutamine acts as an ammonia-donor, this is indicated by adding in parentheses (*glutamine-hydrolysing*) to a ligase name.

(Systematic Names)

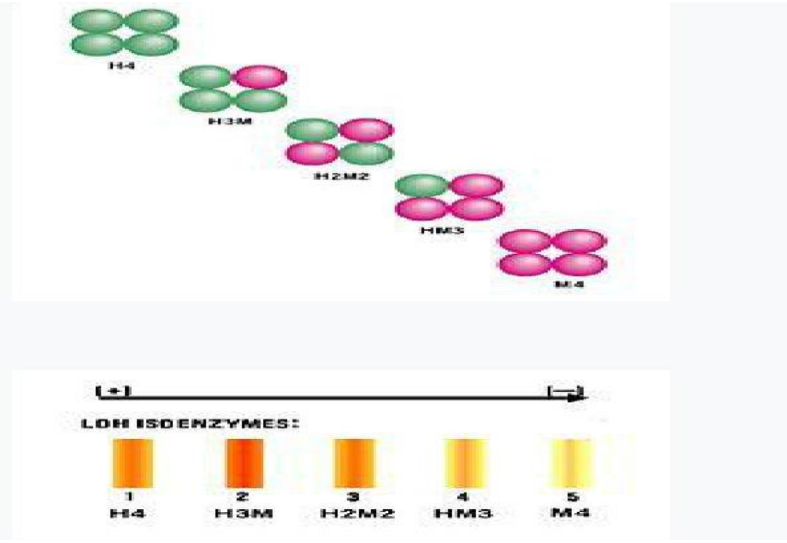
In this case, the name *amido-ligase* should be used in the systematic nomenclature.

Isozyme

Isozymes (also known as isoenzymes or more generally as multiple forms of enzymes) are enzymes that differ in amino acid sequence but catalyze the same chemical reaction. These enzymes usually display different kinetic parameters (e.g. different KM values), or different regulatory properties. The existence of isozymes permits the fine-tuning of metabolism to meet the particular needs of a given tissue or developmental stage (for example lactate dehydrogenase (LDH)). In biochemistry, isozymes (or isoenzymes) are isoforms (closely related variants) of enzymes. In many cases, they are coded for by homologous genes that have diverged over time. Although, strictly speaking, allozymes represent enzymes from different alleles of the same gene, and isozymes represent enzymes from different genes that process or catalyse the same reaction, the two words are usually used interchangeably.

An example of an isozyme is glucokinase, a variant of hexokinase which is not inhibited by glucose 6-phosphate. Its different regulatory features and lower affinity for glucose (compared to other hexokinases), allows it to serve different functions in cells of specific organs, such as control of insulin release by the beta cells of the pancreas, or initiation of glycogen synthesis by liver cells. Both of these processes must only occur when glucose is abundant, or problems occur.

The 5 isozymes of LDH



The differences between 5 isozyme when use Electrophoresis

The enzyme lactate dehydrogenase is made of two (H-form and M-Form) different sub units, combines in different combinations depending on the tissue in which it is present as shown in the table below,

Type	Composition	Location
LDH_1	HHHH	Heart and Erythrocyte
LDH_2	HHHM	Heart and Erythrocyte
LDH_3	HHMM	Brain and Kidney
LDH_4	HMMM	Skeletal Muscle and Liver
LDH_5	MMMM	Skeletal Muscle and Liver

Properties and Characteristics of Enzymes

Enzymes are proteins and the properties and characteristics they exhibit reflect properties of protein. They are organic catalysts that speed up biological reactions especially in digestion and metabolism of food substances. Below are the properties and characteristics of enzymes.

1. **Enzymes Generally Work Very Rapidly:–** The action and speed of an enzyme is expressed as its turnover number. The turnover number is the number of substrate molecules which one mole of the enzyme turns into products per minute. It can be observed that the turnover numbers of different enzymes vary from 100 to several millions and for a greater majority of enzymes, this figure hovers around several thousand. Catalase is the fastest known enzyme and it is found in hydrogen peroxide where it catalyzes its

decomposition into water and oxygen. Catalase has a turnover number of 6million and its action can be demonstrated by dropping a piece of liver into a beaker of hydrogen peroxide—- the fizzing that occurs as the oxygen is given off is a wonderful demonstration of an enzyme in action.

2. **2. Enzymes Can Work In Either Direction:–** Metabolic reactions arereversible and the direction in which these reactions proceed depends largely on the relative amounts of the reacting substrates and the products formed. The reaction either to the left or to the right until an equilibrium is reached between the substrates and the products formed. Therefore, an enzyme that catalyzes the formation of products from substrates would also cause the products to be split into the component substrates when the product is in excess to maintain an equilibrium.

3. **3. Enzymes Are Not Destroyed Or Altered By The Reactions They Catalyze:– E**nzymes can re re-used because they are not destroyed by thereactions they catalyze. However, enzymes cannot be used indefinitely because they are quite unstable as they can be inactivated by heat, acids, and alkaline substances and so on and by this, they are different from inorganic catalysts that stable and that can be re-used over and over again indefinitely.

4. **4. Enzymes Are Sensitive to pH Changes:–** Enzymes operate at specific pHranges and any alterations can adversely affect their action and efficiency. Most intracellular enzymes function best at neutral pH. Interestingly, certain digestive enzymes prefer a distinctly alkaline or acidic environment. This accounts for why the protein splitting enzyme known as pepsin function only in an acidic medium at a pH of about 2.0 is found in the stomach where the environment is acidic.

Trypsin can only function in an alkaline medium at about a pH of 8.5 and often found in the duodenum where the conditions are alkaline.

5. **Enzymes Are Specific In Their Action:–** Enzymes are much more specific inthe reactions they catalyze than inorganic catalysts. However, the degree of catalytic specificity varies from one enzyme to the other. Most intracellular enzymes work on a particular substrate while some digestive enzymes work on a comparatively wide range of substrates that are related. For instance, catalase would only split hydrogen peroxide but an enzyme like the **pancreatic lipase** is much less specific and would digest a variety of different fats.

Coenzymes

These are reusable non-protein molecules that contain carbon (organic). They bind loosely to an enzyme at the active site to help catalyze reactions. Most are vitamins, vitamin derivatives, or form from nucleotides.

Cofactors

Unlike coenzymes, true cofactors are reusable non-protein molecules that do not contain carbon (inorganic). Usually, cofactors are metal ions such as iron, zinc, cobalt, and copper that loosely bind to an enzyme's active site. They must also be supplemented in the diet as most organisms do not naturally synthesize metal ions.

CATALYTIC POWER

In common with all catalysts, enzymes are subject to the normal laws concerning the catalysis of reactions. Thus, the catalyst cannot speed up a reaction that would not occur in its absence, e.g. because the reaction is not thermodynamically possible.

The catalyst is not consumed during the reaction, and so relatively few catalyst molecules are capable of catalysing the reaction a great many times. Lastly, the catalyst cannot alter the equilibrium position of a given reaction.

The vast majority of reactions proceed, eventually, to a state of equilibrium in which the rate of the forward reaction is equal to the rate of the reverse reaction. At equilibrium the substrate and product have specific equilibrium concentrations that are a special characteristic of the reaction. The position of the equilibrium may lie strongly to the product side, for example 1% substrate : 99% product, or more toward the substrate side 80% : 20% or near the middle 50% : 50%.

For example the isomerization of glucose to produce the isomer fructose is catalysed by enzyme glucose isomerase. Starting from 100% glucose the reaction proceeds to equilibrium, which in this reaction is 45% fructose and 55% glucose. The catalyst cannot change the equilibrium position of the reaction, but it can reduce the time that the reaction normally takes to reach equilibrium. In these respects enzymes are no different from other catalysts.

During any reaction the reactants briefly enter a state in which the susceptible substrate is not completely broken and the new bonds in the product are not

completely formed. This transient condition is called the transition state, and it is energy dependent because it requires energy to make and break chemical bonds (350 kJ per mole for a covalent bond). This represents an energy barrier to successful reaction, and is the reason why the vast majority of reactions proceed extremely slowly in the absence of external help.

Reactants can be helped towards the transition state by supplying heat energy, high pressure or extreme pH to weaken bonds or by the addition of catalysts. Enzyme catalysts are more effective than chemical catalysts at reducing the energy barrier to enable transition state formation, and thereby increase the rate of a reaction.

The efficiency of enzyme catalysis varies, but most enzymes can enhance the rate of an uncatalysed reaction by a factor of many millions. One of the most efficient enzymes is carbonic anhydrase, which catalyses the hydration of up to 600,000 molecules of carbon dioxide per second under optimal conditions.

Carbonic anhydrase is found mostly in red blood cells where it plays a vital role in maintaining the acid-base balance in the body. The enzyme enables rapid transport of molecular CO2, formed by cellular respiration, from the site of formation (tissues) to the lungs for expulsion.

An indication of catalytic power is provided by the turnover number of an enzyme. When an enzyme is fully saturated with its substrate, then the turnover number is the number of substrate molecules converted to product per second.

The catalytic power of enzymes is due to the precise molecular interactions that occur at the active site, which lower the energy barrier and enable formation of the transition state. There are at least **four types of interaction** that can accomplish this effect, and they may operate singly or in combination.

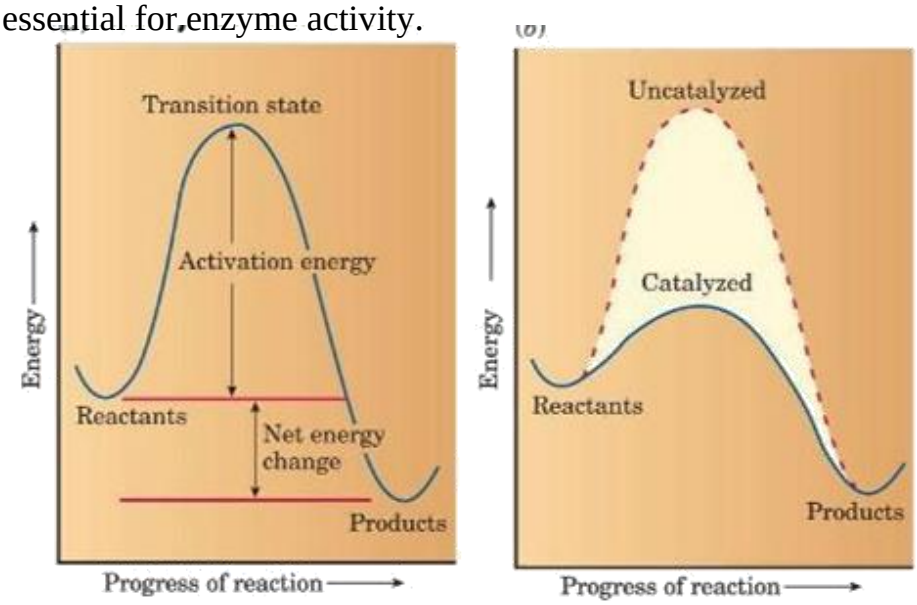
First, the active site in many enzymes provides a non-polar micro-environment, and the removal of a substrate molecule from an aqueous polar solution into a non-polar phase may alter the comformation (shape) of the substrate towards the transition state. Also, a non-polar environment is useful for excluding water molecules, which may interfere in a reaction.

Second, the precise alignment of substrate molecules in the active site presents the susceptible bonds at the correct angle so that a collision between reactants will result in the formation of a transition state.

Third, the substrate molecule is normally held firmly in the active site by a number of non-covalent interactions, and small movements in the conformation of the enzyme molecule can be transmitted to the active site causing a distortion of the substrate structure, weakening the susceptible bond, and reducing the amount of energy required to form a transition state.

Lastly, the site amino acid residues contribute catalytic functional groups to participate directly in the reaction. By adding and/or withdrawing electrons, protons (H+) or other groups, the catalytic functional groups at the active site push the substrate molecules towards the transition state, thereby increasing the rate of reaction. Amino acid side chains such as hydroxyl (-OH), sulphydryl (-SH), carboxyl (-COOH) and amino (-NH2) can function as proton donors or acceptors depending on the state of ionization of the side chain.

Other side chains exert different chemical influences, and many enzymes employ extra help in the form of a cofactor to supply further chemical influence. For example, in the hydration reaction of CO2 discussed earlier, one atom of zinc per enzyme molecule is essential for enzyme activity.



Catalytic Strategies

Enzymes are proteins that catalyze a reaction by stabilizing the transition state and therefore, lowering the activation energy of the reaction. To achieve this, enzymes use different classes of reactions known as catalytic strategies. Four classes of enzymes are used in catalytic strategies are:

- Serine Proteases: example of catalytic mechanism of chymotrypsin
- Carbonic Anhydrase: make a fast reaction faster Carbonic anhydrases dehydrate HCO3- in blood to form CO2 for exhalation as the blood passes through the lungs.
- Restriction Endonucleases (BamHI): Bam HI: is used for DNA cleavage reaction. This enzyme recognizes particular base sequences, recognition sites, in target DNA and cleaves DNA at defined positions. I co-crystallized the complex BamHI-DNA with divalent cations BamHI binding to nonspecific DNA forms an electrostatic traps that allows sliding along DNA. Catalytic Mechanism: Pre-reactive state (Glutamic acid 113 acts as general base removing a proton from water molecule). Transition state (Pentavalent phosphate intermediate forms with 2 negative charges) Post-reactive state (proton donated by water molecule goes to oxygen in leaving group, phosphodiester bond is broken)
- Nucleoside Monophosphate Kinase NMP kinase is enzyme that aids in transferring the phosphoryl group at the end of a nucleoside triphosphate to the phosphoryl group that is on a nucleoside monophosphate

NMP + ATP --> NDP + ADP

The strategies used to catalyze a reaction are:

- **Covalent Catalysis**

Whereas most enzymes simply have an active site that changes the shape of a substrate, in covalent catalysis, the enzymes (or cofactors) covalently bond to the substrate as the first step. The active sites contain a reactive group, usually a nucleophile that forms a covalent bond with the substrate. Then, the enzyme goes through a mechanism, eventually breaking down the substrate and reforming itself. Chymotrypsin, for example, can catalyze reactions by utilizing covalent modification. It can employ a serine residue as a nucleophile to attact the unreactive carbonyl group of a substrate.

Other proteases like chymotrypsin, such as trypsin, can use a catalytic triad (containing aspartate, histidine and serine residues) to activate enzymatic activity and break peptide bonds. Proteases usually involves a nucleophile that attacts a peptide

carbonyl group. Cystein proteases is activated by histidine to attack the carbonyl group. Aspartyl proteases is activated by aspartate pairs, which exists in deprotonated and protonated forms to attack a water molecule and carbonyl group, respectively. Metalloproteases is activated by a bound metal ion (usually zinc) to activate the water molecule, which acts as a nucleophile to attack the peptide carbonyl group.

- **Acid-Base Reactions**

These enzymes generally use a molecule other than water to donate or accept protons as a nucleophile. An example is that of a Zinc ion - Histidine complex in carbonic anhydrase that breaks down the H2CO4 into hydrogen ions and bicarbonate ions. The zinc attracts a water molecule which then deprotonates. The oxygen acts as a nucleophile and attacks a carbon dioxide molecule to create a complicated coordination complex. Another then replaces the complex, releasing the bicarbonate ion.

- **Catalysis by Approxmiation**

The close proximity of two substrates can increase the rate of reaction between the two. Generally, when two molecules combine to become one, entropy decreases. An enzyme that brings the two molecules together decreases the entropy. This increase in rate is similar to increasing the concentration of the reactants. However, catalysis by approximation generally increases the reaction rate more than simply increasing the concentration of the reactants since the enzyme generally makes the reaction pseudo-intramolecular.

- **Metal Ion Catalysis**

Metal ions can directly facilitate the formation of bonds. Because they are electrophillic, they can also act to stabilize the charges on the intermediates in the reaction.

The type of strategy that is employed is based on the enzyme's structural properties and the reaction that the enzyme will catalyze. Many times a combination of strategies is used to in catalytic reactions.

Specificity of Enzymes

One of the properties of enzymes that makes them so important as diagnostic and research tools is the specificity they exhibit relative to the reactions they catalyze. A few enzymes exhibit absolute specificity; that is, they will catalyze only one particular

reaction. Other enzymes will be specific for a particular type of chemical bond or functional group. In general, there are four distinct types of specificity:

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

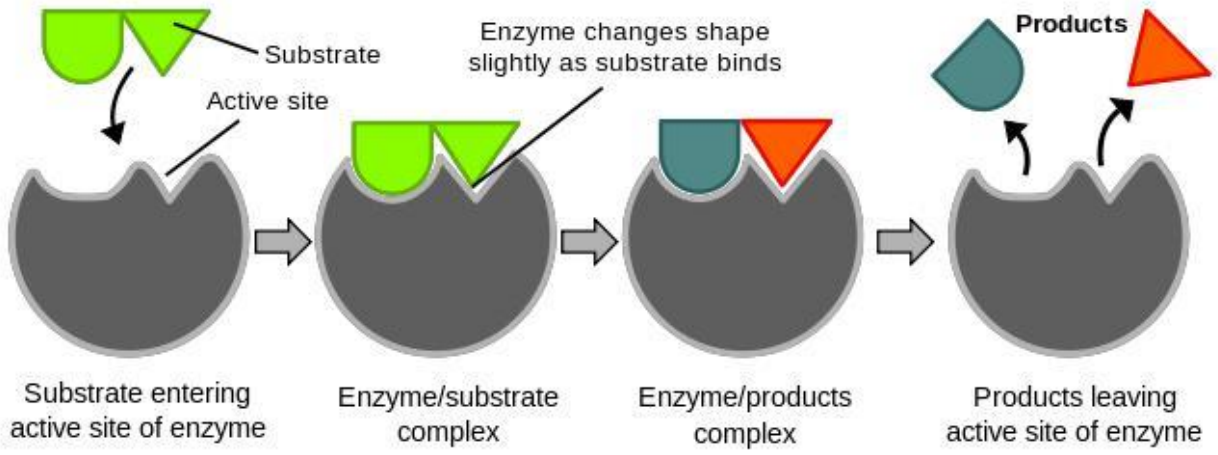
Though enzymes exhibit great degrees of specificity, cofactors may serve many apoenzymes. For example, nicotinamide adenine dinucleotide (NAD) is a coenzyme for a great number of dehydrogenase reactions in which it acts as a hydrogen acceptor. Among them are the alcohol dehydrogenase, malate dehydrogenase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

Lock-and-key mechanism:

A mechanism proposed in 1890 by Emil Fischer (1852–1919) to explain binding between the active site of an enzyme and a substrate molecule. The active site was thought to have a fixed structure (the lock), which exactly matched the structure of a specific substrate (the key). Thus the enzyme and substrate interact to form an enzyme–substrate complex. The substrate is converted to products that no longer fit the active site and are therefore released, liberating the enzyme. Observations made by X-ray diffraction studies have shown that the active site of an enzyme is more flexible than the lock-and-key theory would suggest. Compare induced-fit model.

What it is?

The specific action of an enzyme with a single substrate can be explained using a **Lock and Key** analogy first postulated in 1894 by Emil Fischer. In this analogy, the **lock** isthe enzyme and the **key** is the substrate. Only the correctly sized **key** (substrate) fits into the **key** hole (active site) of the **lock** (enzyme).



Induced fit model

In 1958, Daniel Koshland suggested a modification to the lock and key model: since enzymes are rather flexible structures, the active site is continuously reshaped by interactions with the substrate as the substrate interacts with the enzyme. As a result, the substrate does not simply bind to a rigid active site; the amino acid side-chains that make up the active site are melded into the precise positions that enable the enzyme to perform its catalytic function. In some cases, such as glycosidases, the substrate molecule also changes shape slightly as it enters the active site. The active site continues to change until the substrate is completely bound, at which point the final shape and charge distribution is determined. Induced fit may enhance the fidelity of molecular recognition in the presence of competition and noise via the conformational proofreading mechanism.

Dynamics

Protein dynamics

Enzymes are not rigid, static structures; instead they have complex internal dynamic motions – that is, movements of parts of the enzyme's structure such as individual amino acid residues, groups of residues forming a protein loop or unit of secondary structure, or even an entire protein domain. These motions give rise to a conformational ensemble of slightly different structures that interconvert with one another at equilibrium. Different states within this ensemble may be associated with different aspects of an enzyme's function. For example, different conformations of the

enzyme dihydrofolate reductase are associated with the substrate binding, catalysis, cofactor release, and product release steps of the catalytic cycle.

Allosteric modulation

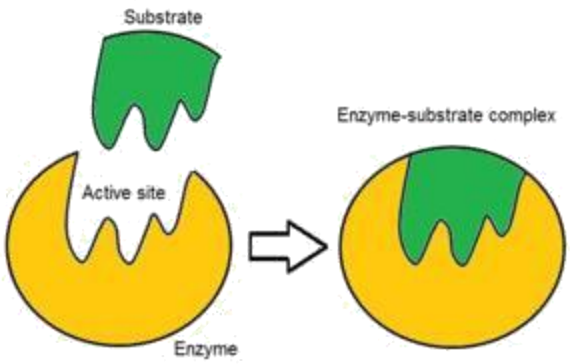
Allosteric regulation

Allosteric sites are pockets on the enzyme, distinct from the active site, that bind to molecules in the cellular environment. These molecules then cause a change in the conformation or dynamics of the enzyme that is transduced to the active site and thus affects the reaction rate of the enzyme.[51] In this way, allosteric interactions can either inhibit or activate enzymes. Allosteric interactions with metabolites upstream or downstream in an enzyme's metabolic pathway cause feedback regulation, altering the activity of the enzyme according to the flux through the rest of the pathway.

Active site

In biology, the active site is the region of an enzyme where substrate molecules bind and undergo a chemical reaction. The active site consists of residues that form temporary bonds with the substrate (binding site) and residues that catalyse a reaction of that substrate (catalytic site). Although the active site is small relative to the whole volume of the enzyme (it only occupies 10~20% of the total volume),[1] it is the most important part of the enzyme as it directly catalyzes the chemical reaction. It usually consists of three to four amino acids, while other amino acids within the protein are required to maintain the protein tertiary structure of the enzyme.

Each active site is specially designed in response to their substrates, as a result, most enzymes have specificityand can only react with particular substrates. This specificity is determined by the arrangement of amino acids within the active site and the structure of the substrates. Sometimes enzymes also need to bind with some cofactors to fulfil their function. The active site is usually a groove or pocket of the enzyme which can be located in a deep tunnel within the enzyme,[or between the interfaces of multimeric enzymes. An active site can catalyse a reaction repeatedly as residues are not altered at the end of the reaction (they may change during the reaction, but are regenerated by the end).This process is achieved by lowering the activation energy of the reaction, so more substrates have enough energy to undergo reaction.



Active site

Substrate binding

Enzymes must bind their substrates before they can catalyse any chemical reaction. Enzymes are usually very specific as to what substrates they bind and then the chemical reaction catalysed. Specificity is achieved by binding pockets with complementary shape, charge and hydrophilic/hydrophobic characteristics to the substrates. Enzymes can therefore distinguish between very similar substrate molecules to be chemoselective, regioselective and stereospecific.

Some of the enzymes showing the highest specificity and accuracy are involved in the copying and expression of the genome. Some of these enzymes have "proof-reading" mechanisms. Here, an enzyme such as DNA polymerase catalyzes a reaction in a first step and then checks that the product is correct in a second step. This two-step process results in average error rates of less than 1 error in 100 million reactions in high-fidelity mammalian polymerases. 5.3.1 Similar proofreading mechanisms are also found in RNA polymerase,] aminoacyl tRNA synthetases and ribosomes.

Conversely, some enzymes display enzyme promiscuity, having broad specificity and acting on a range of different physiologically relevant substrates. Many enzymes possess small side activities which arose fortuitously (i.e. neutrally), which may be the starting point for the evolutionary selection of a new function.

Role of catalytic amino acid residues

The MACiE database contains 223 distinct step-wise enzyme reaction mechanisms and holds representatives from each EC sub-subclass where there is a crystal structure and sufficient evidence in the literature to support a mechanism. Each catalytic step of every reaction sequence in MACiE is fully annotated so that it includes the function of

the catalytic residues involved in the reaction and the mechanism by which substrates are transformed into products. Using MACiE as a knowledge base, we have seen that the top 10 most catalytic residues are histidine, aspartate, glutamate, lysine, cysteine, arginine, serine, threonine, tyrosine and tryptophan. Of these only seven (cysteine, histidine, aspartate, lysine, serine, threonine and tyrosine) dominate catalysis and provide essentially five functional roles that are essential. Stabilisation is the most common and essential role for all classes of enzyme, followed by general acid/base (proton acceptor and proton donor) functionality, with nucleophilic addition following closely behind (nucleophile and nucleofuge). We investigated the occurrence of these residues in MACiE and the Catalytic Site Atlas and found that, as expected, certain residue types are associated with each functional role, with some residue types able to perform diverse roles. In addition, it was seen that different EC classes of enzyme have a tendency to employ different residues for catalysis. Further, we show that whilst the differences between EC classes in catalytic residue composition are not immediately obvious from the general classes of In gold mechanisms, there is some weak correlation between the mechanisms involved in a given EC class and the functions that the catalytic amino acid residues are performing.

The mechanism of enzyme catalysis

In order for a reaction to occur, reactant molecules must contain sufficient energy to cross a potential energy barrier, the **activation energy**. All molecules possess varying amounts of energy depending, for example, on their recent collision history but, generally, only a few have sufficient energy for reaction. The lower the potential energy barrier to reaction, the more reactants have sufficient energy and, hence, the faster the reaction will occur. All catalysts, including enzymes, function by forming a transition state, with the reactants, of lower free energy than would be found in the uncatalysed reaction (Figure 1.1). Even quite modest reductions in this potential energy barrier may produce large increases in the rate of reaction (e.g., the activation energy for the uncatalysed breakdown of hydrogen peroxide to oxygen and water is 76 kJ M^{-1} whereas, in the presence of the enzyme catalase, this is reduced to 30 kJ M^{-1} and the rate of reaction is increased by a factor of 10^8, sufficient to convert a reaction time measured in years into one measured in seconds).

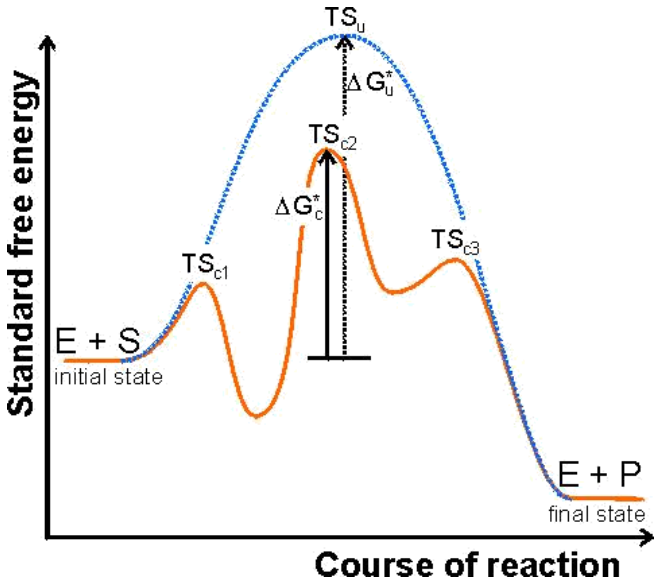


Figure 1.1. A schematic diagram showing the free energy profile of the course of anenzyme catalysed reaction involving the formation of enzyme-substrate (ES) and enzyme-product (EP) complexes, i.e.

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

The catalysed reaction pathway goes through the transition states TS_{c1}, TS_{c2} and TS_{c3}, with standard free energy of activation G_c^*, whereas the uncatalysed reaction goes through the transition state TS_u with standard free energy of activation G_u^*. In this example the rate limiting step would be the conversion of ES into EP. Reactions involving several substrates and products, or more intermediates, are even more complicated. The Michaelis-Menten reaction scheme **[1.7]** would give a similar profile but without the EP-complex free energy trough. The schematic profile for the uncatalysed reaction is shown as the dashed line. It should be noted that the catalytic effect only concerns the lowering of the standard free energy of activation from G_u^* to G_c^* and has no effect on the overall free energy change (i.e., the difference between the initial and final states) or the related equilibrium constant.

There are a number of mechanisms by which this activation energy decrease may be achieved. The most important of these involves the enzyme initially binding the substrate(s), in the correct orientation to react, close to the catalytic groups on the active enzyme complex and any other substrates. In this way the binding energy is used partially in order to reduce the contribution of the considerable activation

entropy, due to the loss of the reactants' (and catalytic groups') translational and rotational entropy, towards the total activation energy. Other contributing factors are the introduction of strain into the reactants (allowing more binding energy to be available for the transition state), provision of an alternative reactive pathway and the desolvation of reacting and catalysing ionic groups.

The energies available to enzymes for binding their substrates are determined primarily by the complementarity of structures (i.e., a good 3-dimensional fit plus optimal non-covalent ionic and/or hydrogen-bonding forces). The specificity depends upon minimal steric repulsion, the absence of unsolvated or unpaired charges, and the presence of sufficient hydrogen bonds. These binding energies are capable of being quite large. As examples, antibody-antigen dissociation constants are characteristically near 10^{-8} M (free energy of binding is 46 kJ M^{-1}), ATP binds to myosin with a dissociation constant of 10^{-13} M (free energy of binding is 75 kJ M^{-1}) and biotin binds to avidin, a protein found in egg white, with a dissociation constant of 10^{-15} M (free energy of binding is 86 kJ M^{-1}). However, enzymes do not use this potential binding energy simply in order to bind the substrate(s) and form stable long-lasting complexes. If this were to be the case, the formation of the transition state between ES and EP would involve an extremely large free energy change due to the breaking of these strong binding forces, and the rate of formation of products would be very slow. They must use this binding energy for reducing the free energy of the transition state. This is generally achieved by increasing the binding to the transition state rather than the reactants and, in the process, introducing an energetic strain into the system and allowing more favourable interactions between the enzyme's catalytic groups and the reactants.

Examples of catalytic mechanisms

In reality, most enzyme mechanisms involve a combination of several different types of catalysis.

Triose phosphate isomerase

Triose phosphate isomerase (EC 5.3.1.1) catalyses the reversible interconvertion of the two triose phosphates isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate.

Trypsin

Trypsin (EC 3.4.21.4) is a serine protease that cleaves protein substrates after lysine or arginine residues using a catalytic triad to perform covalent catalysis, and an oxyanion holeto stabilise charge-buildup on the transition states.

Aldolase

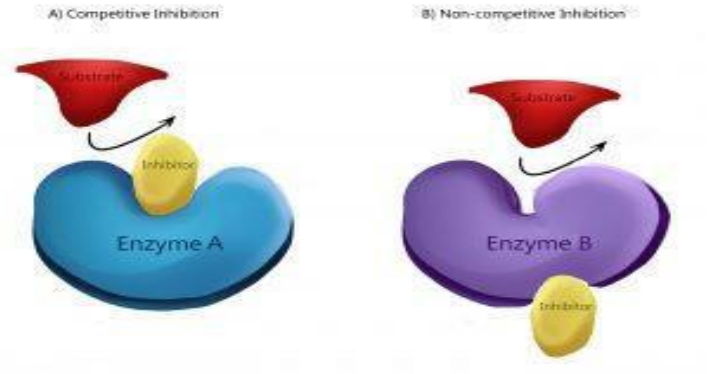
Aldolase (EC 4.1.2.13) catalyses the breakdown of fructose 1,6-bisphosphate (F-1,6-BP) into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP).

Enzyme inhibitor

Enzymes are required for most, if not all, of the processes required for life. Enzymes catalyse a reaction by reducing the activation energy needed for the reaction to occur. However, enzymes need to be tightly regulated to ensure that levels of the product do not rise to undesired levels. This is accomplished by enzyme inhibition.

Types on inhibitor

Reversible and irreversible inhibitors are chemicals which bind to an enzyme to suppress its activity. One method to accomplish this is to almost permanently bind to an enzyme. These types of inhibitors are called irreversible. However, other chemicals can transiently bind to an enzyme. These are called **reversible.** Reversible inhibitors either bind to an active site (competitive inhibitors), or to another site on the enzyme (non-competitive inhibitors).



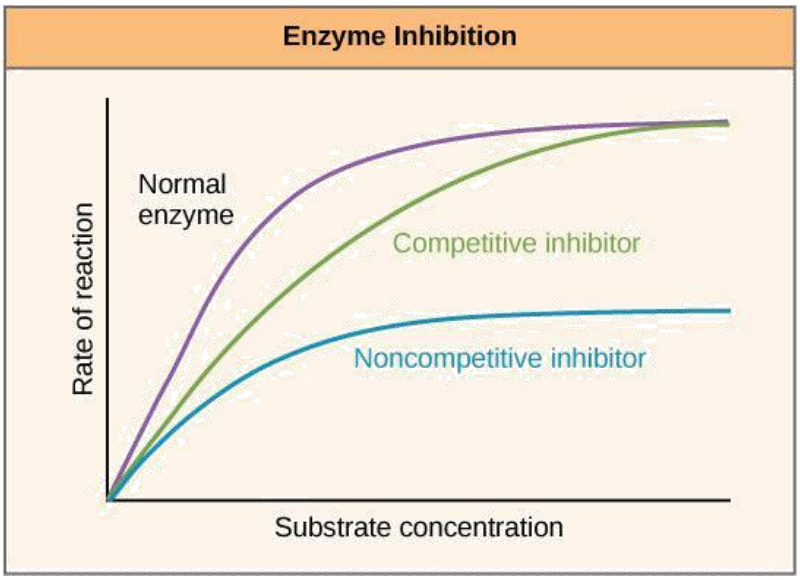
Competitive Inhibitors

Competitive inhibitors compete with the substrate at the active site, and therefore increase **Km** (the Michaelis-Menten constant). However, Vmax is unchanged because, with enough substrate concentration, the reaction can still complete. The graph plot of enzyme activity against substrate concentration would be shifted to the right due to the increase of the Km, whilst the Lineweaver-Burke plot would be steeper when compared with no inhibitor.

Non-Competitive Inhibitors

Non-competitive inhibitors bind to another location on the enzyme and as such decrease$\mathbf{V_{MAX}}$**.** However, K_M is unchanged. This is demonstrated by a lower maximum on a graph plotting enzyme activity against substrate concentration and a higher y-intercept on a Lineweaver-Burke plot when compared with no inhibitor.

Competitive	**Non-Competitive**	
Where does it act?	Active Site	Alternative site
Change of Km	Increase	Unchanged
Change of Vmax	Unchanged	Decrease



Enzyme kinetics

Enzyme kinetics is the study of the chemical reactions that are catalysed by enzymes. In enzyme kinetics, the reaction rate is measured and the effects of varying the conditions of the reaction are investigated. Studying an enzyme's kinetics in this way can reveal the catalytic mechanism of this enzyme, its role in metabolism, how its activity is controlled, and how a drug or an agonist might inhibit the enzyme.

Enzymes are usually protein molecules that manipulate other molecules—the enzymes' substrates. These target molecules bind to an enzyme's active site and are transformed into products through a series of steps known as the enzymatic mechanism

$E + S \rightleftarrows ES \rightleftarrows ES^* \rightleftarrows EP \rightleftarrows E + P$

These mechanisms can be divided into single-substrate and multiple-substrate mechanisms. Kinetic studies on enzymes that only bind one substrate, such as triose phosphate isomerise, aim to measure the affinity with which the enzyme binds this substrate and the turnover rate. Some other examples of enzymes are phosphofructokinase and hexokinase, both of which are important for cellular respiration (glycolysis).

Kinetics of Enzyme-Catalyzed Reactions

Reaction Velocity. The time course of an enzymatic reaction permits one to deduce the substrate affinity, the catalytic mechanism in the active center, and the efficiency of the enzyme (maximum rate, turnover number). The rate of an enzyme-catalyzed single reactant reaction depends on the concentration of substrate and product, respectively. The velocity of the reaction V is:

$$V = -dS/dt = dP/dt \qquad (2)$$

where first term is the rate of dissapearance of substrate S and second term is the rate of appearance of product P (both S and P are in concentration).

Behavior of Initial Rates. The initial rate (V_o) is determined by extrapolating the slope of the time course of the substrate or product concentration to time zero (Fig. 3.5). The dependence of V_o on the substrate concentration, S (at constant enzyme

concentration), is shown in Fig. 3.6. It reflects the typical substrate saturation. At first, V_o increases proportionally to the amount of substrate. Upon further enhancement of substrate concentration V_o levels off. The curve asymptotically approaches a maximum value, V_{max}. When this plateau is reached, a change of S does not lead to a measurable change of V_o: the enzyme is saturated by substrate and has thus reached the limit of its efficiency.

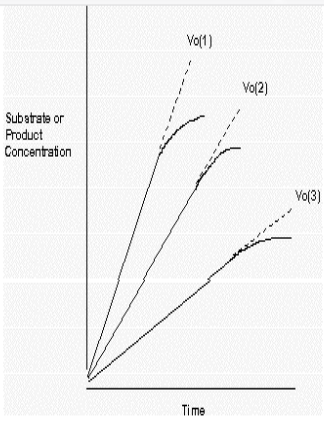


Fig. 3.5. Determination of initial rates at different substrate concentrations.

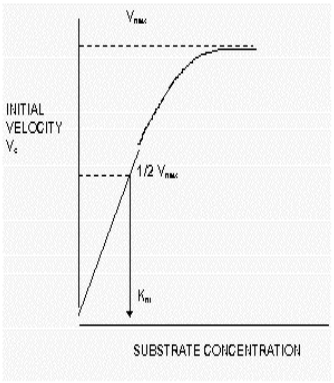


Fig. 3.6. A plot of V_o vs. substrate concentration S.

Micahaelis-Menten Kinetics. These kinetics result from the fast and reversible formation of an enzyme-substrate complex, ES, which dissociates in a second, slower reaction under liberation of the product, P (Fig. 3.7):

E + S ES E + P (3)

Because the second reaction is rate-limiting, at very high substrate concentration almost all enzyme is present as enzyme-substrate complex. Under these conditions a steady state is reached in which the enzyme is steadily saturated by substrate and the initial rate is at a maximum (V_{max}). This relation between substrate concentration and reaction rate may be described by the **Michaelis-Menten equation**:

$$V_o = \frac{V_{max} S}{K_M + S} \qquad (4)$$

where K_M is the Michaelis constant of the enzyme for the given sub-strate. K_M may also be described by:

$$K_M = \frac{k_{-1} + k_{+2}}{k_{+1}} \quad (5)$$

Meaning of K_M The relevance of K_M becomes evident at S = K_M. Then $V_o = V_{max}/2$, i.e., K_M is the substrate concentration at which the reaction rate is half maximum (Fig. 3.6). The K_M value characterizes the affinity between the substrate and the enzyme. At known K_M and V_{max}, V_o can be calculated for each value of substrate concentration. A low K_M value reflects high affinity. At substrate concentrations S << K_M, the reaction rate is directly proportional to the substrate concentration (first order reaction); at high substrate concentration (S >> K_M) the reaction is zero order and is no longer dependent on the substrate concentration but only on the enzyme activity.

Enzyme Kinetics

(Step 1) $E + S \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} E\text{-}S$

(Step 2) $E\text{-}S \xrightarrow{k_2} P$

Rate of reaction:

$$V = -\frac{d[S]}{dt} = \frac{d[P]}{dt} = k_2[ES]$$

Net formation rate of ES:

$$\frac{d[ES]}{dt} = k_1[E][S] - k_{-1}[ES] - k_2[ES]$$

During reaction, the # of active sites occupied by S is constant:

$$\frac{d[ES]}{dt} = 0$$

Total enzyme sites = occupied sites + free sites:

$$[E_o] = [ES] + [E] \quad \text{or} \quad [E] = [E_o] - [ES]$$

Therefore,

$$[ES] = \frac{[E_o][S]}{(k_{-1} + k_2)/k_1 + [S]} = \frac{[E_o][S]}{K_M + [S]}$$

$$V = -\frac{d[S]}{dt} = k_2[ES] = \frac{k_2[E_o][S]}{K_M + [S]} = \frac{V_{max}[S]}{K_M + [S]}$$

Fig. 3.17. Enzyme kinetics.

Lineweaver-Burk Plot. To calculate K_M and V_{max} (and inhibitor constants) it is advantageous to transform the Michaelis-Menten relation so as to obtain linear relationships between S and V_o that can be evaluated graphically. An example is the Lineweaver-Burk equation, containing the reciprocal values of V_o and S:

$$\frac{1}{V_o} = \frac{1}{V_{max}}\left(1 + \frac{K_M}{S}\right) \qquad (6)$$

An example of Lineweaver-Burk plot is shown in Fig. 3.8.

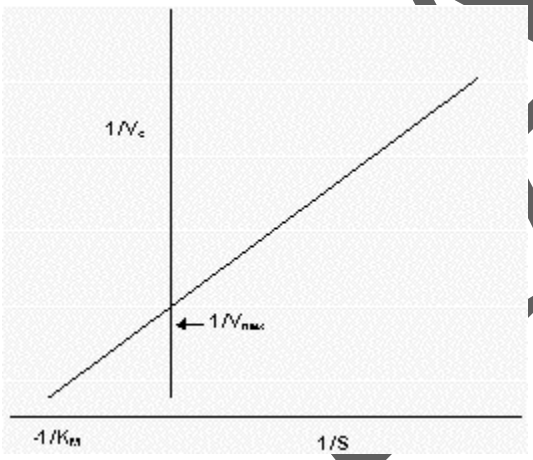


Fig. 3.8. Lineweaver-Burk plot.

Turnover Number. In addition to K_M, and V_{max}, the turnover number (molar activity) and the specific activity are important parameters for the characterization of enzyme reactions. Both are determined under substrate saturation. With highly purified enzymes the **turnover number**

reflects the number of substrate molecules converted in unit time by a single enzyme molecule (or a single active center). Catalase, one of the most potent enzymes, has a turnover number of 2 x 10^5/s.

Specific Activity. The specific activity of enzymes is given in units. One international unit (IU) is the amount of enzyme consuming or forming 1 µmol sub-strate or 1 µmol product per minute under standard conditions. The base unit is 1 katal, corresponding to the amount of enzyme converting 1 mol substrate per second:

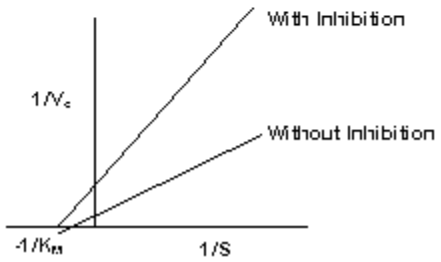
1 kat= $6x10^7$ IU,

1 IU = 16.67 nkat.

Usually U is used instead of IU. For the quantitative determination of enzyme activity, initial rates are measured at different enzyme concentrations and near substrate saturation, in a suitable temperature range (25-37_C) and at optimal pH. In a certain range the enzyme activity is proportional to the enzyme concentration. The enzyme activity of a sample can be estimated from the linear part of the plot.

(a)

(b)



pH and Temperature Dependence

pH Effect. Each enzyme has a characteristic pH optimum at which its activity is at a maximum. In the range of this optimum essential proton-donating or proton-accepting groups in the active center of the enzyme are in the ionized state required for the enzyme to function. Outside this range, substrate binding is no longer possible, and at extreme pH values the enzyme may be irreversibly denatured. The pH optimum depends on the composition of the medium, the temperature, and the enzyme's stability in acid and alkaline environments. The pH stability does not necessarily coincide with the pH optimum of the reaction rate.

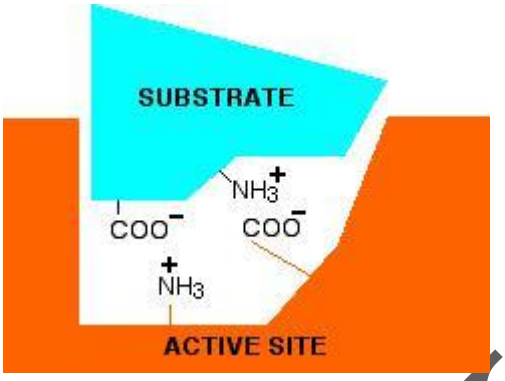
Temperature Effect. As with all chemical reaction rates, those of enzyme reactions increase with increasing temperature (by a factor of 1.4 - 2.0 per 10 K), a limit being set by the thermal stability of the protein. The optimum temperature may be in a wide range, roughly between 30 and 80°C.

The Effect of pH on Enzyme Kinetics

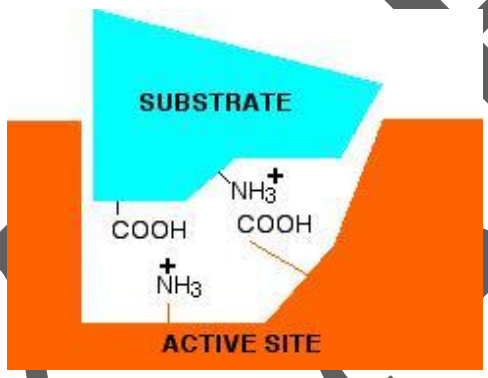
In the same way that every enzyme has an optimum temperature, so each enzyme also has an optimum pH at which it works best. For example, trypsin and pepsin are both enzymes in the digestive system which break protein chains in the food into smaller bits - either into smaller peptide chains or into individual amino acids. Pepsin works in the highly acidic conditions of the stomach. It has an optimum pH of about 1.5. On the other hand, trypsin works in the small intestine, parts of which have a pH of around 7.5. Trypsin's optimum pH is about 8.

If you think about the structure of an enzyme molecule, and the sorts of bonds that it may form with its substrate, it isn't surprising that pH should matter. Suppose an enzyme has an optimum pH around 7. Imagine that at a pH of around 7, a substrate attaches itself to the enzyme via two ionic bonds. In the diagram below, the groups allowing ionic bonding are caused by the transfer of a hydrogen ion from a -COOH group in the side chain of one amino acid residue to an $-NH_2$ group in the side chain of another.

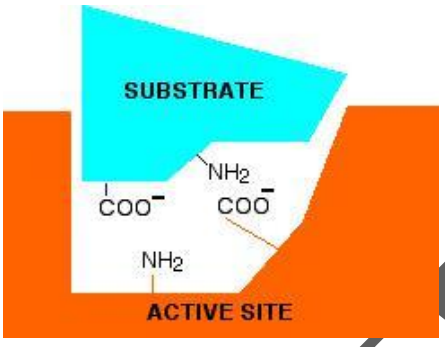
In this simplified example, that is equally true in both the substrate and the enzyme.



Now think about what happens at a lower pH - in other words under acidic conditions. It won't affect the $-NH_3^+$ group, but the $-COO^-$ will pick up a hydrogen ion. What you will have will be this:



You no longer have the ability to form ionic bonds between the substrate and the enzyme. If those bonds were necessary to attach the substrate and activate it in some way, then at this lower pH, the enzyme won't work. What if you have a pH higher than 7 - in other words under alkaline conditions. This time, the $-COO^-$ group won't be affected, but the $-NH_3^+$ group will lose a hydrogen ion. That leaves . . .



Again, there is no possibility of forming ionic bonds, and so the enzyme probably won't work this time either. At extreme pH's, something more drastic can happen. Remember that the tertiary structure of the protein is in part held together by ionic bonds just like those we've looked at between the enzyme and its substrate. At very high or very low pH's, these bonds within the enzyme can be disrupted, and it can lose its shape. If it loses its shape, the active site will probably be lost completely. This is essentially the same as denaturing the protein by heating it too much.

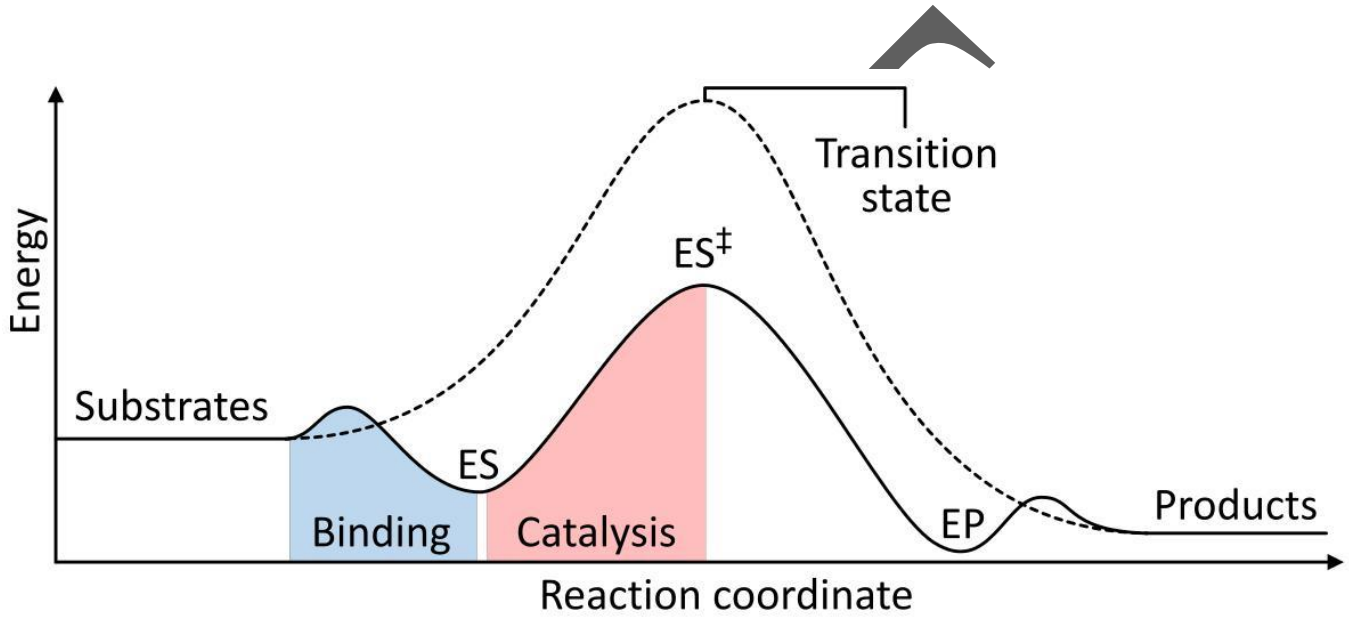
Thermodynamics

As with all catalysts, enzymes do not alter the position of the chemical equilibrium of the reaction. In the presence of an enzyme, the reaction runs in the same direction as it would without the enzyme, just more quickly. For example, carbonic Anhydrase catalyzes its reaction in either direction depending on the concentration of its reactants (in tissues; high CO2 concentration) (1)

(in lungs; low CO2 concentration) (2)

The rate of a reaction is dependent on the activation energy needed to form the transition state which then decays into products. Enzymes increase reaction rates by lowering the energy of the transition state. First, binding forms a low energy enzyme-substrate complex (ES). Secondly the enzyme stabilises the transition state such that it requires less energy to achieve compared to the un catalyzed reaction (ES‡). Finally the enzyme-product complex (EP) dissociates to release the products.

Enzymes can couple two or more reactions, so that a thermodynamically favorable reaction can be used to "drive" a thermodynamically unfavourable one so that the combined energy of the products is lower than the substrates. For example, the hydrolysis of ATP is often used to drive other chemical reactions.


Energy
Transition state
ES‡
Substrates
ES
Binding
Catalysis
EP
Products
Reaction coordinate

UNIT – II Properties of Enzymes:
Thermal stability and catalytic efficiency of enzyme, site directed mutagenesis and enzyme engineering– selected examples, Delivery system for protein pharmaceuticals, structure function relationship in enzymes, structural motifs and enzyme evolution. Methods for analysis of secondary and tertiary structures of enzymes. Protein folding *in vitro* & *in vivo*.

THERMAL STABILITY AND CATALYTIC EFFICIENCY OF ENZYMES

THERMAL STABILITY

Thermostability is the quality of a substance to resist irreversible change in its chemical or physical structure, often by resisting decomposition or polymerization, at a high relative temperature.

Thermostable materials may be used industrially as fire retardants. A *thermostable plastic*, an uncommon and unconventional term, is likely to refer to a thermosetting plastic that cannot be reshaped when heated, than to a thermoplastic that can be remelted and recast.

Thermostability is also a property of some proteins. To be a thermostable protein means to be resistant to changes in protein structure due to applied heat.

Thermostable proteins

Most life-forms on Earth live at temperatures of less than 50 °C, commonly from 15 to 50 °C. Within these organisms are macromolecules (proteins and nucleic acids) which form the three-dimensional structures essential to their enzymatic activity. Above the native temperature of the organism, thermal energy may cause the unfolding and denaturation, as the heat can disrupt the intramolecular bonds in the tertiary and quaternary structure. This unfolding will result in loss in enzymatic activity, which is understandably deleterious to continuing life-functions. An example of such is the denaturing of proteins in albumen from a clear, nearly colourless liquid to an opaque white, insoluble gel.

Proteins capable of withstanding such high temperatures compared to proteins that cannot, are generally from microorganisms that are hyperthermophiles. Such organisms can withstand above 50 °C temperatures as they usually live within environments of 85 °C and above. Certain thermophilic life-forms exist which can withstand temperatures above this, and have corresponding adaptations to preserve protein function at these temperatures. These can include altered bulk properties of the cell to stabilize all proteins, and specific changes to individual proteins. Comparing homologous proteins present in these thermophiles and other organisms reveal some differences in the protein structure. One notable difference is the presence of extra hydrogen bonds in the thermophile's proteins—meaning that the protein structure is more resistant to unfolding. Similarly, thermostable proteins are rich in salt

bridges or/and extra disulfide bridges stabilizing the structure. Other factors of protein thermostability are compactness of protein structure, oligomerization, and strength interaction between subunits.

Uses and Applications of Thermostable Proteins

Polymerase Chain Reactions

Thermostable enzymes such as Taq polymerase and Pfu DNA polymerase are used in polymerase chain reactions (PCR) where temperatures of 94 °C or over are used to melt apart DNA strands in the denaturation step of PCR. This resistance to high temperature allows for DNA polymerase to elongate DNA with a desired sequence of interest with the presence of dNTP's.

Protein Purification

Knowledge of an enzyme's resistance to high temperatures is especially beneficial in protein purification. In the procedure of heat denaturation, one can subject a mixture of proteins to high temperatures, which will result in the denaturation of proteins that are not thermostable, and the isolation of the protein that is thermodynamically stable. One notable example of this is found in the purification of alkaline phosphatase from the hyperthermophile *Pyrococcus abyssi*. This enzyme is known for being heat stable at temperatures greater than 95 °C, and therefore can be partially purified by heating when heterologously expressed in *E. coli*. The increase in temperature causes the *E. coli* proteins to precipitate, while the *P. abyssi* alkaline phosphatase remains stably in solution.

Glycoside Hydrolases

Another important group of thermostable enzymes are glycoside hydrolases. These enzymes are responsible of the degradation of the major fraction of biomass, the polysaccharides present in starch and lignocellulose. Thus, glycoside hydrolases are gaining great interest in biorefining applications in the future bioeconomy. Some examples are the production of monosaccharides for food applications as well as use as carbon source for microbial conversion in fuels (ethanol) and chemical intermediates, production of oligosaccharides for prebiotic applications and production of surfactants alkyl glycoside type. All of these processes often involve thermal treatments to facilitate the polysaccharide hydrolysis, hence give thermostable variants of glycoside hydrolases an important role in this context.

Approaches to improve thermostability of proteins

Protein engineering can be used to enhance the thermostability of proteins. A number of site-directed and random mutagenesis techniques, in addition to directed evolution, have been used to increase the thermostability of target proteins. Comparative methods have been used to increase the stability of mesophilic proteins based on comparison to thermophilic homologs. Additionally, analysis of the protein unfolding by molecular dynamics can be used to understand the process of unfolding and then design stabilizing mutations. Rational protein engineering for increasing protein thermostability includes mutations which truncate loops, increase salt bridges or hydrogen bonds, introduced disulfide

bonds. In addition, ligand binding can increase the stability of the protein, particularly when purified. There are various different forces that allow for the thermostability of a particular protein. These forces include hydrophobic interactions, electrostatic interactions, and the presence of disulfide bonds. The overall amount of hydrophobicity present in a particular protein is responsible for its thermostability. Another type of force that is responsible for thermostability of a protein is the electrostatic interactions between molecules. These interactions include salt bridges and hydrogen bonds. Salt bridges are unaffected by high temperatures, therefore, are necessary for protein and enzyme stability. A third force used to increase thermosability in proteins and enzymes is the presence of disulfide bonds. They present covalent cross-linkages between the polypeptide chains. These bonds are the strongest because they're covalent bonds, making them stronger than intermolecular forces.

Thermostable toxins

Certain poisonous fungi contain thermostable toxins, such as amatoxin found in the death cap and autumn skullcap mushrooms and patulin from molds. Therefore, applying heat to these will not remove the toxicity and is of particular concern for food safety.

ENZYME CATALYSIS

Enzyme catalysis is the increase in the rate of a chemical reaction by the active site of a protein. The protein catalyst(enzyme) may be part of a multi-subunit complex, and/or may transiently or permanently associate with a Cofactor (e.g. adenosine triphosphate). Catalysis of biochemical reactions in the cell is vital due to the very low reaction rates of the uncatalysed reactions at room temperature and pressure. A key driver of protein evolution is the optimization of such catalytic activities via protein dynamics.

The mechanism of enzyme catalysis is similar in principle to other types of chemical catalysis. By providing an alternative reaction route the enzyme reduces the energy required to reach the highest energy transition state of the reaction. The reduction of activation energy (Ea) increases the amount of reactant molecules that achieve a sufficient level of energy, such that they reach the activation energy and form the product. As with other catalysts, the enzyme is not consumed during the reaction (as a substrate is) but is recycled such that a single enzyme performs many rounds of catalysis.

Induced fit

The favored model for the enzyme-substrate interaction is the induced fit model. This model proposes that the initial interaction between enzyme and substrate is relatively weak, but that these weak interactions rapidly induce conformational changes in the enzyme that strengthen binding.

The advantages of the induced fit mechanism arise due to the stabilizing effect of strong enzyme binding. There are two different mechanisms of substrate binding: uniform binding, which has strong substrate binding, and differential binding, which has strong transition state binding. The stabilizing effect of uniform binding increases both substrate and transition state

binding affinity, while differential binding increases only transition state binding affinity. Both are used by enzymes and have been evolutionarily chosen to minimize the activation energy of the reaction. Enzymes that are saturated, that is, have a high affinity substrate binding, require differential binding to reduce the energy of activation, whereas small substrate unbound enzymes may use either differential or uniform binding.

These effects have led to most proteins using the differential binding mechanism to reduce the energy of activation, so most substrates have high affinity for the enzyme while in the transition state. Differential binding is carried out by the induced fit mechanism - the substrate first binds weakly, then the enzyme changes conformation increasing the affinity to the transition state and stabilizing it, so reducing the activation energy to reach it.

It is important to clarify, however, that the induced fit concept cannot be used to rationalize catalysis. That is, the chemical catalysis is defined as the reduction of $Ea^{\ddagger}$ (when the system is already in the $ES^{\ddagger}$) relative to $Ea^{\ddagger}$ in the uncatalyzed reaction in water (without the enzyme). The induced fit only suggests that the barrier is lower in the closed form of the enzyme but does not tell us what the reason for the barrier reduction is.

Induced fit may be beneficial to the fidelity of molecular recognition in the presence of competition and noise via the conformational proofreading mechanism .

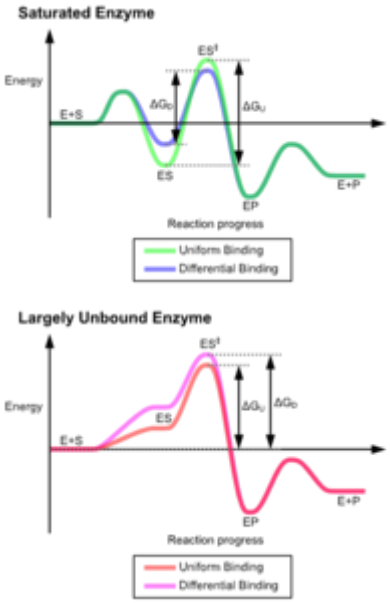


Figure: Different mechanisms of substrate binding

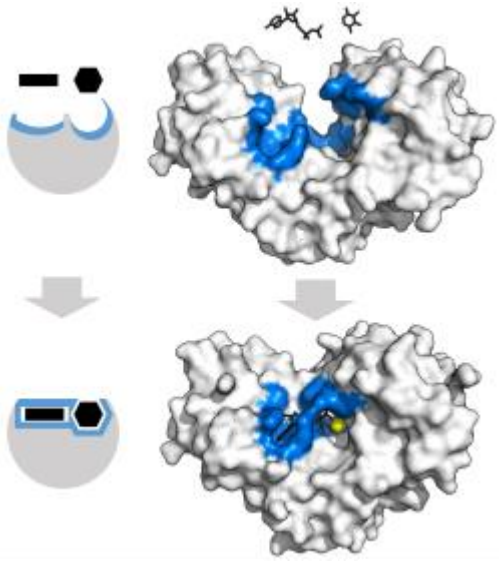


Figure : Enzyme changes shape by induced fit

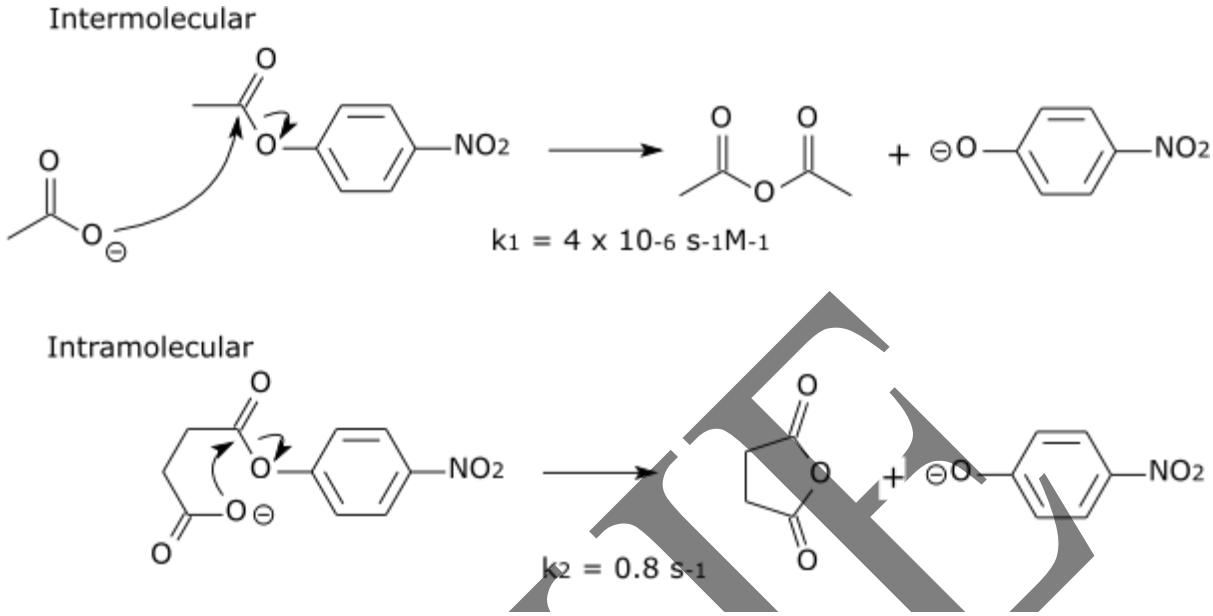
Mechanisms of an alternative reaction route

These conformational changes also bring catalytic residues in the active site close to the chemical bonds in the substrate that will be altered in the reaction. After binding takes place, one or more mechanisms of catalysis lowers the energy of the reaction's transition state, by providing an alternative chemical pathway for the reaction. There are six possible mechanisms of "over the barrier" catalysis as well as a "through the barrier" mechanism:

Proximity and orientation

Enzyme-substrate interactions align the reactive chemical groups and hold them close together in an optimal geometry, which increases the rate of the reaction. This reduces the entropy of the reactants and thus makes addition or transfer reactions less unfavorable, since a reduction in the overall entropy when two reactants become a single product.

This effect is analogous to an effective increase in concentration of the reagents. The binding of the reagents to the enzyme gives the reaction intramolecular character, which gives a massive rate increase.



However, the situation might be more complex, since modern computational studies have established that traditional examples of proximity effects cannot be related directly to enzyme entropic effects. Also, the original entropic proposal[8] has been found to largely overestimate the contribution of orientation entropy to catalysis.

Proton donors or acceptors

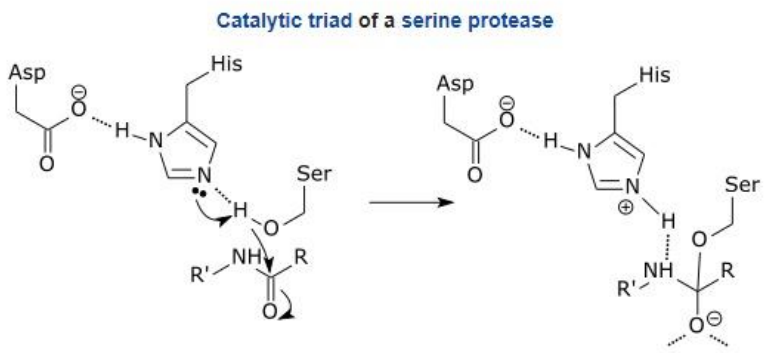
Proton donors and acceptors, i.e. acids and base may donate and accept protons in order to stabilize developing charges in the transition state.This typically has the effect of activating nucleophile and electrophile groups, or stabilizing leaving groups. Histidine is often the residue involved in these acid/base reactions, since it has a pKa close to neutral pHand can therefore both accept and donate protons.

Many reaction mechanisms involving acid/base catalysis assume a substantially altered pKa. This alteration of pKa is possible through the local environment of the residue.

Conditions	Acids	Bases
Hydrophobic environment	Increase pKa	Decrease pKa
Adjacent residues of like charge	Increase pKa	Decrease pKa
Salt bridge (and hydrogen bond) formation	Decrease pKa	Increase pKa

pKa can also be influenced significantly by the surrounding environment, to the extent that residues which are basic in solution may act as proton donors, and vice versa.

For example:



The initial step of the serine protease catalytic mechanism involves the histidine of the active site accepting a proton from the serine residue. This prepares the serine as a nucleophile to attack the amide bond of the substrate. This mechanism includes donation of a proton from serine (a base, pKa 14) to histidine (an acid, pKa 6), made possible due to the local environment of the bases.

It is important to clarify that the modification of the pKa's is a pure part of the electrostatic mechanism. Furthermore, the catalytic effect of the above example is mainly associated with the reduction of the pKa of the oxyanion and the increase in the pKa of the histidine, while the proton transfer from the serine to the histidine is not catalyzed significantly, since it is not the rate determining barrier.

Electrostatic catalysis[edit]

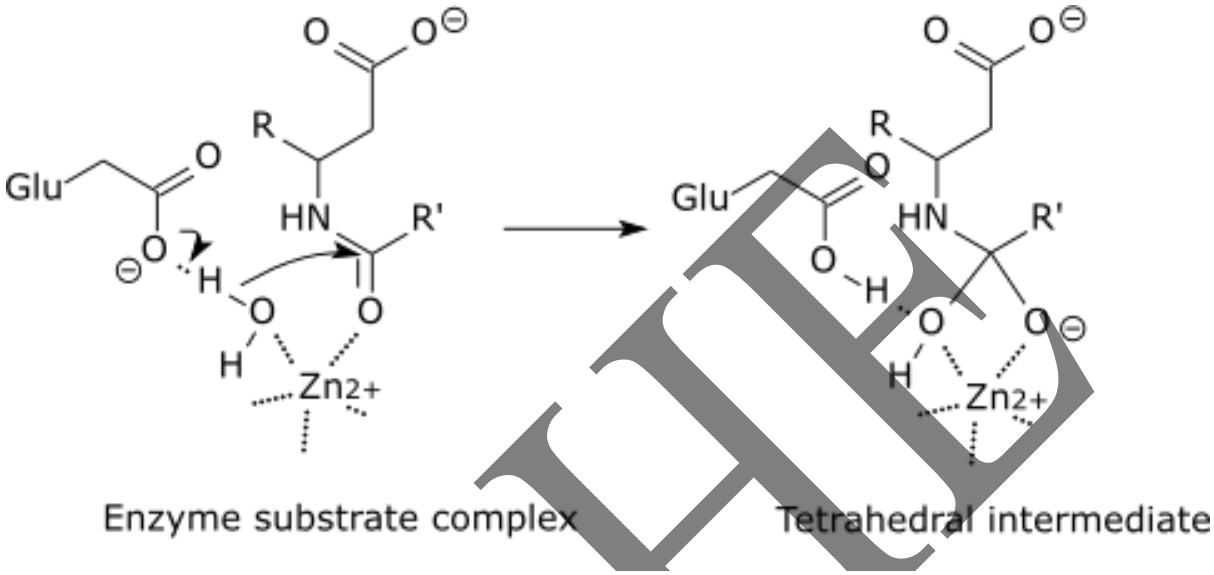
Stabilization of charged transition states can also be by residues in the active site forming ionic bonds (or partial ionic charge interactions) with the intermediate. These bonds can either come from acidic or basic side chains found on amino acids such as lysine, arginine, aspartic acid or glutamic acid or come from metal cofactors such as zinc. Metal ions are particularly effective and can reduce the pKa of water enough to make it an effective nucleophile.

Systematic computer simulation studies established that electrostatic effects give, by far, the largest contribution to catalysis. In particular, it has been found that enzyme provides an environment which is more polar than water, and that the ionic transition states are stabilized by fixed dipoles. This is very different from transition state stabilization in water, where the water molecules must pay with "reorganization energy". In order to stabilize ionic and charged states. Thus, the catalysis is associated with the fact that the enzyme polar groups are preorganized.

The magnitude of the electrostatic field exerted by an enzyme's active site has been shown to be highly correlated with the enzyme's catalytic rate enhancement.

Binding of substrate usually excludes water from the active site, thereby lowering the local dielectric constant to that of an organic solvent. This strengthens the electrostatic interactions between the charged/polar substrates and the active sites. In addition, studies have shown that

the charge distributions about the active sites are arranged so as to stabilize the transition states of the catalyzed reactions. In several enzymes, these charge distributions apparently serve to guide polar substrates toward their binding sites so that the rates of these enzymatic reactions are greater than their apparent diffusion-controlled limits.



Covalent catalysis

Covalent catalysis involves the substrate forming a transient covalent bond with residues in the enzyme active site or with a cofactor. This adds an additional covalent intermediate to the reaction, and helps to reduce the energy of later transition states of the reaction. The covalent bond must, at a later stage in the reaction, be broken to regenerate the enzyme. This mechanism is utilised by the catalytic triad of enzymes such as proteases like chymotrypsin and trypsin, where an acyl-enzyme intermediate is formed. An alternative mechanism is schiff base formation using the free amine from a lysine residue, as seen in the enzyme aldolase during glycolysis.

Some enzymes utilize non-amino acid cofactors such as pyridoxal phosphate (PLP) or thiamine pyrophosphate (TPP) to form covalent intermediates with reactant molecules.Such covalent intermediates function to reduce the energy of later transition states, similar to how covalent intermediates formed with active site amino acid residues allow stabilization, but the capabilities of cofactors allow enzymes to carryout reactions that amino acid side residues alone could not. Enzymes utilizing such cofactors include the PLP-dependent enzyme aspartate transaminase and the TPP-dependent enzyme pyruvate dehydrogenase.

Rather than lowering the activation energy for a reaction pathway, covalent catalysis provides an alternative pathway for the reaction (via to the covalent intermediate) and so is distinct from true catalysis. For example, the energetics of the covalent bond to the serine molecule in chymotrypsin should be compared to the well-understood covalent bond to the nucleophile in the uncatalyzed solution reaction. A true proposal of a covalent catalysis (where the barrier is

lower than the corresponding barrier in solution) would require, for example, a partial covalent bond to the transition state by an enzyme group (e.g., a very strong hydrogen bond), and such effects do not contribute significantly to catalysis.

Metal ion catalysis

A metal ion in the active site participates in catalysis by coordinating charge stabilization and shielding. Because of a metal's positive charge, only negative charges can be stabilized through metal ions. However, metal ions are advantageous in biological catalysis because they are not affected by changes in pH. Metal ions can also act to ionize water by acting as a Lewis acid. Metal ions may also be agents of oxidation and reduction.

Bond strain

This is the principal effect of induced fit binding, where the affinity of the enzyme to the transition state is greater than to the substrate itself. This induces structural rearrangements which strain substrate bonds into a position closer to the conformation of the transition state, so lowering the energy difference between the substrate and transition state and helping catalyze the reaction.

However, the strain effect is, in fact, a ground state destabilization effect, rather than transition state stabilization effect. Furthermore, enzymes are very flexible and they cannot apply large strain effect. In addition to bond strain in the substrate, bond strain may also be induced within the enzyme itself to activate residues in the active site.

For example:

Substrate, bound substrate, and transition state conformations of lysozyme.

The substrate, on binding, is distorted from the half chair conformation of the hexose ring (because of the steric hindrance with amino acids of the protein forcing the equatorial c6 to be in the axial position) into the chair conformation[26][page needed]

Quantum tunneling

These traditional "over the barrier" mechanisms have been challenged in some cases by models and observations of "through the barrier" mechanisms (quantum tunneling). Some enzymes operate with kinetics which are faster than what would be predicted by the classical $\Delta G^{\ddagger}$. In "through the barrier" models, a proton or an electron can tunnel through activation barriers. Quantum tunneling for protons has been observed in tryptamine oxidation by aromatic amine dehydrogenase. Quantum tunneling does not appear to provide a major catalytic advantage, since the tunneling contributions are similar in the catalyzed and the uncatalyzed reactions in solution. However, the tunneling contribution (typically enhancing rate constants by a factor of ~1000 compared to the rate of reaction for the classical 'over the barrier' route) is likely crucial to the viability of biological organisms. This emphasizes the general importance of tunneling reactions in biology.

In 1971-1972 the first quantum-mechanical model of enzyme catalysis was formulated.

Active enzyme

The binding energy of the enzyme-substrate complex cannot be considered as an external energy which is necessary for the substrate activation. The enzyme of high energy content may firstly transfer some specific energetic group X_1 from catalytic site of the enzyme to the final place of the first bound reactant, then another group X_2 from the second bound reactant (or from the second group of the single reactant) must be transferred to active site to finish substrate conversion to product and enzyme regeneration. We can present the whole enzymatic reaction as a two coupling reactions:

It may be seen from reaction (**1**) that the group X_1 of the active enzyme appears in the product due to possibility of the exchange reaction inside enzyme to avoid both electrostatic inhibition and repulsion of atoms. So we represent the active enzyme as a powerful reactant of the enzymatic reaction. The reaction (**2**) shows incomplete conversion of the substrate because its group X_2 remains inside enzyme. This approach as idea had formerly proposed relying on the hypothetical extremely high enzymatic conversions (catalytically perfect enzyme). The crucial point for the verification of the present approach is that the catalyst must be a complex of the enzyme with the transfer group of the reaction. This chemical aspect is supported by the well-studied mechanisms of the several enzymatic reactions. Let us consider the reaction of peptide bond hydrolysis catalyzed by a pure protein α-chymotrypsin (an enzyme acting without a cofactor), which is a well-studied member of the serine proteases family, see.

We present the experimental results for this reaction as two chemical steps:

where S_1 is a polypeptide, P_1 and P_2 are products. The first chemical step (**3**) includes the formation of a covalent acyl-enzyme intermediate. The second step (**4**) is the deacylation step. It is important to note that the group H+, initially found on the enzyme, but not in water, appears in the product before the step of hydrolysis, therefore it may be considered as an additional group of the enzymatic reaction.

Thus, the reaction (**3**) shows that the enzyme acts as a powerful reactant of the reaction. According to the proposed concept, the H transport from the enzyme promotes the first reactant conversion, breakdown of the first initial chemical bond (between groups P_1 and P_2). The step of hydrolysis leads to a breakdown of the second chemical bond and regeneration of the enzyme.

The proposed chemical mechanism does not depend on the concentration of the substrates or products in the medium. However, a shift in their concentration mainly causes free energy changes in the first and final steps of the reactions (**1**) and (**2**) due to the changes in the free energy content of every molecule, whether S or P, in water solution. This approach is in accordance with the following mechanism of muscle contraction. The final

step of ATP hydrolysis in skeletal muscle is the product release caused by the association of myosin heads with actin. The closing of the actin-binding cleft during the association reaction is structurally coupled with the opening of the nucleotide-binding pocket on the myosin active site.

Notably, the final steps of ATP hydrolysis include the fast release of phosphate and the slow release of ADP. The release of a phosphate anion from bound ADP anion into water solution may be considered as an exergonic reaction because the phosphate anion has low molecular mass.

Thus, we arrive at the conclusion that the primary release of the inorganic phosphate $H_2PO_4^-$ leads to transformation of a significant part of the free energy of ATP hydrolysis into the kinetic energy of the solvated phosphate, producing active streaming. This assumption of a local mechano-chemical transduction is in accord with Tirosh's mechanism of muscle contraction, where the muscle force derives from an integrated action of active streaming created by ATP hydrolysis.

Examples of catalytic mechanisms

In reality, most enzyme mechanisms involve a combination of several different types of catalysis.

Triose phosphate isomerase

Triose phosphate isomerase (EC 5.3.1.1) catalyses the reversible interconvertion of the two triose phosphates isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate.

Trypsin

Trypsin (EC 3.4.21.4) is a serine protease that cleaves protein substrates after lysine or arginine residues using a catalytic triad to perform covalent catalysis, and an oxyanion holeto stabilise charge-buildup on the transition states.

Aldolase

Aldolase (EC 4.1.2.13) catalyses the breakdown of fructose 1,6-bisphosphate (F-1,6-BP) into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP).

Enzyme diffusivity

The advent of single-molecule studies led in the 2010s to the observation that the movement of untethered enzymes increases with increasing substrate concentration and increasing reaction enthalpy. Subsequent observations suggest that this increase in diffusivity is driven by transient displacement of the enzyme's center of mass, resulting in a "recoil effect that propels the enzyme".

Reaction similarity

Similarity between enzymatic reactions (EC) can be calculated by using bond changes, reaction centres or substructure metrics (EC-BLAST).

SITE-DIRECTED MUTAGENESIS

Site-directed mutagenesis is a molecular biology method that is used to make specific and intentional changes to the DNA sequence of a gene and any gene products. Also called **site-specific mutagenesis** or **oligonucleotide-directed mutagenesis**, it is used for investigating the structure and biological activity of DNA, RNA, and protein molecules, and for protein engineering.

Site-directed mutagenesis is one of the most important techniques in laboratory for introducing a mutation into a DNA sequence. There are numerous methods for achieving site-directed mutagenesis, but with decreasing costs of oligonucleotide synthesis, artificial gene synthesis is now occasionally used as an alternative to site-directed mutagenesis. Since 2013, the development of the CRISPR/Cas9 technology, based on a prokaryotic viral defense system, has also allowed for the editing of the genome, and mutagenesis may be performed *in vivo* with relative ease.

HISTORY

Early attempts at mutagenesis using radiation or chemical mutagens were non-site-specific, generating random mutations. Analogs of nucleotides and other chemicals were later used to generate localized point mutations, examples of such chemicals are aminopurine,[4] nitrosoguanidine, and bisulfite [1]Site-directed mutagenesis was achieved in 1974 in the laboratory of Charles Weissmann using a nucleotide analogue N^4-hydroxycytidine, which induces transition of GC to AT. These methods of mutagenesis, however, are limited by the kind of mutation they can achieve, and they are not as specific as later site-directed mutagenesis methods.

In 1971, Clyde Hutchison and Marshall Edgell showed that it is possible to produce mutants with small fragments of phage ϕX174 and restriction nucleases. Hutchison later produced with his collaborator Michael Smith in 1978 a more flexible approach to site-directed mutagenesis by using oligonucleotides in a primer extension method with DNA polymerase. For his part in the development of this process, Michael Smith later shared the Nobel Prize in Chemistry in October 1993 with Kary B. Mullis, who invented polymerase chain reaction.

BASIC MECHANISM

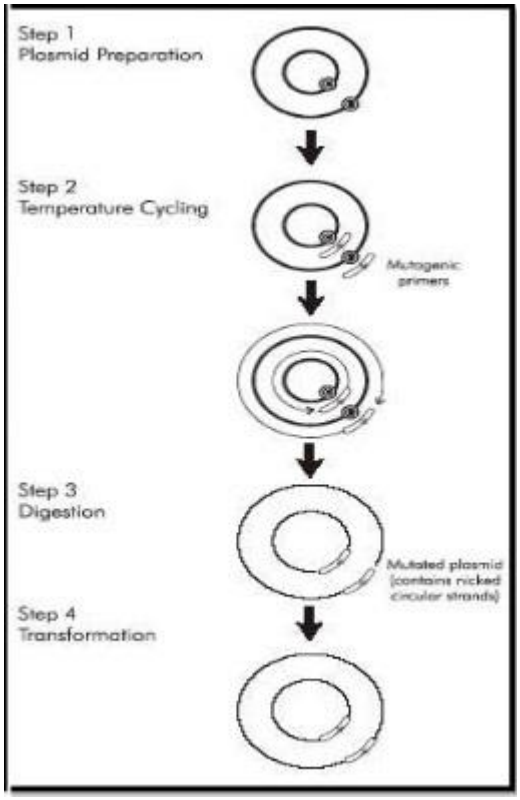


Fig 1: Basic mechanism of site-directed mutagenesis

The basic procedure requires the synthesis of a short DNA primer. This synthetic primer contains the desired mutation and is complementary to the template DNA around the mutation site so it can hybridize with the DNA in the gene of interest. The mutation may be a single base change (a point mutation), multiple base changes, deletion, or insertion. The single-strand primer is then extended using a DNA polymerase, which copies the rest of the gene. The gene thus copied contains the mutated site, and is then introduced into a host cell as a vector and cloned. Finally, mutants are selected by DNA sequencing to check that they contain the desired mutation.

The original method using single-primer extension was inefficient due to a low yield of mutants. This resulting mixture contains both the original unmutated template as well as the mutant strand, producing a mixed population of mutant and non-mutant progenies. Furthermore, the template used is methylated while the mutant strand is unmethylated, and the mutants may be counter-selected due to presence of mismatch repairsystem that favors the methylated template DNA, resulting in fewer mutants. Many approaches have since been developed to improve the efficiency of mutagenesis.

APPROACHES

A large number of methods are available to effect site-directed mutagenesis, although most of them are now rarely used in laboratories since the early 2000s, as newer techniques allow for simpler and easier ways of introducing site-specific mutation into genes.

Kunkel's method

In 1985, Thomas Kunkel introduced a technique that reduces the need to select for the mutants.[13] The DNA fragment to be mutated is inserted into a phagemid such as M13mp18/19 and is then transformed into an *E. coli* strain deficient in two enzymes, dUTPase (*dut*) and uracil deglycosidase (*udg*). Both enzymes are part of a DNA repair pathway that protects the bacterial chromosome from mutations by the spontaneous deamination of dCTP to dUTP. The dUTPase deficiency prevents the breakdown of dUTP, resulting in a high level of dUTP in the cell. The uracil deglycosidase deficiency prevents the removal of uracil from newly synthesized DNA. As the double-mutant *E. coli* replicates the phage DNA, its enzymatic machinery may, therefore, misincorporate dUTP instead of dTTP, resulting in single-strand DNA that contains some uracils (ssUDNA). The ssUDNA is extracted from the bacteriophage that is released into the medium, and then used as template for mutagenesis. An oligonucleotide containing the desired mutation is used for primer extension. The heteroduplex DNA, that forms, consists of one parental non-mutated strand containing dUTP and a mutated strand containing dTTP. The DNA is then transformed into an E. coli strain carrying the wildtype *dut* and *udg* genes. Here, the uracil-containing parental DNA strand is degraded, so that nearly all of the resulting DNA consists of the mutated strand.

Cassette mutagenesis

Unlike other methods, cassette mutagenesis need not involve primer extension using DNA polymerase. In this method, a fragment of DNA is synthesized, and then inserted into a plasmid.[14] It involves the cleavage by a restriction enzyme at a site in the plasmid and subsequent ligation of a pair of complementary oligonucleotides containing the mutation in the gene of interest to the plasmid. Usually, the restriction enzymes that cut at the plasmid and the oligonucleotide are the same, permitting sticky ends of the plasmid and insert to ligate to one another. This method can generate mutants at close to 100% efficiency, but is limited by the availability of suitable restriction sites flanking the site that is to be mutated.

PCR site-directed mutagenesis

The limitation of restriction sites in cassette mutagenesis may be overcome using polymerase chain reaction with oligonucleotide "primers", such that a larger fragment may be generated, covering two convenient restriction sites. The exponential amplification in PCR produces a fragment containing the desired mutation in sufficient quantity to be separated from the original, unmutated plasmid by gel electrophoresis, which may then be inserted in the original context using standard recombinant molecular biology techniques. There are many variations of the same technique. The simplest method places the mutation

site toward one of the ends of the fragment whereby one of two oligonucleotides used for generating the fragment contains the mutation. This involves a single step of PCR, but still has the inherent problem of requiring a suitable restriction site near the mutation site unless a very long primer is used. Other variations, therefore, employ three or four oligonucleotides, two of which may be non-mutagenic oligonucleotides that cover two convenient restriction sites and generate a fragment that can be digested and ligated into a plasmid, whereas the mutagenic oligonucleotide may be complementary to a location within that fragment well away from any convenient restriction site. These methods require multiple steps of PCR so that the final fragment to be ligated can contain the desired mutation. The design process for generating a fragment with the desired mutation and relevant restriction sites can be cumbersome. Software tools like SDM-Assist can simplify the process.

Whole plasmid mutagenesis

For plasmid manipulations, other site-directed mutagenesis techniques have been supplanted largely by techniques that are highly efficient but relatively simple, easy to use, and commercially available as a kit. An example of these techniques is the Quikchange method,[16] wherein a pair of complementary mutagenic primers are used to amplify the entire plasmid in a thermocycling reaction using a high-fidelity non-strand-displacing DNA polymerase such as *pfu* polymerase. The reaction generates a nicked, circular DNA. The template DNA must be eliminated by enzymatic digestion with a restriction enzyme such as *Dpn*I, which is specific for methylated DNA. All DNA produced from most *Escherichia coli* strains would be methylated; the template plasmid that is biosynthesized in *E. coli* will, therefore, be digested, while the mutated plasmid, which is generated *in vitro* and is therefore unmethylated, would be left undigested. Note that, in these double-strand plasmid mutagenesis methods, while the thermocycling reaction may be used, the DNA need not be exponentially amplified as in a PCR. Instead, the amplification is linear, and it is therefore inaccurate to describe them as a PCR, since there is no chain reaction.

Note that *pfu* polymerase can become strand-displacing at higher extension temperature (≥70 °C) which can result in the failure of the experiment, therefore the extension reaction should be performed at the recommended temperature of 68 °C. In some applications, this method has been observed to lead to insertion of multiple copies of primers. A variation of this method, called SPRINP, prevents this artifact and has been used in different types of site directed mutagenesis.

***In vivo* site-directed mutagenesis methods**

- *Delitto perfetto*
- Transplacement "pop-in pop-out"
- Direct gene deletion and site-specific mutagenesis with PCR and one recyclable marker
- Direct gene deletion and site-specific mutagenesis with PCR and one recyclable marker using long homologous regions
- *In vivo* site-directed mutagenesis with synthetic oligonucleotides

CRISPR

Since 2013, the development of CRISPR-Cas9 technology has allowed for the efficient introduction of point mutations into the genome of a wide variety of organisms. The method does not require a transposon insertion site, leaves no marker, and its efficiency and simplicity has made it the preferred method for genome editing.

APPLICATIONS

Site-directed mutagenesis is used to generate mutations that may produce a rationally designed protein that has improved or special properties (i.e.protein engineering).

Investigative tools – specific mutations in DNA allow the function and properties of a DNA sequence or a protein to be investigated in a rational approach. Furthermore, single amino-acid changes by site-directed mutagenesis in proteins can help understand the importance of post-translational modifications. For instance changing a particular serine (phosphoacceptor) to an alanine (phospho-non-acceptor) in a substrate protein blocks the attachement of a phosphate group, thereby allows the phosphorylation to be investigated. This approach has been used to uncover the phosphorylation of the protein CBP by the kinase HIPK2.

Commercial applications – Proteins may be engineered to produce mutant forms that are tailored for a specific application. For example, commonly used laundry detergents may contain subtilisin, whose wild-type form has a methionine that can be oxidized by bleach, significantly reducing the activity the protein in the process. This methionine may be replaced by alanine or other residues, making it resistant to oxidation thereby keeping the protein active in the presence of bleach.

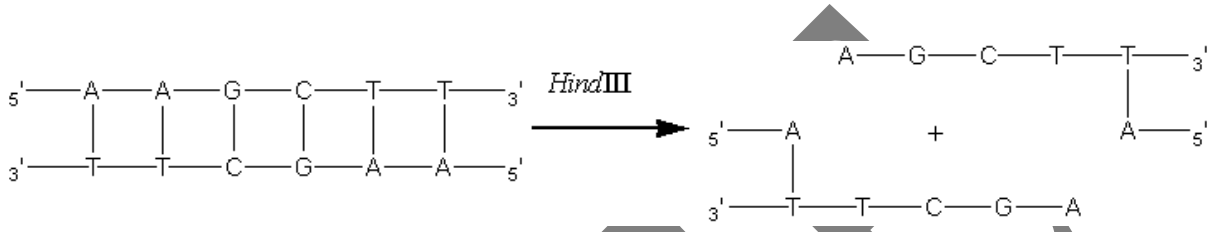
GENE SYNTHESIS

As the cost of DNA oligonucleotides synthesis falls, artificial synthesis of a complete gene is now a viable method for introducing mutation into gene. This method allows for extensive mutagenesis over multiples sites, including the complete redesign of the codon usage of gene to optimise it for a particular organism.

ENZYME ENGINEERING

A most exciting development over the last few years is the application genetic engineering techniques to enzyme technology. A full description this burgeoning science is beyond the scope of this text but some suitable references are given at the end of this chapter. There are a number of properties which may be improved or altered by genetic engineering including the yield and kinetics of the enzyme, the ease of downstream processing and various safety aspects. Enzymes from dangerous or unapproved microorganisms and from slow growing or limited plant or animal tissue may be cloned into safe high-production microorganisms. In the future, enzymes may be redesigned to fit more appropriately into industrial processes; for example, making glucose isomerase less susceptible to inhibition by the Ca^{2+}present in the starch saccharification processing stream.

The amount of enzyme produced by a microorganism may be increased by increasing the number of gene copies that code for it. This principle has been used to increase the activity of penicillin-G-amidase in*Escherichia coli*. The cellular DNA from a producing strain is selectively cleaved by the restriction endonuclease HindIII. This hydrolyses the DNA at relatively rare sites containing the 5'-AAGCTT-3' base sequence to give identical 'staggered' ends.



intact DNA ↦ cleaved DNA

The total DNA is cleaved into about 10000 fragments, only one of which contains the required genetic information. These fragments are individual cloned into a cosmid vector and thereby returned to *E. coli*. These colonies containing the active gene are identified by their inhibition of a 6-amino-penicillanic acid-sensitive organism. Such colonies are isolated and the penicillin-G-amidase gene transferred on to pBR322 plasmids and recloned back into *E. coli*. The engineered cells, aided by the plasmid amplification at around 50 copies per cell, produce penicillin-G-amidase constitutively and in considerably higher quantities than does the fully induced parental strain. Such increased yields are economically relevant not just for the increased volumetric productivity but also because of reduced downstream processing costs, the resulting crude enzyme being that much purer.

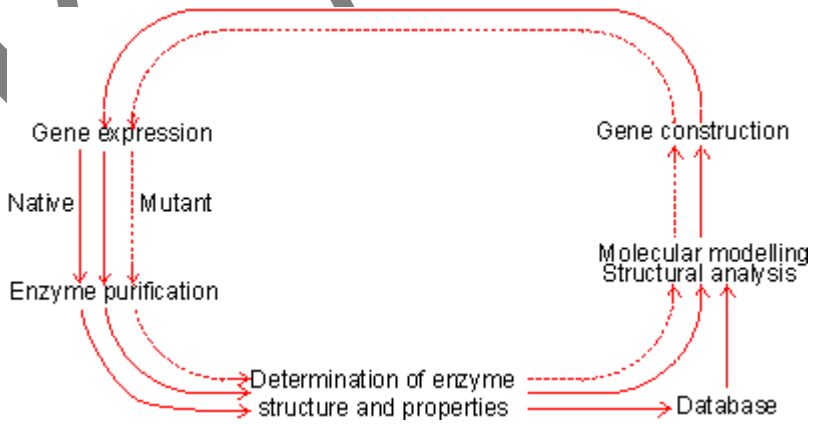


Figure 2: The protein engineering cycle. The process starts with the isolation and characterisation of the required enzyme. This information is analysed together with the database of known and putative structural effects of amino acid substitutions to produce a

possible improved structure. This factitious enzyme is constructed by site-directed mutagenesis, isolated and characterised. The results, successful or unsuccessful, are added to the database, and the process repeated until the required result is obtained.

Another extremely promising area of genetic engineering is protein engineering. New enzyme structures may be designed and produced in order to improve on existing enzymes or create new activities. An outline of the process of protein engineering. Such factitious enzymes are produced by site-directed mutagenesis. Unfortunately from a practical point of view, much of the research effort in protein engineering has gone into studies concerning the structure and activity of enzymes chosen for their theoretical importance or ease of preparation rather than industrial relevance. This emphasis is likely to change in the future.

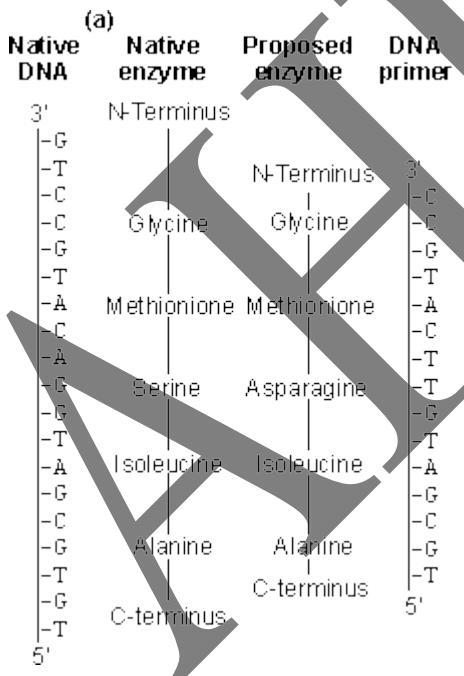
As indicated by the method used for site-directed mutagenesis, the preferred pathway for creating new enzymes is by the stepwise substitution of only one or two amino acid residues out of the total protein structure. Although a large database of sequence-structure correlations is available, and growing rapidly together with the necessary software, it is presently insufficient accurately to predict three-dimensional changes as a result of such substitutions. The main problem is assessing the long-range effects, including solvent interactions, on the new structure. As the many reported results would attest, the science is at a stage where it can explain the structural consequences of amino acid substitutions after they have been determined but cannot accurately predict them. Protein engineering, therefore, is presently rather a hit or miss process which may be used with only little realistic likelihood of immediate success. Apparently quite small sequence changes may give rise to large conformational alterations and even affect the rate-determining step in the enzymic catalysis. However it is reasonable to suppose that, given a sufficiently detailed database plus suitable software, the relative probability of success will increase over the coming years and the products of protein engineering will make a major impact on enzyme technology.

Much protein engineering has been directed at subtilisin (from *Bacillus amyloliquefaciens*), the principal enzyme in the detergent enzyme preparation, Alcalase. This has been aimed at the improvement of its activity in detergents by stabilising it at even higher temperatures, pH and oxidant strength. Most of the attempted improvements have concerned alterations to:

1. the P_1 cleft, which holds the amino acid on the carbonyl side of the targeted peptide bond;
2. the oxyanion hole (principally Asn_{155}), which stabilises the tetrahedral intermediate;
3. the neighbourhood of the catalytic histidyl residue (His_{64}), which has a general base role; and
4. the methionine residue (Met_{222}) which causes subtilisin's lability to oxidation.

It has been found that the effect of a substitution in the P_1 cleft on the relative specific activity between substrates may be fairly accurately predicted even though predictions of the absolute

effects of such changes are less successful. Many substitutions, particularly for the glycine residue at the bottom of the P_1cleft (Gly_{166}), have been found to increase the specificity of the enzyme for particular peptide links while reducing it for others. These effects are achieved mainly by corresponding changes in the K_m rather than the V_{max}. Increases in relative specificity may be useful for some applications. They should not be thought of as the usual result of engineering enzymes, however, as native subtilisin is unusual in being fairly non-specific in its actions, possessing a large hydrophobic binding site which may be made more specific relatively easily (e.g. by reducing its size). The inactivation of subtilisin in bleaching solutions coincides with the conversion of Met_{222} to its sulfoxide, the consequential increase in volume occluding the oxyanion hole. Substitution of this methionine by serine or alanine produces mutants that are relatively stable, although possessing somewhat reduced activity.



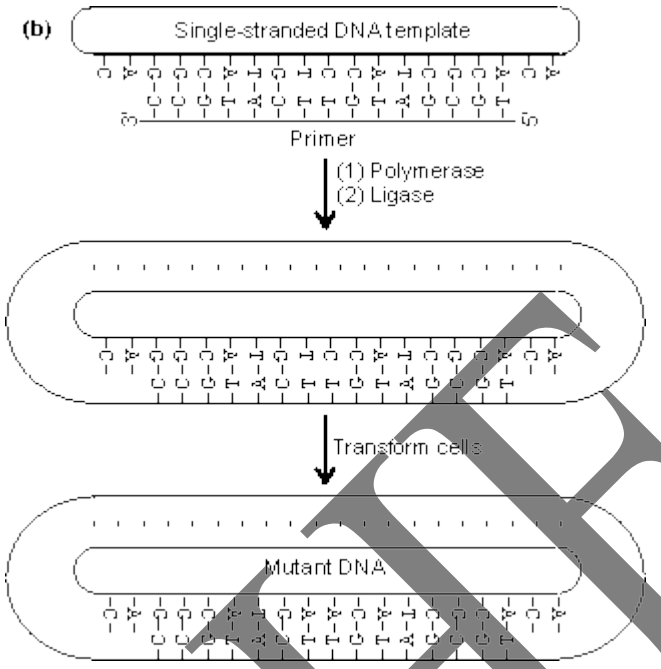


Figure 3: An outline of the process of site-directed mutagenesis, using a hypothetical example. (a) The primary structure of the enzyme is derived from the DNA sequence. A putative enzyme primary structure is proposed with an asparagine residue replacing the serine present in the native enzyme. A short piece of DNA (the primer), complementary to a section of the gene apart from the base mismatch, is synthesised. (b) The oligonucleotide primer is annealed to a single-stranded copy of the gene and is extended with enzymes and nucleotide triphosphates to give a double-stranded gene. On reproduction, the gene gives rise to both mutant and wild-type clones. The mutant DNA may be identified by hybridisation with radioactively labelled oligonucleotides of complementary structure.

An example of the unpredictable nature of protein engineering is given by trypsin, which has an active site closely related to that of subtilisin. Substitution of the negatively charged aspartic acid residue at the bottom of its P_1 cleft (Asp_{189}), which is used for binding the basic side-chains of lysine or arginine, by positively charged lysine gives the predictable result of abolishing the activity against its normal substrates but unpredictably also gives no activity against substrates where these basic residues are replaced by aspartic acid or glutamic acid.

Considerable effort has been spent on engineering more thermophilic enzymes. It has been found that thermophilic enzymes are generally only 20-30 kJ more stable than their mesophilic counterparts. This may be achieved by the addition of just a few extra hydrogen bonds, an internal salt link or extra internal hydrophobic residues, giving a slightly more hydrophobic core. All of these changes are small enough to be achieved by protein

engineering. To ensure a more predictable outcome, the secondary structure of the enzyme must be conserved and this generally restricts changes in the exterior surface of the enzyme. Suitable for exterior substitutions for increasing thermostability have been found to be aspartate ⇔glutamate, lysine ⇔ glutamine, valine ⇔ threonine, serine ⇔ asparagine, isoleucine ⇔ threonine, asparagine ⇔ aspartate and lysine ⇔ arginine. Such substitutions have a fair probability of success. Where allowable, small increases in the interior hydrophobicity for example by substituting interior glycine or serine residues by alanine may also increase the thermostability. It should be recognised that making an enzyme more thermostable reduces its overall flexibility and, hence, it is probable that the factitious enzyme produced will have reduced catalytic efficiency.

DELIVERY SYSTEM FOR PROTEIN PHARMACEUTICALS

The Protein and Peptide is a Novel Drug Delivery System and it is a Novel approach of drug delivery system. Protein and Peptides are the Most Abundant Material of Living system and Biological cell. act has Hormones, Enzymes, Structural Elements and Immunoglobulin's.It is also important take part in Several Metabolic Process, Immunogenic Defense as well as its take part in several Biological activities. Proteins are the one of the most abundant Organic molecule in Biological System, the term Protein first used has Berzelius. The term Protein is derived from a Greek word Proteios Means Holding the first Place. Proteins are the high molecular weight mixed polymer of Alpha amino acids joined together the Peptide Linkages. In Protein mainly contain Carbon, Nitrogen, Oxygen and Sulphur Molecule. Protein are the compounds having linear chain amino acids are held Together by the Covenant Linkages is called has Peptide Bonds. Peptides are the Condensation Product of Alpha Amino acids. The alpha amino group of one molecule of amino acid are condensed alpha carboxyl group of another amino acids. Protein are occurs in every part of all living cells for giving nutritional activity for providing a body building ability. It is Important Molecule for the Plant and Animal cells. In Protein is mainly act has Enzyme for catalysis of Biochemical reactions, It is applicable for the Transportation of Metabolites and Gene. It is applicable for giving a definite shape, strength to the cell and tissues. It is having a One of the Most Important Applicability to control the Metabolic Pathways, PH, Osmotic Pressure and Temperature. The Protein Insulin Regulates the Blood sugar level. It is Important for the muscle formation and Mechanical work. In case of Peptide the two amino acids are condensed to from dipeptides, three forms Tripeptides, Four to from Tetra peptide and Peptide for the 2-20 amino acids are Polypeptides. The Polymers of 100 and more than 100 Amino acid called has Proteins. The Proteins are classified into two types first is depending on the solubility of proteins and another is complexity in structure of proteins. In fist case on the basis of solubility they are classified into two types Globular Protein and Fibrous Proteins, The proteins are soluble in water or common salts known has Globular proteins and the the proteins are insoluble in Water and common solvents are called has Fibrous Proteins. In second case on the basis complexity Proteins are classified in three types Fist is simple protein it can contains only one amino acids, second is conjugated proteins it can contains amino acids and non protein parts, and third Derived Proteins it is

hydrolysis product formed by the action of the physiological agents like heat, chemical agent, and enzymatic actions on the Protein molecules. The structure of Protein is mainly classified into four types First is Primary Structure of Protein the Primary structure of protein is referred as the number, nature and sequence of amino acids along with polypeptide chain, In this structure the N terminal of amino acids always shown in left end of Polypeptide and C terminal of amino acid shown in right side, The best example of Pri. Structure is an Insulin Molecule. The Secondary structure of protein in which the Long Polypeptide chain are folded or collided in a different Geometric arrangements. The two types of arrangements of secondary structure of Protein Alpha helical Structure and Beta Pleated sheet. In tertiary structure of proteins are the three dimensional coiling and folding of the chain, stabilized by the interaction between the sequences of amino acids, this folding results the (R-) group is side chai amino acids, these interaction are mainly (H-) bonded Interactions. The final shape of the tertiary structure of protein is an elapsed, globe and any other irregular shape. In Quaternary structure of Proteins are the two or more polypeptide chain hold together by non covalent bond to give the quaternary structure of the proteins, Hemoglobin has Example of Quaternary structure of Proteins. Proteins and Peptide are applicable Endogenous functioning to maintain the Biological Environments. The discovery of Numerous Hormones and Peptides are Applicable for the Pharmaceutical and Biopharmaceuticals, It is applicable in Pathophysiology of the Human diseases, The important application in Protein and Peptide in Medical Practices, Drug discovery Processes and Research activities.

NEED OF PROTEIN AND PEPTIDE DRUG DELIVERY SYSTEM

1. The protein and peptides are very important in biological cells and Organic Molecules. 2. In the Absence of proteins and peptides causes diseases like Diabetes mellitus. (Caused due to the lack of protein called INSULIN)[39] 3. Now a days R-DNA technology and hybridoma techniques also used in protein and peptide based pharmaceuticals.

ADVANTAGES OF PROTEIN AND PEPTIDE DRUG DELIVERY SYSTEM

1. Erythropoietin is mainly used for production of RBC. 2. The protein Tissue plasminogen activator is used for Heart attack, Stroke. 3. Oxytocin is used in management of labor pain. 4. Bradykinin increases the peripheral circulation. 5. Somatostatin decrease bleeding in gastric ulcer. 6. Gonadotropin induce ovulation. 7. Insulin maintain blood sugar level.

FUNCTIONS OF PROTEIN AND PEPTIDE DRUG DELIVERY SYSTEM

1. Transport and storage of small molecules and biological molecules. 2. Coordinated motion via muscle contraction. 3. The

Mechanical support from fibrous protein. 4.
Generation and transmission of nerve impulses. 5.
Enzymatic catalysis in biochemical reactions. 6. The
Immune protection through antibodies. 7. The
Control of growth and differentiation via hormones.

ROUTES OF ABSORPTION

The Proteins and Peptide drug delivery system in which Most of the Pharmaceutical Proteins and Peptides Formulations are the Formulated as a Solution, suspension, Emulsions and they are delivered in Invasive or Parenteral Route such as Intra muscular route (IM), Intravenous route (IV) and Subcutaneous route (SC) Injections. But, These all routes are arises its own Difficulties such as, Poor Patient Compliance, The pain and discomfort associated in this route (to inject injection in same site again and again it can arises Pain) and it is a Inconvenience to treat the Paediatric Patients. The oral route of administration in protein and peptide is suitable as compared to parenteral route, The Oral route having a One of the most convenient route of drug administration, in this type of route no pain and discomfort was arises and Maintained the Higher Patient Compliances or Acceptance. But, The Development oral Protein and Peptide Drug delivery arises several Problems for their Oral Administration of Drugs. This Problem is arises There Unfavourable and Undesirable Physicochemical Properties are such as The Large molecular size of the drug molecules, drug undergoes susceptibility to Biological and Enzymatic degradations, The oral drug having a short Plasma Half Life as compared to other drugs, it can having high Immunogenicity, The tendency of Protein undergoes Aggregations, Adsorption and it can undergoes Denaturation's, The Major Problem Orally Administered Proteins and Peptides are having a Lesser Bioavailability or Less Bioavailability is having a less than 1%. The other route of administration of protein and peptide is arises success for the administration of Proteins and Peptide drugs, the routes are Oral, Buccal administration, Intranasal administration, Pulmonary administrations, Transdermal, Rectal and Ocular administrations of Proteins and Peptide.

PROPERTIES OF PROTEINS AND PEPTIDES

The Protein are the most abundant biological and organic molecule they are soluble in water and it can formed a Colloidal solution with water. Protein and Peptides are the physicochemically and Metabolically Stable System. In case oral administration of Protein and Peptide Drug delivery system Several Properties can affect the rate of absorption of Protein and Peptide in oral drug delivery system, the properties are such as, Absorption Properties, In case of Absorption Properties Molecular weight and size of the particle, Conformational studies and Steriospecification of Three Dimensional Arrangements in Space, Immunogenicity of drug molecules. Are affected the rate of Absorption of Protein and

Peptide in Oral drug delivery systems. Another one is Physicochemical Properties such as, solubility and Lipophilicity of drug is major Criteria of absorption of drug, The aggregations and Hydrogen bonding of drug in oral administrations, The Physicochemical Properties are the major Criteria for the drug absorption in oral drug delivery systems, The drug absorption oral drug delivery system it an mainly arises two main Problems are the Metabolic degradation of Various forms of Protein and Peptides by interaction with the various Proteolytic Enzymes, and it is having Less Membrane Penetration Abilities. This all Criteria associated in Properties of Protein and Peptide drug delivery system is Applicable for determination of various Problem associated in oral drug delivery system and it is important to give idea on the basis Properties to prevent the problems in drug administration in oral Protein and Peptide in Oral drug delivery Systems.

PHARMACEUTICAL APPROACHES

The protein and Peptides are having Four Approaches they has Follows

1. CHEMICAL MODIFICATION
2. ENZYME INHIBITORS
3. PENETRATION ENHANCERS
4. FORMULATION VEHICLE
5. MUCOADHESIVE POLYMERIC SYSTEM

1. CHEMICAL MODIFICATION (PRODRUG APPROACH)

The Chemical Modification of Protein and Peptide Drug Delivery System of Drugs is Important to Improve the Enzymatic Stability as well as Membrane Permeations. It is Applicable for the reducing the Immunogenicity.

The Chemical Modification is Includes in Two Types of Modifications as Follows:

1. Amino acid Modification
2. Hydrophobization

1. Amino acid Modifications: The Modification of amino acid is one of the important approach in which the Substitution of the D- amino acid and L- amino acid is important to alter the Physiological Properties of Protein and Peptide Drug Delivery Systems. **Example:** Desmopressin and Deaminovasopressin are the two important analogs of vasopressin, former involves deamination of first amino acid and replacement of last Larginine D-arginine to give Deaminovasopressin. **Application:** The Amino acid modification is important to enhance the Membrane Permeability and Maintain the Enzymatic Stability.

2. Hydrophobization: It is having an important approach for the Lipophilic Moieties. **Example:** NOBEX INSULIN by the Palmitoylatios. **Description of Example:** Conjugation of the Insulin Molecule to the 1, 3-dipalmitoylglycerol containing a free amino acid groups of glycine, Phenylalanine and Lysine molecule to from mono and insulin is important to facillated the transfer the insulin across the mucosal membrane of the large intestines. It is important to improve the Stability against the enzymatic degradations.

ENZYME INHIBITORS (PROTEASE)

The enzyme (protease) inhibitors are the enzymatic approach of the Protein and Peptide drug delivery systems. GIT and Liver is play important role in Metabolization of the Protein and Peptides into smaller fragments of the two to ten amino acids with the help of the variety of Proteolytic Enzymes. This Protease inhibitors are CO- administered with Protein and Peptide to alter the Environment for the Enzyme stability to supress the Proteolytic activity. The enzyme proteases inhibiters are divided into four types they are Aspartic Proteases (Pepsin, Rennin), Cystinyl Proteases (Papain, Endopeptidase), Serinyl Proteases (Thrombin, Trypsin), and Metallo Proteases (Carboxypeptidase).

PENETRATION ENHANCERS

Penetration enhancers are the one of the most important Component of Protein and Peptides formulation is responsible for the Disruption of the Mucosal Barriers and applicable to improve the Membrane Permeations of Large Macromolecular substances lie Proteins and Peptides. The Several classes of compounds are mainly used has a permeation enhancers are such as Surfactant (Polysorbate, SLS, Pluronic F-68), Chelating agent (EDTA), Fatty acids (Sodium Carprate), Mucoadhesive Polymeric systems (Thiomers, Cellulose derivatives), Phospholipids (PC). The basic Mechanism of Penetration enhancers are the, detergent and surfactant molecules are the increases the transcellular transport of the drug material is responsible to disrupting the structure of the lipid bilayer of lipid membrane are having more permeability. Another mechanism is the calcium chelates are the responsible for the Exert the action of complex formation of the calcium ions and they are passing through the tight junctions and they are facillated the Paracellular transport of the hydrophilic drugs materials. Fatty acids are the important for the improving the paracellular absorption by phospholipases C activations and upregulation of intracellular Calcium ions, is leading to the contraction of actine myosin filaments.

FORMULATION VEHICLES

The Protein and Peptide Drug Delivery system is important for the Oral Delivery of Protein and Peptides can be successfully achieved by using various carrier systems are like

1. Dry Emulsion
2. Microspheres
3. Liposomes
4. Nanoparticles

1. Dry Emulsion: It is important application in drug delivery system s to prevent the instabilities of the long term storage of multiple emulsions. The novel approach at which multiple emulsion is replaced by dry emulsions. Dry Emulsion is prepared by the Spray drying, Lyophollization and evaporation Techniques. In dry emulsion preparation application of the PH responsive polymers like HPMCP, is important for the emulsions are the enteric coated and site specific achieved.
2. Microspheres: The uniform distribution of drug in oral drug delivery in Protein peptides drug are known as Microspheres. The PH responsive microspheres are the mainly used in oral delivery for the protection of the stomach from proteolytic degradations and Protection upper portion of small intestine from proteolytic degradations.
3. Liposomes: Liposomes are the small microscopic vesicles in which aqueous volume is entirely enclosed by the membrane composed lipid molecules. Liposomes in drug delivery system, the encapsulation of the insulin with sugar chain portion of mucin and PEG completely suppressed the degradation of the insulin molecules in intestinal fluid. The uncoated from of liposomes are suppressed it on partially surface coating of the liposomes molecules in PEG or mucin gained resistances against dagestion by salts and increased the stability of GI tract.
4. Nanoparticles: Nanoparticles are Nano sized colloidal structure having size is 10-1000nm. The particles in nanometric sized range of the particles are absorbed intact by the intestinal epithelium and they are the less prone towards the enzymatic degradations. The particle size surface charges are the influencing the uptake of nanoparticle system in GI tract.

MUCOADHESIVE POLYMERIC SYSTEMS

The mucoadhesive polymeric system is important to prevent the problem associated in Presystemic Metabolism or first pass metabolism and maintain its therapeutic efficacy. The residence time of this drug delivery systems at the site of action and the increasing or decreasing the drug clearance rate.
Examples: Thiomers, polyacrylic acid derivatives and cellulose derivatives. The stronger mucoadhesive properties of thiomers are believed to be based on covalent bonds between thiol groups of the thiomer and cystein- rich domains of mucus glycoproteins. (Higher amount of thiol groups is responsible for the stronger mucoadhesive properties).

INCORPORATION INTO DRUG DELIVERY MATRIX

The drug incorporate in the Protein and Peptide drug delivery system undergoes Three methods they as follows
1. EMULSIFICATION
2. EXTRUSION AND SPRAY DRYING
3. POLYMERIZATION

1. EMULSIFICATION: In this Process water soluble drugs is first dissolved in the aqueous (water solution) and it is soluble in Organic solvent. The two solutions are mixed with the appropriate Proportion to produce w/o emulsion. This prepared Primary emulsion is emulsified into aqueous solution containing emulsifier to produced w/o/w emulsion. Finally the organic solvent is mainly removed from emulsion by evaporation of solvent under reduced pressure by the filtration and increasing the Temperature.
2. EXTRUSION AND SPRAY DRYING :The extrusion and Spraying is employed to from microspheres and the core material or matrix containing drug, incorporated as Solution and the Particulate is mainly ejected from the orifice of fine tubes, syringe or nozzles to from micro droplets. The size of droplet is mainly depends upon the Properties of Liquid (melt, solution and suspension) and Orifice diameter to jet velocity.
3. POLYMERIZATION :Polymeriasation in hydrogels having a polymeric drug delivery system preparation by the mixing of monomer with the drug an initiator and a cross linking agents. The Intravascular delivery of the protein via hydro system that is photo polymerized in situ on the inner surface of blood vessel. The gamma radiation are producing deleterious effect on integrity of protein molecules one of the drawback of Protein and Peptide drug delivery systems.

STABILITY ASPECTS

In stability of protein and peptide is determined by the Protein degradations Pathways. In This drug delivery system under two Pathways of degradation of Protein and Peptide Molecules They has Follows,
1. Physical Degradation Pathways (Instability Aspects)
2. Chemical Degradation Pathways (Instability Aspects) The chemical degradation Pathways the Native or original structure of protein is changes by the modification of their Primary Structure of Protein Molecules. The Physical Degradation Pathways the Native or original structure of Protein is Changes or Modified to from Higher order Structure of Proteins (secondary, tertiary or quaternary structure).
1. Physical Instabilities: In case of Physical Degradation the Primary Sign of Physical instability of the Protein Molecules. In case of globular Protein, the hydrophobic residue are buried in the interior and the hydrophilic residues. It is having interaction with the aq. Solvents. The denaturation of Protein Molecule refers to the loss or damage of the globular structure of protein molecule leads to protein unfolding. The physical denaturation is may be caused the changes in the environment of protein molecules such as temperature, pH, introduction of hydrophobic surfaces or by introduction of interfaces by the addition of organic solvents. **2. Chemical Instabilities:** The chemical instability of the protein and Peptide can causes following four types of reactions.

1. Oxidation
2. Deamination
3. Peptide bond hydrolysis
4. Disulphide exchange

1. Oxidation: Oxidation is one of the most important chemical instability of Protein and peptide molecules. The Amino acid side chain of the protein and peptide are susceptible to oxidation, the oxidation is arises by the atmospheric oxygen molecule, various types of the metal ions like copper or iron, several reagents such as Hydrogen Peroxides. **Example:** The Methionine residues under acidic conditions are especially prone to oxidation by reagents like hydrogen peroxide, producing methionine sulfoxide. (Hydrogen peroxide is used to sterilize formulation vessels or the formulation area).

2. Deamination: In this type of Instability is arises in hydrolysis of amide side chain of certain amino acid residue are mainly includes Glutamine and Aspargine, is known has Deamination. Some conditions are like changes in Temperature and PH are mainly shown to Facillated the Process of Deamination's of Biological Therapeutic Protein and Peptides.

3. Peptide bond Hydrolysis: In this Peptide bond Hydrolysis Process the aspartic acid residues are heated at 90-1000 C, in PH 4 (acetate), the hydrolysis of the Asp-X bonds are leads to loss of the Biological activity.

4. Disulphide exchange: The Therapeutics Protein contain cystein residues that from disulfide bonds. These formed bonds are important components of the structural integrity of the Proteins. The incorrect linkage of peptide bonds lead to changes in the three dimensional structure of Protein Molecules and their biological activity.

APPLICATION

1. CVS acing drugs Protein and Peptides: (Angiotensin 2 antagonist, Bradykinin, Captopril) is important for the Lowering blood pressure and improving peripheral circulation for Heart failure management. **2. CNS active Protein and Peptides** (Cholecystokinin, B-endorphin) is important for the Suppressing appetite and Relieving pain. **3. GI-active Protein and Peptides** (Gastrin antagonist, pancreatic enzymes) is important for the Reducing secretion of gastric acid and it is important for Digestive supplement. **4. Immunomodulation of the Protein and Peptides** (Bursin, Cyclosporin, and Interferon) is important for Selective B-cell differentiatingharmone Inhibits functions of T-lymphocyte Enhancing activity of killer cells. **5. Metabolism modulating Protein and Peptides** (Insulin, Vasopressin) is important for treating diabetes mellitus and treating diabetes insipidus.

RECENT ADVANCES

PEGylation: PEGylation is a Recent Advancement of Protein and Peptide Drug Delivery systems, PEGylation is a process of attaching the strands of the polymer PEG to most typical peptides fragments that can help to meet the protein and challenges of improving the safety and efficiency of many therapeutic macromolecules such as Protein and Peptides. It is widely used for the modification of proteins and peptides, antibody fragments and oligonucleotides. PEG are the Non-toxic. And non –immunogenic, it is having a specified Hydrophilicity and it

is having high Flexibility. PEGylation is important to increases the Bioavailability, it is applicable for the optimized Pharmacokinetics, it is important for Decreasing Immunogenicity, It is important to Decreases the Frequency of administration. The PEGylation is important Mechanism for increasing the molecular weight of the molecules, it can increases the drug solubility and it is applicable for the protection against Proteolytic degradations, it is having an important mechanism to reducing the dosing frequency and maintain therapeutic activity.

Depo-Foam TECHNOLOGY : Therapeutic Proteins and Peptides are administered in IV or SC are often too rapid fro of the Circulation and it is need to inject to the frequent order of administration for maintaining their therapeutic level of the blood. Various types of liposomal formulations have been utilized as drug delivery vehicles for sustained release of proteins and peptides like unilamellar or Multilamellar vesicle systems but few deals with the mulltivesicular liposomes are called as ―DepoFoam particles.‖ The DepoFoam technology is capable of accommodating high drug loading and high recovery of drug material, it is having a high Encapsulation efficiency, it is important type of technique is applicable for the sustained delivery of macromolecular drugs. A unique feature of DepoFoam system is that inside each DepoFoam particle, discontinuous internal aqueous chambers ,bounded by a continuous network of lipid membranes render a higher aqueous volume to lipid ratio and much larger particle diameter as compared to SUV's or MLV's.

Protein and peptide based pharmaceuticals are rapidly becoming a very important class of therapeutic agents and are likely to replace many existing organic based pharmaceuticals in the very near future. Peptide and protein drugs will be produced on a large scale by biotechnology processes and will become commercially available for therapeutic use. This poses an urgent challenge to the pharmaceutical industry to develop viable delivery systems for the efficient delivery of these complex therapeutic in biologically active form. Their need in the clinical & therapeutic regions has intensified the investigation for their convenient & effective delivery through noninvasive system.

STRUCTURE FUNCTION RELATIONSHIP IN ENZYMES

Structural Studies of Enzymes

An enzyme's function is intrinsically linked to its three dimensional structure, determining how it performs substrate binding, catalysis and regulation. X-ray crystallography has been the most important technique in the development of our understanding of enzyme structure and hence enzyme function. Nuclear magnetic resonance (NMR) has also been used successfully to study many structures, but crystallography remains the principle technique for structure elucidation. The first enzyme to be crystallised and have its structure successfully solved was chicken egg lysozyme in 1965. Importantly, as well as the structure of the free enzyme, it was possible to crystallise lysozyme with a substrate analog bound in the active site. This structure, allowed the proposal of a chemical mechanism for the enzyme, based on positioning of groups around the site of substrate cleavage. The use of crystal structures with bound substrate and transition state analogs has helped to reveal the catalytic mechanisms of

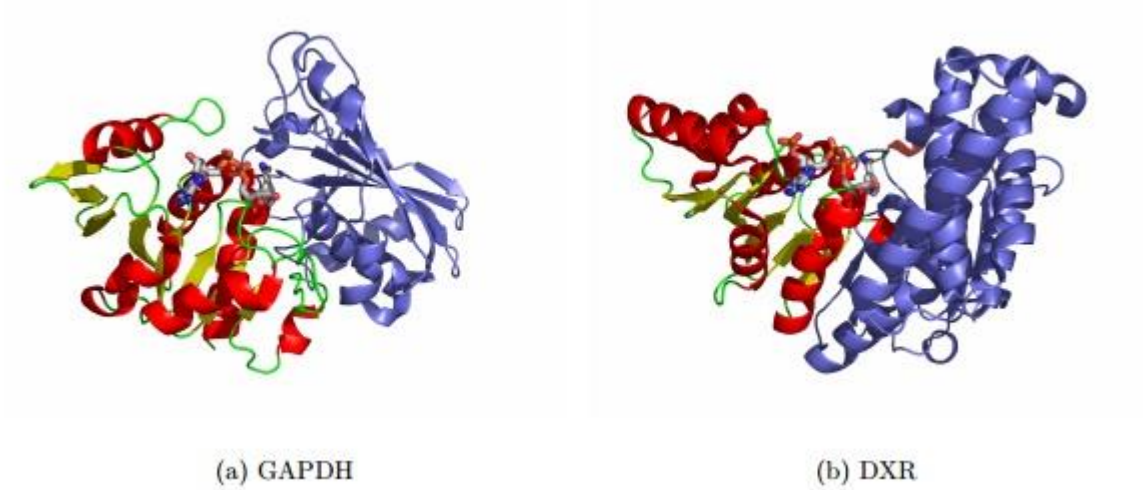
countless enzymes since. The structure of lysozyme was solved to a resolution of 2˚A; at this resolution it is possible to accurately place the residue side chains and the plane of each peptide bond. However, individual atoms are not generally well resolved. 'Atomic' resolution (1.2˚A resolution or higher) allows the placement of atoms with fewer geometrical restraints and so gives a better picture of the 'true' protein structure. In recent years, advances in X-ray sources and cryocrystallography have led to increasing numbers of structures solved at these high resolutions. Any structure, no matter what the resolution, contains a certain amount of error. Quite substantial errors, that have only been identified at a later date, have been found in some published protein structures. It has also been found that different areas of a crystal structure can have quite different amounts of error. Part of the reason for this lies in the physical nature of the crystal, some parts, particularly the loop regions, may be naturally flexible and so do not lie in a single conformation. This leads to these regions diffracting poorly and so the atoms within them are placed less accurately. Disorder in the crystal is measured by the temperature factor associated with each atom that specifies the positional variability of that atom. It has also been found that some important parts of protein structures, such as ligands bound within an enzyme, are prone to error, because the geometrical restraints used in refining these regions are of a lower quality compared to those used to refine the protein itself[18]. Given the errors that all crystal structures are subject to, methods for validating protein structures are of great importance. There are two types of validation: validating the model given the experimental data collected, and validating the model against 'typical' structures that have already been solved. A full discussion of these techniques is beyond the scope of this introduction, but both types of validation are reviewed elsewhere. The Protein Data Bank (PDB) was set up in 1971 to provide a central repository of solved protein structures. At this time, structure determination by protein crystallography was an extremely time consuming process, and only a few structures were solved each year. However, since 1990 the rate of structure determination has steadily increased. Over 5000 new structures were deposited in 2004 and, as of June 2005, the PDB contained over 30,000 entries, though many of these structures are different forms of the same protein.

Domains

Larger proteins tend to fold into a series of smaller domains, each of which forms a self contained structural unit. These domains are often described as the units of evolution because they can often be swapped between proteins without disturbing the folding of other parts of the protein and thus novel functions can be created by novel combinations of domains within a single protein.

In enzymes, certain functions are often contained within a domain. For instance, the nucleotide binding Rossmann domain[21] is found combined with a diverse range of separate catalytic domains, allowing each enzyme to bind similar nucleotide cofactors such as nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH) and flavin mono-nucleotide (FMN), but perform quite different chemistry. Figure 1.9 shows two different Rossmann domain containing enzymes: glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[22] (EC: 1.2.1.12) and 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR)[23] (EC: 1.1.1.267). Both enzymes contain the Rossmann domain

with a common 3 parallel strand β sheet flanked by α helices. This sheet binds to the cofactor NAD in the case of GAPDH and NADP in the case of DXR. The remainder of the enzymes structure are completely unrelated, and contain quite different catalytic residues which allow them to catalyse their different reactions.



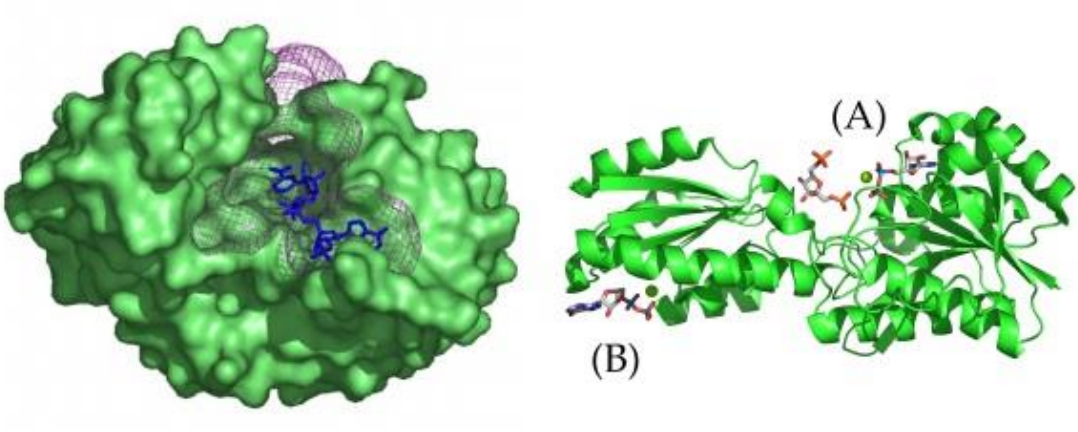
(a) GAPDH (b) DXR

Figure 4: The common Rossmann domains of GAPDH and DXR are shown in red helices and yellow sheets. The NAD(P) cofactors are shown in sticks. The remainder of each protein, which is unrelated, is coloured blue.

Active Sites and Clefts

Although enzymes are often large molecules comprising many hundreds of amino acids, the functional regions of an enzyme are generally restricted to clefts on the surface that comprise only a small part of the enzyme's overall volume. The most important of these regions is the active site - the pocket or cleft in which the enzyme binds the substrate and in which the catalytic chemistry of the enzyme is performed. Analysis of enzyme structures have shown that active sites tend to be formed from the largest cleft on the surface of the protein.Figure 5(a) shows the surface of tetrahydrofolate dehydrogenase with an NADP molecule bound in the active site cleft.

Other clefts in the enzyme can be responsible for binding regulatory molecules. Phosphofructokinase, shown in Figure 5(b), catalyses the phosphorylation of Dfructose 6-phosphate, converting ATP into ADP in the process. It is regulated by binding of ATP to an allosteric site, quite distinct from the active site, that inhibits the enzyme.These regulatory clefts as well as being able to bind regulatory molecules, also require the ability to transmit binding information from themselves to the active site, so that catalytic activity can be regulated.



(a) Tetrahydrofolate dehydrogenase with the largest cleft marked and NADP

(b) Phosphofructokinase with the active site (A) and allosteric site (B) marked. Substrate and inhibitor molecules are also shown.

Figure 5: The active site and other functional sites are usually formed from clefts in the enzyme surface.

Determining the Function of Enzymes

To fully appreciate an enzyme's role in biology, as well as the structure, it is also necessary to understand its chemistry, kinetics and thermodynamics. The chemistry of an enzyme is largely defined by those protein residues involved in the catalytic mechanism, and can be studied by a number of techniques. Chemical labelling can identify catalytic residues by forming covalent attachments to those residues directly involved in the mechanism of the enzyme. The catalytic machinery of the serine proteases was elucidated by the use of inhibitors such as diisopropylfluorophosphate (DFP) and tosyl-L-phenylalanine chloromethyl ketone (TPCK), which form covalent links to serine and histidine residues respectively, that are involved in the mechanism.pH rate profiles and NMR experiments can also suggest the involvement of different chemical groups in the active site, by tracking protonation changes and subsequent changes in the enzymes activity.

Another important experimental technique for determining the catalytic residues of an enzyme, is site directed mutagenesis. When searching for catalytic residues the alanine scanning approach can be used, whereby each residue is replaced by alanine and the effects on the reaction is measured.It has been noted however, that in many cases, the removal of an 'essential' catalytic residue does not completely abolish catalysis, but rather causes the enzyme to use an alternative (and usually slower) mechanism.More in-depth understanding can come from considered mutations of certain residues. In studies of the protein tyrosine phosphatases (PTPs) for instance, the replacement of an active site arginine with a lysine was found to reduce kcat 8200-fold, whilst leaving KM unchanged. This implies that the change has disrupted catalysis, without disturbing the substrate binding ability of the

enzyme. Since lysine, like arginine, provides a positive charge to the active site, but does so using an amino group rather than a guanidinium group, it was deduced that the guanidinium group of the arginine must make specific contacts to the transition state which aided catalysis.

Even without mutagenesis or labelling experiments, measuring the kinetic parameters KM and kcat can reveal aspects of the enzyme mechanism, including those residues which are important for catalysis, binding or both. Measuring these parameters requires methods for determining the rate of formation of products or depletion of substrates; such as spectrophotometry, spectrofluorimetry and radioactivity assays. These techniques can then be used to determine the number and sequence of intermediate processes in an enzyme mechanism. An example of this is the detection of a burst of product release observed in the catalysis of the hydrolysis of p-nitrophenyl acetate by chymotrypsin. This burst is due to the quick formation of an intermediate (during which time p-nitrophenol is released), which then breaks down relatively slowly. During the burst, the enzyme molecules in the reaction are saturated with the intermediate and subsequently turnover much more slowly.

Structural and Functional Enzyme Classification

The wealth of structural information that emerged in the 1990's has led to the need for schemes for the classification of protein structures. Although these schemes are designed to be used for all protein structures, they have proved extremely useful for classifying enzymes, and tracing the evolution of different enzyme functions. Both manual and automatic methods for structural classification have been developed. Automatic schemes, such as FSSP and SSM have the advantage of easily staying up to date and having a clearly defined methodology. However, those systems with at least some manual curation still add value by identifying groups of structures, and distinctions between groups, that are missed by the automatic schemes.

The CATH[37] and SCOP[38] classifications are both hierarchical systems that require some manual intervention. The highest level (first digit) of the CATH classification describes the class of the protein according to the secondary structure content: mainly alpha, mainly beta, mixed alpha/beta and no secondary structure. The next level, the architecture, describes the shape of the domain as determined by the orientation of the secondary structures, but not the connectivity. The next level: topology, describes the connectivity of the secondary structures. The SSAP structural comparison program[39] is used to find structural similarities at this level. The final homologous superfamily level groups proteins known to have a common ancestor, using both structural and sequence comparisons. Some example structures from the first three levels of CATH.
SCOP uses a similar classification scheme with a top level class division according to secondary structure content, a fold level which describes structural similarities without implying homology, corresponding to the architecture and topology levels of CATH, and a superfamily level which groups related proteins together.

In addition to the structural classifications which, at the lower levels, reflect evolutionary relationships, there is also a need for a classification that describes enzyme function, since enzymes with the same common ancestor can evolve to catalyse quite

different reactions. The Enzyme Commission (EC) classification and the ENZYME database describe an enzyme reaction using a four level classification. The first level describes the general class of the reaction: oxidoreductase, transferase, hydrolase, lyase, isomerase or ligase. The second and third levels then describe different properties of the reaction depending on the top level class. The fourth level generally describes the substrate specificity of the enzyme, so that each unique EC number describes a single enzyme catalysed reaction. The difficulty in using the EC classification for studying enzymes, is that it does not specify the direction of the reaction catalysed or the mechanism that the enzyme uses to catalyse the reaction. This means that just as two related enzymes in the same CATH superfamily can catalyse quite different reactions, it is also possible for two enzymes to share the same EC code without being related or even using a similar reaction mechanism. Some of the beta-lactamases (EC: 3.5.2.6) for instance, that are responsible for the hydrolysis of beta-lactamase antibiotics such as penicillin, use a zinc dependent mechanism, whilst others use a serine hydrolase type mechanism.

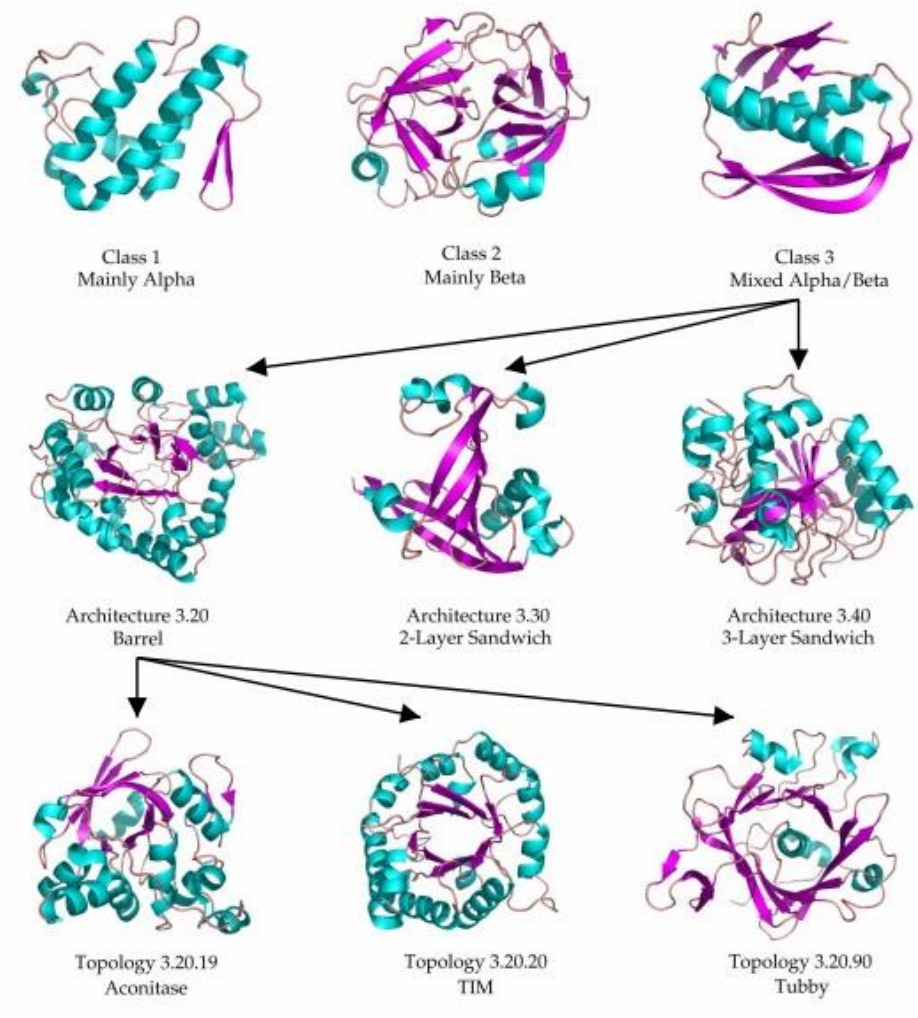


Figure 6: The top level shows examples of the three main classes of structures in CATH (class 4 has few secondary structures). The middle level shows three different architectures within the mixed alpha-beta class. The bottom level shows three different topologies within the barrel architecture. Each topology is then broken down into homologous superfamilies that represent the final structure based level of CATH.

STRUCTURAL MOTIFS

In a chain-like biological molecule, such as a protein or nucleic acid, a **structural motif** is a supersecondary structure, which also appears in a variety of other molecules. Motifs do not allow us to predict the biological functions: they are found in proteins and enzymes with dissimilar functions.

Because the relationship between primary structure and tertiary structure is not straightforward, two biopolymers may share the same motif yet lack appreciable primary structure similarity. In other words, a structural motif does not have to be associated with a sequence motif. Also, the existence of a sequence motif does not necessarily imply a distinctive structure. In most DNA motifs, for example, it is assumed that the DNA of that sequence does not deviate from the normal "double helical" structure.

In proteins

In proteins, a structural motif describes the connectivity between secondary structural elements. An individual motif usually consists of only a few elements, e.g., the 'helix-turn-helix' motif which has just three. Note that, while the *spatial sequence* of elements may be identical in all instances of a motif, they may be encoded in any order within the underlying gene. In addition to secondary structural elements, protein structural motifs often include loops of variable length and unspecified structure.

- Beta hairpin:

Extremely common. Two antiparallel beta strands connected by a tight turn of a few amino acids between them.

- Greek key:

Two beta strands folded over into a sandwich shape.

- Omega loop:

A loop in which the residues that make up the beginning and end of the loop are very close together.

- Helix-loop-helix:

Consists of alpha helices bound by a looping stretch of amino acids. This motif is seen in transcription factors.

- Zinc finger:

Two beta strands with an alpha helix end folded over to bind a zinc ion. Important in DNA binding proteins.

- Helix-turn-helix:

Two α helices joined by a short strand of amino acids and found in many proteins that regulate gene expression.

- Nest:

Extremely common. Three consecutive amino acid residues form an anion-binding concavity.

- Niche:

Extremely common. Three or four consecutive amino acid residues form a cation-binding feature.

ENZYME EVOLUTION

Enzymes, like any biological system, are under evolutionary pressures which cause them to evolve over time. There are a number of different ways in which enzyme function can evolve:

1. Evolution of a new catalytic function: The evolution of a completely novel catalytic function is perhaps the most dramatic change in enzyme function that is possible, though paradoxically it may not require a large number of individual mutations. This is because of the nature of the active site, where only a few residues are directly involved in catalysis.
2. Evolution of substrate specificity: While keeping the same basic catalytic activity an enzyme may adapt to act upon different substrates. Serine proteases are an example of this, where the catalytic triad is used to hydrolyse many different peptides. In this case, the catalytic residues remain the same whilst the binding residues mutate.
3. Evolution of stability: Extremophile species require enzymes that are stable at extremes of temperature or pH. This can be achieved by mutations in areas other than the active site, so that the scaffold on which the active site is built is maintained.
4. Evolution of rate: Some enzyme have evolved to perform catalysis as fast as is physically possible.However, not all enzymes need to perform catalysis at the highest possible rate and it may be that in many cases the evolutionary pressure on the enzyme, to increase its rate, abates before the enzyme reaches the fastest rates possible.
5. Evolution of regulatory features: Enzyme activity must be regulated, so that the metabolism of an organism can adapt to changing surroundings. There are a host of regulatory mechanisms such as post-translational modification and allosteric control that modulate the rate at which an enzyme performs catalysis.
6. Evolutionary drift: Even without evolutionary pressure, random mutations will cause a slow divergence between two enzymes. Evolution may maintain the structure of the crucial functional regions in these cases, whilst the remainder of the protein changes.

There are a number of different mechanisms by which these functions can evolve, varying from simple point mutations, to gene duplication and fusion events which may combine whole domains in novel configurations. These evolutionary events usually lead to divergent evolution whereby enzymes with a common ancestor gradually differentiate in their functions. Some families of enzymes are particularly ‘functionally promiscuous’ with members catalysing a wide range of different reactions.

In a study of 31 structural superfamilies, Todd et al found they performed almost 200 different functions. The types of changes which these families undergo were also found to vary. The non-heme di-iron carboxylate proteins, for instance, tended to undergo large scale changes: domain enlargements, domain re-organisations and oligomerisation changes; whilst, the α/β barrel proteins, of which triose phosphate isomerase (TIM) is the canonical example, varied only through the catalytic residues in the active site, while the scaffold of protein structure around the active site remained constant. Example of this with the TIM barrel enzymes triose phosphate isomerase (5.3.1.1) and xylose isomerase (EC: 5.3.1.5).

The opposite of divergent evolution, which leads to enzymes with evolutionary relationships performing different functions, is convergent evolution, whereby unrelated enzymes converge on the same solution to a catalytic 'problem'. The best known example of this being the Ser-His-Asp catalytic triad, which appears to have separately evolved on a number of occasions.

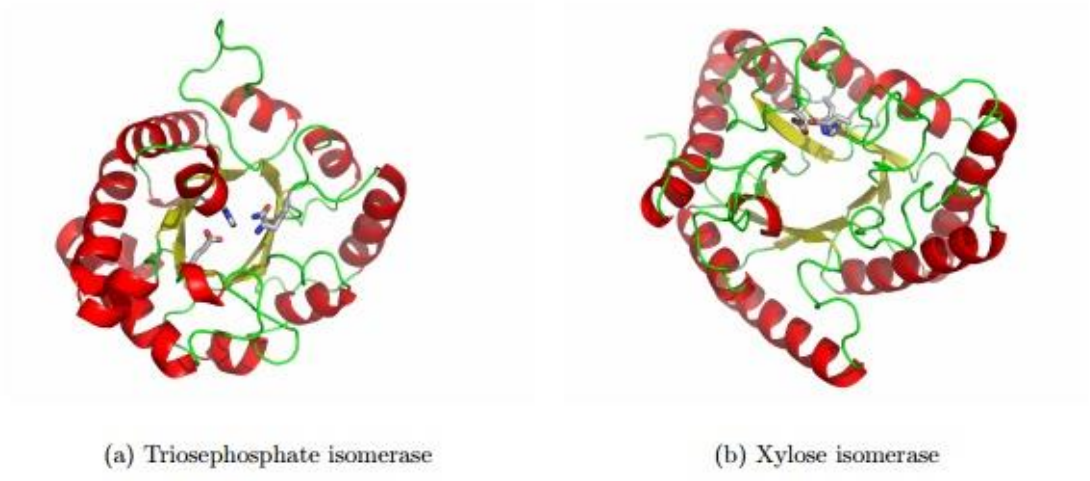


Figure 7: The TIM barrels of triosephosphate isomerase and xylose isomerase. The catalytic residues shown in sticks are different in each case but similarly placed at one end of the barrel.

METHODS FOR ANALYSIS OF SECONDARY AND TERTIARY STRUCTURES OF ENZYMES

Increasingly, drug developers are looking to large molecules and particularly proteins as a therapeutic option. Formulation of a protein drug product can be quite a challenge, but without a good understanding of the nature of protein structure and the conformational characteristics of the specific protein being formulated, the results can be

ruinous. This technical brief aims to give the reader a quick overview of protein structure. It will also cover briefly how protein structure can be affected during formulation and some of the analytical methods which can be used both to determine the structure and analyze the stability of the protein.

The term *structure* when used in relation to proteins, takes on a much more complex meaning than it does for small molecules. Proteins are macromolecules and have four different levels of structure – primary, secondary, tertiary and quaternary.

Primary Structure

There are 20 different standard L-α-amino acids used by cells for protein construction. Amino acids, as their name indicates, contain both a basic amino group and an acidic carboxyl group. This difunctionality allows the individual amino acids to join together in long chains by forming *peptide bonds:* amide bonds between the -NH2 of one amino acid and the -COOH of another. Sequences with fewer than 50 amino acids are generally referred to as *peptides*, while the terms *protein* or *polypeptide*are used for longer sequences. A protein can be made up of one or more polypeptide molecules. The end of the peptide or protein sequence with a free carboxyl group is called the *carboxy-terminus* or *C-terminus*. The terms *amino-terminus* or *N-terminus* describe the end of the sequence with a free α-amino group.

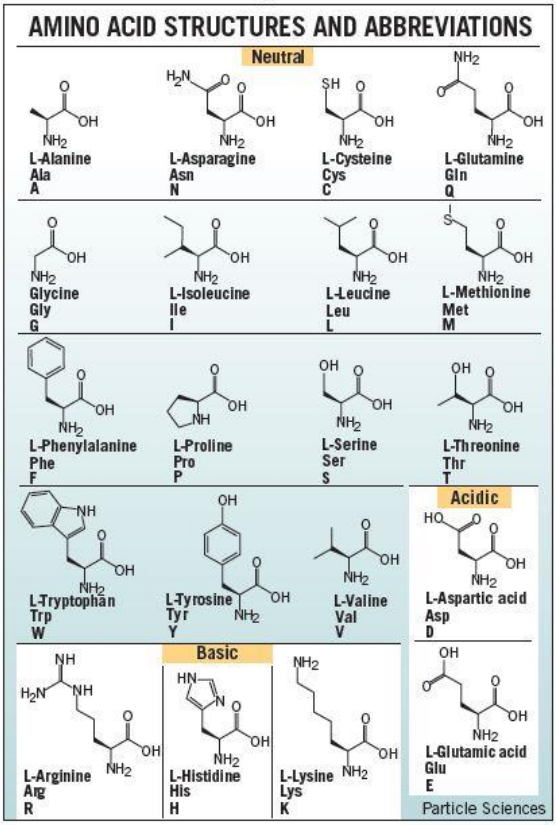
The amino acids differ in structure by the substituent on their side chains. These side chains confer different chemical, physical and structural properties to the final peptide or protein. The structures of the 20 amino acids commonly found in proteins. Each amino acid has both a one-letter and three-letter abbreviation. These abbreviations are commonly used to simplify the written sequence of a peptide or protein.

Depending on the side-chain substituent, an amino acid can be classified as being acidic, basic or neutral. Although 20 amino acids are required for synthesis of various proteins found in humans, we can synthesize only 10. The remaining 10 are called essential amino acids and must be obtained in the diet.

The amino acid sequence of a protein is encoded in DNA. Proteins are synthesized by a series of steps called transcription (the use of a DNA strand to make a

complimentary messenger RNA strand - mRNA) and translation (the mRNA sequence is used as a template to guide the synthesis of the chain of amino acids which make up the protein). Often, post-translational modifications, such as glycosylation or phosphorylation, occur which are necessary for the biological function of the protein. While the amino acid sequence makes up the ***primary structure*** of the protein, the chemical/biological properties of the protein are very much dependent on the three-dimensional or tertiary structure.

Figure 1



Secondary Structure

Stretches or strands of proteins or peptides have distinct characteristic local structural conformations or ***secondary structure***, dependent on hydrogen bonding. The two main types of secondary structure are the α-helix and the ß-sheet.

The *α-helix* is a right-handed coiled strand. The side-chain substituents of the amino acid groups in an α-helix extend to the outside. Hydrogen bonds form between the oxygen of the C=O of each peptide bond in the strand and the hydrogen of the N-H group of the peptide bond four amino acids below it in the helix. The hydrogen bonds make this

structure especially stable. The side-chain substituents of the amino acids fit in beside the N-H groups.

The hydrogen bonding in a *ß-sheet* is between strands (inter-strand) rather than within strands (intra-strand). The sheet conformation consists of pairs of strands lying side-by-side. The carbonyl oxygens in one strand hydrogen bond with the amino hydrogens of the adjacent strand. The two strands can be either parallel or anti-parallel depending on whether the strand directions (N-terminus to C-terminus) are the same or opposite. The anti-parallel ß-sheet is more stable due to the more well-aligned hydrogen bonds.

Tertiary Structure

The overall three-dimensional shape of an entire protein molecule is the ***tertiary structure***. The protein molecule will bend and twist in such a way as to achieve maximum stability or lowest energy state. Although the three-dimensional shape of a protein may seem irregular and random, it is fashioned by many stabilizing forces due to bonding interactions between the side-chain groups of the amino acids.

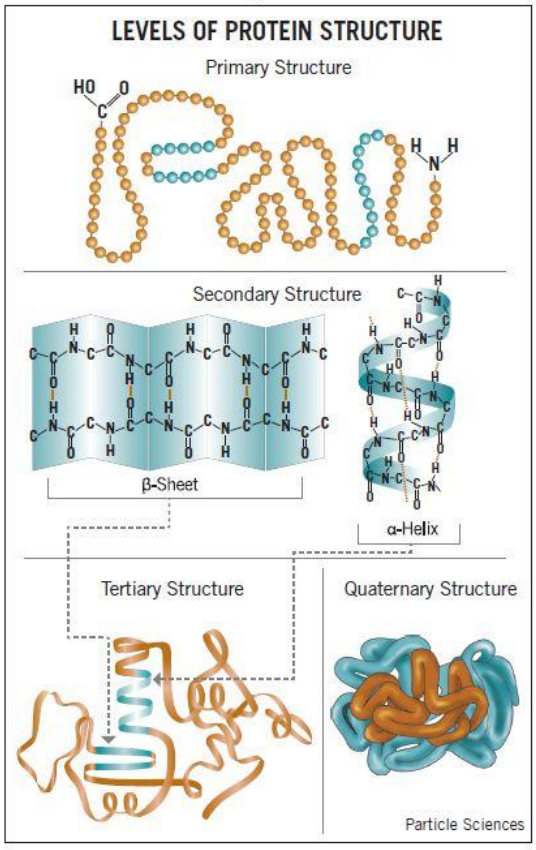
Under physiologic conditions, the hydrophobic side-chains of neutral, non-polar amino acids such as phenylalanine or isoleucine tend to be buried on the interior of the protein molecule thereby shielding them from the aqueous medium. The alkyl groups of alanine, valine, leucine and isoleucine often form hydrophobic interactions between one-another, while aromatic groups such as those of phenylalanine and tryosine often stack together. Acidic or basic amino acid side-chains will generally be exposed on the surface of the protein as they are hydrophilic.

The formation of disulfide bridges by oxidation of the sulfhydryl groups on cysteine is an important aspect of the stabilization of protein tertiary structure, allowing different parts of the protein chain to be held together covalently. Additionally, hydrogen bonds may form between different side-chain groups. As with *disulfide bridges*, these hydrogen bonds can bring together two parts of a chain that are some distance away in terms of sequence. *Salt bridges*, ionic interactions between positively and negatively charged sites on amino acid side chains, also help to stabilize the tertiary structure of a protein.

Quaternary Structure

Many proteins are made up of multiple polypeptide chains, often referred to as *protein subunits*. These subunits may be the same (as in a homodimer) or different (as in a heterodimer). The ***quaternary structure*** refers to how these protein subunits interact with each other and arrange themselves to form a larger aggregate protein complex. The final shape of the protein complex is once again stabilized by various interactions, including hydrogen-bonding, disulfide-bridges and salt bridges.

Figure 2



Protein Stability

Due to the nature of the weak interactions controlling the three-dimensional structure, proteins are very sensitive molecules. The term native state is used to describe the protein in its most stable natural conformation in situ. This native state can be disrupted by a number of external stress factors including temperature, pH, removal of water, presence of hydrophobic surfaces, presence of metal ions and high shear. The loss of secondary, tertiary

or quaternary structure due to exposure to a stress factor is called denaturation. Denaturation results in unfolding of the protein into a random or misfolded shape.

A denatured protein can have quite a different activity profile than the protein in its native form, usually losing biological function. In addition to becoming denatured, proteins can also form aggregates under certain stress conditions. Aggregates are often produced during the manufacturing process and are typically undesirable, largely due to the possibility of them causing adverse immune responses when administered.

In addition to these physical forms of protein degradation, it is also important to be aware of the possible pathways of protein chemical degradation. These include oxidation, deamidation, peptide-bond hydrolysis, disulfide-bond reshuffling and cross-linking. The methods used in the processing and the formulation of proteins, including any lyophilization step, must be carefully examined to prevent degradation and to increase the stability of the protein biopharmaceutical both in storage and during drug delivery.

Protein Structure Analysis

The complexities of protein structure make the elucidation of a complete protein structure extremely difficult even with the most advanced analytical equipment. An amino acid analyzer can be used to determine which amino acids are present and the molar ratios of each. The sequence of the protein can then be analyzed by means of peptide mapping and the use of Edman degradation or mass spectroscopy. This process is routine for peptides and small proteins, but becomes more complex for large multimeric proteins.

Peptide mapping generally entails treatment of the protein with different protease enzymes in order to chop up the sequence into smaller peptides at specific cleavage sites. Two commonly used enzymes are trypsin and chymotrypsin. Mass spectroscopy has become an invaluable tool for the analysis of enzyme digested proteins, by means of peptide fingerprinting methods and database searching. Edman degradation involves the cleavage, separation and identification of one amino acid at a time from a short peptide, starting from the N-terminus.

One method used to characterize the secondary structure of a protein is circular dichroism spectroscopy (CD). The different types of secondary structure, α-helix, ß-sheet and random coil, all have characteristic circular dichroism spectra in the far-uv region of the spectrum (190-250 nm). These spectra can be used to approximate the fraction of the entire protein made up of each type of structure.

A more complete, high-resolution analysis of the three-dimensional structure of a protein is carried out using X-ray crystallography or nuclear magnetic resonance (NMR) analysis. To determine the three-dimensional structure of a protein by X-ray diffraction, a large, well-ordered single crystal is required. X-ray diffraction allows measurement of the short distances between atoms and yields a three-dimensional electron density map, which can be used to build a model of the protein structure.

The use of NMR to determine the three-dimensional structure of a protein has some advantages over X-ray diffraction in that it can be carried out in solution and thus the protein is free of the constraints of the crystal lattice. The two-dimensional NMR techniques generally used are NOESY, which measures the distances between atoms through space, and COESY, which measures distances through bonds.

Protein Structure Stability Analysis

Many different techniques can be used to determine the stability of a protein. For the analysis of unfolding of a protein, spectroscopic methods such as fluorescence, UV, infrared and CD can be used. Thermodynamic methods such as differential scanning calorimetry (DSC) can be useful in determining the effect of temperature on protein stability. Comparative peptide-mapping (usually using LC/MS) is an extremely valuable tool in determining chemical changes in a protein such as oxidation or deamidation. HPLC is also an invaluable means of analyzing the purity of a protein. Other analytical methods such as SDS-PAGE, iso-electric focusing and capillary electrophoresis can also be used to determine protein stability, and a suitable bioassay should be used to determine the potency of a protein biopharmaceutical. The state of aggregation can be determined by following “particle” size and arrayed instruments are now available to follow this over time under various conditions.

The variety of methods for determining protein stability again emphasizes the complexity of the nature of protein structure and the importance of maintaining that structure for a successful biopharmaceutical product.

PROTEIN FOLDING IN VITRO AND IN VIVO

Protein folding in vitro

Early studies of protein folding involved small proteins which could be denatured and refolded in a reversible fashion. A two state model, D <===> N, was assumed. The denaturants were heat, urea, or guanidine HCl. Since the denatured states are less compact than the native state, the viscosity of the solution can be used as a measure of denaturation/renaturation. Likewise, the amino acid side chains in the differing states would be in different environments. The aromatic amino acid Trp, Phe, and Tyr absorb UV light. After excitation, the electrons decay to the ground state through several processes. Some vibrational relaxation occurs, bringing the electrons to lower vibrational energy levels. Some of the electrons can then fall to various vibrational levels at lower principle energy states through a radiative process. The photons emitted are lower in energy and hence longer in wavelength. The emitted light is termed fluorescence. The wavelength of maximum fluorescent intensity and the lifetime of the fluorescence decay is very sensitive to the environment of the amino acids. Hence fluorescence can also be used to measure changes in protein conformation. Other spectral techniques like CD spectroscopy as well as simple absorbance measurements, are used. For small, single domain proteins (such as RNase) undergoing reversible denatuation, graphs showing the extent of denaturation using each technique above, are superimposable, giving strong validity to the two state model.

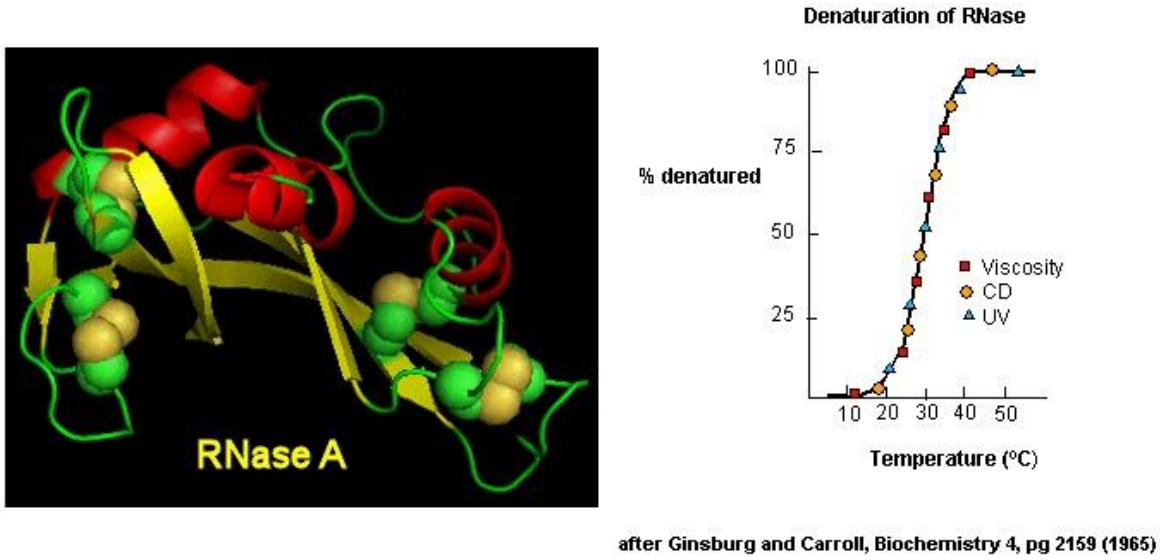


Figure 8: Reversible denaturation

Proteins that fold without easily discernable, long lived intermediates and following a simple two state model, D <=> N are said to undergo cooperative folding. This simple model needed to be expanded as more proteins were studied. Some intermediates in the process were detected.

- Some proteins show two steps, one slow, one quick, in refolding studies, suggesting an intermediate. The longer a protein is kept in the denatured state, the more likely it is to display an intermediate. One accepted explanation for this phenomena is that during an extended time in the D state, some X-Pro bonds might isomerize from trans to the cis state, to form an intermediate. Alternatively, as in the case of RNase, which has a cis X-Pro bond in the native state, denaturation causes an isomerization to the trans state. In the case of RNase, to refold, the accumulating intermediate I must reisomerize in a slow step to the cis state, followed by a quick return to the N state.
- Some proteins which contain multiple disulfide bonds that must reform correctly after reductive denaturation can refold into intermediates with the wrong S-S partner. Such intermediates can be trapped by stopping further S-S formation during refolding with the addition of iodoacetamide.

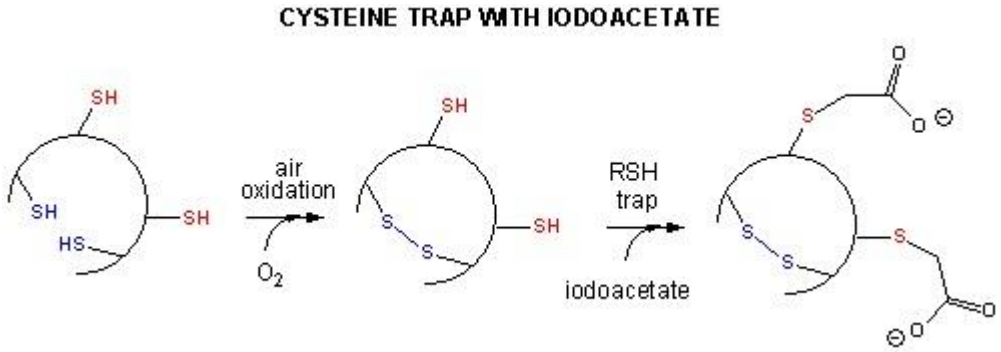


Figure 9: addition of iodoacetamide

As an example consider the following data on bovine pancreatic trypsin inhibitor.

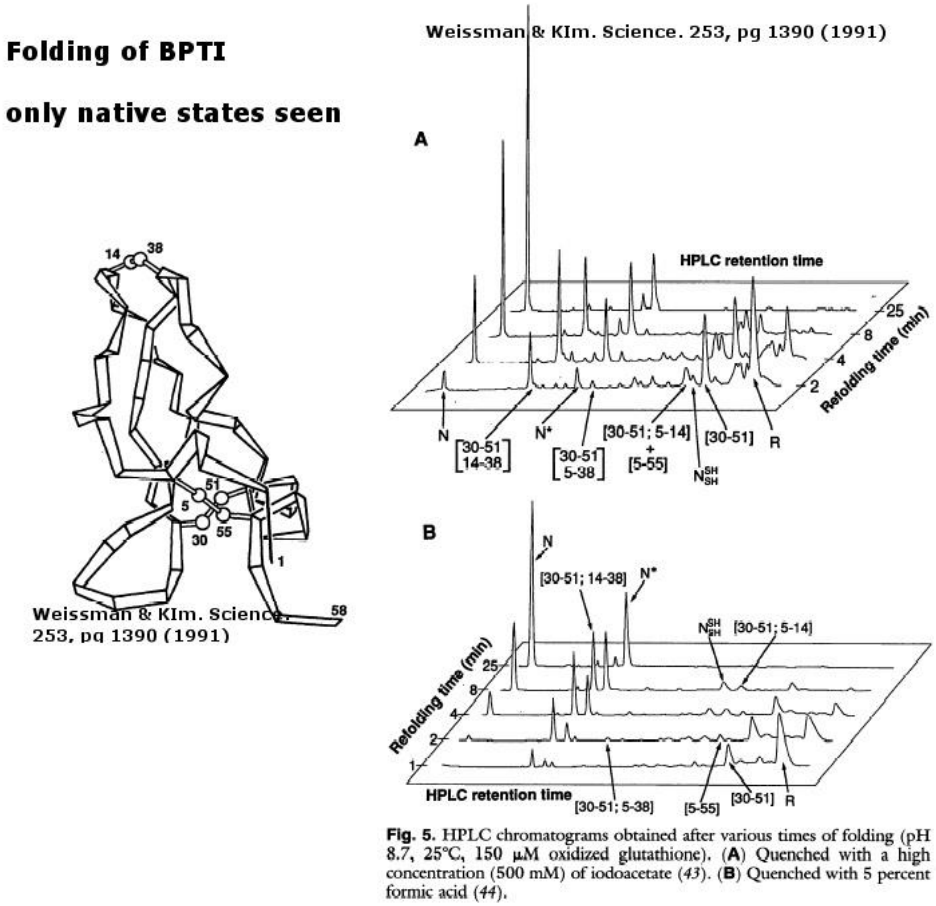


Fig. 5. HPLC chromatograms obtained after various times of folding (pH 8.7, 25°C, 150 μM oxidized glutathione). (**A**) Quenched with a high concentration (500 mM) of iodoacetate (*43*). (**B**) Quenched with 5 percent formic acid (*44*).

Figure 10: Bovine pancreatic trypsin inhbitor (BPTI): Folding Kinetics - only native disulfide structures seem to form.

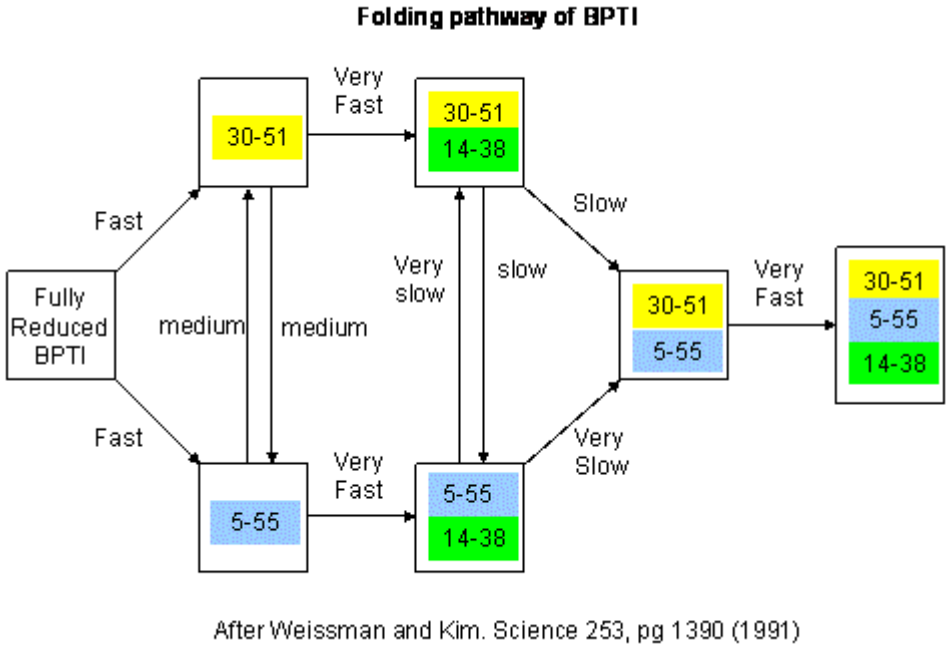


Figure 11: BPTI Folding Pathway In Vitro - gives possible scheme of folding intermediates

- Some proteins form partially folded but stable intermediates when folded under partially denaturing conditions. A good example is lactalbumin, which under mildly acidic conditions (pH 4), low levels of guanidine HCl, or neutral pH and low ionic strength in the absence of calcium (which normally binds to the protein), forms a stable, isolatable intermediate (I) called the molten globule (MG). The image below shows the folded state with two calcium ions bound.

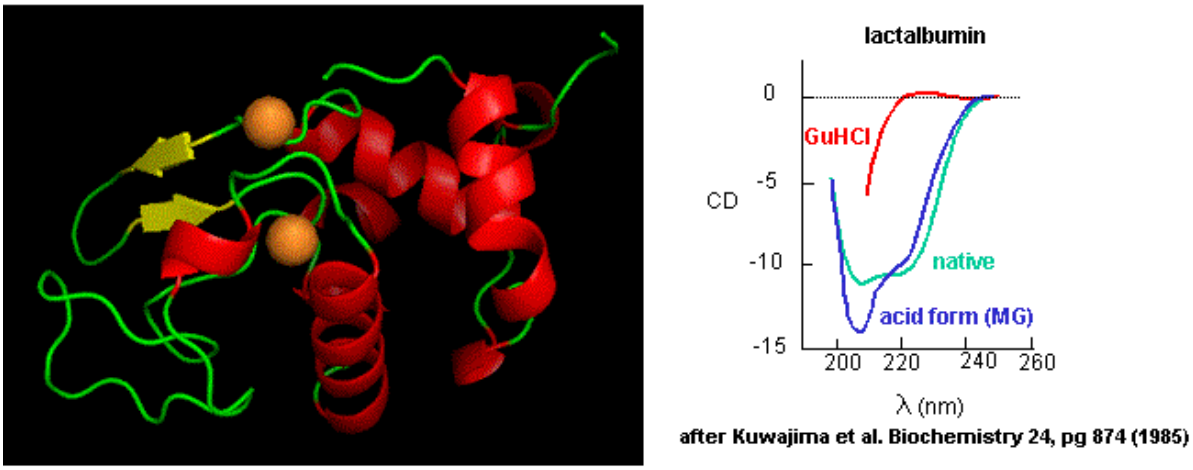


Figure 12: lactalbumin (image made with Pymol)

Data show that the MG is about 50% larger in volume than the N state. This compares to the denatured state, which can be 300% larger than the native state. Hence, it is more like the native state as studied by hydrodynamic techniques, but with more solvent accessibility of hydrophobic side chains. The MG has a similar CD spectra as the native state, but the aromatic side chains display the same UV absorption and fluorescent characteristics as the protein in 6 M guanidine HCl, suggesting that the final tertiary state has not yet completely formed. The secondary structure in the MG may not be the same as in the native state

NMR can also be used to detect folding intermediates. Using this technique, proteins are unfolded in D2O, which will cause the exchange of all Cs with ionizable protons, including, the amide Hs. An amine is a weak base (pKb around 3.5) so its conjugate acid, the protonated amine, has a pKa of around 9.5. An amide or peptide bond would be a weaker base than an amine since it's lone pair is less available (due to delocalization through resonance) for sharing with a proton. The pKa for the conjugate acid of the amide (in which the amide N is protonated and has a plus charge) is much lower, around -0.5, than the pKa for the conjugate acid of an amine. At 2 pH units greater than its pKa, the charged amide N is close to 100% deprotonated The pka of the protonated group is important since the rate of H exchange is related to the pKa, holding other variables constant. The pka of an unprotonated amine (RNH2 -> RNH- is very high (30s) and hence deprotonation of the RNH2 amine to form RNH- is not likely under normal conditions.

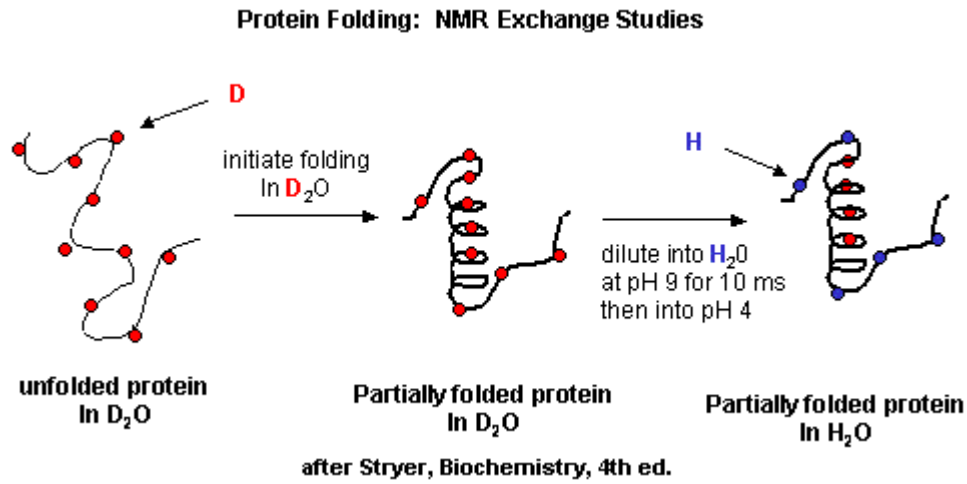


Figure 13: Exchange of all Cs with ionizable protons, including the amide Hs

Refolding is initiated by diluting the protein into a solution without the denaturatant, but still in D2O. As the protein folds and becomes more compact, the buried atoms are now sequestered from the solvent, and no longer readily exchange Ds. Then the protein is placed in H2O at pH 9.0 for 10 ms, after which the pH is changed to pH 4.0. D --> H exchange is promoted at high pH, and quenched for the amide Ds and Hs at low pH. Amide H's that continue to exchange must be accessible to water. Those that aren't are usually buried in secondary structure.

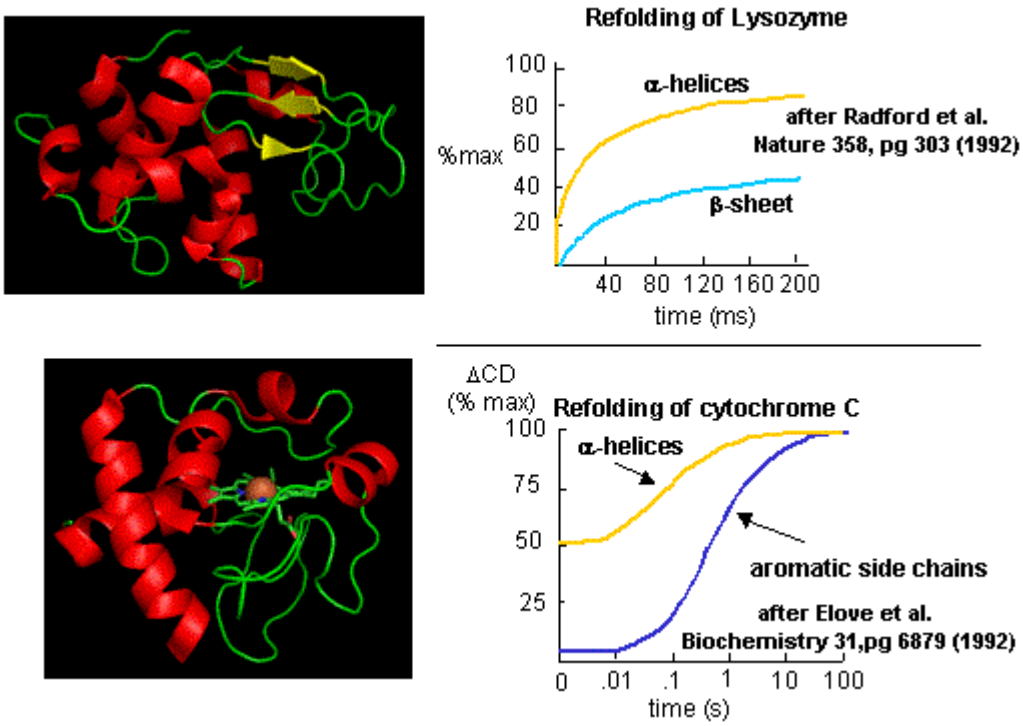


Figure 14: Experimental data on model proteins.

When the same techniques are applied to large, multidomain or oligomeric proteins, only a few percent refold in vitro. Incorrect intermolecular interactions and heterogeneous aggregation seems to be the main problems which prevent correct protein folding in vitro.

Protein folding in vivo

There are many differences between how a protein might fold or unfold in a cell compared to a test tube.

- The total concentration of all the proteins and nucleic acids in cells are estimated to be about 350 g/L, or 350 mg/ml. Most measurement in the lab are conducted in the range of 0.1 to 10 mg/ml
- Proteins are synthesized in cells from an N to C terminal direction. Hence the nascent protein, as it emerges from its site of synthesis (the ribosome), might fold into intermediate structures since not all of the protein sequence is yet available to direct folding.
- Proteins are synthesized in the cytoplasm, but they have to find their final place in the cell. Some end up in membranes, some must translocate across one or even two

different membranes to end up in specific organelles like the Golgi, mitochondria, chloroplasts (in plant cells), nuclei, lysosomes, peroxisomes, etc.

Additional evidence suggests that protein folding/translocation requires assistance (i.e. catalysis) in the cell.

- Mutant cells defective in certain proteins can lead to the accumulation in the cells of misfolded and aggregated proteins.
- eukaryotic genes (taken from higher cells which contain nuclei and internal organelles), when transferred into prokaryotes (bacteria, like E. Coli), can be expressed to form protein, but they often misfold and aggregate in the bacterial cells and form structures called inclusion bodies.

Hence recombinant proteins expressed in vivo have the same problems in folding as larger proteins in vitro. In both cases, conditions favor accumulation of nonnative proteins with exposed hydrophobic groups leading to aggregation. Aggregation also occurs in vivo when a protein is over-expressed or expressed at a higher temperature than normal. Why? Mutant cells have been selected that actually suppress inclusion bodies in vivo. This effect was mediated by a class of proteins which are expressed by the bacteria and other cells when their temperature is raised. The function of these proteins, called heat shock proteins (Hsp), was unknown until it was realized that they facilitate correct protein folding, in part, by binding to denatured proteins in the cells before they aggregate into inclusion bodies. Further studies discovered a large number of proteins that seem to facilitate protein folding and prevent aggregation in vivo. These proteins are now called molecularchaperones. They are classified on the basis of their molecular weight) and can be divided into at least two families, the Hsp-70 chaperone family and the chaperonin and Hsp 90 families as illustrated and summarized in the figure and text below.

Hsp-70 Family: This family includes DnaK/DnaJ and GrpE proteins in prokaryotes and immunolglobulin heavy chain binding protein (BiP) and alpha crystalline in eukaryotes. Alpha crystalline comprises 30% of the lens proteins in the eye, where it functions, in part, to prevent nonspecific, irreversible aggregates. These proteins have molecular weights of about 70K and :

- bind to growing polypeptide chains as they are synthesized on ribosomes.
- express activity as monomers.
- have ATPase activity - i.e. they cleave the phosphoanhydride ATP (which can drive reactions).
- bind short, extended peptides, which stimulates the ATPase activity
- release bound peptides after ATP cleavage

A figure showing

In prokaryotes, a protein called trigger factor (TF) binds in a co-translational process to proteins as they begin to emerge from the ribosome and catalyzes correct folding of about 70% of bacterial protein. The rest requires additional chaperones, including DnaJ and DnaK which bind proteins during synthesis in a cotranslational process. Upon interaction with the DnaJ-bound protein, DnaK hydrolyzes bound ATP, resulting in the formation of a stable complex between DnaJ and DnaK. GrpE, a nucleotide exchange factor for DnaK, facilitates the releases ADP from DnaK. Rebinding of ATP to DnaK then triggers the release of the substrate protein. This cycle repeats itself until the protein is fully folded. For about 20% of proteins in E. Coli, the DnaK/DnaJ/GrpE cycle leads to complete post-translational folding of proteins . Eukaryotes utilize an analogous set of proteins Hsp70 complex proteins including If folding is still incomplete after several rounds, the fully synthesized yet incompletely folded protein interacts with an amazing catalyst of protein folding, the chaperonin system.

Chaperonins- including chaperonin 60 (or GroEL in E. Coli) and chaperonin 10 (or GroES in E. Coli) in chloroplasts, mitochondria and bacteria, and TCP-1 in eukaryotic cytoplasm.

These proteins:

- bind to proteins after they have left the ribosome or have been transported into organelles like mitochondria.
- express activity as multimers. GroEL consist of two stacks of rings of monomers, with 7 monomers in each ring (each monomer around 60K MW), forming a hollow cylinder. GroES consist of one single ring of 7 monomers (each 10K MW). The GroES complex forms a lid over one open end of the GroEL cylinder. Proteins can fold within the cavity in GroEL (lined with hydrophobic patches) without "fear" of aggregation. GroEL also binds and cleaves ATP, leading to conformational changes inside the barrel and hiding of the hydrophobic patches in Gro EL, which leads to the releases of the unfolded peptide. The process proceeds until the folding protein passes through the barrel and is released in its correct folded state.
- bind nonnative proteins at the GroEL opening of a complex of GroEL and GroES, which has a large hydrophobic cavity.

GroEL has also been shown to bind in its hydrophobic cavity a fluorescent CdS semiconductor nanoparticle which can be released on addition and cleavage of ATP. There are two classes of chaperonins:

- Class I: Those found in bacteria, chloroplasts and mitochondria. The have structures analogous to GroEL (two rings of 7 identical monomers) and Gro ES.
- Class II: Those found in archebacteria and in the cytoplasm of eukaryotic cells. These contain two rings of 8-9 subunits which may not be identical.

Other chaperons have proven to be of clinical significance. Hsp 90 is a chaperone that is expressed both in normal and tumor cells. It appears to have special importance in tumor cells in helping key proteins involved in malignancy (signal transduction proteins such as HER-2/ErbB2, Akt, Raf-1, Bcr-Abl, and p53) to maintain their shapes under conditions of drug exposure and the inherent genetic instability present in the cells. Drugs that bind to and inhibit Hsp90 appear to have much greater effect on tumor cells, making this protein a new chemotherapeutic target to treat cancer. Recent studies by Kamal et al. have shown the drug 17-AAG binds Hsp90 about 100 times as strongly in tumor cells than in normal cells. Hsp 90 appears to be complexed to other "co-chaperones" in the tumor cells which lead to higher drug binding affinity. The chaperone complex may actually induce the drug to adopt a different conformation. A comparison of chaperone catalyzed folding in prokaryotes and eukaroytes is shown below.

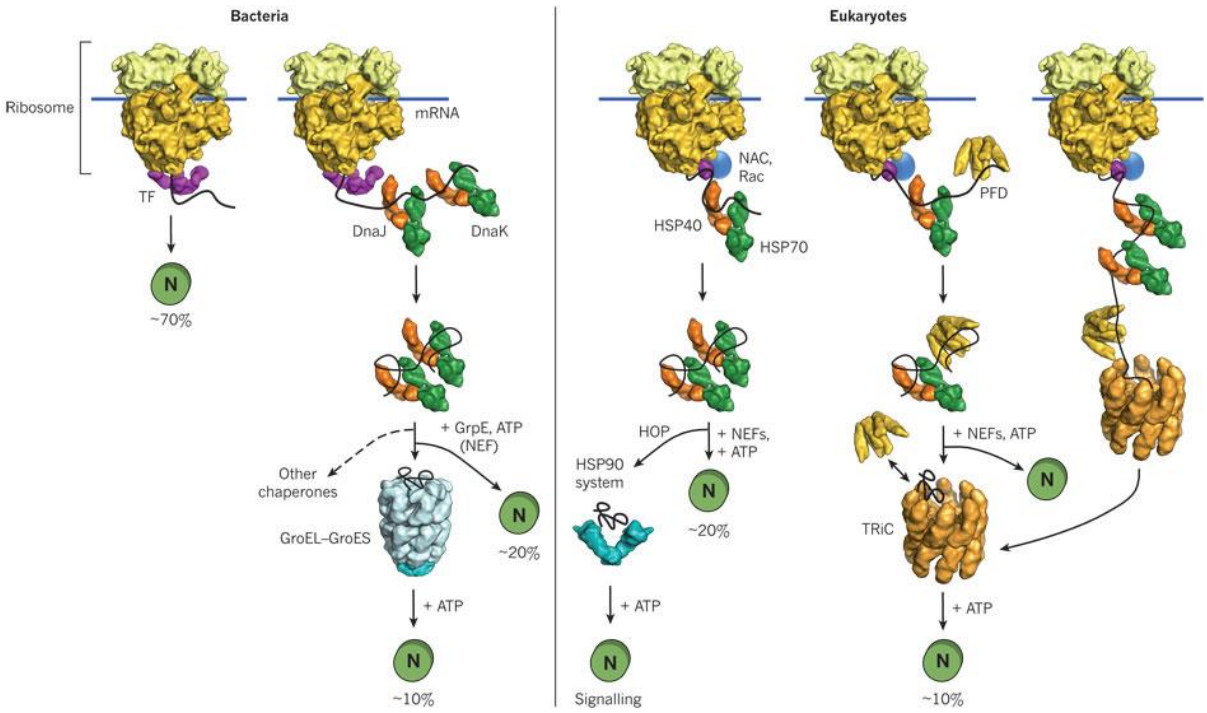


Figure 15: Comparison of cytosolic prokaryotic and eukaryotic chaperone pathways

Additional Proteins Which Catalyze Protein Folding: Chaperons function to minimize protein aggregation, which increases the efficiency of the entire process. Other proteins in the cell actually catalyze specific steps. Here are two examples:

- Protein Disulfide Isomerase (PDI) - catalyzes the conversion of incorrect to correct disulfides. The active site consists of 2 sets of the the following sequence - Cys-Gly-His-Cys, in which the pKa of the Cys are much lower (7.3) than normal (8.5). How would this facilitate disulfide isomerization?

- Peptidyl Prolyl-Isomerase (PPI) - catalyses X-Pro isomerization, by a mechanism which probably involves bending the X-Pro peptide bond. How would this facilitate the process?

Many proteins have been found to possess PPI activity. One class is the immunophilins. These are small proteins found in the cytoplasm that bind anti-rejections drugs used to prevent tissue rejection after transplanation. The immunophilin FK506 binding protein (FKBP) binds FK506 while the protein cyclophilin binds that anti-rejection drug cyclosporin. The complex of cyclophilin:cyclosporin or FKBP:FK506 binds to an inhibits calcineurin, an important protein (with phosphatase activity) in immune cells (T cells) required for T cell function. In this case, immunophilin:drug binding to calcineurin inhibits the activity of the T cell, preventing immune attack on the transplanted tissue, preventing rejection. The immunosuppressant drugs (FK506 and cyclosporin) inhibit the PPI activity of their respective immunnophilin. The extent to which the PPI activity of cyclophiin is required for its activity is unclear, but it seems to be important for some of its biological effects.

As the site responsible for folding of membrane proteins and proteins destined for secretion, as well as the major site for lipid synthesis, the endoplasmic reticulum (ER) must be able to maintain homeostatic conditions to ensure proper protein formation. Plasma cells that synthesize antibodies for secretion as part of the immune activation, show large increases in protein chaperones and ER membrane size

The main pathway controlling ER biology is the unfolded protein response (UPR) signaling pathway. If demand for protein synthesis in the ER exceeds capacity, unfolded proteins accumulate. This ER stress conditions activates a protein called IRE1, a transmembrane Ser/Thr protein kinases (which phosphorylates proteins). IRE1 activates a transcription factor that controls transcription of many genes associated with protein folding in the ER. Another protein, ERAD (ER-associated degradation) which moves unfolded proteins back into the cytoplasm where they are degraded by the proteasome. Proteins involved in lipid synthesis are also activated as lipids are needed for membranes as the ER increases in size. If the stress can not be mitigated the signaling pathway leads to programmed cell death (apoptosis).

Schuck at al investigated the specific role and importance of UPR in the homeostasis of ER as modeled by the yeast Saccharomyces cervisiae. The UPR signaling pathway was analyzed using light and electron microscopy to visualize and quantify ER growth under various stress conditions. Western blotting procedures were performed to determine chaperone protein concentrations after stress induction and association with ER expansion after the ER was exposed to various treatment conditions. The authors found ER membrane expansion occurred through lipid synthesis since stress induction increased concentrations of proteins responsible for promoting lipid synthesis and expansion failed when the proteins were absent and lipid concentration was low. In addition, these lipid

synthesis proteins were activated by the UPR signaling pathway. By separating ER size control and UPR signaling, they found that expansion occurred regardless of chaperone protein concentrations. However, if lipid synthesis genes were not available, raising the ER chaperone level helped alleviate stress levels in ER.

UNIT – III Improvement of enzymes:

Strategies for the discovery of improved and novel enzymes for industrial applications (homology and structure based approaches, screening methods, use of mutants). Optimization of industrial enzymes by mutagenesis; Protein engineering strategies to improve enzyme stability, specificity and activity; Enzyme immobilization - types, advantages, drawbacks and applications; Artificial enzymes; Isolation and purification of industrially important enzymes.

Strategies for the discovery of improved and novel enzymes for industrial application

Due to ongoing research by biotechnologists, enzymes now have a large number of commercial applications. They carry many advantages, with one important one being that enzymes are specific to only one catalytic reaction and so they therefore do not produce a range of unwanted by-products.

Commercial applications of enzymes:

Enzymes are widely used in the textile industry. They are used for improving production methods and for fabric finishing. In this industry, a very common application is the use of the enzyme amylase in order to remove starch size. The threads (the longitudinal threads) of the fabrics are often coated in starch. This prevents them from breaker when weaving takes place. In the textile industry, a process called scouring is used (the cleaning of fabrics by removing any impurities such as waxes, pectins and any mineral salts from cellulose fibers). Pectin can act as a glue between the core of the fibers and the waxes, but this can be destroyed by an alkaline called pectinase. Cellulases have quite recently become the tool for fabric finishing. This began in denim finishing where it was discovered that cellulases could achieve the fashionable stonewashed look traditionally achieved through the abrasive action of pumice stones. Cellulases are also quite often used in order to prevent pilling and improve the smoothness and color brightness of cotton fabrics. In addition, a softer handle is obtained. Catalases can also be used for degrading residual hydrogen peroxide after the bleaching of

cotton. Hydrogen peroxide has to be removed before dyeing. Protease enzymes are used for wool treatment and the degumming of raw silk. So, examples of enzymes that may be used in the textile industry:

Cellulase – for stonewashing denim, polishing of cotton

Catalase – removing hydrogen peroxide

Pectinase – for bioscouring (a way to scour fabrics)

Alpha amylase – for desizing at low temperatures

The food and drink industry has to be one of the largest markets for enzymes. In the baking industry, enzymes are added to the dough when baking bread to ensure that the bread is high in quality and has a better volume (that there is more of it). Enzymes also have the ability to preserve bread; keeping it fresh for a longer period of time and therefore increasing its shelf life. In the dairy industry, enzymes are used in cheese making to help bring about the coagulation of milk. In these applications, enzymes from microbial and animal sources are used. Industrial enzymes are added to control the brewing process in alcohol making and the brewing industry. This also helps to produce consistent and high quality beer. When making wines and juices, enzymes are used to break down cell walls of plants when extracting plant material. This use of enzymes would give higher juice yields and also improves the color and smell of the extracted substances.

Fungal alpha amylase – for dough improvement in the bread making industry

Glucoamylase – used in fermentation

Papain enzymes – for fermentation in the brewing industry

Beta glucanse – for filtration

Protease – used in biscuit production

Enzymes are also used in the pulp and paper industry. Amylase is used for modification of starch coating and xylanases to reduce the consumption of bleach chemicals are very well known applications, but nowadays 'lipases for is used for pitch control, esterases is used for stickies removal and amylases and cellulases are used for improved deinking and cellulases for fiber modification have become an integral part of the chemical solutions used in the pulp and paper mills. In the manufacturing of coated papers, a starch-based coating formulation is used in order to coat the surface of the paper. Compared with the uncoated paper, the coating provides a number of benefits, including; improved gloss, a smoother texture, and printing properties.

Cellulase – can be used for pulp deinking and pulp refining

Xylanase – for pulp bleaching

Alpha amylase – starch modification

Enzymes are used in detergents and in personal care and hygiene. They are used in many household and industrial detergents. This industry, in addition to the food processing industry is currently one of the largest application areas for enzymes. This is because the enzymes are very effective at relatively low temperatures and pH values. They contribute to a: better overall cleaning performance; they are biodegradable so they do not really effect the environment that much; they reduce water consumption through more effective release of soil.

So, a few of the most common enzymes that are used commercially are:

Bioethanol is a type of biofuel. It may be used when adding fuel to a vehicle. This biofuel is able to be produced from starchy plant materials with the use of enzymes that are capable of

efficiently making this conversion. At the moment, corn is widely used as a source of starch, however increasing interest in bioethanol is raising concerns as corn prices rise and corn as a food supply is being threatened. Other plants including wheat, bamboo, or other grasses are possible candidate sources of starch for bioethanol production. Bioethanol production (the growing of crops, shipping and manufacturing) still requires a large input of non-renewable resources. Technological research and manipulation of enzymes to make the process more efficient, thus requiring less plant material or consuming less fossil fuels, are in the works, to improve on this area of biotechnology.

Protease enzyme – used in the manufacturing of baby foods to pre digest proteins

Lipase – can be used in conjunction with protease in biological detergents in order to break down and digest the substances in stains into smaller and more water soluble substances

Carbohydrase – Can be used to convert starch syrup into sugar syrup. This is done during the manufacturing of sports drinks ; sugar syrup is much more valuable than starch syrup, which is relatively cheap

Isomerase – Can be used in slimming foods/weight loss products. It converts the glucose syrup into fructose syrup (fructose is much sweeter than glucose so it can be used in much smaller amounts, thus saving money during the production processes).

Zymase in alcohol manufacturing – fermentation is another method used for manufacturing alcohol. During the fermentation process, carbohydrates are converted into ethanol (with carbon dioxide as a by product). The carbohydrate is usually a sugar or a starch .The ethanol that is produced during the fermentation process may have an alcohol concentration of up to 14%. The fermentation process is carried out at quite low temperatures using used. It is the zymase enzymes that are present in the yeast that actually catalyse the fermentation reactions. The reaction takes places at temperatures between 25◦C and 37◦C. This is because zymase would begin to denature at temperatures above 37◦C and it would therefore begin to lose its function and efficiency, whereas at temperature bellow 25◦C the reaction would be too slow. Zymase also stops functioning at an alcohol concentration of above 14%.

Optimization Of Industrial Enzymes By Mutagenesis

Site Directed Mutagenesis

Site-directed mutagenesis (SDM) is a method to create specific, targeted changes in double stranded plasmid DNA. There are many reasons to make specific DNA alterations (insertions, deletions and substitutions), including:

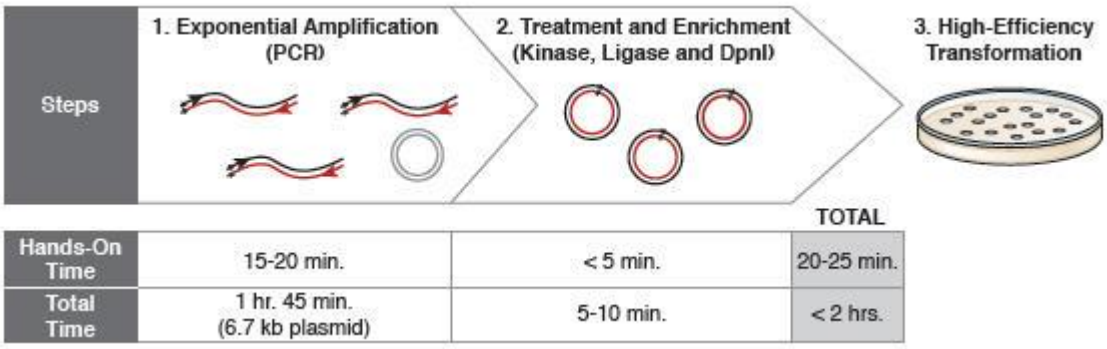
- To study changes in protein activity that occur as a result of the DNA manipulation.
- To select or screen for mutations (at the DNA, RNA or protein level) that have a desired property
- To introduce or remove restriction endonuclease sites or tags

Method :

SDM is an in vitro procedure that uses custom designed oligonucleotide primers to confer a desired mutation in a double-stranded DNA plasmid. Formerly, a method pioneered by Kunkel (Kunkel, 1985) that takes advantage of a strain deficient in dUTPase and uracil deglycosylase so that the recipient E. coli degrades the uracil-containing wild-type DNA was widely used. Currently, there are a number of commercially available kits that also require specific modification and/or unique E. coli strains. The most widely-used methods do not require any modifications or unique strains and incorporate mutations into the plasmid by inverse PCR with standard primers. For these methods, primers can be designed in either an overlapping or a back-to-back orientation (Figure 1). Overlapping primer design results in a product that will re-circularize to form a doubly-nicked plasmid. Despite the presence of these nicks, this circular product can be directly transformed into E. coli, albeit at a lower efficiency than non-nicked plasmids. Back-to-back primer design methods not only have the advantage of transforming non-nicked plasmids, but also allow exponential amplification to generate significantly more of the desired product (Figure 2). In addition, because the primers do not overlap each other, deletions sizes are only limited by the plasmid and insertions are

only limited by the constraints of modern primer synthesis. Currently, by splitting the insertion between the two primers, insertions up to 100 bp can routinely be created in one step using this method.

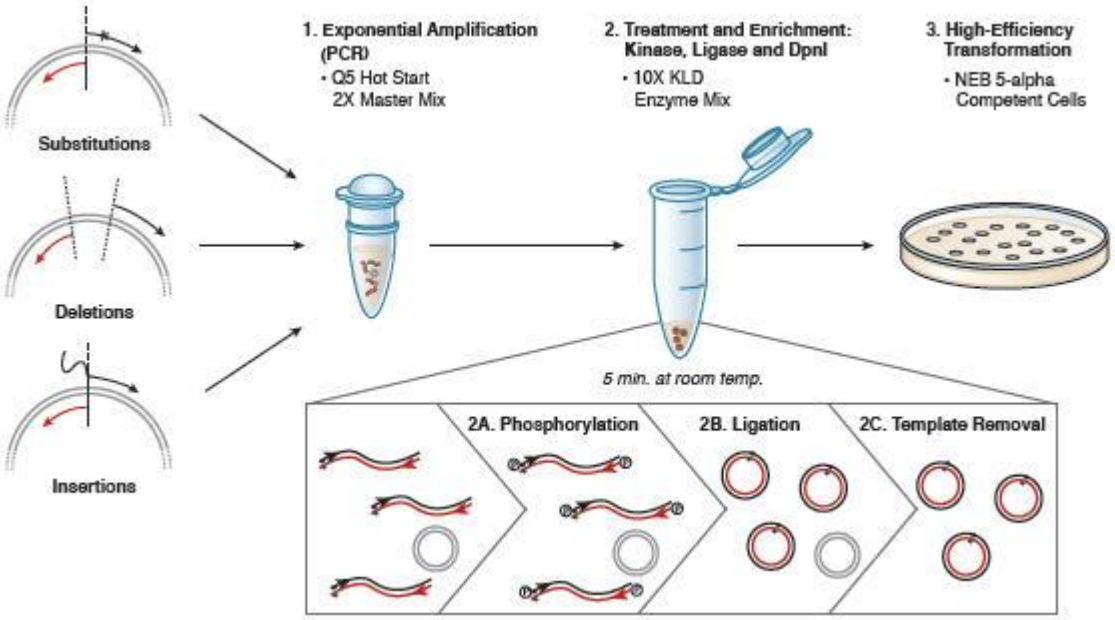
Figure 1: Site-specific mutagenesis proceeds in less than 2 hours



			TOTAL
Hands-On Time	15-20 min.	< 5 min.	20-25 min.
Total Time	1 hr. 45 min. (6.7 kb plasmid)	5-10 min.	< 2 hrs.

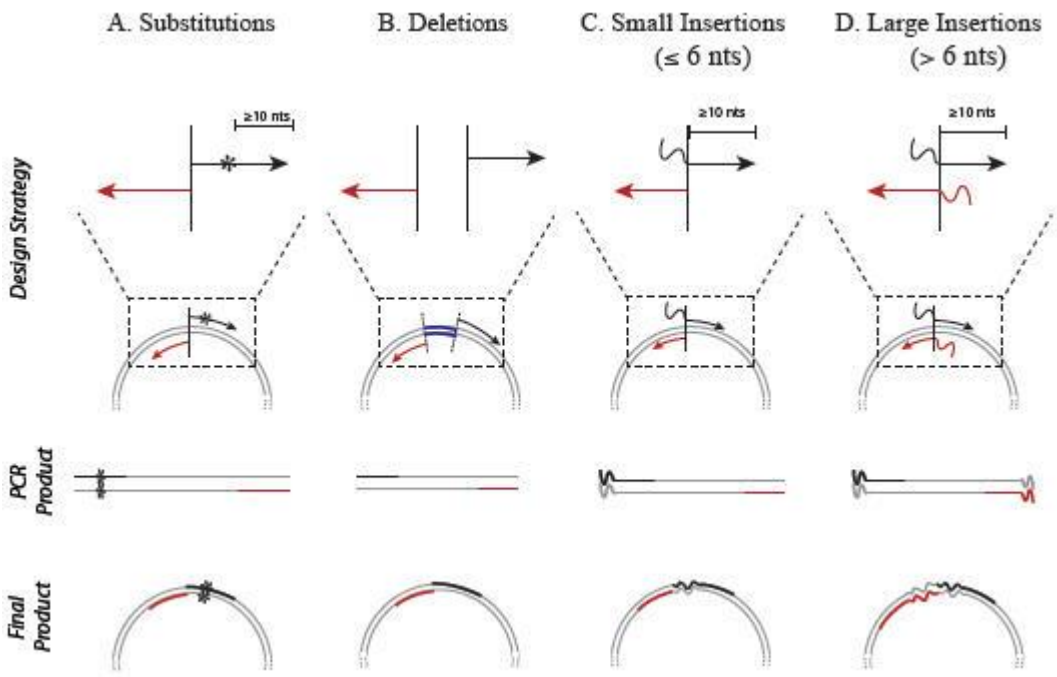
The use of a master mix, a unique multi-enzyme KLD enzyme mix, and a fast polymerase ensures that, for most plasmids, the mutagenesis reaction is complete in less than two hours.

Figure 2: Q5 Site-Directed Mutagenesis Kit



This kit is designed for rapid and efficient incorporation of insertions, deletions and substitutions into doublestranded plasmid DNA. The first step is an exponential amplification using standard primers and a master mix fomulation of Q5 Hot Start High-Fidelity DNA Polymerase. The second step involves incubation with a unique enzyme mix containing a kinase, a ligase and DpnI. Together, these enzymes allow for rapid circularization of the PCR product and removal of the template DNA. The last step is a high-efficiency transformation into chemicallycompetent cells.

Figure 3: Primer Design for the Q5 Site-Directed Mutagenesis Kit



Substitutions, deletions and insertions are incorporated into plasmid DNA through the use of specifically designed forward (black) and reverse (red) primers. Unlike kits that rely on linear amplification, primers designed for the Q5 Site-Directed Mutagenesis Kit should not overlap to ensure that the benefits of exponential amplification are realized. A) Substitutions are created by incorporating the desired nucleotide change in the center of the forward primer,

including at least 10 complementary nucleotides on the 3´side of the mutation(s). The reverse primer is designed so that the 5´ ends of the two primers anneal back-to- back. B) Deletions are engineered by designing standard, non-mutagenic forward and reverse primers that flank the region to be deleted. C) Insertions less than or equal to 6 nucleotides are incorporated into the 5´ end of the forward primer while the reverse primer anneals back-to-back with the 5´ end of the complementary region of the forward primer. D) Larger insertions can be created by incorporating half of the desired insertion into the 5´ ends of both primers. The maximum size of the insertion is largely dictated by oligonucleotide synthesis limitations.

Protein Engineering Strategies To Improve Enzyme Stability

One of the major barriers to the use of enzymes in industrial biotechnology is their insufficient stability under processing conditions. The use of organic solvent systems instead of aqueous media for enzymatic reactions offers numerous advantages, such as increased solubility of hydrophobic substrates or suppression of water-dependent side reactions. For example, reverse hydrolysis reactions that form esters from acids and alcohols become thermodynamically favorable. However, organic solvents often inactivate enzymes. Industry and academia have devoted considerable effort into developing effective strategies to enhance the lifetime of enzymes in the presence of organic solvents. The strategies can be grouped into three main categories:

(i) isolation of novel enzymes functioning under extreme conditions.

(ii) modification of enzyme structures to increase their resistance toward nonconventional media.

(iii) modification of the solvent environment to decrease its denaturing effect on enzymes.

Here we discuss successful examples representing each of these categories and summarize their advantages and disadvantages. Finally, we highlight some potential future research directions in the field, such as investigation of novel nanomaterials for immobilization, wider application of computational tools for semirational prediction of stabilizing mutations, knowledge-driven modification of key structural elements learned

from successfully engineered proteins, and replacement of volatile organic solvents by ionic liquids and deep eutectic solvents.

Immobilization of Enzymes

Immobilization of enzymes (or cells) refers to the technique of confining/anchoring the enzymes (or cells) in or on an inert support for their stability and functional reuse. By employing this technique, enzymes are made more efficient and cost-effective for their industrial use. Some workers regard immobilization as a goose with a golden egg in enzyme technology. Immobilized enzymes retain their structural conformation necessary for catalysis.

Advantages of immobilized enzymes:

a. Stable and more efficient in function.

b. Can be reused again and again.

c. Products are enzyme-free.

d. Ideal for multi-enzyme reaction systems.

e. Control of enzyme function is easy.

f. Suitable for industrial and medical use.

g. Minimize effluent disposal problems.

Disadvantages of immobilization:

a. The possibility of loss of biological activity of an enzyme during immobilization or while it is in use.

b. Immobilization is an expensive affair often requiring sophisticated equipment.

Immobilized enzymes are generally preferred over immobilized cells due to specificity to yield the products in pure form. However, there are several advantages of using immobilized multi-enzyme systems such as organelles and whole cells over immobilized enzymes. The

immobilized cells possess the natural environment with cofactor availability (and also its regeneration capability) and are particularly suitable for multiple enzymatic reactions.

Methods of Immobilization:

The commonly employed techniques for immobilization of enzymes are—adsorption, entrapment, covalent binding and cross-linking.

Adsorption:

Adsorption involves the physical binding of enzymes (or cells) on the surface of an inert support. The support materials may be inorganic (e.g. alumina, silica gel, calcium phosphate gel, glass) or organic (starch, carboxymethyl cellulose, DEAE-cellulose, DEAE-sephadex).

Adsorption of enzyme molecules (on the inert support) involves weak forces such as van der Waals forces and hydrogen bonds (Fig. 21.3). Therefore, the adsorbed enzymes can be easily removed by minor changes in pH, ionic strength or temperature. This is a disadvantage for industrial use of enzymes.

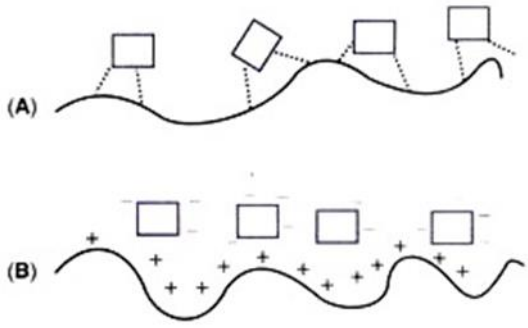


*Fig. 21.3 : Immobilization of enzymes by adsorption (A) By van der Waals forces (B) By hydrogen bonding (**Note** : Cloured blocks represent enzymes)*

Entrapment:

Enzymes can be immobilized by physical entrapment inside a polymer or a gel matrix. The size of the matrix pores is such that the enzyme is retained while the substrate and product

molecules pass through. In this technique, commonly referred to as lattice entrapment, the enzyme (or cell) is not subjected to strong binding forces and structural distortions.

Some deactivation may however, occur during immobilization process due to changes in pH or temperature or addition of solvents. The matrices used for entrapping of enzymes include polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone and rubber. Enzymes can be entrapped by several ways.

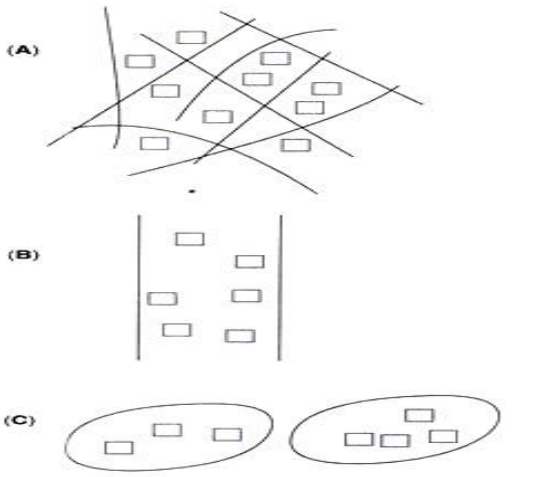


*Fig. 21.4 : Immobilization of enzymes by entrapment (A) Inclusion in gels (B) Inclusion in fibres (C) Inclusion in microcapsules (**Note** : Coloured blocks represent enzymes)*

1**. Enzyme inclusion in gels:**

This is an entrapment of enzymes inside the gels (Fig. 21.4A).

2. **Enzyme inclusion in fibres:**

The enzymes are trapped in a fibre format of the matrix (Fig. 21.4B).

3**. Enzyme inclusion in microcapsules:**

In this case, the enzymes are trapped inside a microcapsule matrix (Fig. 21.4C). The hydrophobic and hydrophilic forms of the matrix polymerise to form a microcapsule containing enzyme molecules inside. The major limitation for entrapment of enzymes is their

leakage from the matrix. Most workers prefer to use the technique of entrapment for immobilization of whole cells. Entrapped cells are in use for industrial production of amino acids (L-isoleucine, L-aspartic acid), L-malic acid and hydroquinone.

Microencapsulation:

Microencapsulation is a type of entrapment. It refers to the process of spherical particle formation wherein a liquid or suspension is enclosed in a semipermeable membrane. The membrane may be polymeric, lipoidal, lipoprotein-based or non-ionic in nature. There are three distinct ways of microencapsulation.

1. Building of special membrane reactors.

2. Formation of emulsions.

3. Stabilization of emulsions to form microcapsules.

Microencapsulation is recently being used for immobilization of enzymes and mammalian cells. For instance, pancreatic cells grown in cultures can be immobilized by microencapsulation. Hybridoma cells have also been immobilized successfully by this technique.

Covalent Binding:

Immobilization of the enzymes can be achieved by creation of covalent bonds between the chemical groups of enzymes and the chemical groups of the support (Fig. 21.5). This technique is widely used. However, covalent binding is often associated with loss of some enzyme activity. The inert support usually requires pretreatment (to form pre-activated support) before it binds to enzyme. The following are the common methods of covalent binding.

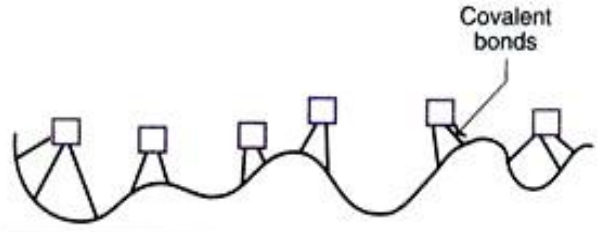


*Fig. 21.5 : A general representation of immobilization of enzymes by covalent binding (**Note** : coloured blocks represent enzymes).*

1. Cyanogen bromide activation:

The inert support materials (cellulose, sepharose, sephadex) containing glycol groups are activated by CNBr, which then bind to enzymes and immobilize them (Fig. 21.6A).

2. Diazotation:

Some of the support materials (amino benzyl cellulose, amino derivatives of polystyrene, aminosilanized porous glass) are subjected to diazotation on treatment with NaNO2 and HCI. They, in turn, bind covalently to tyrosyl or histidyl groups of enzymes (Fig. 21.6B).

3. Peptide bond formation:

Enzyme immobi-lization can also be achieved by the formation of peptide bonds between the amino (or carboxyl) groups of the support and the carboxyl (or amino) groups of enzymes (Fig. 21.6C). The support material is first chemically treated to form active functional groups.

4. Activation by bi- or poly-functional reagents:

Some of the reagents such as glutaraldehyde can be used to create bonds between amino groups of enzymes and amino groups of support (e.g. aminoethylcellulose, albumin, amino alkylated porous glass).

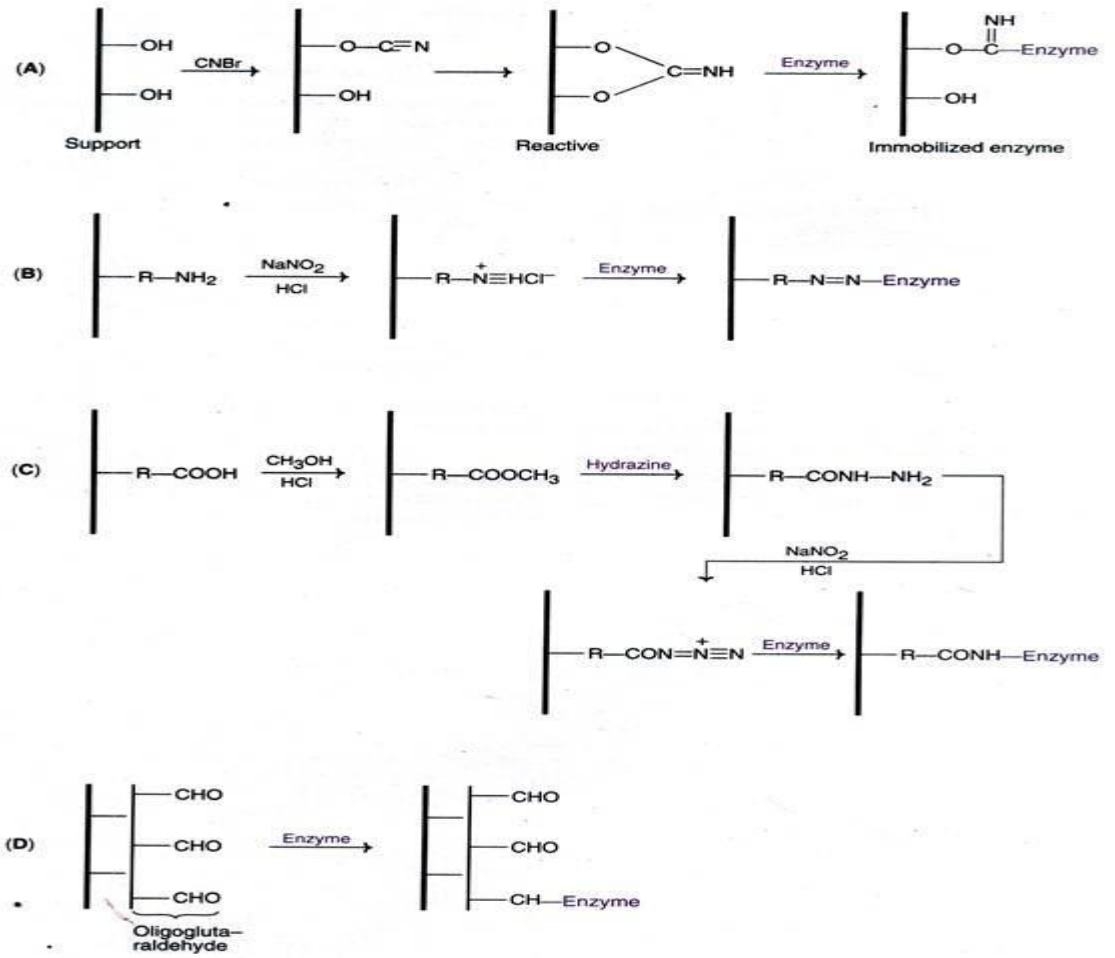


Fig. 21.6 : Immobilization of enzymes by covalent binding (A) Cyanogen bromide activation, (B) Diazotation, (C) Peptide bond formation, (D) Activation by bifunctional agent.

Cross-Linking:

The absence of a solid support is a characteristic feature of immobilization of enzymes by cross- linking. The enzyme molecules are immobilized by creating cross-links between them, through the involvement of poly-functional reagents. These reagents in fact react with the enzyme molecules and create bridges which form the backbone to hold enzyme molecules (Fig. 21.7). There are several reagents in use for cross-linking. These include glutaraldehyde, diazobenzidine, hexamethylene diisocyanate and toluene di- isothiocyanate.

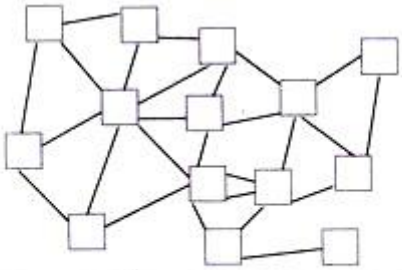


Fig. 21.7 : Immobilization of enzyme molecules by cross linking.

Glutaraldehyde is the most extensively used cross-linking reagent. It reacts with lysyl residues of the enzymes and forms a Schiff's base. The cross links formed between the enzyme and glutaraldehyde are irreversible and can withstand extreme pH and temperature. Glutaraldehyde cross- linking has been successfully used to immobilize several industrial enzymes e.g. glucose isomerase, penicillin amidase. The technique of cross-linking is quite simple and cost-effective. But the disadvantage is that it involves the risk of denaturation of the enzyme by the poly-functional reagent.

Artificial Enzyme

Protein engineering technologies has developed to design and synthesize molecules with the attributes of enzymes (selective, proficient, green, nontoxic, and biodegradable) for non-natural reactions.

- Scientists from the University of Bristol have designed an artificial enzyme that functions as well as (and in some cases better than) a vital class of natural enzymes.
- Enzymes are fundamentally important biological molecules that perform the bulk of the chemical reactions in all living organisms.
- These reactions power cellular life and are involved in a great number of processes necessary to give cells their chemical and physical characteristics.
- Furthermore, many enzymes participate in chemical reactions which have commercial or medical value, and for which we have no good manmade substances that can catalyse such reactions with the same precision and efficiency as enzymes.

- The artificial enzyme was synthesised in a fully functioning form by E. coli bacteria and could be of significant interest to the biotech industry due to its industrially-relevant catalytic activities, thermal stability and chemical resistance.
- They could even be used as replacements for certain natural enzymes that are more complex and difficult to produce on a larger scale.

Isolation and Purification of Industrially important enzymes:

Purification

Enzyme purification is directly related to product quality, in addition to regulatory requirements. Therefore, enzyme purification must be thoroughly considered and cautiously operated for both research and production purposes. However, the task is not straightforward. Many factors could change the efficiency, the yield, and stability of activity during purification, and the effects of these factors vary largely from one enzyme to another. At **Creative Enzymes**, we depend on the knowledgeable scientists and their years of experiences to design and perform the most suitable purification process for each enzyme.

Almost all samples need to be prepared before the actual purification. For the enzymes from cell sources, they need to be fractionated into components before purification. The first step usually involves homogenization of cells, which disrupt the cell wall to release the enzyme into the homogenate, along with other components. Depending on the cell type, the homogenization could be easy as in the case of mammalian tissue without rigid cell wall, or it may need harsher conditions such as abrasion, freezing, and high pressure due to the rigid cell wall of the plant tissue. Sometimes, additional hydrolytic enzymes or detergents are added for better extraction. The mixture is then fractionated by centrifugation, yielding a dense pellet of heavy material at the bottom of the centrifuge tube and a lighter supernatant above (Figure 1). The supernatant is again centrifuged at a greater force to yield yet another pellet and supernatant. The procedure, called differential centrifugation, yields several fractions of decreasing density, each still containing hundreds of different proteins, which are subsequently assayed for the activity being purified. Usually, one fraction will be enriched

for such activity, and it then serves as the source of material to which more discriminating purification techniques are applied. The choice of temperature, pH, buffering salt, buffer strength, ionic strength, osmolarity, additives (EDTA, SDS, non-ionic detergents etc.), and homogenization technique are important of the success of purification.

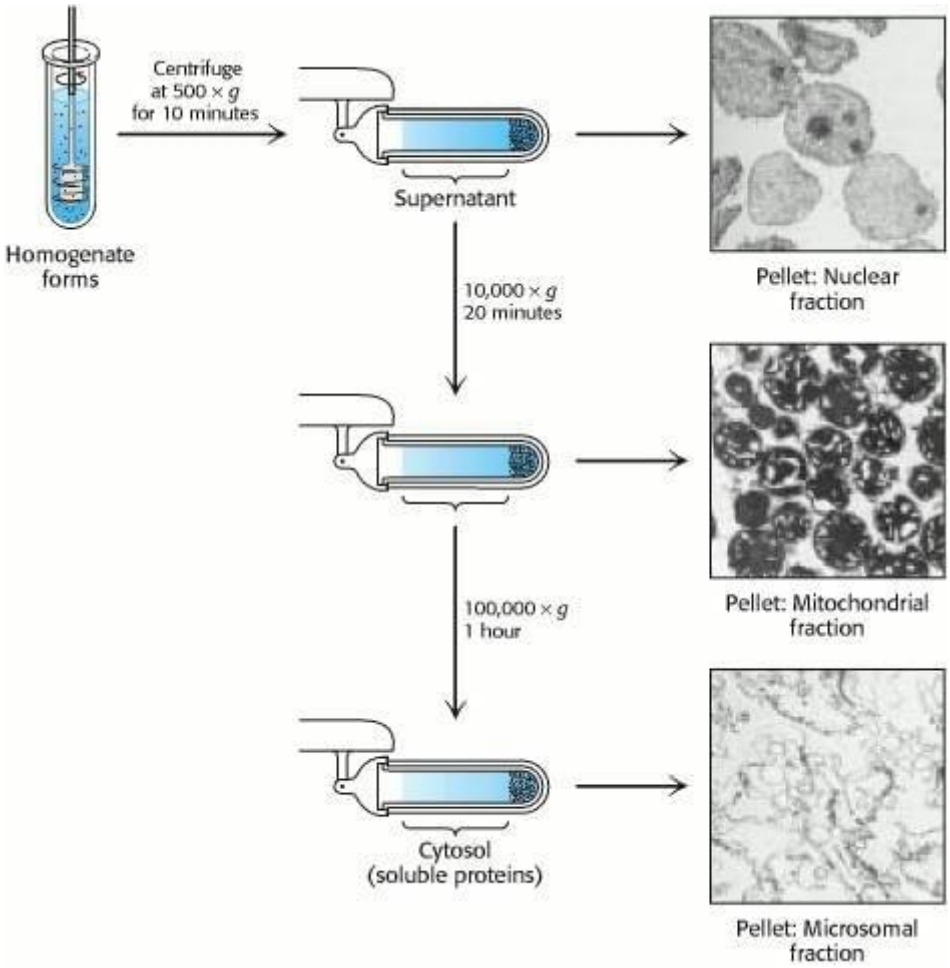


Figure 1. Differential Centrifugation.

Purification and separation of enzymes are generally based on solubility, size, polarity, and binding affinity. The production scale, timeline, and properties of the enzymes should all be considered when choosing the proper separation method.

- **Solubility based separation**

The principle of the type of separation is that enzyme solubility changes drastically when the pH, ionic strength, or dielectric constant changes. For example, most proteins are less soluble at high salt concentrations, an effect called salting out. The salt concentration at which a protein precipitates differs from one protein to another. Hence, salting out can be used to fractionate proteins. Salting out is also useful for concentrating dilute solutions of proteins, including active fractions obtained from other purification steps. Addition of water-miscible organic solvents such as ethanol or acetone will change the dielectric constant of the solvent and therefore precipitate the desired enzyme. Neutral water-soluble polymers can also be used for the same purpose instead of organic solvents. However, the risks of losing enzyme activity during precipitation and further separation of the added salt or polymer need to be considered.

- **Size or mass based method**

Because enzymes are relatively large molecules, separation based on the size or mass of molecules favors purification of enzymes, especially the ones with high molecular weight. Dialysis is a commonly used method, where semipermeable membranes are used to remove salts, small organic molecules, and peptides (Figure 2). The process usually needs a large volume of dialysate, the fluid outside the dialysis dag, and a period of hours or days to reach the equilibrium. Countercurrent dialysis cartages can also be used, in which the solution to be dialyzed flow in one direction, and the dialysate in the opposite direction outside of the membrane. Similarly, ultrafiltration membranes, which are made from cellulose acetate or other porous materials, can be used to purify and concentrate an enzyme larger than certain molecular weight. The molecular weight is called the molecular weight cutoff and is available in a large range from different membranes. The ultrafiltration process is usually carried out in a cartridge loaded with the enzyme to be purified. Centrifugal force or vacuum is applied to accelerate the process. Both dialysis and ultrafiltration are quick but somewhat vague on distinguishing the molecular weight, whereas size exclusion chromatography gives fine fractionation from the raw mixture, allowing separation of the desired enzyme from not only small molecules but also other enzymes and proteins. Size exclusion chromatography, also known as gel-filtration chromatography, relies on polymer beads with defined pore sizes that

let particles smaller than a certain size into the bead, thus retarding their egress from a column. In general, the smaller the molecule, the slower it comes out of the column. Size exclusion resins are relatively "stiff" and can be used in high pressure columns at higher flow rates, which shortens the separation time. Other factors including the pore size, protein shape, column volumes, and ionic strength of the eluent could also change the result of purification.

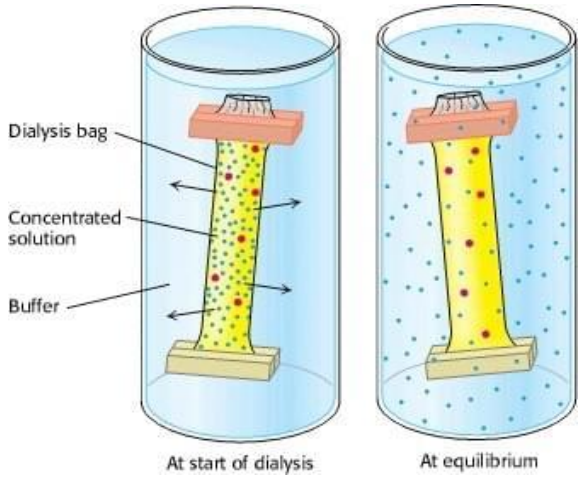


Figure 2. The scheme of dialysis. Enzyme molecules (red dots) are retained in the dialysis bag and separated from other smaller molecules (blue dots).

- **Polarity based separation**

Like other proteins, enzymes can be separated on the basis of polarity, more specifically, their net charge, charge density, and hydrophobic interactions. In ion-exchange chromatography, a column of beads containing negatively or positively charged functional groups are used to separate enzymes. The cationic enzymes can be separated on anionic columns, and anionic enzymes on cationic column.

Electrophoresis is a procedure that uses an electrical field to cause permeation of ions through a solid or semi-solid matrix or surface resulting in separations on constituents on the basis of charge density. The most commonly used methods with a SDS-PAGE matrix are quite well standardized and do not differ much between labs. The distance a protein migrates in SDS-PAGE is inversely proportional to the log of its molecular radius, which is roughly proportional to molecular weight. Similarly, a matrix with gradient pH can be used in

isoelectric focusing separation. A protein moves under the influence of an electrical field and stops upon reaching the pH which is the pI for the protein (net charge = 0). The matrix used can be liquid or a gel poured into either a cylindrical shape, or a flat plate.

Hydrophobic interaction chromatography (HIC) employs hydrophobic interactions to distinguish different enzymes, which are adsorbed on matrices such as octyl- or phenyl-Sepharose. A gradient of decreasing ionic strength, or possibly increasing non-polar solvent concentration can be used to elute the proteins, giving fractions that usually contain relatively high-pure enzymes. High-pressure liquid chromatography (HPLC) uses the same principle of separation of HIC, which is filled with more finely divided and tuned materials and thus allows more choices of eluents and results in better separation. Note that HPLC could be based on polarity, affinity, or both.

- **Affinity or ligand based purification**

Affinity chromatography is another powerful and generally applicable means of purifying enzymes. This technique takes advantage of the high affinity of many enzymes for specific chemical groups. In general, affinity chromatography can be effectively used to isolate a protein that recognizes a certain group by (1) covalently attaching this group or a derivative of it to a column, (2) adding a mixture of proteins to this column, which is then washed with buffer to remove unbound proteins, and (3) eluting the desired protein by adding a high concentration of a soluble form of the affinity group or altering the conditions to decrease binding affinity. Affinity chromatography is most effective when the interaction of the enzyme and the molecule that is used as the bait is highly specific. A special example of ligand-affinity chromatography is the Ni-NTA (nickel – nitrolotriacetic acid-agaraose) affinity chromatography. This ligand binds tightly to a 6 amino acid peptide consisting only of histidines (His6). The cDNA sequence for His6 can be appended to the cDNA coding for a given recombinant protein, thus yielding a recombinant protein which contains a His-TAG. This allows the affinity-purification of such a protein using Ni-NTA without having to design a special ligand-affinity column. Other forms of affinity chromatography include dye-ligand chromatography, immunoadsorption chromatography, and covalent chromatography.

UNIT – IV Enzyme Technology:

Methods for large scale production of enzymes. Immobilized enzyme and their comparison with soluble enzymes, Methods for immobilization of enzymes. Immobilized enzyme reactors. Application of Immobilized and soluble enzyme in health and industry. Application to fundamental studies of biochemistry. Enzyme electrodes.

Enzyme Technology

For thousands of years natural enzymes made by microorganisms have been used to make products such as cheese, bread, wine, and beer. Enzymes are now used in a wide range of industrial processes. The study of industrial enzymes and their uses is called **enzyme technology.**

The advantages and disadvantages of using enzymes are **directly related to their properties:**

Advantages	Disadvantages
They are **specific in their action** and are therefore **less likely to produce unwanted by-Products**	They are **highly sensitive to changes in physical and chemical conditions** surrounding them.
They are **biodegradable** and therefore **cause less environmental pollution**	They are **easily denatured** by even a small increase in temperature and are **highly susceptible to poisons and changes in pH**. Therefore the **conditions in which they work must be tightly controlled**.
They **work in mild conditions**, i.e. low temperatures, neutral pH and normal atmospheric pressure, and therefore **are energy Saving**	The enzyme substrate mixture **must be uncontaminated with other substances** that might affect the reaction.

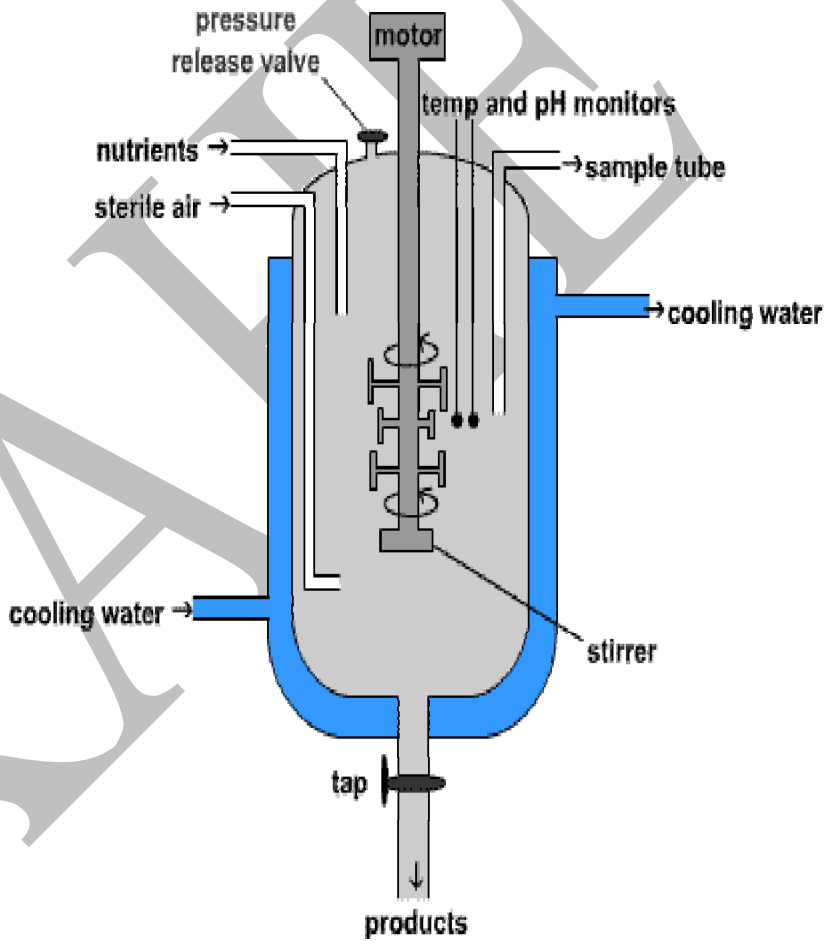
Microbes are still the most common source of industrial enzymes. Microorganisms produce enzymes inside their cells (**intracellular** enzymes) and may also secrete enzymes for action outside the cell (**extracellular** enzymes). The microorganisms selected are usually cultured in large fermentation chambers (*known as **fermenters** – see later*) under controlled conditions to maximise enzyme production. The microorganisms may have specific genes introduced into

their DNA through ***genetic engineering***, so that they produce enzymes naturally made by other organisms - this is explained in further detail under the genetic engineering section of this unit.

Growing microbes in a fermentor

Given a suitable nutrient medium and the right conditions (temperature, pH, oxygen levels (many microbes are *obligate anaerobes*, i.e. are killed by oxygen), it is easy to grow microbes on a laboratory scale in Petri dishes, test tubes and flasks. However, producing substances such as penicillin from microbes on an industrial scale causes serious problems because massive numbers of organisms have to be grown for commercial use.

The microorganisms are grown in very large vessels called **fermenters** – as shown in this simplified diagram:



The large stainless steel cavity is filled with a sterile nutrient solution,
which is then **inoculated** with a pure culture of the carefully selected fungus or bacterium.

Paddles rotate the mixture so that the suspension is mixed well. As the nutrients are used up, more can be added. Probes monitor the mixture and changes in pH,
oxygen concentration and temperature are all computer controlled. A water jacket surrounding the fermenter contains fast flowing cold water to cool the fermenter since fermentation is a heat generating process. Most of the air, including carbon dioxide and other gases produced by cell metabolism, leave the fermenter by an exhaust pipe.

Requirements for the production of microbes in fermenters:

- **Oxygen** is needed for aerobic respiration of (some) micro-organisms – others are strict anaerobes and oxygen must be excluded
- a source of **Carbohydrate** is needed as an energy source for respiration to release energy needed for growth.

- a source of **Nitrogen** is needed need nitrogen for protein synthesis – **Ammonia** (NH_3) and **urea (**($NH_2)_2CO$) are both widely used as (cheap) sources of useable nitrogen

Isolating the Enzyme

Pure enzymes are needed for commercial use; therefore microbes must be grown in **aseptic conditions**, free from contaminants - such as unwanted chemicals - and other microbes. It is necessary to prevent contamination with other bacteria since:

- there may be competition for nutrients;
- the required enzyme may not be produced as readily;
- the end-product may be contaminated and unsafe.

The required enzyme that is finally produced must also be isolated from the microbial cells.

- **Extracellular** enzymes are present in the culture outside the microbial cells, since they have been secreted. They are often soluble in water, so they can readily be extracted from the culture medium and purified. Less common in Nature (though genetic engineering can be used to modify cells to promote this), these enzymes are cheaper to produce and tend to be more stable – they are therefore the preferred choice, **when available!**
- To obtain an **intracellular** enzyme, the microbe cells are harvested *(by filtration or centrifugation)* from the culture and are then broken up. The mixture is next centrifuged to remove large cell fragments and the enzymes (**all** of them!) are precipitated from solution by a salt or alcohol. The **required** enzyme must then be purified by techniques such as electrophoresis or column chromatography.

This last process is complicated and expensive, so these enzymes are only used when no other alternative is available. By their very nature, they tend to be more sensitive to their operating conditions, which makes their commercial use less easy. On the other hand, they are much more common in Nature!

Table comparing intra- and extra-cellular enzymes:

Intracellular enzymes	Extracellular enzymes
More difficult to isolate	Easier to isolate
Cells have to be broken apart to release them	No need to break cells – secreted in large amounts into medium surrounding cells
Have to be separated out from cell debris and a mixture of many enzymes and other chemicals	Often secreted on their own or with a few other Enzymes
Often stable only in environment inside intact cell	More stable
Purification/downstreaming processing is difficult/expensive	Purification/downstreaming processing is easier/cheaper

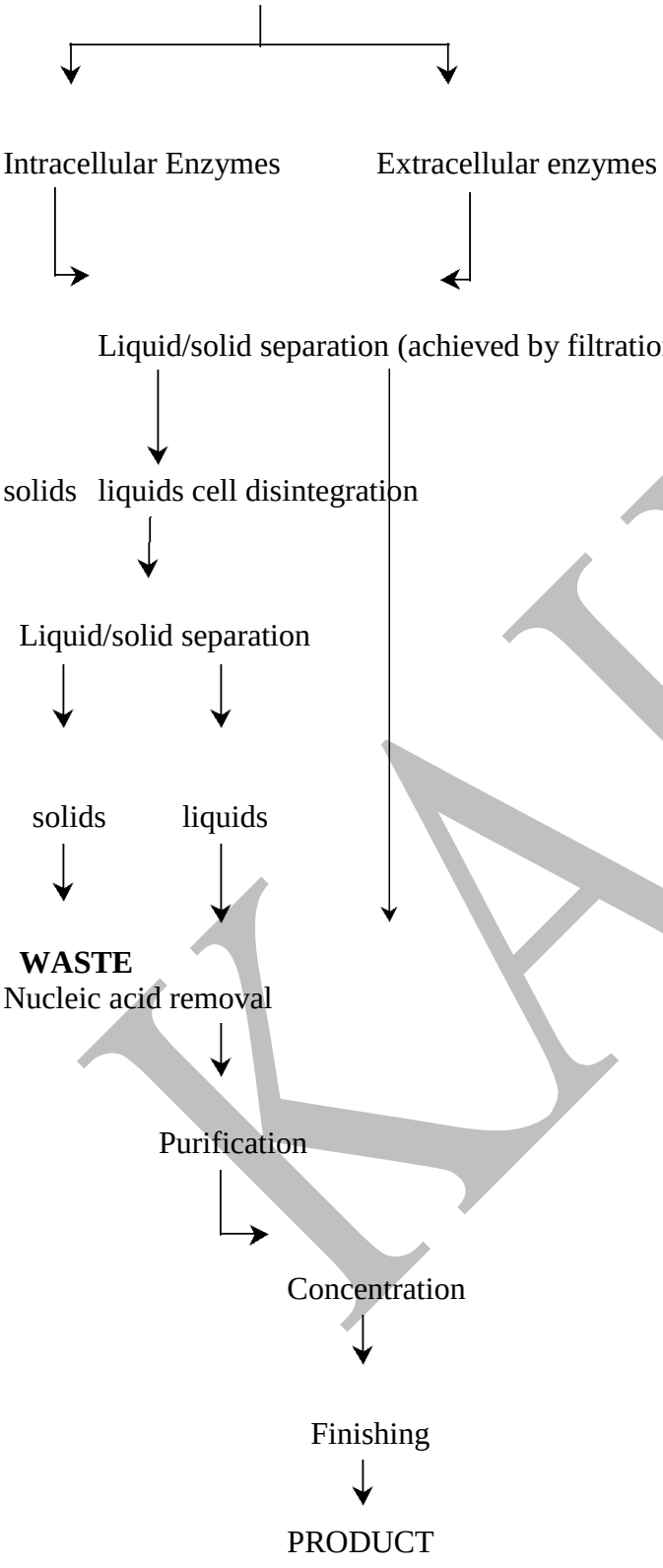
Microorganisms such as bacteria and fungi are **saprobionts** i.e. they feed **saprophytically,** secreting enzymes onto their food – making them a good source of extracellular enzymes. For example, the fungus *Aspergillus niger* produces an enzyme called ***pectinase,*** which breaks down pectin, a substance found in the cell walls of plant cells. The fruit juice industry uses pectin widely, since when fruit is crushed to extract the juice, pectin prevents some being released and also makes the juice cloudy.

The stainless steel fermenter with complicated control systems is not actually the most expensive part of the process. Almost 80% of the cost is accounted for by **downstream processing:** the isolation, extraction, and purification of the product at the end of the culture in the fermenter.

Downstreaming uses a variety of techniques. In the first stage cells need to be separated from the liquid part of the suspension. This can be done by sedimentation, centrifugation or filtration. If the cells themselves are the desired product (e.g. for single-cell protein production for animal feed) then they need to be sterilised, washed, dried and packaged. If the desired product is a chemical within the cells, the cells have to be broken apart to release the chemical and the cellular components removed. The desired chemical is then extracted and purified by a number of techniques such as precipitation and chromatography.

Finally, the purified chemical has to be dried and packaged in a suitable form. In the case of the enzymes in biological washing powders, this means coating the granules with wax to ensure that they remain dry until used – otherwise the enzymes would digest themselves!

Microbial Fermentations



Large Scale production of enzymes.

Among various enzymes produced at large scale are proteases (subtilisin, rennet), hydrolases (pectinase, lipase, lactase), isomerases (glucose isomerase), and oxidases (glucose oxidase). These enzymes are produced using overproducing strains of certain organisms. Separation and purification of an enzyme from an organism require disruption of cells, removal of cell debris and nucleic acids, precipitation of proteins, ultrafiltration of the desired enzyme, chromatographic separations (optional), crystallization, and drying. The process scheme varies depending on whether the enzyme is intracellular or extracellular. In some cases, it may be more advantageous to use inactive (dead or resting) cells with the desired enzyme activity in immobilized form. This approach eliminates costly enzyme separation and purification steps and is therefore economically more feasible.

The first step in the large-scale production of enzymes is to cultivate the organisms producing the desired enzyme. Enzyme production can be regulated and fermentation conditions can be optimized for overproduction of the enzyme. Proteases are produced by using overproducing strains of Bacillus, Aspergillus, Rhizopus, and Mucor; pectinases are produced by Aspergillus niger; lactases are produced by yeast and Aspergillus; lipases are produced by certain strains of yeasts and fungi; glucose isomerase is produced by Flavobacterium arborescens or Bacillus coagulans. After the cultivation step, cells are separated from the media usually by filtration or sometimes by centrifugation. Depending on the intracellular or extracellular nature of the enzyme, either the cells or the fermentation broth is further processed to separate and purify the enzyme. The recovery of intra cellular enzymes is more complicated and involves the disruption of cells and removal of cell debris and nucleic acids.

In some cases, an enzyme may be both intracellular and extracellular, which requires processing of both broth and cells. Intracellular enzymes may be released by increasing the permeability of the cell membrane. Certain salts, such as CaCl2, and other chemicals, such as dimethylsulfoxide (DMSO), and pH shift may be used for this purpose. If enzyme release is not complete, then cell disruption may be essential.

Enzymes have been significant industrial products for more than a hundred years. However, the range of potential applications is increasing rapidly. With the advent of recombinant DNA technology, it has become possible to make formerly rare enzymes in large quantities and hence to reduce cost. Also, in pharmaceutical manufacturing, the desire to make chirally pure compounds is leading to new opportunities. Chirality is important in a product; in a racemic mixture, one enantiomer is often therapeutically useful while the other may cause side effects and add no therapeutic value. The ability of enzymes to recognize chiral isomers and react with only one of them can be a key component in pharmaceutical synthesis. Processes that depend on a mixture of chemical and enzymatic synthesis are being developed for a new generation of pharmaceuticals.

Immobilzation

As enzymes are catalytic molecules they are not directly used up by the process in which they are used. However due to denaturation, they do loose activity with time. Therefore they should be stabilised against denaturation. When the enzymes are used in a soluble form they can

contaminate the product, and its removal may involve extra purification costs. In order to eliminate wastage and improve productivity the enzyme and product can be separated during the reaction. The enzyme can be imprisoned allowing it to be reused but also preventing contamination of the product – this is known as **immobilisation**.

Unstable enzymes may be **immobilised** by being attached to or located within an insoluble support, therefore the enzyme is not free in solution. Once attached, an enzyme's stability is increased, possibly because its ability to change shape is reduced.

The restriction of enzyme mobility in a fixed space is known as enzyme immobilization. Immobilization of enzymes provides important advantages, such as enzyme reutilization and elimination of enzyme recovery and purification processes and may provide a better environment for enzyme activity. Because enzymes are expensive, catalyst reuse is critical for many processes. Since some of the intracellular enzymes are membrane bound, immobilized enzymes provide a model system to mimic and understand the action of some membrane-bound intracellular enzymes. Product purity is usually improved, and effluent-handling problems are minimized by immobilization.

Compared with free enzymes, immobilised enzymes have several other advantages:

Advantages of immobilisation	Disadvantages of immobilisation
1. Easier to separate enzyme and products	1. Immobilisation may alter shape of enzyme
2. Allows catalysis in unfavourable media	2. May alter catalytic ability
3. Increases stability and can be manipulated easily	3. Enzyme may become detached
4. Allows continuous production/enzyme used for longer	4. Expensive
5. Enzyme can be recovered and reused	
6. Enzyme does not contaminate product/no purification required	

Methods of Immobilization

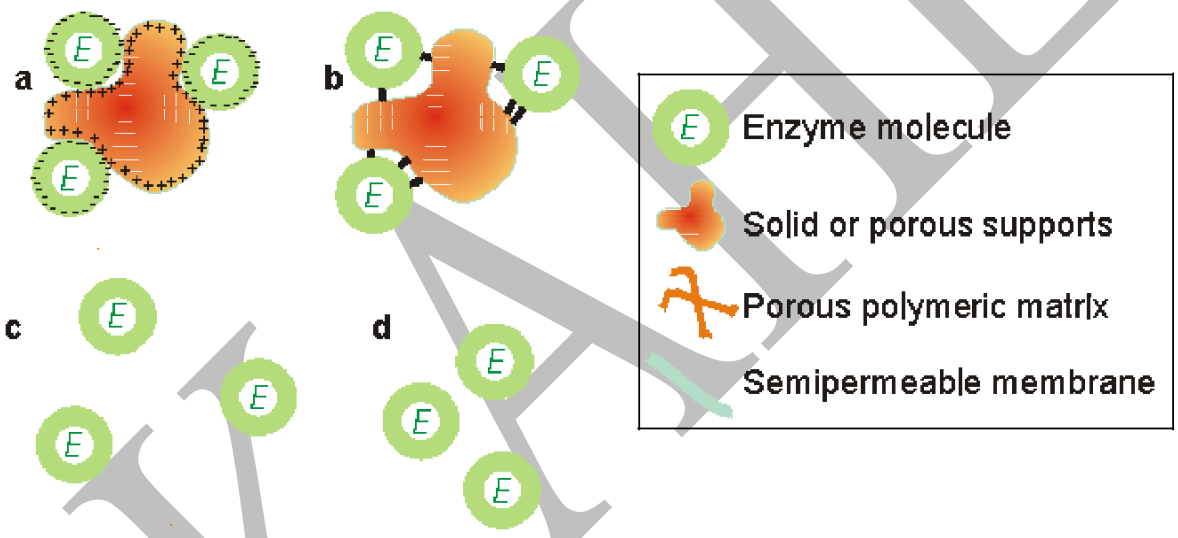
There are four main methods available for immobilising enzymes:

a. Adsorption in glass or alginate beads – enzyme is attached to the outside of an inert material

b. Cross-linkage to another chemical e.g. cellulose or glyceraldehydes.

c. Entrapment in a silica gel – enzyme is held in a mesh or capsule of an inert material.

d. Membrane confinement



Entrapment is the physical enclosure of enzymes in a small space. Matrix entrapment and membrane entrapment, including microencapsulation, are the two major methods of entrapment.

Matrices used for enzyme immobilization are usually polymeric materials such as calcium-alginate, agar, k-carrageenin, polyacrylamide, and collagen. However, some solid matrices, such as activated carbon, porous ceramic, and diatomaceous earth, can also be used for this purpose. The matrix can be a particle, a membrane, or a fiber. When immobilizing in a polymer matrix, enzyme solution is mixed with polymer solution before polymerization takes place. Polymerized gel-containing enzyme can be extruded, or else a template is used to

shape the particles from a liquid polymer–enzyme mixture. Entrapment and surface attachment may be used in combination in some cases.

Membrane entrapment of enzymes is possible; for example, hollow fiber units have been used to entrap an enzyme solution between thin, semipermeable membranes. Membranes of nylon, cellulose, polysulfone, and polyacrylate are commonly used. Configurations, other than hollow fibers, are possible, but in all cases a semipermeable membrane is used to retain high-molecular-weight compounds (enzyme) while allowing small-molecular-weight compounds (substrate or products) access to the enzyme.

A special form of membrane entrapment is microencapsulation. In this technique, microscopic hollow spheres are formed. The spheres contain the enzyme solution, and the spheres are enclosed within a porous membrane. The membrane can be polymeric or an enriched interfacial phase formed around a microdrop.
, enzyme entrapment may have its inherent problems, such as enzyme leakage into solution, significant diffusional limitations, reduced enzyme activity and stability, and lack of control of microenvironmental conditions. Enzyme leakage can be overcome by reducing the molecular weight cutoff of membranes or the pore size of solid matrices. Diffusion limitations can be eliminated by reducing the particle size of matrices and/or capsules. Reduced enzyme activity and stability are due to unfavorable microenvironmental conditions, which are difficult to control. However, by using different matrices and chemical ingredients, by changing processing conditions, and by reducing particle or capsule size, more favorable microenvironmental conditions can be obtained. Diffusion barrier is usually less significant in microcapsules as compared to gel beads **.**

Adsorption is the attachment of enzymes on the surfaces of support particles by weak physical forces, such as van der Waals or dispersion forces. The active site of the adsorbed enzyme is usually unaffected, and nearly full activity is retained upon adsorption. However, desorption of enzymes is a common problem, especially in the presence of strong hydrodynamic forces, because binding forces are weak. Adsorption of enzymes may be stabilized by cross-linking with glutaraldehyde. Glutaraldehyde treatment can denature some proteins. Support materials used for enzyme adsorption can be inorganic materials, such as alumina, silica, porous glass, ceramics, diatomaceous earth, clay, and bentonite, or organic materials, such as cellulose (CMC, DEAE-cellulose), starch, activated carbon, and ion-exchange resins, such as Amberlite, Sephadex, and Dowex. The surfaces of the support materials may need to be pretreated (chemically or physically) for effective immobilization.

Covalent binding is the retention of enzymes on support surfaces by covalent bond formation. Enzyme molecules bind to support material via certain functional groups, such as amino, carboxyl, hydroxyl, and sulfhydryl groups. These functional groups must not be in the active site. One common trick is to block the active site by flooding the enzyme solution with

a competitive inhibitor prior to covalent binding. Functional groups on support material are usually activated by using chemical reagents, such as cyanogen bromide, carbodiimide, and glutaraldehyde. Support materials with various functional groups. Binding groups on the protein molecule are usually side groups (R) or the amino or carboxyl groups of the polypeptide chain.The cross-linking of enzyme molecules using agents such as glutaraldehyde, bis-diazobenzidine, and 2,2-disulfonic acid is another method of enzyme immobilization. Cross-linking can be achieved in several ways: enzymes can be cross-linked with glutaraldehyde to form an insoluble aggregate, adsorbed enzymes may be cross-linked, or cross-linking may take place following the impregnation of porous support material with enzyme solution. Cross-linking may cause significant changes in the active site of enzymes, and severe diffusion limitations may result.

The most suitable support material and immobilization method vary depending on the enzyme and particular application. Two major criteria used in the selection of support material are the binding capacity of the support material, which is a function of charge density, functional groups, porosity, and hydrophobicity of the support surface; and stability and retention of enzymatic activity, which is a function of functional groups on support material and microenvironmental conditions.

If immobilization causes some conformational changes on the enzyme, or if reactive groups on the active site of the enzyme are involved in binding, a loss in enzyme activity can take place upon immobilization. Usually, immobilization results in a loss in enzyme activity and stability. However, in some cases, immobilization may cause an increase in enzyme activity and stability due to more favorable microenvironmental conditions. Because enzymes often have more than one functional site that can bind the surface, an immobilized enzyme preparation may be very heterogeneous. Even when binding does not alter enzyme structure, some enzyme can be bound with the active site oriented away from the substrate solution and toward the support surface, decreasing the access of the substrate to the enzyme. Retention of activity varies with the method used.

IMMOBILIZED ENZYME BIOREACTORS:

The methods for the heterogenisation (or localization) of enzymes

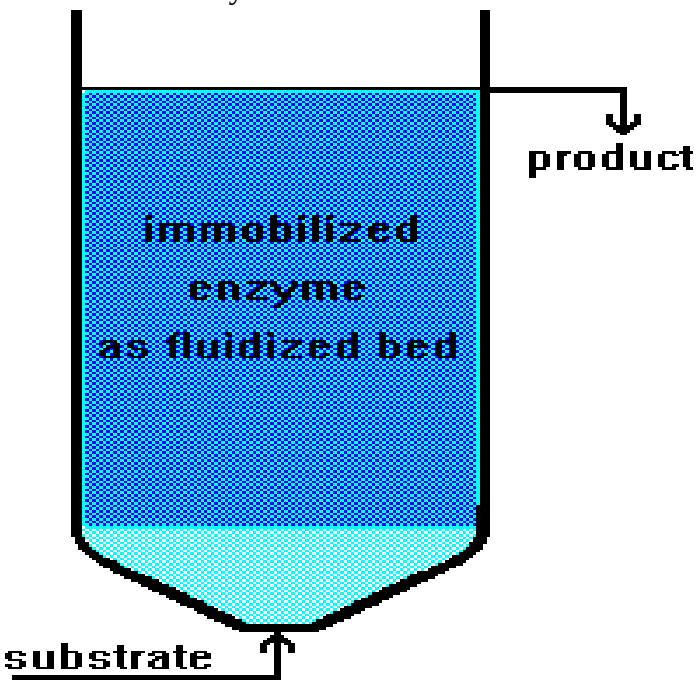
- by coupling them to insoluble supports or
- by entrapment.

Reactors for immobilized enzymes.

- Batch reactors
- Stirred-tank reactor
- Fixed-bed reactor
- Fluidized-bed reactor
- Continous reactor

Fluidized Bed Reactor:

- - a high viscosity substrate solution
- a gaseous substrate or product in a continuous reaction system
- care must be taken to avoid the destruction and decomposition of immobilized enzymes



CONTINOUS BIOREACTORS

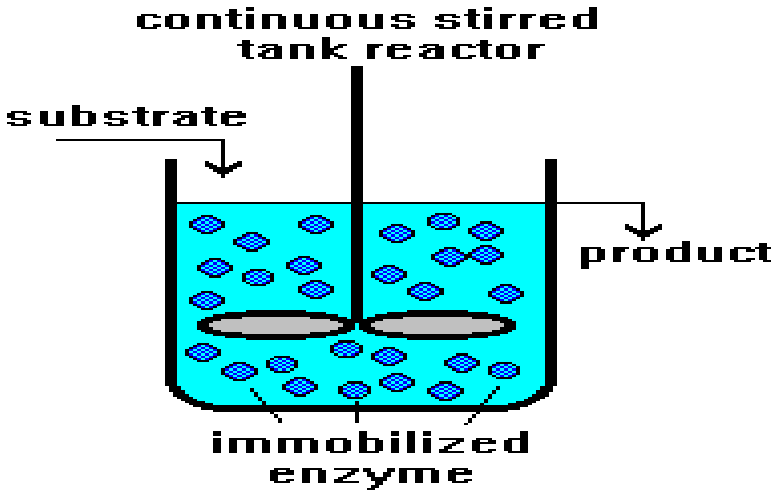
- An immobilized enzyme tends to decompose upon physical stirring.

- The batch system is generally suitable for the production of rather small amounts of chemicals.

Reactants are continuously fed into the reactor and emerge as continuous stream of product. Continuous reactors are used for a wide variety of chemical and biological processes within the food, chemical and pharmaceutical industries. A survey of the continuous reactor market will throw up a daunting variety of shapes and types of machine. Beneath this variation however lies a relatively small number of key design features which determine the capabilities of the reactor. When classifying continuous reactors, it can be more helpful to look at these design features rather than the whole system.

BENEFITS

- The rate of many chemical reactions is dependent on reactant concentration. Continuous reactors are generally able to cope with much higher reactant concentrations due to their superior heat transfer capacities. Plug flow reactors have the additional advantage of greater separation between reactants and products giving a better concentration profile.
- The small size of continuous reactors makes higher mixing rates possible.
- The output from a continuous reactor can be altered by varying the run time. This increases operating flexibility for manufacturers



BATCH FERMENTOR

The batch reactor is the generic term for a type of vessel widely used in the process industries. Its name is something of a misnomer since vessels of this type are used for a variety of process operations such as solids dissolution, product mixing, chemical reactions, batch distillation, crystallization, liquid/liquid extraction and polymerization. In some cases, they are not referred to as reactors but have a name which reflects the role they perform (such as crystallizer, or bioreactor).

A typical batch reactor consists of a tank with an agitator and integral heating/cooling system. These vessels may vary in size from less than 1 litre to more than 15,000 litres. They are usually fabricated in steel, stainless steel, glass-lined steel, glass or exotic alloy. Liquids and solids are usually charged via connections in the top cover of the reactor. Vapors and gases also discharge through connections in the top. Liquids are usually discharged out of the bottom.

The advantages of the batch reactor lie with its versatility. A single vessel can carry out a sequence of different operations without the need to break containment. This is particularly useful when processing toxic or highly potent compounds.

AGITATION

The usual agitator arrangement is a centrally mounted driveshaft with an overhead drive unit. Impeller blades are mounted on the shaft. A wide variety of blade designs are used and typically the blades cover about two thirds of the diameter of the reactor. Where viscous products are handled, anchor shaped paddles are often used which have a close clearance between the blade and the vessel walls.

Most batch reactors also use baffles. These are stationary blades which break up flow caused by the rotating agitator. These may be fixed to the vessel cover or mounted on the interior of the side walls.

Despite significant improvements in agitator blade and baffle design, mixing in large batch reactors is ultimately constrained by the amount of energy that can be applied. On large vessels, mixing energies of more than 5 Watts per litre can put an unacceptable burden on the cooling system. High agitator loads can also create shaft stability problems. Where mixing is a critical parameter, the batch reactor is not the ideal solution. Much higher mixing rates can be achieved by using smaller flowing systems with high speed agitators, ultrasonic mixing or static mixers.

Heating and cooling systems

Products within batch reactors usually liberate or absorb heat during processing. Even the action of stirring stored liquids generates heat. In order to hold the reactor contents at the desired temperature, heat has to be added or removed by a cooling jacket or cooling pipe. Heating/cooling coils or external jackets are used for heating and cooling batch reactors. Heat transfer fluid passes through the jacket or coils to add or remove heat.

Within the chemical and pharmaceutical industries, external cooling jackets are generally preferred as they make the vessel easier to clean. The performance of these jackets can be defined by 3 parameters:

- response time to modify the jacket temperature
- uniformity of jacket temperature
- stability of jacket temperature.

It can be argued that heat transfer coefficient is also an important parameter. It has to be recognized however that large batch reactors with external cooling jackets have severe heat transfer constraints by virtue of design. It is difficult to achieve better than 100 Watts/litre even with ideal heat transfer conditions. By contrast, continuous reactors can deliver cooling

capacities in excess of 10,000 W/litre. For processes with very high heat loads, there are better solutions than batch reactors.Fast temperature control response and uniform jacket heating and cooling is particularly important for crystallization processes or operations where the product or process is very temperature sensitive. There are several types of batch reactor cooling jackets:

Single external jacket

Batch reactor with single external cooling jacket

The single jacket design consists of an outer jacket which surrounds the vessel. Heat transfer fluid flows around the jacket and is injected at high velocity via nozzles. The temperature in the jacket is regulated to control heating or cooling.

The single jacket is probably the oldest design of external cooling jacket. Despite being a tried and tested solution, it has some limitations. On large vessels, it can take many minutes to adjust the temperature of the fluid in the cooling jacket. This results in sluggish temperature control. The distribution of heat transfer fluid is also far from ideal and the heating or cooling tends to vary between the side walls and bottom dish. Another issue to consider is the inlet temperature of the heat transfer fluid which can oscillate (in response to the temperature control valve) over a wide temperature range to cause hot or cold spots at the jacket inlet points.

Half coil jacket

Batch reactor with half coil jacket

The half coil jacket is made by welding a half pipe around the outside of the vessel to create a semi circular flow channel. The heat transfer fluid passes through the channel in a plug flow fashion. A large reactor may use several coils to deliver the heat transfer fluid. Like the single jacket, the temperature in the jacket is regulated to control heating or cooling.

The plug flow characteristics of a half coil jacket permits faster displacement of the heat transfer fluid in the jacket (typically less than 60 seconds). This is desirable for good temperature control. It also provides good distribution of heat transfer fluid which avoids the problems of non uniform heating or cooling between the side walls and bottom dish. Like the single jacket design however the inlet heat transfer fluid is also vulnerable to large oscillations (in response to the temperature control valve) in temperature.

Constant flux cooling jacket

Batch reactor with constant flux (Coflux) jacket

The constant flux cooling jacket is a relatively recent development. It is not a single jacket but has a series of 20 or more small jacket elements. The temperature control valve operates by opening and closing these channels as required. By varying the heat transfer area in this way, the process temperature can be regulated without altering the jacket temperature.

The constant flux jacket has very fast temperature control response (typically less than 5 seconds) due to the short length of the flow channels and high velocity of the heat transfer fluid. Like the half coil jacket the heating/cooling flux is uniform. Because the jacket operates at substantially constant temperature however the inlet temperature oscillations seen in other jackets are absent. An unusual feature of this type jacket is that process heat can be measured very sensitively. This allows the user to monitor the rate of reaction for detecting end points, controlling addition rates, controlling crystallization etc.

Applications

Batch reactors are often used in the process industry. Batch reactors also have many laboratory applications, such as small scale production and inducing fermentation for beverage products. They also have many uses in medical production. Batch reactors are generally considered expensive to run, as well as variable product reliability. They are also used for experiments of reaction kinetics, volatiles and thermodynamics. Batch reactors are also highly used in waste water treatment. They are effective in reducing BOD (biological oxygen demand)of influent untreated water.

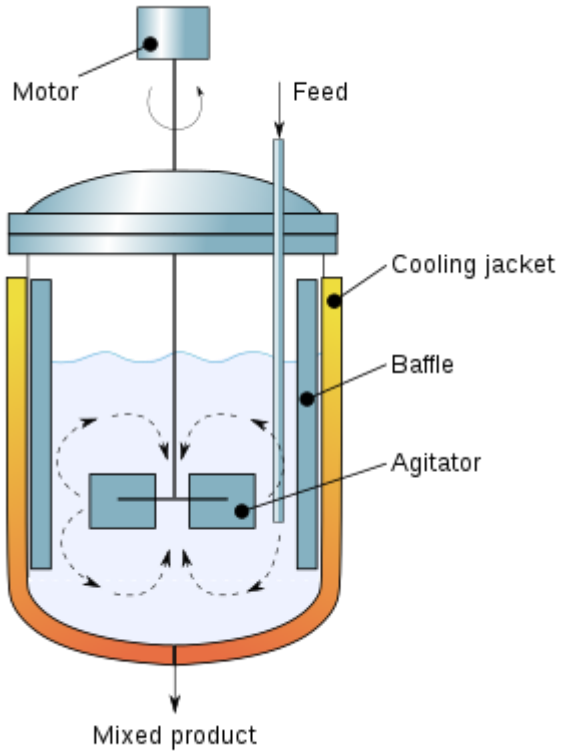
CONTINOUS REACTOR

Continous reactors carry material as a flowing stream. Reactants are continuously fed into the reactor and emerge as continuous stream of product. Continuous reactors are used for a wide variety of chemical and biological processes within the food, chemical and pharmaceutical industries. A survey of the continuous reactor market will throw up a

daunting variety of shapes and types of machine. Beneath this variation however lies a relatively small number of key design features which determine the capabilities of the reactor. When classifying continuous reactors, it can be more helpful to look at these design features rather than the whole system.

Benefits of continuous reactors

- The rate of many chemical reactions is dependent on reactant concentration. Continuous reactors are generally able to cope with much higher reactant concentrations due to their superior heat transfer capacities. Plug flow reactors have the additional advantage of greater separation between reactants and products giving a better concentration profile.
- The small size of continuous reactors makes higher mixing rates possible.
- The output from a continuous reactor can be altered by varying the run time. This increases operating flexibility for manufacturers.



FIXED BED REACTOR

A fluidized bed reactor (FBR) is a type of reactor device that can be used to carry out a variety of multiphase chemical reactions. In this type of reactor, a fluid (gas or liquid) is passed through a solid granular material (usually a catalyst possibly shaped as tiny spheres) at high enough velocities to suspend the solid and cause it to behave as though it were a fluid. This process, known as fluidization, imparts many important advantages to the FBR. As a result, the fluidized bed reactor is now used in many industrial applications.

Principles

The solid substrate (the catalytic material upon which chemical species react) material in the fluidized bed reactor is typically supported by a porous plate, known as a distributor. The fluid is then forced through the distributor up through the solid material. At lower fluid velocities, the solids remain in place as the fluid passes through the voids in the material. This is known as a packed bed reactor. As the fluid velocity is increased, the reactor will reach a stage where the force of the fluid on the solids is enough to balance the weight of the solid material. This stage is known as incipient fluidization and occurs at this minimum fluidization velocity. Once this minimum velocity is surpassed, the contents of the reactor bed begin to expand and swirl around much like an agitated tank or boiling pot of water. The reactor is now a fluidized bed. Depending on the operating conditions and properties of solid phase various flow regimes can be observed in this reactor.

Advantages

The increase in fluidized bed reactor use in today's industrial world is largely due to the inherent advantages of the technology.

- **Uniform Particle Mixing:** Due to the intrinsic fluid-like behaviour of the solid material, fluidized beds do not experience poor mixing as in packed beds. This complete mixing allows for a uniform product that can often be hard to achieve in other reactor designs. The elimination of radial and axial concentration gradients also allows for better fluid-solid contact, which is essential for reaction efficiency and quality.
- **Uniform Temperature Gradients:** Many chemical reactions require the addition or removal of heat. Local hot or cold spots within the reaction bed, often a problem in packed beds, are avoided in a fluidized situation such as an FBR. In other reactor types, these local temperature differences, especially hotspots, can result in product degradation. Thus FBRs are well suited to exothermic reactions. Researchers have also learned that the bed-to-surface heat transfer coefficients for FBRs are high.
- **Ability to Operate Reactor in Continuous State:** The fluidized bed nature of these reactors allows for the ability to continuously withdraw product and introduce new reactants into the reaction vessel. Operating at a continuous process state allows manufacturers to produce their various products more efficiently due to the removal of startup conditions in batch process.

Disadvantages

- **Increased Reactor Vessel Size:** Because of the expansion of the bed materials in the reactor, a larger vessel is often required than that for a packed bed reactor. This larger vessel means that more must be spent on initial capital costs.
- **Pumping Requirements and Pressure Drop:** The requirement for the fluid to suspend the solid material necessitates that a higher fluid velocity is attained in the reactor. In order to achieve this, more pumping power and thus higher energy costs are needed. In addition, the pressure drop associated with deep beds also requires additional pumping power.
- **Particle Entrainment:** The high gas velocities present in this style of reactor often result in fine particles becoming entrained in the fluid. These captured particles are then carried out of the reactor with the fluid, where they must be separated. This can be a very difficult and expensive problem to address depending on the design and function of the reactor. This may often continue to be a problem even with other entrainment reducing technologies.
- **Lack of Current Understanding:** Current understanding of the actual behavior of the materials in a fluidized bed is rather limited. It is very difficult to predict and calculate the complex mass and heat flows within the bed. Due to this lack of understanding, a pilot plant for new processes is required. Even with pilot plants, the scale-up can be very difficult and may not reflect what was experienced in the pilot trial.
- **Erosion of Internal Components:** The fluid-like behavior of the fine solid particles within the bed eventually results in the wear of the reactor vessel. This can require expensive maintenance and upkeep for the reaction vessel and pipes.
- **Pressure Loss Scenarios:** If fluidization pressure is suddenly lost, the surface area of the bed may be suddenly reduced. This can either be an inconvenience (e.g. making bed restart difficult), or may have more serious implications, such as runaway reactions (e.g. for exothermic reactions in which heat transfer is suddenly restricted)

APPLICATION:
MEDICAL USES:
Diagnostic:

Reagent strips have been designed to perform rapid and semi-quantitative analysis for glucose. They are easy to use and require no addition laboratory equipment or reagents. A **Clinistix** contains molecules of two enzymes fixed onto the end of a plastic strip. When this is dipped into a sample, the first, **glucose oxidase,** converts any glucose molecules, by reaction with atmospheric oxygen, into gluconic acid and hydrogen peroxide. The second enzyme, **peroxidase,** then enables the hydrogen peroxide to react with an indicator to give a purple colour. A colour chart on the strip will match the shade of purple to the glucose concentration. The idea of fixing an enzyme to a plastic support is the basic principle of

biosensors - mobile, cheap and accurate sensors which can monitor a number of biochemicals in blood, urine and also in food and soil. Over the next few years, the use of biosensors is likely to increase dramatically.

Step 1: *Glucose oxidase*

Glucose ----------> Gluconic Acid + Hydrogen Peroxide

Step 2:

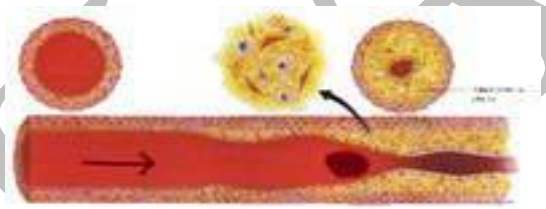
Peroxidase

Hydrogen peroxide + (reduced) orthotolidine ----------> Water + (oxidized) orhotolidine

Brown *Blue*

Treatment:

The approved treatments for strokes are i.v. prescription drugs Urokinase, Streptokinase and t-PA (tissue-plasminogen-activator). All three are available in the form of intravenous infusion only. To work best, they must be given **within three hours from the onset of the attack**.



A floating time bomb, an embolism could be trapped and block any artery. Fibrin filaments wrap around and entrap a single red blood cell.

The industrial use of enzymes (using the whole microbe)

Historically, three examples of the industrial use of microbes (and their enzymes) are:
Brewing:

In which yeast (*Saccharomyces cerevisiae)* reacts with the sugars in fruit or malted barley to produce ethanol and carbon dioxide. In Nature, the yeast is competing with bacteria for the available sugar in the wild fruit. Its response is a form of 'chemical warfare', since the ethanol it makes it poisonous to many bacteria and, indeed, ethanol can be used as a disinfectant (though it stings a lot!). The process of fermentation takes several days or weeks and results in a product with a maximum alcohol content of about 12% - above which the yeast is itself killed. More alcoholic beverages can only be made by distilling the raw brew. From wine we get brandy, cider gives Calvados, ale gives whisky.

Surplus yeast could then be used to mix with flour and water to make (leavened) bread. Hence brewing and baking are closely related. In baking, the carbon dioxide is the important product, since it makes the dough rise. the ethanol evaporates off in the baking, so you cannot get drunk by eating bread! The reason why 'in-store bakeries' are so popular in supermarkets is that the smell of baking bread (and the ethanol) in the air circulates throughout the store and this stimulates our 'hunger centre' and so we buy more - quite true! These days the surplus yeast is heated and processed to make Marmite.

Vinegar production:

Louis Pasteur was employed by Emperor Napoleon III in 1864 to research why (sometimes) wine went 'off' or turned to vinegar. Pasteur soon showed that the historic 'spontaneous generation' theory was wrong – substances did not spontaneously go 'bad'; instead he formulated the modern 'germ theory'. This states that it is the existence of microbes which makes food rot. The secret to keeping wine was thus to keep the microbes out i.e. bottle it, rather than storing it in open casks. To make vinegar, wine is slowly pored over oak chips in a tall tower, open to the air. Bacteria (*Acetobacter)* on the wood oxidise the ethanol in the wine and turn it into ethanoic acid or vinegar, giving out a great deal of heat as well. If the vinegar is made from fermented raisins and stored in oak vats (similar to the *solera system* used for making sherry) then the sweet, highly-prized ***Balsamic vinegar*** is made – mainly around Modena in Italy. Note how different forms of 'sweet and sour' dishes are a part of local cuisine from all over the World!

Yoghurt production:

Milk goes sour within a few hours in the hot conditions common in the Middle East. If stored in a leather bag and mixed with a suitable starter culture, however, it rapidly turns into yoghurt, which will keep for several days. This happy accident led to the development of the modern industry, which thus has its roots in Biblical times (Abraham was said owe his longevity to drinking yoghurt). In Russia, *Kefir* is a similar ancient product with a fascinating modern commercial history, beginning in 1908 with the attempted seduction of a Prince Barcharov, the kidnapping of a beautiful maiden (Irina) and a court case with a fine of 'the

Prophet's Grains', which were the sacred starter culture for Kefir. In 1973 the Minister for Food in the USSR wrote to Irena thanking her for bringing Kefir to the Soviet people!

The industrial use of enzymes (not using the whole microbe)

Leather:

The earliest example of enzymes in industry is a colourful one! To make leather soft, it has to be **bated,** which means that some of the protein fibres are removed. Otherwise, the leather will be hard - perfect for the soles of shoes but of little use for anything else.

The Roman writer Pliny reported the use of pigeon droppings for this process over 2000 years ago. Later, leather was bated by smearing it with dog excrement! People used to go around the streets collecting dog turds and then rubbed them into the skins by hand, paddle or by trampling it in by foot.

By the early 1900's it was known that the excrement was rich in bacteria which produced p**roteases**, which degraded part of the leather. It was a highly skilled job to prevent the enzymes damaging the leather, which is largely made up of protein. But thanks to the The German scientist Rŝhm, developed a standardized ***bate*** in 1908, based on an extract from the pancreases of slaughtered animals. This contained trypsin - one of a mixture of enzymes found in the digestive system. Since then, all bates have been based on enzyme preparations, though now bacterial and fungal enzymes are used instead.

Washing powders:

Rŝhm was quite a genius - he was the first to examine the chemical composition of dirt on laundry and he came up with the idea of using the pancreatic extract to wash clothes. His wife tested trypsin at home on their dirty underwear - and found it was excellent! When soaked overnight, their clothes became clean and the water became dirty. So, he patented his idea and in 1914, developed the first enzymatic washing agent. It was so effective that only a small quantity was required: it was sold as a spot remover. Unfortunately German housewives were used to bulky washing powders that produced lots of lather so they regarded it with suspicion. In 1915, some people even thought it was a hoax. The product was investigated by scientists who found that it really did work – indeed, it was about 50 years ahead of its time: it wasn't until the 1960s that enzymatic detergents gained widespread acceptance.

The mass-production of an alkaline protease suitable for wash conditions began in 1962. Unlike trypsin, this wasn't an animal extract but a product of microbial fermentation. This

new enzyme was initially shunned by detergent manufacturers but there were exceptions. In 1963, it was incorporated into ***Bio-tex,*** which took the market by storm. Industry began to take notice of enzymes and by 1967 their widespread use in domestic detergents was commonplace.

Enzymes used:

These are produced from *Bacillus licheniformis*. They are usable at high pH and temperatures up to 60^{o} C and are all relatively non-specific proteases. They attack the C-terminal of carboxyl amino acids producing small peptides which can be readily dissolved by the detergent. There is currently considerable interest in developing better proteases for washing powders through protein engineering, particularly in engineering oxidation-resistance into the proteases.

Engineered Subtilisin for improved wash performance

Not just proteases

Since the 1990's, amylases have also been added to detergents to remove stains from spaghetti, sauces, oatmeal and baby foods. In 1988 the first detergent lipase was released - the first commercial enzyme to be produced from a genetically-modified organism (GMO). Today more than 90% of detergent enzymes are made from GMOs.

The detergent industry has been the largest market for industrial enzymes for over 25 years, accounting for 37% of world sales of enzymes. Apart from laundry detergents, many automatic dishwashing detergents now also contain enzymes.

To maximise the effectiveness and to be as economical as possible in the production process the enzyme molecules must be brought into maximum contact with the substrate molecules. This can be achieved by mixing the solutions of enzyme and substrate in suitable concentrations. **However t**his means that the enzyme is 'lost' with each batch of product made and that the end-product will be contaminated too – as in cheese manufacture:

Cheese making:

Warm milk is mixed (about 2000:1) with the enzyme **rennin (***rennet)* (formerly extracted from (dead) calves' stomachs, but now produced from bacteria) and allowed to react for several hours. The ***caesinogen*** in the milk is uncoiled and clots to ***casein***. This turns the milk solid. The **curds** (solid) are then cut with a knife and the **whey** (liquid) drained away and fed to animals (remember Miss Tuffet?). The chopped up curd is then salted and placed

in a mould before squeezing to remove any trapped air (a process known as 'cheddaring' – hence Cheddar cheese). Sometimes the cheese is then dipped in brine or a solution of fungal spores to inoculate it and produce a surface 'rind'.

The cheese is then left at a constant, low, temperature (in the old days, in a cave, hence many cheeses are associated with cave-rich districts) to mature. this may take up to a year or more, so cheese-making was an important way of preserving a valuable food through the winter in the days before refrigeration.

The 'blue' in cheeses such as Stilton, is added by pushing spore-covered wires (*Penicillium notatum*- the same fungus that gave us penicillin) into the partially ripened cheese. This fungus needs oxygen to make the blue pigment, so holes have to be made in the cheese – the more holes, the faster the blue veins develop.
The role of the rennin in young mammals is to clot the mother's milk in baby's stomach. This then 'tricks' the stomach into keeping the contents there for several hours, thus allowing protein digestion mother to get some (much-needed) rest! In most mammals the rennin is only made until the animal is weaned, but in Caucasian people, milk was (historically) drunk throughout life and so the enzyme continues to be made, even in adults. Also made is the enzyme **lactase**, which breaks down the milk sugar and stops the bacteria from fermenting it in the colon, with subsequent large volumes of gas produced and embarrassing side–effect.

ENZYME ELECTRODES

Starting from the state of the art, principles for improving the analytical characteristics of enzyme electrodes are discussed. Coupling of appropriate amperometric electrode processes with enzyme systems, e.g. urease or aminopeptidases, results in a simplification of operation. Optimal sample frequencies are realized on the basis of enzyme membranes, with both a small characteristic diffusion time and a high enzyme activity, applied in a well-designed sample-processing system. Coupled enzyme reactions of the sequence or competition type are successfully used for extension to new analytes, e.g. inhibitors, cofactors or alternative substrates. Cyclization of the analyte enhances the sensitivity of enzyme electrodes to the nanomolar concentration range. Enzymic anti-interference layers are a tool for improving the sensor specificity. The operational characteristics of enzyme electrodes are thus adaptable to any given analytical problem.

The principle behind the enzyme electrode concept is that the molecule of interest reacts in an enzyme-catalyzed process that either yields products or involves co-substrates that are readily analyzed electrochemically. In practice, the enzyme electrode is made by placing a membrane containing the immobilized enzyme over a conventional electrode that is sensitive to either

the product or the unconsumed co-substrate. A complete sensor can be constructed by 1incorporating the enzyme electrode and a second, reference electrode to indicate the ambient concentration of the product or co-substrate. The difference between the signals of these two electrodes, determined by an appropriate method, can indicate the concentration of the chemical of interest. This approach provides an indirect means of monitoring certain molecules that cannot be readily analyzed by direct electrochemical methods. Although the enzyme electrode principle is widely known, development of practical sensors has been somewhat limited. This chapter discusses the theory of chemical transport and reaction related to enzyme electrodes. Models are proposed to describe the steady–state response of one and two substrate enzyme electrodes. These models provide a better understanding of electrode operation and lead to recommendations for improved sensor design.

UNIT – V

Applications of enzymes:

Enzymes used in different industries, Enzyme catalysis in organic solvents, enzyme replacement therapy – definition, modes of administration, enzyme deficiency disorders and enzyme therapy; Application of enzymes: Cosmetic benefits, Enzyme-based biosensors; Enzymes in clinical diagnosis: primary and secondary serum enzymes, Intracellular distribution of diagnostic enzymes, Enzyme markers of Xenobiotic toxicity - Pharmacogenomics related to polymorphism of drug metabolizing enzymes, , KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway.

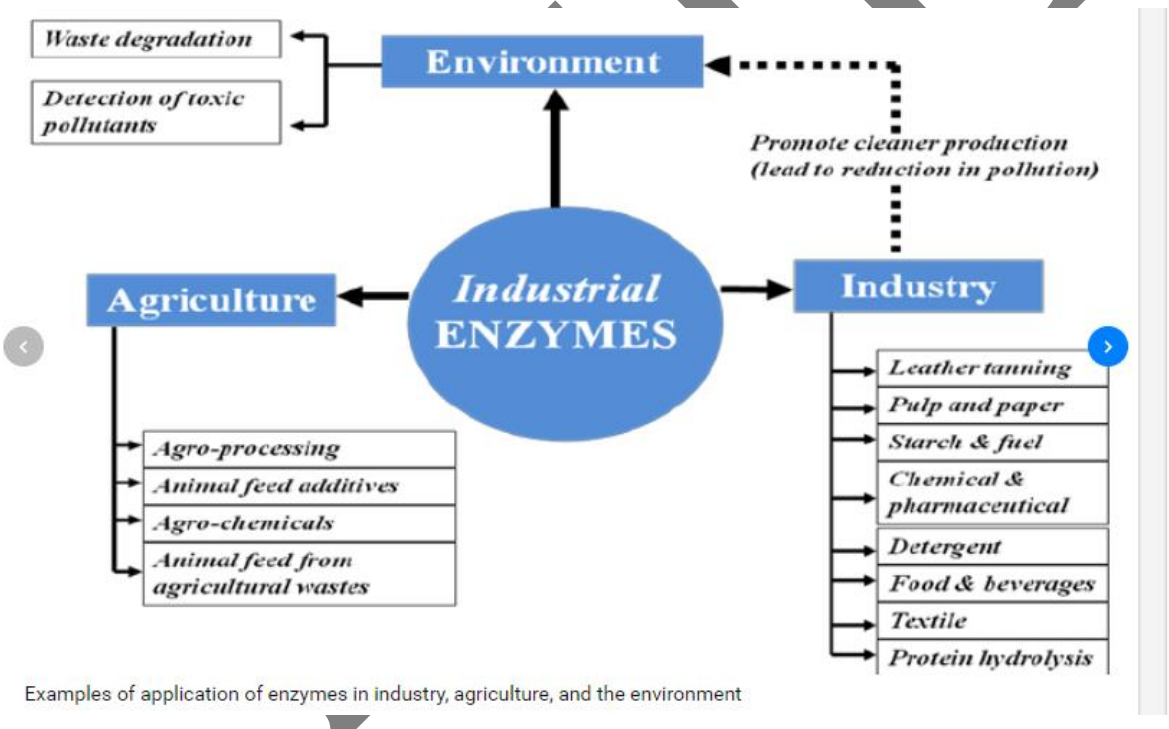
INDUSTRIAL APPLICATION

Enzymes are used in the chemical industry and other industrial applications when extremely specific catalysts are required. Enzymes in general are limited in the number of reactions they have evolved to catalyze and also by their lack of stability in organic solvents and at high temperatures. As a consequence, protein engineering is an active area of research and involves attempts to create new enzymes with novel properties, either through rational design or *in vitro* evolution. These efforts have begun to be successful, and a few enzymes have now been designed "from scratch" to catalyze reactions that do not occur in nature

Application	Enzymes used	Uses
Biofuel industry	Cellulases	Break down cellulose into sugars that can be fermented to produce cellulosic ethanol.
	Ligninases	Pretreatment of biomass for biofuel production.
Biological detergent	Proteases, amylases, lipases	Remove protein, starch, and fat or oil stains from laundry and dishware.
	Mannanases	Remove food stains from the common food additive guar gum
Brewing	Amylase, glucanases, prote	Split polysaccharides and proteins in

industry	ases	the malt.
	Betaglucanases	Improve the wort and beer filtration characteristics.
	Amyloglucosidase and pull ulanases	Make low-calorie beer and adjust fermentability.
	Acetolactate decarboxylase (ALDC)	Increase fermentation efficiency by reducing diacetylformation.
Culinary uses	Papain	Tenderize meat for cooking.
Dairy industry	Rennin	Hydrolyze protein in the manufacture of cheese.
	Lipases	Produce Camembert cheese and blue cheeses such as Roquefort.
Food processing	Amylases	Produce sugars from starch, such as in making high-fructose corn syrup
	Proteases	Lower the protein level of flour, as in biscuit-making.[
	Trypsin	Manufacture hypoallergenic baby foods
	Cellulases, pectinases	Clarify fruit juices.
Molecular	Nucleases, DNA	Use restriction digestion and the polymerase chain reaction to

biology	ligase and polymerases	create recombinant DNA
Paper industry	Xylanases, hemicellulases a nd lignin peroxidases	Remove lignin from kraft pulp
Personal care	Proteases	Remove proteins on contact lenses to prevent infections
Starch industry	Amylases	Convert starch into glucose and various syrups



Examples of application of enzymes in industry, agriculture, and the environment

Enzyme Deficiency

One way to sell a product is to persuade people they lack something and can get it by buying whatever you sell. Michael Pinkus, D.C., and MediaPower, Inc., would like you to believe that "enzyme deficiency" is widespread and can be remedied by a product called Nu-Zymes™. Media Power,located in Portland, Maine, provides services to media hosts and guests through InterviewWorld and sells various products through its outlet MPDirect. Pinkus, a chiropractor who lectures and writes about "alternative health care," is said to have

many movie stars and prominent athletes among his clients. His biographical sketch says that "his extensive research into nutritional solutions for chronic pain and illness has resulted in the special formulation of a complete line of natural health products" and that he has had more than 500 radio and television talk show appearances.

Nu-Zymes is said to contain amylase, protease, lactase, lipase, cellulase, *Lacobacillus acidophilus*, and CereCalase (a blend of phytase, hemicellulase, and beta-glucanase). The claims for Nu-Zymes are elaborated on the Web sites Dr. Pinkus.com and MediaPower.com and in Pinkus's booklet "Ultimate Health," which Media Power published in 2001. Individually and collectively, they assert that:

- Many Americans suffer from "enzyme deficiency" that can be corrected by taking Nu-Zymes.
- The American diet is generally enzyme-deficient because most of the food we consume has been cooked or processed.
- Lack of enzymes in food strains the human body.
- Enzyme deficiency causes heart disease, joint pain, obesity, and many other health problems that Nu-Zymes can correct.

Enzyme therapy

Enzyme replacement therapy (ERT) is a medical treatment which replaces an enzyme that is deficient or absent in the body. Usually, this is done by giving the patient an intravenous (IV) infusion of a solution containing the enzyme.

ERT is currently available for some lysosomal storage diseases: Gaucher disease, Fabry disease, MPS I, MPS II (Hunter syndrome), MPS VI and Pompe disease. ERT does not correct the underlying genetic defect, but it increases the concentration of the enzyme that the patient is lacking. ERT has also been used to treat patients with severe combined immunodeficiency (SCID) resulting from an adenosine deaminase deficiency (ADA-SCID)

Other treatment options for patients with enzyme or protein deficiencies include substrate reduction therapy, gene therapy, and bone-marrow derived stem cell transplantation.

Medical uses

Lysosomal storage diseases are fatal group of diseases and a main application of ERT. Lysosomes are cellular organelles that are responsible for the metabolism of many different macromolecules and proteins. They use enzymes to break down macromolecules, which are recycled or disposed. As of 2012, there are 50 lysosomal storage diseases, and more are still being discovered. These disorders arise because of genetic mutations that prevent the production of certain enzymes used in the lysosomes.The missing enzyme often leads to a build-up of the substrate within the body. This can result in a variety of symptoms, many of which are severe and can affect the skeleton, brain, skin, heart, and the central nervous system.[Increasing the concentration of the missing enzyme within the body has been shown to improve the body's normal cellular metabolic processes and reduce substrate concentration in the body.

ERT has also been successful in treating severe combined immunodeficiency caused by an adenosine deaminase deficiency (ADA-SCID). This is a fatal childhood disease that requires early medical intervention.When the enzyme adenosine deaminase is deficient in the body, the result is a toxic build-up of metabolites that impair lymphocytedevelopment and function Many ADA deficient children with SCID have been treated with the polyethylene glycol-conjugated adenosine deaminase (PEG-ADA) enzyme. This is a form of ERT that has resulted in healthier, longer lives for patients with ADA-SCID

Nine lysosomal storage diseases and corresponding enzyme therapies. Information in this table is from pivotal clinical trials

Disease	Enzyme	Administration and Dosage in Pivotal Clinical Trials
Fabry disease	Agalsidase beta	IV *Age range 16–61*: 1 mg/kg every 2 weeks
Fabry disease	Agalsidase alfa*	IV *Adult males*: 0.2 mg/kg every 2 weeks
Gaucher disease	Imiglucerase	IV *Age range 12–69*: 15–60 U/kg every 2 weeks
Gaucher disease	Taliglucerase alfa	IV *Age range 19–74*: 11–73 U/kg every 2 weeks
Gaucher disease	Velaglucerase alfa	IV *Age range 4–62*: 15–60 U/kg every 2 weeks
Gaucher disease type I	Alglucerase	IV *Age range 7–42*: 2.5 U/kg 3 times a week, up to 60 U/kg as frequently as once a week or as infrequently as every 4 weeks
Lysosomal acid lipase deficiency (Wolman disease/CESD)	Sebelipase alpha	IV *Age range 1–6 months*: 1 mg/kg every 2

		weeks *Age range 4–58*: 1–3 mg/kg weekly
MPS I	Laronidase	IV *Age range 6–43*: 100 U/kg weekly
MPS II	Idursulfase	IV *Age range 5–31*: 0.5 mg/kg weekly
MPS IVA	Elosulfase alpha	IV *Age range 5–57*: 2 mg/kg weekly
MPS VI	Galsulfase	IV *Age range 5–29*: 1 mg/kg weekly
Pompe disease	Alglucosidase alpha (160L bioreactor)	IV *Age range 1–3.5*: 20 mg/kg every 2 weeks
Pompe disease	Alglucosidase alpha (4000L bioreactor)	IV *Age range 10–70*: 20 mg/kg every 2 weeks

Administration

ERT is administered by IV infusion. Typically, infusions occur every week or every two weeks.] For some types of ERT, these infusions can occur as infrequently as every four weeks

Complications

ERT is not a cure for lysosomal storage diseases, and it requires lifelong IV infusions of the therapeutic enzymeThis procedure is expensive; in the United States, it may cost over $200,000 annually.] The distribution of the therapeutic enzyme in the body (biodistribution) after these IV infusions is not uniform. The enzyme in less available to certain areas in the body, like the bones, lungs, brain. For this reason, many symptoms of lysosomal storage diseases remain untreated by ERT, especially neurological symptoms. Additionally, the efficacy of ERT is often reduced due to an unwanted immune response against the enzyme, which prevents metabolic function

Other treatments for enzyme deficiencies

Substrate reduction therapy is another method for treating lysosomal storage diseases. In this treatment, the accumulated compounds are inhibited from forming in the body of a patient

suffering from a lysosomal storage disease The accumulated compounds are responsible for the symptoms of these disorders, and they form via a multi-step biological pathway. Substrate reduction therapy uses a small molecule to interrupt this multi-step pathway and inhibit the biosynthesis of these compounds This type of treatment is taken orally. It does not induce an unwanted immune response, and a single type of small molecule could be used to treat many lysosomal storage diseases.] Substrate reduction therapy is FDA approved and there is at least one treatment available on the market.

Gene therapy aims to replace a missing protein in the body through the use of vectors, usually viral vectorsIn gene therapy, a gene encoding for a certain protein is inserted into a vector. The vector containing the therapeutic gene is then injected into the patient. Once inside the body the vector introduces the therapeutic gene into host cells, and the protein encoded by the newly inserted gene is then produced by the body's own cells. This type of therapy can correct for the missing protein/enzyme in patients with lysosomal storage diseases.

Hematopoietic stem cell (HSC) transplantation is another treatment for lysosomal storage diseases. HSCs are derived from bone-marrow. These cells have the ability to mature into the many cell types that comprise blood, including red blood cells, platelets, and white blood cells. Patients suffering from enzyme deficiencies often undergo HSC transplantations in which HSCs from a healthy donor are injected. This treatment introduces HSCs that regularly produce the deficient enzyme since they have normal metabolic function. This treatment is often used to treat the central nervous system of patients with some lysosomal storage disease.

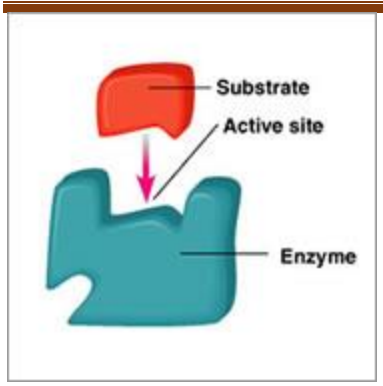
Application of enzymes

Cosmetic benefits

Enzymes are effective cellular catalysts responsible for controlling thousands of reactions in the cell. Discovered in 1833, it was not until 1926 that it was established that enzymes were special, active proteins.

Diastase the first to be extracted (from malt) was eventually shown to be amylase, an enzyme that converts starch to sugar.

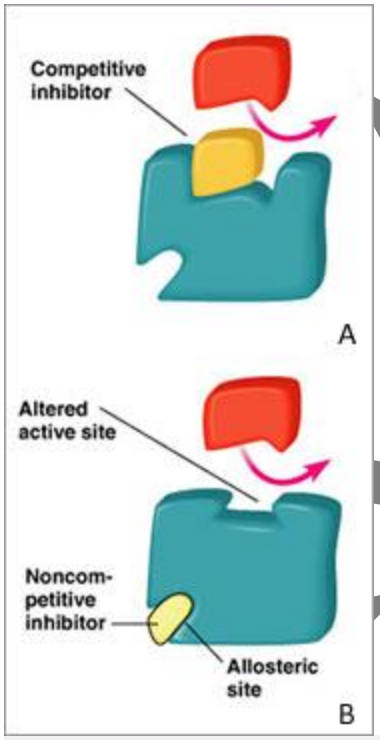
The next enzymes discovered, also in the late 19th century, were isolated from the stomach and identified as pepsin and trypsin. These are proteases, which are enzymes that digest protein by breaking down peptide bonds.



At the temperature and pH usually present in the cells, most chemical reactions would not proceed fast enough to maintain cell viability without enzymes.

Enzymes are specific for the type of reaction they catalyse, and they may be specific for the type of substrate they use: therefore, there are no by-products and no side effects.

Enzymes work by a shape recognition method, the substrate must form a complex with the enzyme. When the enzyme locks onto the substrate a reaction (energy) will occur. When this happens, the substrate then binds with the enzymes reactive site.



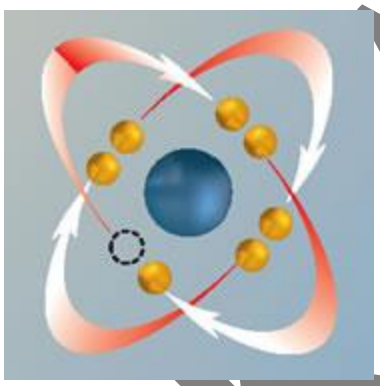
Action of enzyme inhibitors Medical science has shown that it is possible to synthesize powerful inhibitors that closely resemble the transition state and bind to the enzyme. Most commonly, inhibition occurs when one molecule interferes with the binding of the substrate to the enzyme.
Interest in the use of inhibitors in cosmetics has increased, and much of it has been directed toward those with and anti-tyrosinase activity for whitening of the skin, those who exhibit

anti-elastase activity, which prevents elastin from cross-linking and results in the skin losing its flexibility.
These cosmetic inhibitors work by non-competitively binding to the enzyme, altering its structure, so that it either does not work as well, or it does not work at all.
Enzymes in cosmetic formulations and clinic treatments
The use of coenzymes and cofactors in cosmetics may represent a safe way to promote the efficient functions of the enzymes in the skin to help maintain the healthy appearance of the skin.

The coenzymes and cofactors are stable, low in molecular weight and should penetrate through the stratum corneum to help activate the enzymes present. They are relatively easy to formulate into cosmetics and most important, these materials offer a good degree of safety when topically applied.

The use of many enzymes themselves in cosmetic formulations if fraught with difficulty as high molecular weight entities require a specific shape to function effectively. This is difficult to maintain when adding to a cosmetic formulation.

The cosmetic industry however, has for some time now been using the proteolytic (dissolves protein) enzymes, such as papain, bromelaine and others for resurfacing and skin smoothing. These substances have proven to be a very useful tool for the skin treatment therapist in treating many the skin conditions related to skin aging, acne, congestion and pigmentation.



Enzymes as anti-free radicals
One area where the topical application of enzymes has been shown to have significant benefits is in skin protection.
Some materials with excellent cosmetic stability exist. They are enzymes with the ability to capture free radicals, preventing damage to the skin caused by environmental pollution, bacteria, smoke, sunlight and other harmful factor. Here the enzyme can work successfully on the surface of the skin.

There is no need for them to penetrate down to the living cells (although it might be helpful). Perhaps one of the most ubiquitous protective enzymes is superoxide dismutase. (SOD) This is found in almost all-living organisms and works to protect the cell from free radical oxygen attack in the aqueous environment.

Superoxide Dismutase (SOD) and Catalase:

SOD in combination with catalase is responsible for protecting the proteins from aging due to oxidation. SOD works by dismutation, a process by which a dangerous highly reactive oxygen free radical is converted to a less reactive form.
It is important to aerobic cells that the oxygen molecule be completely reduced to two water molecules by accepting four electrons.
If oxygen is only partially reduced by accepting one electron, the product is the superoxide radical.
Superoxide radicals are extremely toxic to cells because they attack unsaturated fatty acid components of membrane lipids, thus damaging membrane structure, causing cell injury.
There are possible benefits for using enzymes in cosmetics; they may help to slow the visible signs of aging and the damaging effects of the environment on the epidermis. Research scientists will continue exploring the possibilities until the mystery, benefits and use of all enzymes that may be used in cosmetics is known. Maybe that key to the fountain of youth is just around the corner.

Enzyme based biosensors

Enzyme - based sensors are more specific than cell based sensors. They have faster responds due to shorter diffusion paths. They are expensive to produce due to the problem of isolating the enzyme. Glucose biosensor is mostly used biosensor. An enzyme biosensor is an analytical device that combines an enzyme with a transducer in order to produce a signal proportional to target analyte concentration. Optimal enzyme activity is essential for maintenance of physiological homeostasis. Both non-genetic and genetic disruptions can excessively activate or silence intrinsic enzyme activities, with pathological outcomes. The pharmacological agents are activators and inhibitors of enzymes. It is essential in the development of drugs as enzyme activators and inhibitors that enzyme activities be accurately measured under physiological and pathological conditions. Different biochemical assays have been developed for this, some of which are based on nanostructured materials. There has been an increased interest in the use of electrochemical sensors in clinical diagnostics. The coupling of enzymes with electrochemical sensors permits the simple determination of metabolites, therapeutic drugs, antigens, and antibodies. Signal amplification in conventional enzyme-based biosensors is not enough to achieve the ultrasensitive detection of biomolecules. Signal amplification has been improved by combining enzymatic reactions with redox cycling or employing multienzyme labels per detection probe. Electrochemical redox cycling allows ultrasensitive detection simply by including one or two more chemicals in a solution without the use of an additional enzyme. Multiple horseradish peroxidase labels on magnetic bead carriers provide high signal enhancement along with a multiplex detection possibility.
Enzyme-based chemical biosensors are based on biological recognition. In order to operate, the enzymes must be available to catalyze a specific biochemical reaction and be stable under the normal operating conditions of the biosensor. Design of biosensors is based on knowledge about the target analyte, as well as the complexity of the matrix in which the analyte has to be quantified. This article reviews the problems resulting from the interaction of enzyme-based amperometric biosensors with complex biological matrices containing the target analyte(s). One of the most challenging disadvantages of amperometric enzyme-based biosensor detection is signal reduction from fouling agents and interference from chemicals present in the sample matrix. This article, therefore, investigates the principles of functioning of

enzymatic biosensors, their analytical performance over time and the strategies used to optimize their performance. Moreover, the composition of biological fluids as a function of their interaction with biosensing will be presented.

Enzyme in clinical diagnosis

Clinical enzymology is branch off medical science deals with the usage of enzymes for diagnosis prognosis of various diseases. In general, each enzyme of clinical significance is found in many tissues of the body, and in healthy individuals, these enzyme exhibit very low levels in serum. In certain disease states or with cell injury, these intracellular enzymes are released into the blood and are indicative of the presence of a pathological condition. Quantification of enzyme levels in serum is useful in determining the presence of disease. Based on the individual's physical symptoms, several enzymes may be chosen for analysis to determine if a pattern develops that aids in identifying the tissue source of the enzyme elevation in the serum(2). The understanding of enzyme kinetics allows for laboratory measurement of plasma levels. Damaged or dying cells within organ can release enzymes into the circulation, these plasma enzyme levels can be used to develop a differential diagnosis of a patient with respect to specific organ disease and dysfunction(1).

Like others analyte use for clinical chemistry analysis, specific pre-analytical influences have to be taken into consideration. Analysis of enzyme measurement would involve the process from the start to the end that comprises the pre-analytical factors, analytical and post analytical factors. Pre- analytical issues in the enzyme measurement include the types of specimens, the specific anticoagulants and preservative in the tubes and the specimen collection procedure. Table 2.0 describe the type of enzymes, the specimen of choice and the pre-analytical factors that can affect the enzyme measurement.

Slight hemolysis can be accepted as there is no CK ain rbc, however severe or moderate hemolysis can cause enzymes and intermediates (adenylate kinase,ATP,glucose – 6-phosphatea) liberated from the erytrocytes and may affect the lag phase and the side reactions occurring in the assay system(3)

Lactate Dehydrogenase LDH

Serum or heparanised plasma

Plasma containing anticoagulant especially oxalate, should not be used. Haemolysed specimen 150 times LDH in rbc than serum(3)

Alkaline Phosphatare ALP

Serum or haparinized plasma

ALP-, free hemolysis. Complexing anticoagulants such as citrate,oxalate, and edta must be avoided.

Storage and doing test later than 4 hours can cause loss of activity

EDTA concentration in the sample-reagent mixture, causing chelation of metallic cations, and this can affect the activity of the alkaline phosphatase

Gamma Glutamyl Transferase GGT

Serum free from hemolysis preferred.

EDTA -plasma (up to 1 mg/mL blood) can be used

Heparin produces turbidity in the reaction mixture; citrate, oxalate and fluoride depress activity by 10 – 15 %

The rate of disappearance of substrate or the rate of appearance of product had been utilized for enzyme measurement. Usually, measuring small increase in product it is much easier than

to measure small decrease in a large amount of substrate. In some enzyme measurements, neither the product not the substrate of a chemical reaction can be measured conveniently. In such cases the enzymatic reaction can be 'coupled' to another reaction that uses the product of the enzyme catalyzed reaction to produce an indicator substance.

The rate of change in concentration of substrate or product is the principle of 'kinetic' method for most of the enzyme measurement. The accuracy of Kinetic makes it easier to detect changes in reaction conditions and samples requiring dilution. In a kinetic reaction, the rate of reaction can be expressed as $\Delta P/\Delta T$, the change in amount of per unit time. The amount of enzyme in a sample is measured by the rate of reaction catalyzed by the enzyme. This rate is directly proportioned to the amount of enzyme and is expressed in enzyme unit, IU/L.

Substrate depletion phase is a period during an enzyme assay when the concentration of substrate is falling and the assay is not following zero-order kinetics. The amount of substrate must be present in sufficient quantity, so that the reaction rate is limited only by the amount of enzymes. In order to get optimal method of enzyme measurement, the substrate concentration is one of the important parameters. It is essential for the concentration of the substrate(s) is saturating during the measured period of the reaction. At saturating substrate concentrations, the reaction velocity is pseudo zero order with respect to the substrate and the velocity is proportional only to the enzyme concentration. Figure 1.0; describe the importance of substrate depletion in enzyme measurement.

- Enzyme activity
- High
- Moderate
- Low
- Substrate depletion
- Substrate depletion
- Lag phase
- Absorbance
- Time

Figure 1.0 – Enzyme activity can be calculated from a plot of absorbance versus time when monitoring an enzyme-catalysed reaction. When reagents and serum are mixed, there may initially be a period of a time when mixing and any preliminary reactions occur; this is termed the lag period. Following this phase, the reaction will proceed at zero-order kinetics (V max); at this point, the rate of appearance of product (as measured from the slope of the line, $\Delta A/\Delta T$) is directly proportional to the enzyme activity present. As the reaction proceeds and substrate is depleted, the rate of reaction will fall below V max and the plot is no longer linear. At this point, the reaction is no longer zero order with respect to substrate concentration; rate of reaction is now dependent on both amount of substrate (which is declining) and amount of enzyme present, making it difficult to calculate amount of enzyme present. (Adapted from Henry's Clinical Diagnostic and Management by Laboratory Methods)

An organic component of enzymes is called coenzyme. Coenzymes participate in many of the enzyme analyses performed in the clinical laboratory. As the coenzyme make up a part of the active site, the role of this coenzyme in enzymatic transamination is crucial as an example the use of pyridoxal phosphate for expression of enzyme activity for aspartate aminotransferase

and alanine aminotransferase measurement. Table 3.0 describe the enzyme, the coenzyme and the clinical relevance of the enzyme measurement for laboratory diagnosis.

In conclusion, the type of assay method, sample preparation, age and storage conditions are the variables that have to be taken into consideration in the determination of enzyme activity. Other important variables in determining enzyme activity include temperature, pH, concentration of substrate, concentration of cofactors of the assay, use of other enzyme reactions as indicators, and whether the forward or backward reaction is used to measure the enzyme. All of these variables can lead to significant differences in enzyme activity between methods.

Primary and secondary serum enzymes

The enzymes which are detectable in serum are derived from the cells of tissues and are essentially of two sorts: those whose primary physiological function and site of action is in the serum or plasma, and those whose presence in serum under normal, or even abnormal, circumstances would appear not to be of physiological importance.

PRIMARY SERUM ENZYMES

The enzymes of the first sort may suitably be called the primary serum enzymes. They are usually present as precursors and require to be activated to exert their physiological effect. Examples are: (a) Lipoprotein lipase, which is activated by heparin and heparin-like substances, and which catalyzes the conversion of the triglycerides of plasma lipoproteins to free fatty acids and to di- and mono-glycerides. The enzyme thus assists in the removal of chylomicrons from plasma after a fatty meal. It has been isolated from heart and from adipose tissue. The serum lipoprotein lipase level is reduced in a rare sub-group of patients with essential familial hyperlipaemia. (b) Caeruloplasmin, which is an a2-globulin to which 98 per cent of the circulating serum copper in man is bound. The protein is an oxidase containing eight copper atoms per molecule with a molecular weight of about 150,000. Its precise in vivo oxidative function is unknown, aldiough it will oxidize, in vitro, substances such as epinephrine and serotonin. The normal serum caeruloplasmin, as assayed by its oxidase activity, is 20 to 40 mg/100 ml (Cumings, 1968). The level is greatly reduced in Wilson's disease to values from 0 to 8 mg/100 ml and determinations of caeruloplasmin are thus much used in the differential diagnosis of this disease. Normal values have been reported in about 7 per cent of cases, and decreased values are occasionally found in clinically normal heterozygotes. Other primary enzymes are plasmin (fibrinolysin) and the several enzymes concerned in blood coagulation, i.e. in the formation of fibrin. The serum levels of these physiologically important enzymes are not usually determined directly in the diagnosis of coagulation defects.

SECONDARY SERUM ENZYMES

Enzymes of the second sort, the secondary serum enzymes, are of much greater importance diagnostically than are the primary serum enzymes, because in diseases affecting individual tissues, enzymes from the tissue cells may be released into the serum in increased

or reduced amounts. Usually, in normal health, small quantities of these enzymes find their way, by diffusion or otherwise, across the cell and capillary membranes and into the plasma.

CAUSES OF AN INCREASED SERUM ENZYME CONCENTRATION

All increased serum enzyme concentration may arise by one or more of the following mechanisms: (1) Increased synthesis of enzymes in a tissue, the cell membranes remaining normal. Examples are the serum alkaline phosphatase and the serum pepsinogen. The former is raised in normally growing children and in conditions such as osteomalacia, associated with increased osteoblastic activity of bone. The serum pepsinogen is often raised in duodenal ulcer. In these instances there is probably an increased number of cells in the bone and stomach respectively, rather than an increased production of enzymes within individual cells. The rate of passage of enzymes from cell to plasma is normal, but since more cells are involved the serum values are raised. (2) Increased release of enzymes into the serum. This may arise for several reasons. (a) Increased permeability of cell membranes and of capillary membranes to intracellular enzymes, as in inflammation. Thus in acute pancreatitis the serum a-amylase rises rapidly. (b) Increased release of enzymes may also occur when there is tissue necrosis from trauma or vascular occlusion, so that cell membranes are ruptured. Thus the serum aspartate amino-transferase (SGOT) may rise rapidly in coronary thrombosis (vide infra). (c) A third cause of increased release occurs when a malignant neoplasm metastasizes via the blood stream, releasing enzymes into the plasma. For example, a metastasizing carcinoma of prostate is responsible for a raised serum acid phosphatase. (3) Decreased or diverted excretion of enzymes. In obstructive jaundice, for example, alkaline phosphatase, normally excreted in bile, is retained hi the circulation and the serum alkaline phosphatase rises. The serum amylase may also increase slightly when there is impaired renal function, as this enzyme, arising in the pancreas (and salivary glands), is excreted in the urine. Diagnostic Significance of a raised Serum Enzyme Concentration. Generally speaking, the higher the serum concentration of an enzyme, the more severe are the events producing it, and the more likely it is that estimation of the enzyme will yield information of diagnostic value. Conversely, the smaller the lesion, the less likely it is that the serum concentration of appropriate enzymes will rise significantly. Thus the procedure of muscle biopsy does not significantly affect the serum concentrations of aldolase or creatine kinase (Buxton and Taylor, 1968), so that it is perhaps not surprising that quite small cardiac infarcts do not always cause a rise in serum creatine kinase or in the serum amino-transferases (SGOT and SGPT).

CAUSES OF A DECREASED SERUM ENZYME CONCENTRATION

Decreased concentrations of enzymes in serum always arise from decreased synthesis in the cells of the tissue of origin. Decreased synthesis may arise for several reasons. (1) Atrophy of a tissue. A good example of this is the fall of serum pepsinogen which occurs in pernicious anaemia, a disease in which the fundic glands of the stomach are atrophic. (2) Surgical ablation of a tissue. Again the stomach may be taken as an example. After total gastrectomy, the serum pepsinogen is zero. (3) Congenital defects. Many inherited diseases result from defective synthesis of one or more enzymes. Usually their diagnosis does not, at present, involve analyses of serum enzymes. One good example, however, of a hereditary disease which is diagnosed by determination of a serum enzyme, and which indeed is named

enzymically, is hypophosphatasia, in which the serum alkaline phosphatase is unduly low. For further examples of importance to anaesthetists, the reader is referred to the paper by Lehmann and Liddell (1969) in this issue. (4) Diminished synthesis. Little is known about the acquired, as distinct from the congenital, failure of synthesis of specific intracellular enzymes, but an example may be the low serum cholinesterase values reported in acute hepatitis and in hepatic cirrhosis.

Different times been used in the diagnosis of muscle disease: the two amino-transferases, lactate dehydrogenase, (ketose-1-phosphate) aldolase, creatine kinase and iditol (sorbitol) dehydrogenase. It is now generally agreed that creatine kinase is, singly, the most useful diagnostically. There are three principal reasons for this: it is the most abundant of the above enzymes in muscle and therefore the most sensitive indicator of muscle damage; although found in heart and in liver it is present in much smaller quantities than in muscles: unlike lactate dehydrogenase and ketose-1-phosphate aldolase, it is not present in erythrocytes, so that the determination is not affected by small amounts of haemolysis into serum. Creatine Kinase. Reaction catalyzed: ATP + creatine^=^ ADP + phosphocreatine. The normal range for creatine kinase is 3.5 to 65 mU/ml (Griffiths, 1964). Severe muscular work may cause the serum level to rise, so that blood samples should be taken from the resting patient. Most anticoagulants inhibit the enzyme, so that the assay should always be carried out on serum. Severe muscle trauma also causes raised values, but a simple muscle biopsy usually does not.

SERUM ENZYMES IN THE DIAGNOSIS OF DISEASE

The serum creatine kinase is raised in muscular dystrophy. This is invariably so in the Duchenne type when values of 5 or more times the upper normal range are not uncommon. The frequency of raised values is less in the limb girdle and facioscapulo-humeral types, and when raised values are recorded they are not often more than twice the upper normal limit. The highest values in the Duchenne type occur at the onset of the illness, and the levels fall, sometimes into the normal range, as the disease progresses over the years with increasing loss of muscle fibres. The disease may be suspected and to some extent predicted in the brothers of affected patients by finding a moderately raised serum creatine kinase value. Known female carriers (mothers) may have moderately raised serum creatine kinase values, the incidence varying in different series from 25 to 85 per cent, depending on factors such as the precise level of the upper normal range. In the unmarried female siblings of affected males, raised values are also seen, but the incidence is less than in mothers. A female sibling may wish to know if she is likely to be a carrier. If the serum creatine kinase is persistently raised it seems likely in our present state of knowledge that she will be; if it is not raised the chances are probably less, but the possibility is by no means excluded. Polymyositis; dermatomyositis. In these two diseases increased levels of serum creatine kinase may be found and often the increase is marked. Successful treatment with steroids causes a fall in the serum creatine kinase paripassu with the clinical improvement. Treatment should probably not be discontinued before a normal level is restored. In muscular dystrophy, steroids do not produce a progressive lowering of the serum creatine kinase. Other muscular and neuromuscular diseases.

INTRACELLULAR DISTRIBUTION OF DIAGNOSTIC ENZYMES

Enzymes are produced by cellular anabolism, the naturally occurring biological process of making more complex molecules from simpler ones. Source organisms include bacteria, fungi, higher plants and animals Enzymes may be extracted from a given source organism by a number of different methods. Most of the organisms that produce commercial enzymes are fungi. These organisms are molds Rhizopusoryzae, Aspergillusniger, Rhizomucormeihei, blights such as Endothiaparasitica and yeasts such as Saccharomyces and Candida sp.

In cells and organisms most reactions are catalyzed by enzymes, which are regenerated during the course of a reaction. These biological catalysts are physiologically important because they speed up the rates of reactions that would otherwise be too slow to support life Enzymes increase reaction rates, sometimes by as much as one million-fold, but more typically by about one thousand fold. Catalysts speed up the forward and reverse reactions proportionately so that, although the magnitude of the rate constants of the forward and reverse reactions is are increased, the ratio of the rate constants remains the same in the presence or absence of enzyme. Since the equilibrium constant is equal to a ratio of rate constants, it is apparent that enzymes and other catalysts have no effect on the equilibrium constant of the reactions they catalyze.

Enzymes increase reaction rates by decreasing the amount of energy required to form a complex of reactants that is competent to produce reaction products. This complex is known as the activated state or transition state complex for the reaction. Enzymes and other catalysts accelerate reactions by lowering the energy of the transition state. The free energy required to form an activated complex is much lower in the catalyzed reaction. The amount of energy required to achieve the transition state is lowered; consequently, at any instant a greater proportion of the molecules in the population can achieve the transition state. The result is that the reaction rate is increased.

While it is clear that enzymes are responsible for the catalysis of almost all biochemical reactions, it is important to also recognize that rarely, if ever, do enzymatic reactions proceed in isolation. The most common scenario is that enzymes catalyze individual steps of multi-step metabolic pathways, as is the case with glycolysis, gluconeogenesis or the synthesis of fatty acids. As a consequence of these lock-step sequences of reactions, any given enzyme is dependent on the activity of preceding reaction steps for its substrate. In humans, substrate concentration is dependent on food supply and is not usually a physiologically important mechanism for the routine regulation of enzyme activity. Enzyme concentration, by contrast, is continually modulated in response to physiological needs. Three principal mechanisms are known to regulate the concentration of active enzyme in tissues: (1) Regulation of gene expression controls the quantity and rate of enzyme synthesis. (2) Proteolytic enzyme activity determines the rate of enzyme degradation. (3) Covalent modification of preexisting pools of inactive proenzymes produces active enzymes.

Enzyme synthesis and proteolytic degradation are comparatively slow mechanisms for regulating enzyme concentration with response times of hours, days or even weeks. Proenzyme activation is a more rapid method of increasing enzyme activity but, as a

regulatory mechanism, it has the disadvantage of not being a reversible process. Proenzymes are generally synthesized in abundance, stored in secretory granules and covalently activated upon release from their storage sites. Examples of important proenzymes include pepsinogen, trypsinogen and chymotrypsinogen, which give rise to the proteolytic digestive enzymes. Likewise, many of the proteins involved in the cascade of chemical reactions responsible for blood clotting are synthesized as proenzymes. Other important proteins, such as peptide hormones and collagen, are also derived by covalent modification of precursors.

Another mechanism of regulating enzyme activity is to sequester enzymes in compartments where access to their substrates is limited. For example, sequestering these enzymes within the lysosome controls the proteolysis of cell proteins and glycolipids by enzymes responsible for their degradation.

In contrast to regulatory mechanisms that alter enzyme concentration, there is an important group of regulatory mechanisms that do not affect enzyme concentration, are reversible and rapid in action and actually carry out most of the moment to moment physiological regulation of enzyme activity. These mechanisms include allosteric regulation, regulation by reversible covalent modification and regulation by control proteins such as calmodulin. Reversible covalent modification is a major mechanism for the rapid and transient regulation of enzyme activity. The best examples, again, come from studies on the regulation of glycogen metabolism where phosphorylation of glycogen synthase and glycogen phosphorylase kinase results in the stimulation of glycogen degradation while glycogen synthesis is coordinately inhibited. Numerous other enzymes of intermediary metabolism are affected by phosphorylation, either positively or negatively). These covalent phosphorylations can be reversed by a separate sub-subclass of enzymes known as phosphatases. Recent research has indicated that the aberrant phosphorylation of growth factor and hormone receptors, as well as of proteins that regulate cell division, often leads to unregulated cell growth or cancer. The usual sites for phosphate addition to proteins are the serine, threonine and tyrosine R group hydroxyl residues.

CLASSIFICATION OF ENZYME AND CO ENZYMES

This is a special commission of the International Union of Biochemistry (IUB) that made recommendations for the classification and naming of enzymes and for the definitions of the mathematical constants used in enzymology. The recommendations were first published in 1964 and were published in revised form in 1972, 1978 and 1984. This is the systematic arrangement and the naming of enzymes that is based on the 1972 recommendations of the Enzyme Commission of the International Union of Biochemistry Reactions and the enzymes that catalyze them form 6 classes, each having 4-13 subclasses. The enzyme name has 2 parts. The first Soetan et al. 383 names the substrate or substrates. The second, ending in-ase, indicates the type of reaction catalysed. A number composed of four figures denotes each enzyme. The first figure denotes one of the six main divisions: oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. The second figure denotes the subclass and the third figure denotes the sub-subclass. The last figure denotes the serial number of the enzyme in its sub-subclass. The enzyme number is preceded by the abbreviation E.C. For example, E.C.2.7.1.1 denotes class 2 (a transferase), subclass 7 (transfer of phosphate), subsubclass 1 (an alcohol is the phosphate acceptor). The final digit

denotes hexokinase, or ATP: D-hexose 6 -Phos-photransferase, an enzyme catalyzing phosphate transfer from ATP to the hydroxyl group on carbon 6 of glucose. There are 6 classes of enzymes as follows:

Oxidoreductases: These enzymes are involved in oxidations and reductions of their substrates e.g., alcohol dehydrogenase, lactate dehydrogenase, xanthine oxidase, glutathione reductase, glucose-6-phosphate dehydrogenase.

Transferases: These enzymes catalyze the transfer of a particular group from one substrate to another e.g., aspartate amino transferase (AST), alanine aminotransferase (ALT), hexokinase, phosphoglucomutase, hexose- 1-phosphate uridyltransferase, ornithine carbamoyltransferase etc.

Hydrolases: These enzymes bring about hydrolysis e.g., glucose- 6 -phosphatase, pepsin, trypsin, esterases, glycoside hydrolases etc.

Lyases: These are enzymes that facilitate the removal of small molecule from a large substrate e.g., fumarase, arginosuccinase, histidine decarboxylase.

Isomerases: These enzymes are involved in isomerization of substrate e.g., UDP-glucose, epimerase, retinal isomerase, racemases, triose phosphate isomerase.

Ligases: These enzymes are involved in joining together 2 substrates e.g., alanyl-t-RNA synthetase, glutamine synthetase, DNA ligases.

Many enzymes require a coenzyme which functions as group transfer reagents. Many enzymes that catalyze group transfer and other reactions require, in addition to their substrate, a second organic molecule known as a coenzyme, without which they are inactive. Coenzymes expand the repertoire of the catalytic capabilities of an enzyme far beyond those offered by the functional groups alone of the amino acids that constitute the bulk of the enzyme. Coenzymes that are tightly associated with an enzyme through either covalent bonding or non covalent forces are often referred to as prosthetic groups. Coenzymes that are freely diffusible generally serve as continually recycled carriers of hydrogen, flavin adenine dinucleotide (reduced) (FADH), hydride nicotinamide adenine dinucleotide (reduced) (NADH) and nicotinamide adenine dinucleotide phosphate (reduced) (NADPH), or chemical units such as acyl groups (coenzyme A) or methyl groups (folates), shuttling them between their points of generation and consumption. These latter coenzymes can thus be considered as second substrates. Enzymes that require coenzymes include those which catalyzeoxidoreductions, group transfer and isomerization reactions and reactions that form covalent bonds (IUB classes 1, 2, 5 and 6). Lytic reactions, including the hydrolytic reactions catalyzed by the digestive enzymes, do not require coenzymes.

Coenzymes can be classified according to the group whose transfer they facilitate based on the above concept, coenzymes may be classified as follows: Coenzymes involved in transfer of groups other than hydrogen: Biotin, CoA-SH, cobamide (B12) coenzymes, folate coenzymes, pyridoxal phosphate, lipoic acid, sugar phosphates, thiamine pyrophosphate and Coenzymes involved in transfer of hydrogen. Nicotinamide adenine dinucleotide (oxidized)

(NAD+), Nicotinamide adenine dinucleotide phosphate (oxidized) (NADP+). Flavin mononucleotide (FMN), Flavin Adenine Dinucleotide (FAD), lipoic acid, coenzyme Q.

Exonuclease: This is an enzyme that removes nucleotides from the ends of DNA fragments. A 5I ----3I exonuclease removes nucleotides from the 5I end, while a 3I ----5I exonuclease removes nucleotides from the 3Iend. This enzyme recognizes the terminal 5 I - phosphate of dsDNA for its exonuclease activity. Its primary use is the removal of protruding 5I terminus from dsDNA which is needed for the terminal transferase tailing of DNA .

Exonuclease III: This enzyme is used for generating linear template DNA for the dideoxysequencing technique and generating staggered ends on dsDNA due to its 3I ---- 5I exonuclease activity.

Ligases: It seals single-stranded gaps (nicks) in double-stranded DNA. It is also used for the formation of recombinant DNA molecules in gene cloning. T4 DNA Ligase: This enzyme catalysis the formation of a Phosphodiester bond between 3I-OH and 5I- phosphate ends in DNA using DNA molecules with cohesive ends as substrate.

Alkaline phosphatase: In gene cloning, this enzyme is used to remove phosphate groups from the 5I end of DNA molecules and also used as a reporter gene for identification of secretion signals.

Polynucleotide kinase: It transfers a phosphate group from ATP to the 5I OH end of DNA or RNA. T4 Polynucleotide kinase is an enzyme is isolated from T4 infected E. coli and catalyzes the transfer of-phosphate of ATP to a 5I -OH end in DNA or RNA. It is also used for the labeling of 5I termini of DNA for Maxam and Gilbert DNA sequencing and the phosphorylation of DNA lacking 5I P termini.

Reverse transcriptase: This is RNA-directed DNA polymerase. It synthesizes DNA (complementary DNA) using mRNA template. For example Reverse transcriptase is an enzyme coded for by avian myeloblastosis virus which catalyses the synthesis of cDNA from an RNA template. It can also be used for the labeling of termini of DNA with extended 5I ends.

Topoisomerase: This is a class of enzymes that alters the conformation of DNA, for example by changing the degree of winding or super coiling.

Transposase: This enzyme catalysis the initial steps in transpositions.

Terminal transferase: This adds nucleotides to the 3I end of DNA, without requiring a template strand.

Terminal deoxynucleotidyltransferase: This enzyme is isolated from calf thymus and catalyses the addition of dNTP to the 3 I -OH of DNA molecules. One of the primary uses of terminal transferase is the tailing of vectors and cDNA with complementary bases, thus permitting the cloning of the cDNA fragments. It can also be used for labeling of 3I ends of DNA fragments.

IMMOBILIZED ENZYME

Since the second half of the last century, numerous efforts have been devoted to the development of insoluble immobilized enzymes for a variety of applications these applications can clearly benefit from use of the immobilized enzymes rather than the soluble counterparts, for instance as reusable heterogeneous biocatalysts, with the aim of reducing production costs by efficient recycling and control of the process as stable and reusable devices for analytical and medical applications as selective adsorbents for purification of proteins and enzymes as fundamental tools for solid-phase protein chemistry and as effective micro devices for controlled release of protein drugs

This is an enzyme that is physically confined while it carries out its catalytic function. This may occur naturally, as in the case of particulate enzymes, or itmay be produced artificially by chemical or by physical methods .

Fig. 1: Range of application of immobilized enzymes

In the chemical methods, the enzyme is linked covalently to a support. These methods include attachment of the enzyme to a water-insoluble support, incorporation of the enzyme into a growing polymer chain, or cross linking of the enzyme with a multifunctional low molecular weight reagent. In the physical methods, the enzyme is not linked covalently to a support. These methods include adsorption of the enzyme to a water-insoluble matrix, entrapment of the enzyme within either a water-insoluble gel or a microcapsule, or containment of the enzyme within special devices equipped with semi permeable membrane. Expensive enzymes can be recovered and used again. The enzyme can also be used in a variety of configurations of bioreactors that permit continuous operation.

ENZYMES AS MARKERS FOR DISEASE

Some enzymes are found only in specific tissues or in a limited number of such tissues. For example, lactase dehydrogenase (LDH) has 2 different forms, called isozymes, in heart and skeletal muscle. Two forms differ slightly in amino acid composition and Can be separated on the basis of charge as a result. Since LDH is a tetramer of four subunits, it too can exist in 5 different forms depending on the source of the subunits. An increase of any form of LDH in the blood indicates some kind of tissue damage. A heart attack can usually be diagnosed with certainty if there is an increase of LDH from heart. Also, there are different forms of Creatine Kinase (CK), an enzyme that occurs in the brain, heart and skeletal muscle. Appearance of the brain type can indicate a stroke or a brain tumour, whereas the heart type indicates a heart attack. After a heart attack, CK shows up more rapidly in the blood than LDH. Monitoring the presence of both enzymes extends the possibility of diagnosis, which is useful, since a very mild heart attack might be difficult to diagnose. An elevated level of the isozyme from heart in blood is a definite indication of damage to the heart tissue. Another useful enzyme assayed is acetyl cholinesterase (AChE), which is important in controlling certain nerve impulses. Many pesticides affect this enzyme, so farm workers are often tested to be sure that they have not received inappropriate exposure to these important agricultural toxins. There are several enzymes that are typically used in the clinical laboratory to diagnose

diseases. There are highly specific markers for enzymes active in the pancrease, red blood cells, liver, heart, brain, prostate gland and many of the endocrine glands. Since these enzymes are relatively easy to assay using automated techniques, they are part of the standard blood test veterinary and medical doctors are likely to need in the diagnosis and treatment/management of diseases.

ENZYME AS DRUG OR ANTIBIOTICS

New antibiotics that are active against resistant bacteria have lived on earth for several billion years. During this time, they encountered in nature a wide range naturally occurring antibiotics or drugs. To survive, bacteria developed antibiotic resistance mechanism .Enzymes as drugs have two important features that distinguish them from all other types of drugs. First, enzymes often bind and act on their targets with great affinity and specificity. Second, enzymes are catalytic and convert multiple target molecules to the desired products. These two features make enzymes specific and potent drugs that can accomplish therapeutic biochemistry in the body that small molecules cannot. These characteristics have resulted in the development of many enzyme drugs for a wide range of disorders.

ENZYMES IN THE DIAGNOSIS OF PATHOLOGY

The measurement of the serum levels of numerous enzymes has been shown to be of diagnostic significance. This is because the presence of these enzymes in the serum indicates that tissue or cellular damage has occurred resulting in the release of intracellular components into the blood. Hence, when a physician indicates that he/she is going to assay for liver enzymes, the purpose is to ascertain the potential for liver cell damage. Commonly assayed enzymes are the amino transferases: alanine transaminase, ALT (sometimes still referred to as serum glutamate-pyruvate aminotransferase, SGPT) and aspartate aminotransferase, AST (also referred to as serum glutamate-oxaloacetate aminotransferase, SGOT); lactate dehydrogenase, LDH; creatine kinase, CK (also called creatine phosphokinase, CPK); gamma-glutamyltranspeptidase, GGT. Other enzymes are assayed under a variety of different clinical situations. Many enzymes are involved in the clinical diagnoses of various diseases in human and veterinary medicine. These enzymes facilitate or enhance rapid diagnoses of these diseases. These enzymes could be classified into many classes. They are:

Alkaline phosphatase: Alkaline phosphatases were the earliest serum enzymes to be recognized to have clinical significance, when in the 1920s, it was discovered that they increase in bone and liver diseases . Since then, they have been the subject of more publications than any other enzyme .Alkaline phosphates are a group of isoforms which hydrolyse many types of phosphate esters, whose natural substrate or substrates are unknown. The term '>alkaline'= refers to the optimal alkaline pH of this class of phosphatases in vitro. In both humans and animals, the major sources of ALPs are the liver, bone, kidney and placenta. In humans, it is involved in bone and hepatobiliary diseases. ALPs are also of diagnostic importance in animal diseases. Total serum ALP activity has diagnostic value in the hepatic and bone diseases in dogs and cats. It is of little value in hepatic diseases of horses and ruminants because of the broad range of reference values against which the patients' values must be compared. The range of serum ALP value in goats may be 10-fold

with no evidence of hepatic damage. Values within the individual are fairly constant for sequential evaluation.

Creatine kinase: Creatine kinase isozymes are the most organ-specific serum enzymes in clinical use. They catalyse the reversible phosphorylation of creatine by ATP to form creatine phosphate, the major storage form of high-energy phosphate required by muscle. Creatine kinases are found in many parts of the body like the heart, brain, skeletal muscle and smooth muscle but they have their highest specific activity in the skeletal muscle. In humans, Creatine kinase is associated with myocardial infarction and muscle diseases. Increase in Creatine kinase in cerebrospinal fluid has been associated with a number of disorders in dogs, cats, cattle and horses. The Creatine kinase are such sensitive indicators of muscle damage that, generally, only large increases in serum activity are of clinical significance.

Alanine aminotransferase: It was formerly known as Glutamic Puruvate Transaminase; (GPT). It catalyses the reversible transamination of Lalanine and 2-oxoglutarate to pyruvate and glutamate in the cytoplasm of the cell. ALT can be found in the liver, skeletal muscle and heart. The greatest specific activity of ALT in primates, dogs, cats, rabbits and rats is in the liver. It is a well established, sensitive liver-specific indicator of damage. However, ALT in the tissues of pigs, horses, cattle, sheep or goats is too low to be of diagnostic value. It is used as an indicator of hepatopathy in toxicological studies which use small laboratory rodents as well as dogs.

Aspartate aminotransferase: It wasformerly called Glutamic Oxaloacetic Transaminase; (GOT). It catalyses the transamination of L-aspartate and 2-oxoglutarate to oxaloacetate and glutamate. AST is found in skeletal muscle, heart, liver, kidney and erythrocytes and is associated with myocardial, hepatic parenchymal and muscle diseases in humans and animals. The pre-sence of AST in so many tissues make their serum level a good marker of soft tissue but precludes its use as an organspecific enzyme. Red blood cells contain a large amount of AST which leaks into plasma before haemolysis is seen.

Sorbitol dehydrogenase (SDH): It is also called L-iditol dehydrogenase; (IDH). It catalyses the reversible oxidation of D-sorbitol to D-fructose with the cofactor NAD. The plasma activity is low in dog and horse plasma but appreciably greater in cattle, sheep, and goat serum. Aside from the testes, it is found in appreciable amounts only in hepatocytes. As a result of this, an increase in plasma SDH is consistent with hepatocyte damage. SDH is liver specific in humans and all species of animals and hepatic injury appears to be the only source of increased SDH activity. Although SDH is liver specific in all species, the already established usage of ALT in dogs and cats has limited SDH as a diagnostic indicator of hepatocellular damage to horses, cattle, sheep and goats.

Lactate dehydrogenases(LDH): It catalyses the reversible oxidation of pyruvate to L(+) lactate with the cofactor NAD. The equilibrium favours lactate formation, but the preferred assay method is in the direction of pyruvate because pyruvate has an inhibitory effect on LDH. Lactate dehydrogenase has isoenzymes. LDH can be found in the heart, liver, erythrocyte, skeletal muscle, platelets and lymph nodes. In humans, it is involved in myocardial infarction, haemolysis and liver disease. LDH isoenzyme profiles were the first isoenzyme profiles used in clinical veterinary medicine in an attempt to detect specific organ

damage. The introduction of more highly organ-specific procedures has resulted in LDH no longer being in common use in veterinary medicine .

Cholinesterase (ChE): Serum cholinesterase (ChE) activity is composed of two distinct cholinesterases. The major substrate is acetylcholine, the neurotransmitter found at the myoneural junction. Acetylcholinesterase (AChE; EC 3.1.1.7) found at the myoneural junction is the true ChE and is essential in hydrolyzing acetylcholine so that the junction can be reestablished and prepared for additional signals .The myoneural junction AChE is also found in Red Blood Cells (RBC), mouse, pig, brain and rat liver. Only a small amount of AChE is found in plasma. The ChE of plasma is a pseudocholinesterase, butylcholinesterase (ButChE; EC 3.1.1.8), which hydrolyses butyrylcholine four times faster than acetylcholine and is also located in white matter of the brain, liver, pancrease and intestinal mucosa . Decreases in ButChE have been reported in humans with acute infection, muscular dystrophy, chronic renal disease and pregnancy, as well as insecticide intoxification.

Amylase: Amylases are calcium-dependent metalloenzymes that randomly catalyze the hydrolysis of complex carbohydrates, e.g., glycogen at the -1-4 linkages. The products of this action are maltose and limit dextrins. The enzyme is a Ca2+ metalloenzyme which requires one of a number of activator ions such as Cl-or Br-. Amylase can be found in the salivary glands, pancrease and ovaries and is used as a diagnostic aid for pancreatitis .

Glutamyltransferase: This is a carboxypeptidase which cleaves C-terminal glutamyl groups and transfers them to peptides and other suitable acceptors. It is speculated that GGT is associated with glutathione metabolism. The major sources are the liver and kidney and are involved in hepatobiliary disease and alcoholism. Cholestatic disorders of all species examined result in increased serum GGT activity.

Trypsin: Trypsins are serum proteases which hydrolyse the peptide bonds formed by lysine or arginine with other amino acids. The pancreas as the zymogen trypsinogen, which is converted to tyrosine by intestinal enterokinase or trypsin itself, secretes them.

Glutathione peroxidases: These are metalloenzymes containing four atoms of selenium per molecule of enzyme. They catalyze the oxidation of reduced glutathione by peroxide to form water and oxidized glutathione. Because of the high concentration of selenium in glutathione peroxidases, there is a good direct correlation between the amount of red blood cell GPx activity and the selenium concentration of other organs .Other enzymes with disease diagnosis applications are acid phosphatase (ACP), found in prostate and erythrocytes and are used in diagnosis of prostate carcinoma. Aldolase (ALD), found in skeletal muscle and heart and involved in muscle disease. Glutamate dehydrogenase (GLDH), found in the liver is used to diagnose hepatic parenchymal disease. Hydroxybutyrate dehydrogenase (HBD), which is the heart form of lactate dehydrogenase is involved in myocardial infarctionJust as enzyme assay is used to diagnose diseases in humans and animals, it may also be applied to the investigation of diseases in plants. For example, it has been found that an injury (either mechanical or pathogenic) results in a marked, localized increase in the activity of glucose-6-phosphate dehydrogenase, but not of glucose phosphate isomerase, indicating diversion of glucose breakdown from glycolysis to the pentose phosphate pathway.

NZYMES ACTED AS THERAPEUTIC AGENTS

In a few cases enzymes have been used as drugs in the therapy of specific medical problems. Streptokinase is an enzyme mixture prepared from streptococcus. It is usefull in clearing blood clots that occur in the lower extremities. Streptokinase activates the fibrinolyticproenzyme plasminogen that is normally present in plasma. The activated enzyme is plasmin is a serine protease like trypsin that attacks fibrin, cleaving it into several soluble components. Another enzyme of therapeutic importance is asparaginase. Asparaginase therapy is used for some types of adult leukemia. Tumor cells have a nutritional requirement for asparagine and must scavenge it from the host's plasma. By administering asparaginase i.e., the host's plasma level of asparagine is markedly depressed, which results in depressing the viability of the tumor. Enzyme replacement in individuals that are genetically deficient in a particular enzyme are also applications of enzymes as therapeutic agents. Also, enzymes such as u-plasminogen activator, formerly known as urokinase, extracted from human urine, can be infused into the blood stream of patients at risk from a pulmonary embolism (a fragment of a blood-clot lodging in the pulmonary artery): these enzymes stimulate a cascade system responsible for the production of active plasmin, a proteolytic enzyme which digests fibrin, the main structural component of blood-clots. Some enzymes may also be used to restrict the growth of cancer cells by depriving them of essential nutrients: for example, Lasaparaginase may be used in the treatment of several types of leukaemia, since the tumour cells, in contrast to normal cells, have a requirement for exogenous Lasaparagine. Another example of therapeutic application of enzymes is the use of immobilized enzymes as components of artificial kidney machines, which are used to remove urea and other waste products from the body, where kidney disease prevents this being done by natural processes. Urea enters the machine from the blood, by dialysis (termed haemodialysis) and is converted to CO2 and NH4+ by immobilized urease; toxic NH4+ is then either trapped on ion exchange resins or incorporated into glutamate by the action of immobilized glutamate dehydrogenase linked to alcohol dehydrogenase to ensure coenzyme recycling, before the fluid is returned to the blood stream.

KEGG (KYOTO ENCHYCLOPEDIA OF GENE AND GENOMES)

The KEGG database project was initiated in 1995 by Minoru Kanehisa, Professor at the Institute for Chemical Research, Kyoto University, under the then ongoing Japanese Human Genome Program. Foreseeing the need for a computerized resource that can be used for biological interpretation of genome sequence data, he started developing the KEGG PATHWAY database. It is a collection of manually drawn KEGG pathway maps representing experimental knowledge on metabolism and various other functions of the cell and the organism. Each pathway map contains a network of molecular interactions and reactions and is designed to link genes in the genome to gene products (mostly proteins) in the pathway. This has enabled the analysis called KEGG pathway mapping, whereby the gene content in the genome is compared with the KEGG PATHWAY database to examine which pathways and associated functions are likely to be encoded in the genome.

According to the developers, KEGG is a "computer representation" of the biological system. It integrates building blocks and wiring diagrams of the system — more specifically, genetic building blocks of genes and proteins, chemical building blocks of small molecules and reactions, and wiring diagrams of molecular interaction and reaction networks. This concept is realized in the following databases of KEGG, which are categorized into systems, genomic, chemical, and health information.

Systems information

- PATHWAY — pathway maps for cellular and organismal functions
- MODULE — modules or functional units of genes
- BRITE — hierarchical classifications of biological entities
- Genomic information
-
- GENOME — complete genomes
- GENES — genes and proteins in the complete genomes
- ORTHOLOGY — ortholog groups of genes in the complete genomes

- Chemical information
- COMPOUND, GLYCAN — chemical compounds and glycans
- REACTION, RPAIR, RCLASS — chemical reactions
- ENZYME — enzyme nomenclature
- Health information
- DISEASE — human diseases
- DRUG — approved drugs
- ENVIRON — crude drugs and health-related substances

Databases

Systems information

The KEGG PATHWAY database, the wiring diagram database, is the core of the KEGG resource. It is a collection of pathway maps integrating many entities including genes, proteins, RNAs, chemical compounds, glycans, and chemical reactions, as well as disease genes and drug targets, which are stored as individual entries in the other databases of KEGG. The pathway maps are classified into the following sections:

- Metabolism
- Genetic information processing (transcription, translation, replication and repair, etc.)
- Environmental information processing (membrane transport, signal transduction, etc.)
- Cellular processes (cell growth, cell death, cell membrane functions, etc.)
- Organismal systems (immune system, endocrine system, nervous system, etc.)
- Human diseases
- Drug development

The metabolism section contains aesthetically drawn global maps showing an overall picture of metabolism, in addition to regular metabolic pathway maps. The low-resolution global maps can be used, for example, to compare metabolic capacities of different organisms in genomics studies and different environmental samples in metagenomics studies. In contrast, KEGG modules in the KEGG MODULE database are higher-resolution, localized wiring diagrams, representing tighter functional units within a pathway map, such as subpathways conserved among specific organism groups and molecular complexes. KEGG modules are defined as characteristic gene sets that can be linked to specific metabolic capacities and other phenotypic features, so that they can be used for automatic interpretation of genome and metagenome data.

Another database that supplements KEGG PATHWAY is the KEGG BRITE database. It is an ontology database containing hierarchical classifications of various entities including genes, proteins, organisms, diseases, drugs, and chemical compounds. While KEGG PATHWAY is limited to molecular interactions and reactions of these entities, KEGG BRITE incorporates many different types of relationships.

Genomic information

Several months after the KEGG project was initiated in 1995, the first report of the completely sequenced bacterial genome was published.[5] Since then all published complete genomes are accumulated in KEGG for both eukaryotes and prokaryotes. The KEGG GENES database contains gene/protein-level information and the KEGG GENOME database contains organism-level information for these genomes. The KEGG GENES database consists of gene sets for the complete genomes, and genes in each set are given annotations in the form of establishing correspondences to the wiring diagrams of KEGG pathway maps, KEGG modules, and BRITE hierarchies.

These correspondences are made using the concept of orthologs. The KEGG pathway maps are drawn based on experimental evidence in specific organisms but they are designed to be applicable to other organisms as well, because different organisms, such as human and mouse, often share identical pathways consisting of functionally identical genes, called orthologous genes or orthologs. All the genes in the KEGG GENES database are being grouped into such orthologs in the KEGG ORTHOLOGY (KO) database. Because the nodes (gene products) of KEGG pathway maps, as well as KEGG modules and BRITE hierarchies, are given KO identifiers, the correspondences are established once genes in the genome are annotated with KO identifiers by the genome annotationprocedure in KEGG.

Chemical information

The KEGG metabolic pathway maps are drawn to represent the dual aspects of the metabolic network: the genomic network of how genome-encoded enzymesare connected to catalyze consecutive reactions and the chemical network of how chemical structures of substrates and products are transformed by these reactions. A set of enzyme genes in the genome will identify enzyme relation networks when superimposed on the KEGG pathway maps, which in turn characterize chemical structure transformation networks allowing interpretation of biosynthetic and biodegradation potentials of the organism. Alternatively, a set of metabolites identified in the metabolome will lead to the understanding of enzymatic pathways and enzyme genes involved.

The databases in the chemical information category, which are collectively called KEGG LIGAND, are organized by capturing knowledge of the chemical network. In the beginning of the KEGG project, KEGG LIGAND consisted of three databases: KEGG COMPOUND for chemical compounds, KEGG REACTION for chemical reactions, and KEGG ENZYME for reactions in the enzyme nomenclature. Currently, there are additional databases: KEGG GLYCAN for glycans and two auxiliary reaction databases called RPAIR (reactant pair alignments) and RCLASS (reaction class). KEGG COMPOUND has also been expanded to contain various compounds such as xenobiotics, in addition to metabolites.

Health information

In KEGG, diseases are viewed as perturbed states of the biological system caused by perturbants of genetic factors and environmental factors, and drugs are viewed as different types of perturbants. The KEGG PATHWAY database includes not only the normal states but also the perturbed states of the biological systems. However, disease pathway maps cannot be drawn for most diseases because molecular mechanisms are not well understood. An alternative approach is taken in the KEGG DISEASE database, which simply catalogs known genetic factors and environmental factors of diseases. These catalogs may eventually lead to more complete wiring diagrams of diseases.

The KEGG DRUG database contains active ingredients of approved drugs in Japan, the USA, and Europe. They are distinguished by chemical structures and/or chemical components and associated with target molecules, metabolizing enzymes, and other molecular interaction network information in the KEGG pathway maps and the BRITE hierarchies. This enables an integrated analysis of drug interactions with genomic information. Crude drugs and other health-related substances, which are outside the category of approved drugs, are stored in the KEGG ENVIRON database. The databases in the health information category are collectively called KEGG MEDICUS, which also includes package inserts of all marketed drugs in Japan.

Subscription model

In July 2011 KEGG introduced a subscription model for FTP download due to a significant cutback of government funding. KEGG continues to be freely available through its website, but the subscription model has raised discussions about sustainability of bioinformatics databases.

PHARMACOGENOMICS RELATED TO POLYMORPHISM OF DRUG METABOLIZING ENZYMES

Pharmacogenomics is the understanding of how individuals differ in their response to drug therapy and the mechanisms underlying variable drug response by utilizing genomics, proteomics, transcriptomics, and metabolomics based knowledge. Every individual has a different genetic makeup, which influences the risk of developing diseases as well as responses to drugs and environmental factors. Genomic differences between individuals are present approximately every 300–1000 nucleotides with over 14 million single nucleotide polymorphisms (SNPs) distributed throughout the entire human genome . Therefore, identification of DNA variants that most significantly contribute to the population variations in each trait is one of the fundamental objectives of genetics . The understanding of variations in interindividual drug response behaviors has been greatly improved owing to the rapid developments in pharmacogenomics over the last few years. Each individual in a large patient population responds differently, which possibly explains why a treatment that has been proven efficacious in some patients often fails to elicit adequate responses in others. Moreover, such treatment failure in the affected patients may cause some serious side effects or even lead to death, which is inductive of individual variability in drug safety and efficacy. The causative factors for variations in drug response are complex and multifold with direct or

indirect consequences. Among them, stably-inherited genetic factors are the major variables , whereas others include environmental factors like chemicals and radiation exposure, lifestyle factors like drinking, smoking and exercise, and physiological factors like age, sex, liver and kidney function, pregnancy, and starvation . It is evident from previous studies that population variability in drug response is often larger than intrapatient variability (within the same individual at different time points). Drug response of individual patients is primarily determined by the pharmacokinetic and pharmacodynamic properties of prescribed drugs, which is directly or indirectly affected by polymorphisms in drug metabolizing enzymes and transporters. Different populations have varied allele frequencies in genes of both drug metabolizing enzymes and transporters. For precision medicine, the molecular and clinical information is integrated in order to understand the biological basis of disease and develop medications with better outcomes for patients . Therefore, precision medicine will help to improve the selection of disease targets and lead to the identification of patient populations that exhibit better clinical result at normal doses.

disease conditions where it is recommended for the treatment of embolism and thrombosis . Similar observation has also been reported for dose-dependent individual variations in drug response to simvastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) .

The recommended daily maximum dose of simvastatin for the management of blood cholesterol levels is 40 mg. In a cohort study of 156 patients, 95% of them showed reduced levels of low-density lipoprotein (LDL) cholesterol, whereas the remaining 5% exhibited no reduction was observed for the remaining 5% of the patients, even at doses as high as 160 mg/day of simvastatin . It is suggested that the genetic polymorphisms in genes encoding ATP-binding cassette sub-family G member 2 (ABCG2) and HMGCR contribute to the interindividual difference in a dose-dependent manner.

Contributing factors in inter individual drug response.

Individual-specific response to medication can be attributed to many multifold and complex factors including the unique genetic makeup (mutations such as SNPs, gene deletions, and duplications). These genetic factors, as well as physiological conditions (age, gender, body size, and ethnicity); environmental influences (exposure to toxins, diet, and smoking); and pathological factors (liver and renal function, diabetes, and obesity) can work alone or in combination to influence drug responses . According to the hypothesis of Tang et al. , various genetic factors contribute approximately 20%–95% to determining the interindividual variability in drug responses. Furthermore, individual variations in responses related to genetic factors are often permanent, while those influenced by other factors are mostly transient . In support of inheritance being a major determinant of drug response, Vesell et al. found relatively higher population variability of a drug response among all the individuals in a population than the intrapatient variability at different times.

DETERMINANTS OF INTERINDIVIDUAL DRUG RESPONSE

Disease conditions of individuals used to be diagnosed based on signs and symptoms, which may be indicative of several different diseases or somewhat related to the family history. In

the past, clinicians could only attempt to cure or treat disease upon its onset . Currently, more specific and precise diagnostic approaches have been developed to examine genes and the genetic variants known to be associated with altered interindividual drug response or specific diseased conditions. Success of the Human Genome Project (HGP) has contributed considerably in this context. Pharmacogenomics enables scientists to assess specific genetic variants that may be responsible for an individual's particular drug response by identifying the particular genetic loci involved . Whole-genome SNP profiling, haplotyping, multigene analysis, and gene expression studies using biochips or microarrays are recently used to study individual responses to drugs at various levels and could facilitate drug discovery and development .

Genetic polymorphisms may influence a drug's effect by altering its pharmacokinetics, pharmacodynamics, or both, which are two major determinants conferring the interindividual differences in drug responses. Pharmacokinetics deals with how much of a drug is required to reach its target site in the body, while pharmacodynamics deals with how well the targets such as receptors, ion channels, and enzymes respond to various drugs . Genetic polymorphisms in drug transporters and phase-1 drug-metabolizing enzymes can alter the pharmacokinetic and pharmacokinetic properties of the administered drugs, their metabolites or both at the target site, resulting in variability in drug responses. Theoretically, variations at even a single base (SNPs) or sets of closely-related SNPs (haplotypes) in genes involved in the pharmacokinetic and pharmacodynamic pathways at any stage could affect the overall drug response of an individual .

Mutations in the gene coding regions could cause alterations in gene expression or protein structure, leading to variations in protein quantity and quality. In the case of enzymes, such mutations affect both the protein function and the rate and kinetic constants. Changes in drug-receptor or drug–enzyme interactions due to structural alterations of enzymes or receptors could also result in variations in drug responses . Polymorphisms in genes responsible for drug transport can affect pharmacokinetic properties of an administered drug and ultimately its plasma concentration as well as concentrations in the target tissues. In addition, altered drug response could also be attributed to reduced repairing capability for mutations triggered by alkylating agents due to malfunctioning of DNA repair enzymes . Such protective effect could be affected by genetic polymorphisms causing altered protein structure or reduced expression in enzymes responsible for glutathione biosynthesis.

Twin studies have provided evidence supporting the contribution of genetic factors to individuals' varied drug responses. For instance, in the late 1950s, it was found that dizygotic twins exhibited more metabolic variability than did monozygotic twins for isoniazid metabolism . Subsequent investigations of halothane, antipyrine, and phenytoin metabolism in twins revealed the major influence of genetic factors and exposure to disease-favoring environment .

Influence of polymorphisms in genes encoding phase-I drug metabolizing enzymes

Cytochrome P450 2D6

Cytochrome P450 (CYP), which represents a large and diverse group of heme-containing enzyme superfamily, is involved in oxidative metabolism of structurally-diverse molecules like drugs, chemical, and fatty acids. The genetic polymorphism in the genes encoding CYP members was firstly reported for *CYP2D6*. The highly polymorphic *CYP2D6* gene is located on the chromosome 22q13.1, consisting of nine exons and eight introns (GenBank accession No. NM 000106.5) . More than 100 *CYP2D6* genetic variants have been described) to date, resulting from point mutations, duplication, insertions or deletions of single or multiple nucleotides, and even whole-gene deletion. Individuals carrying different CYP2D6 allelic variants have been classified as poor metabolizers (PMs), intermediate metabolizers (IMs), extensive metabolizers (EMs), and ultrarapid metabolizers (UMs) according to the metabolic nature of the drugs and degree of involvement in drug metabolism of these variants . Although constituting only 2%–4% of the total amount of CYPs in the liver, CYP2D6 actively metabolizes approximately 20%–25% of all administered drugs . The drugs metabolized by CYP2D6 include tricyclic antidepressants, serotonin reuptake inhibitors, antiarrhythmics, neuroleptics, and β-blockers .

The extensive presence of polymorphism in the *CYP2D6* gene significantly affects phenotypic drug responses. Up to a 10-fold difference in the required dose was observed in order to achieve the same plasma concentration in different individuals . Dextromethorphan, debrisoquine, bufuralol and sparteine are the probe drugs used for *in vivo* CYP2D6 phenotyping. According to the probe substrate metabolic capabilities among the sampled individuals in a population, patients can be categorized into the following four phenotypic groups: poor, intermediate, extensive, and ultra-rapid metabolizers (PMs, IMs, EMs, and UMs), respectively .The interindividual phenotypic variations depend on the metabolic properties of the *CYP2D6* allelic variants. Simultaneous presence of two null (non-functional) alleles in an individual confers a PM phenotype, whereas individuals with two normally-functioning alleles present with the EM phenotype. In addition, co-existence of a null allele with another allele associated with reduced function gives rise to an IM phenotype, whereas presence of extra *CYP2D6* gene copies with normal activity confers the UM phenotype. According to the CYP2D6 phenotype, the Caucasian population comprises approximately 5%–10% PMs, 10%–17% IMs, 70%–80% EMs, and 3%–5% UMs . The percentages of PMs, IMs, EMs, and UMs differs among different ethnicities due to the significant variability in the *CYP2D6* allele distribution.

Individuals with the UM phenotype can metabolize the administered CYP2D6 substrates in much shorter time than individuals with the IM or PM phenotypes. This leads to very low plasma drug levels with potential loss of drug efficacy. Therefore, higher drug doses would be required to attain effective drug concentrations, which could be fatal when dealing with drugs with narrow therapeutic indexes. Notably, a large number (approximately 10%–30%) of Saudi Arabians and Ethiopians have been reported to have the *CYP2D6*2XN* allele . On the other hand, there is an opposite situation for the individuals with the *CYP2D6*3*, **4*, **5*, and **6* alleles (PM phenotype). These allelic variants lead to inactive CYP2D6. As a result, the affected individuals exhibit high plasma drug levels with increased risks of drug-related side effects and therefore reduced drug dose should be administered . The allelic frequencies

with clinical consequences of *CYP2D6*3* (3.3% in Sardinians), *CYP2D6*4*(23%–33% in Polish and Faroese populations), *CYP2D6*5* (5.9%–6.2% in Spaniards and African Americans), and *CYP2D6*6* (1.9%–3.3% in Faroese and Italians) were also calculated in diverse populations.

The prodrug tamoxifen is a selective estrogen receptor (ER) modulator used to treat ER-positive breast cancer patients Tamoxifen is actively catalyzed to endoxifen and 4-hydroxytamoxifen by various CYPs with CYP2D6 acting as the rate-limiting enzyme . Plasma level of endoxifen in UM patients is usually higher than that in PM and IM patients due to the presence of multiple functional *CYP2D6* copies . The presence of *CYP2D6* null alleles in high frequencies commonly contributes to the CYP2D6 PM phenotype in individuals, as is the case with the *CYP2D6*4* (33%) in the Faroese population .In tamoxifen-treated surgically resected ER-positive breast cancer patients, a much lower (0) prevalence of moderate to severe hot flashes, together with a higher risk of disease relapse, was reported in women with the *CYP2D6*4/*4* genotype than in patients with one or no *CYP2D6*4* alleles (20%) . Codeine is a commonly prescribed analgesic, which is converted to its active metabolite morphine and acts at mu-opioid receptors to induce analgesia. The affinity of morphine to mu-opioid receptors is 200-fold stronger than that of codeine. Interestingly, conversion from codeine to morphine is also catalyzed by CYP2D6, which has been proven as the key enzyme responsible for the analgesic effect of codeine. The CYP2D6 phenotype is therefore a critical determinant in opioid analgesia.

CYP2C9

CYP2C9 is another important member of the CYP superfamily. The gene coding for CYP2C9 is located on chromosome 10q24.2, and spans more than 55 kb in length. CYP2C9 constitutes approximately 18% of the total CYP protein in the human liver microsomes . CYP2C9 metabolizes approximately 25% of clinically-administered drugs including anti-inflammatory agents such as flurbiprofen, hypoglycemic agents such as glipizide and tolbutamide, the anticoagulant S-warfarin, and the anticonvulsant phenytoin . More than 60 variant alleles have been identified for the *CYP2C9* gene). Among them, *CYP2C9*2* (R144C) and *CYP2C9*3* (I359L) are the most common variants associated with highly-reduced CYP2C9 enzymatic activities in comparison with the wild-type allele (*CYP2C9*1*) .

The *CYP2C9*2* variant results in a markedly decreased enzyme activity due to higher km value and lower intrinsic clearance of drugs like S-warfarin The *CYP2C9*2* allelic variant has been reported with up to 25% allelic frequencies in the Iranian population. However, frequencies of heterozygous *CYP2C9*1/*2*, homozygous *CYP2C9*2* or *CYP2C9*3* carriers were lower (0.1%–1%) in the Chinese and Japanese populations compared with those in Caucasians and Iranians. Caucasians have approximately 1% *CYP2C9*2* and 0.4% *CYP2C9*3* homozygotes, respectively . Furthermore, approximately one-third of the Turkish population has either the **1*2* or the **1*3* genotype, while more than 2% have the **2*2*, **2*3*, and **3*3* genotypes . In the Iranian and Pakistani populations, the prevalence of *CYP2C9*2* and *CYP2C9*3* is greater than that in the other studied populations. On the other hand, Chinese, Vietnamese, Korean, Bolivian, and Malaysian populations have a *CYP2C9*1* allelic frequency variant of >90%, whereas allelic *CYP2C9*2* variant was not

detected in the Korean, Chinese, and Vietnamese populations but occurs 1% in the Japanese. Furthermore, no individuals from the South African and Zimbabwean populations have been reported to carry the *CYP2C9*2* allele .

Both CYP2C9 and CYP2C19 are involved in microsomal hydroxylation of phenytoin to its R and S enantiomers . Therefore, *CYP2C9* genotype is an important determinant in *in vivo* phenytoin metabolic studies. Due to the narrow therapeutic range of phenytoin, even minimal variations in CYP2C9 activity can be clinically important . In a study on healthy Turkish individuals with already known CYP2C9 genotypes, Aynacioglu et al. reported that subjects with *CYP2C9*1/*2*, *CYP2C9*1/*3*, and *CYP2C9*2/*2* genotypes had significantly higher phenytoin serum concentrations and lower levels of 5-(4-hydroxyphenyl)-5-phenylhydantoin (phenytoin metabolite) than those with the *CYP2C9*1/*1* genotype. Multiple studies have also shown that the *CYP2C9*3/*3* genotype is associated with reduced metabolisms and altered pharmacokinetic properties of substrates such as phenytoin, warfarin, losartan, and tolbutamide .

CYP2C19

The polymorphic *CYP2C19*, which is located on the chromosome 10q24 encodes another CYP family member. CYP2C19 can metabolize numerous routinely-administered drugs such as anxiolytics (diazepam), proton pump inhibitors (omeprazole), anticonvulsants (S-mephenytoin), and antimalarial biguanides. Up to now, more than 35 *CYP2C19* variants and approximately 2000 SNPs have been identified with continuous increase in SNP numbers reported. Among them, *CYP2C19*2* and *CYP2C19*3* are the most common variants that have been studied extensively. Both of them are null variants and patients carrying these variants are therefore categorized as PMs. *CYP2C19*2* is the most common allelic variant caused by a single nucleotide alteration in exon 5 (G > A), resulting in an abnormal splicing site and conferring reduced enzymatic activities of CYP2C19 ..

The *CYP2C19*2* variant is found at a high allelic frequency (30%) in South Indians, but occurs with the lowest frequency (2.9%) in the Faroeses. In contrast, *CYP2C19*3* is found at higher allelic frequencies in the Japanese (approximately 13%) but lower (0) among the Italians, South Africans, Greeks, European-Americans, and other populations). Approximately 15%–25% of the Korean, Japanese, and Chinese populations have been reported as PMs of the anticonvulsant drug S-mephenytoin . The activity of omeprazole, a drug recommended for treating peptic ulcers and gastroesophageal reflux diseases, was found to be highly patient *CYP2C19* genotypes dependent . Furuta et al.found that after a single dose (20 mg) of omeprazole , the observed intragastric pH values were 4.5, 3.3, and 2.1 for PMs, heterozygous EMs, and EMs individuals, respectively. In another study, Schwab et al. reported lower serum concentrations of lansoprazole, a proton pump inhibitor, and lower rates of *Helicobacter pylori* eradication in Caucasian EM patients following a standard dose of lansoprazole. The individuals with the PM phenotype of CYP2C19 required lower doses of the proton pump inhibitor lansoprazole for beneficial therapy than that required by the patients with the EM phenotype of CYP2C19 .

.

CYP3A4 and CYP3A5

More than 50% of clinically-administered drugs are metabolized by CYP3A4, which is the most abundant CYP enzyme in the liver. Therefore, polymorphisms in *CYP3A4* are of great concern in the study of interindividual altered drug metabolisms and related ADRs. More than 26 *CYP3A4* variants have been identified and most of these variants are responsible for varied enzyme activities ranging from modest to highly reduced catalytic efficiencies among the affected individuals . Comparatively, high frequencies of allelic variants of the *CYP3A4* gene (*CYP3A4*2* and *CYP3A4*3*) were observed in Caucasian whereas high frequencies of allelic variant *CYP3A4**18* were observed in Chinese people .The clinical consequences of different allelic variants of *CYP3A4*are still undefined for many substrates of CYP3A4. Considering the relatively low frequencies, only small changes in the enzyme activity have been caused by *CYP3A4*16* and *CYP3A4*18* variants .

CYP3A5 is one of the factors that contribute to the complexity of CYP3A4. With few exceptions, CYP3A5 can metabolize most drugs that are substrates of and metabolized by CYP3A4. Although slower in most cases , the metabolic activity of CYP3A5 is equal to or even faster than that of CYP3A4 in some cases . *In vivo* studies revealed that the metabolic rates for the drug that are metabolized by both CYP3A4 and CYP3A5 are the sum of the activities of both enzymes. Functionally active variants of *CYP3A5* are expressed in half of the African population and one-fourth of Caucasians . This may partially explain why human studies of the *CYP3A4* allelic variants do not agree with its clinical effects .

CYP oxidoreductase (CYPOR) is the catalytic partner and compulsory element to all CYP-mediated metabolisms. The interaction between the CYP and CYPOR is essential for the metabolic activities of CYPs . CYPOR is required for electron transfer from NADPH to CYP via its FAD and FMN domains, which is crucial for CYP catalytic activities . Therefore, *CYPOR* allele variants like *POR*5*, *POR*13* and *POR*27* can indirectly alter the functional consequences of CYPs . For example, in *POR*27* variant, L577P mutation located in the NADPH-binding domain of CYPOR leads to decreased CYPOR activity, due to changed helix and disrupted NADPH interaction , whereas *POR*5* (A287P) is associated with impaired ability to accept electrons from NADPH .

Effect of polymorphisms in genes encoding drug transporters

A drug could produce a beneficial or toxic effect in a particular patient. The nature and extent of the resulting effect is largely dependent on the absorption, distribution, and excretion rates of the drug. Drug transporters primarily control the movement of all drugs and their active or inactive metabolites into or out of cells. Therefore, polymorphisms of drug transporter genes can modify the absorption, distribution, and excretion rates, and ultimately safety and efficacy of the administered drugs. The ABC and solute-carrier (SLC) transporters are two superfamilies of transport proteins are ubiquitous membrane-bound transport proteins that are involved in the absorption, distribution, and elimination of drugs .

ABCB1

The *ABCB1* gene, also known as the multidrug resistance 1 (*MDR1*), encodes a P-glycoprotein (Pgp), which is involved in the cellular efflux of numerous chemotherapeutic agents, physiological metabolites, and carcinogens . *ABCB1* is highly polymorphic with allelic variants found in varied frequencies in different populations). *ABCB1* polymorphisms were identified firstly by Kioka et al. in different cancer cell lines in 1989 and subsequently by Hoffmeyer et al. and other researchers . As an efflux transporter, ABCB1 is detected on the surface of epithelial cells, preventing intestinal absorption, protecting fetus and brain from xenobiotic exposure and facilitating renal and hepatobiliary excretions . Interestingly, overexpression of the *ABCB1* gene in cancer cells induced resistance to chemotherapeutic agents .

ABCC1 and ABCC2

As the important ABC members, both ABCC1 and ABCC2 are involved in the transport and excretion of several chemotherapeutic agents, toxicants, and organic anion molecules . Glutathione cotransporter is essential for both of them to transport some substrates such as estrone sulfate . In non-Hodgkin lymphoma patients treated with doxorubicin, significant associations between the G671V variant and a V188E-C1515Y haplotype of *ABCC2* and G671V variant with 28% allelic frequency in Caucasians have been reported . V417I is another widely distributed variant in *ABCC2* (Asians 13%–19%, Africans 14%, and Caucasians 22%–26%) that has been extensively studied for its role in drug resistance development in cancer and human immunodeficiency virus type 1 (HIV-1)-infected patients .

cancer patients who were treated with gefitinib, presence of C421A was related to increased drug accumulation and higher prevalence of drug-induced grade 1 or 2 diarrhea , when compared to patients with wild type allele. In another study, Sparreboom et al. reported a 300% elevation in plasma levels of the anticancer drug diflomotecan in individuals with the heterozygous C421A genotype when the drug was administered intravenously . Presence of C421A also affects the pharmacokinetic and therapeutic effects of rosuvastatin in Chinese and Caucasians. Tomlinson et al. reported the significant influence of C421A in reducing LDL cholesterol levels in a gene- and dose-dependent manner in Chinese patients with hypercholesterolemia . Therefore, a systemic analysis of polymorphisms of *ABC* transporters would be essential to enhance the understanding of the genetic impact on pharmacotherapy.

Influence of genetic polymorphisms of drug metabolizing enzymes or transporters on drug–drug interactions

Effects of one drug are modified by other concomitantly administered drugs due to drug–drug interactions, which may be attributed to the altered pharmacokinetic or pharmacodynamic properties of one drug induced by the coadministered drug. The polymorphisms in drug metabolizing and transporter genes are an important risk factor of drug–drug interactions and varied interindividual drug responses . These polymorphisms can lead to decreased levels of a drug-metabolizing enzyme in an individual, which may cause severe adverse drug reactions following the coadministration of enzyme inhibitors . Among the CYPs, CYP2C9, CYP2C19, and CYP2D6 are involved in the metabolism of approximately 40% of routinely

administered drugs . Different CYP allelic variants significantly contribute to the variability of an individual's susceptibility to drug–drug interactions and drug-metabolizing capacities . Different drugs interact with the CYP metabolic machinery differently. The metabolism of some drugs by CYP enzymes is extremely specific, for example, metoprolol is primarily metabolized by CYP2D6 , whereas other drugs such as warfarin may be simultaneously metabolized by several CYPs including CYP2D6, CYP3A4, and CYP1A2 . Polymorphisms related to the altered expression of drug metabolizing and transporter genes will ultimately affect the therapeutic effects of administered drugs . When a drug is metabolized by more than one CYP metabolic pathway and the administered drug acts by inhibiting or inducing CYPs, genetic polymorphisms could redirect the metabolism of drugs via other CYP routes . This could lead to drug–drug interactions. For example, antifungal voriconazole is actively metabolized by CYP3A4 and CYP2C19, whereas ritonavir strongly inhibits CYP3A4 while inducing CYP2C19 metabolic activities. When CYP2C19 PM patients are treated with voriconazole and ritonavir, up to 461% increased AUC of voriconazole was observed, since the patients were unable to metabolize voriconazole owing to reduced CYP2C19 and CYP3A4 activities. In another case, the antiplatelet activity of clopidogrel was reduced when it was administered with proton pump inhibitors such as esomeprazole and omeprazole owing to the inhibition of CYP2C19, whereas an increased activity of clopidogrel was anticipated in the presence of rifampicin and aspirin . Clopidogrel is a prodrug that needs oxidative activation *in vivo* by CYP1A2, CYP2B6 and CYP2C19 for its anti-platelet activity . Genetic polymorphisms in *CYP2C19*, *CYP1A2*, *2B6*6*, and *CYP3A5*3* were found to be associated with the varied degree of drug–drug interactions for clopidogrel, due to its highly-complex pharmacokinetics and variable drug response as compare to other anti-platelet drugs.

Mutations in the drug transporter genes also contribute to drug–drug interactions and adverse drug reactions. HMGCR inhibitors such as atorvastatin, rosuvastatin, and pravastatin are actively transported by OATP1B1 and ABCG2 . The concomitant administration of cyclosporine (a potent inhibitor of OATP1B1 and ABCG2) with statins like rosuvastatin and pitavastatin will result in higher plasma levels of statins, leading to rhabdomyolysis . Digoxin is potently cleared by MDR1, therefore its coadministration with verapamil, clarithromycin, or talinolol that inhibits MDR1 transport activity leads to increased plasma levels due to decreased renal clearance of the drug.