PRACTICAL CLINICAL ENZYMOLOGY

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Preface

For many years assays of some digestive enzymes and of the phosphatases have been performed for the clinically useful information derived therefrom, but in textbooks of clinical biochemistry such procedures have occupied no special position. However, the last decade has witnessed an ever-increasing interest in enzymes, not only for diagnostic purposes but also for the elucidation of the mechanisms and transmission of disease processes. A number of pathologies can now be explained in terms of hereditary enzyme abnormalities and predictably this number will increase. In the field of diagnosis full exploitation of the recent advances in clinical enzymology depends upon the development of sensitive assay procedures which can be applied generally, and the time now seems appropriate for a book on such methodologies.

This monograph is therefore essentially practical and intended primarily for hospital biochemists and medical laboratory technicians. Since such an audience wants to know why as well as how, some enzymic and clinical data are also included. It is hoped that this step may also increase the field of usefulness of the book and will be fully justified if the many recorded instances of conflicting clinical observations and technical shortcomings encourages but one reader to attempt a solution of but one of the numerous problems of this expanding field. To this end a selection of references is given in each section and while in a work of this scope an exhaustive bibliography is neither possible nor desirable most original papers are included and the author has endeavoured to list those later references which most fully cover the subject concerned while regretfully omitting some equally important papers. The technical facilities generally available have dictated the choice of procedures given and where the most satisfactory methods involve instrumentation or skill not universally available alternative techniques are also given which should be within the scope of all hospital laboratories.

Since a basic purpose of this book is to supplement rather than to provide a poor copy of the excellent volumes on clinical enzymology which have appeared in recent years, the treatment of different sections has been dictated by the ready availability of information elsewhere. This may have made for a lack of balance inasmuch as space which could

have been given to enzymes of recognized value which are already well covered in reviews or clinical textbooks has been sacrificed to presenting information on possibly less important enzymes. Similarly, a discussion of the enzymes of the clotting mechanism and of the use of purified enzymes in the estimation of substrates and cofactors which are adequately dealt with in textbooks of haematology and clinical biochemistry respectively has been omitted.

My indebtedness to such standard works as Dixon and Webb's *Enzymes* and Varley's *Practical Clinical Biochemistry* is obvious. Throughout this book I have also been guided by the recommendations of the *Report of the Commission on Enzymes of the International Union of Biochemistry* (Pergamon Press, 1961) from which the classification table (pp. 8-12) is taken.

It is with gratitude that I record my thanks to my wife and family for their forbearance, reading the scripts and help with the preparation of diagrams and index; to Mr. H. Varley for constant valuable advice, editing the typescript and reading the proofs; to Mrs. M. Robinson for secretarial work and typing the manuscript; and to Mr. E. Horwood and the staff of the publishers for unfailing courtesy and encouragement. The labour involved in the writing of this book has been further lightened by many friends and colleagues and I should like particularly to express my thanks to Dr. R. N. Antebi, Dr. J. E. Horrocks, Miss M. P. Ivie, Dr. A. P. B. Waind and Dr. J. Ward. Because of geographical location I have been made aware, perhaps more than most writers, of our dependence upon library facilities, and I gratefully acknowledge the co-operation of Mr. F. M. Sutherland, Mr. R. G. Griffin, Mr. F. Barnes and their staffs at the British Medical Association Library, the Chemical Society Library and the Barrow-in-Furness Public Library respectively.

Despite this wealth of assistance, the undoubted inaccuracies and shortcomings still remaining are the responsibility of the author alone who will welcome any corrections or suggestions for improvement.

J. K.

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Chapter 1

THE NATURE OF ENZYME ACTIVITY

An enzyme is a protein with catalytic properties due to its power of specific activation. This assertion by Dixon and Webb (1958) is, however, academic, as such definitions must be, and may require explanation for readers having only a little knowledge of the subject.

Proteins are complex molecules of high molecular weight built up from a number of some twenty α-amino-acids. The selection of different amino-acids and the total number present in the molecule, as well as the sequence in which these units are linked by peptide bonds and the method of coiling and cross-linking of the resulting chains, make for an unimaginably vast number of individual proteins.

Catalysts by classical definition are agents which affect the velocity of a chemical reaction without themselves appearing in the final products of the reaction. This indicates that a catalyst is not permanently altered in the process of the chemical reaction which it facilitates, but is present in the same quantity and physical and chemical condition at the end of the reaction as at the beginning. Enzymes are not altered by the reaction, except momentarily, but since they are proteins, they are thermolabile and sensitive to changes in physical environment. The attrition of enzymes is much greater than that of other catalysts. It is worth emphasizing that a catalyst only accelerates a chemical reaction—that is, exaggerates a tendency already present. In the case of organic compounds which may undergo many different reactions, the increased velocity given to one pathway by the enzyme selectively channels the reaction in this direction. There is nevertheless some evidence that enzymes may actually initiate certain reactions. The equilibrium mixture of a chemical reaction is determined by Mass Law considerations and a catalyst only hastens the attainment of this equilibrium; it does not change it. In some instances, however, where the enzyme concentration is of the same order as the reactants, as may occur locally in cell structures, the equilibrium may be altered. It is usually accepted that an enzyme accelerates a reaction from both sides of the reaction equation, although in some cases the necessary experimental conditions

may not be attainable. It is often found that in a particular reaction there are two different enzymes catalysing reciprocal processes, one hydrolysing a compound, the other synthesizing it.

One of the most striking features of enzymes, constituting a fundamental difference from other catalysts, is their *specificity*. An enzyme is not indiscriminate in its catalytic action, but is strictly limited to one substance or, at most, a small group of closely related substances, in one particular type of reaction. The substance on which the enzyme acts is called its substrate. When an enzyme acts on one substrate only it is said to have absolute specificity. Urease acts only on urea, not upon substituted ureas or other compounds of similar structure, and hence has absolute specificity. Many naturally occurring organic compounds are optically active. Sugars are usually found to have a D-configuration; amino-acids are predominantly L-isomers. When an enzyme's substrate is optically active it is found that the enzyme only catalyses the reaction for one enantiomorph—that is, enzymes are stereospecific. Further, when an enzyme catalyses the conversion of an optically inactive substance into one which is optically active the product is of one form only, the naturally occurring isomer. Thus lactate dehydrogenase oxidized optically inactive pyruvate to L-lactate. This enzyme can catalyse the reduction, albeit more slowly, of other α-hydroxy-monocarboxylic acids besides lactate. Such enzymes, which can act upon a number of closely related compounds, are said to be group specific. The substrate with which an enzyme shows greatest activity is usually, but not invariably, considered to be its natural substrate. Where an enzyme does not require a definite structure in its substrate, apart from a particular bond, as does lipase, it is said to have low or reaction specificity.

It is difficult to conceive of a material agent affecting a chemical reaction which does not in some manner come in contact, combine, or complex with the reacting substances. That chemical combination does actually happen has been proved experimentally for a number of enzymes. The requirement for a specialized structure in the substrate argues an equally specialized, and complementary, structure in the enzyme itself. The mechanism, however, by which the substrate is activated or made more reactive by the enzyme is one of the basic problems still awaiting solution. It may be helpful, however, to picture the substrate with its particular structure fitting on to a complementary structure on the enzyme—Fischer's analogy of a 'lock and key'. That the interlocking topographies of substrate and enzyme are not necessarily spatial, but more likely electrical, or a combination of both, in no way alters the idea of a fitting together of substrate and enzyme. If it is further imagined that while the fit is sufficiently good to bind the

substrate, it is still not perfect so that the substrate molecule is 'strained', or that the fit is perfect but a consequent alteration in the enzyme protein causes a 'straining' of the substrate, a possible picture of what may occur in *activation* is obtained.

The whole of the enzyme protein is not required for catalytic activity. The enzyme papain may have 70 of its constituent amino-acids removed and still retain its hydrolytic activity and specificity. It therefore appears that not only does an enzyme have a specialized arrangement of its amino-acid residues, but that this arrangement is also localized. Such inferences have given rise to the notion of an 'active centre' or 'catalytic site' on the enzyme protein, and the amino-acid sequences of some of these sites have in fact been determined. Furthermore, it has been proved that some enzyme proteins possess more than one such active centre at which catalytic activation of substrate takes place.

Prosthetic Groups and Activators

Not all enzymes are pure proteins. Some contain non-protein moieties more or less firmly attached to the enzyme protein, and are in fact conjugated proteins. Where the non-protein grouping is necessary for catalytic activity it is termed the prosthetic group and the protein is known as the apoenzyme. The active conjugated protein is called the holoenzyme. Metalloenzymes contain a metal, which is either firmly bound to some group in the protein itself, or is present in the prosthetic group. Dialysis does not remove the metal from true metalloenzymes. There are, however, many enzymes which are catalytically inactive or show greatly reduced activity in the absence of certain metal ions, which are accordingly called activators or accelerators. These are usually monovalent or divalent ions such as Na^{+}, K^{+}, Mg^{++}, Ca^{++}, Zn^{++}, Mn^{++}, Fe^{++}, Co^{++} and Ni^{++}. There is some degree of specific requirement in enzymes activated by these ions, which may function in some instances by forming a link between enzyme and substrate. The distinction between activators in such cases and the metal of true metalloenzymes is then only one of degree, depending upon the firmness of the attachment. However, in some cases the ions activate by reacting with the substrate so that the true substrate is not the substance itself but its metal complex. A few enzymes, such as phosphoglucomutase, require two metal ions for maximal activity.

Anions may also act as unspecific activators, probably due to effects on the ionization of the protein molecule. Amylase and fumarase are two enzymes so activated, the former by monovalent ions such as chloride, bromide or nitrate, and the latter by trivalent ions such as citrate, phosphate or arsenate.

Another type of enzyme activation occurs with some extracellular enzymes—that is, enzymes elaborated by a cell and excreted in an inactive form. The classical illustration of an inactive enzyme precursor, pro-enzyme or zymogen, is trypsinogen, produced by the pancreas and excreted in the pancreatic juice. Either trypsin itself, or enterokinase, an enzyme elaborated by the duodenal mucosa, splits off a hexapeptide from the trypsinogen molecule to produce active trypsin. This process of freeing the active centre is sometimes referred to as unmasking.

A final type of enzyme activation is by the removal or neutralization of substances which 'poison' or inhibit the enzyme, or otherwise interfere with its catalytic action. This is activation by de-inhibition. Removal of products of the reaction is sometimes erroneously referred to as activation under this heading. In this case it is the equilibrium of the reaction, not the enzyme, which is affected.

Coenzymes

In some instances, even where all the necessary activators are present and the substrate bound to the enzyme, no reaction takes place in the absence of a further substance which acts as a donor-acceptor or group-carrier. These substances are termed coenzymes, and two of the most important within the limited scope of the present survey are Coenzyme I, nicotinamide-adenine dinucleotide (NAD) and Coenzyme II, nicotinamide-adenine dinucleotide phosphate (NADP). These substances have previously been called cozymase, codehydrogenase 1, codehydrase 1 or diphosphopyridine nucleotide (DPN) and phosphocozymase or triphosphopyridine nuceotide (TPN) respectively. The structural formulae of these important hydrogen carriers are given in Figure 4.1. Flavin monophosphate (FMN), flavin-adenine diphosphate (FAD) and lipoic acid (6,8-dithioloctanoic acid) are also hydrogen transport coenzymes. Adenosine triphosphate (ATP) and guanosine triphosphate (GTP) are coenzymes in phosphate transport reaction, Coenzyme *A* (Co.A) in acyl group transport, and thiamine pyrophosphate in decarboxylation of α-oxo acids.

The metabolic activity of many vitamins, particularly of the B group, has been shown to be through the formation of prosthetic groups and coenzymes.

It may be worth noting at this point that the terms substrate, prosthetic group, activator and coenzyme are rather loose, general names not rigorously defined. It is sometimes said that the differences are of degree only, that prosthetic groups are more firmly attached to the enzyme protein than coenzymes. Prosthetic groups, however, are

unchanged at the end of an isolated reaction, while coenzymes are altered and only recover their original form by taking part in a second reaction. In certain circumstances then there is little difference between a coenzyme and a substrate. Of the 18 transaminating enzymes listed by Dixon and Webb (1958), 13 require glutamate as substrate and the others can utilize it. Glutamate may quite readily then be called a coenzyme in these reactions, acting as it does as an amino group carrier and being regenerated from α-oxoglutarate in a second reaction. An example of this function is clearly shown in Figure 1.1.

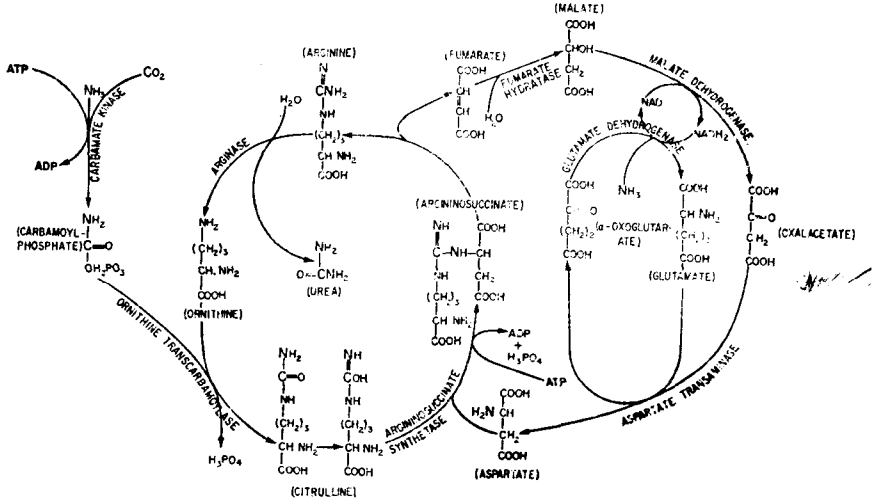


FIGURE 1.1 UREA SYNTHESIS

ATP = Adenosine triphosphate; ADP = Adenosine diphosphate; NAD = Coenzyme I; $NADH_2$ = reduced Coenzyme I

Two molecules of ammonia and one molecule of carbon dioxide (the nitrogens and carbon, shown in larger type throughout) are eliminated in the formation of one molecule of urea. The energy for the process is supplied by the breakdown of two molecules of adenosine triphosphate to adenosine diphosphate. The adenosine triphosphate is regenerated in oxidative pathways such as the glycolysis system (Figure 1.2).

Inhibition

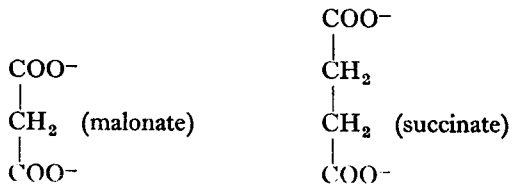
Any condition or agent which denatures protein obviously destroys enzyme activity. Heat, exposure to extremes of pH, X-rays, ultraviolet light, very high pressures, certain sonic waves and mechanical shaking inactivate enzymes. Oxidizing agents and the protein precipitants, heavy metal ions by virtue of their positive charge and the alkaloidal reagents, trichloracetic, tannic and phosphotungstic acids through

their negative charge, also cause enzyme inactivation. Though some enzymes are more resistant to these conditions than others, the final effect is an irreversible inactivation.

Apart from denaturation of proteins there are many substances which limit or curtail enzyme activity. These substances, called inhibitors, may interfere with enzyme action not only by their effect on the enzyme itself, but also by their action on substrate, activator or coenzyme. It is not always easy to decide through which channel inhibition is caused. Cyanide, sodium azide, hydrogen sulphide and carbon monoxide are effective inhibitors of metalloenzymes, particularly those containing iron, copper or zinc. In addition to their ability to complex with metals, these agents may also react with carbonyl groups, or act as oxidizing agents on substrates and cofactors. Compounds which react with essential thiol (-SH) groups of enzymes or cofactors, as do iodoacetate, ethyl iodoacetate, iodoacetamide, chloroacetophenone and acrolein by alkylation, and *p*-chloromercuribenzoate and phenyl mercuriacetate by mercaptide formation, cause varying degrees of inhibition. In the case of the mercaptide forming agents, the inhibition can be alleviated or abolished by chemical means—that is, the inhibition is reversible.

Besides the heavy metals, mainly silver, mercury and copper, which are protein precipitants, there are other metals which have a more specific inhibitory action. These are called ion antagonists because of their mode of action and are classed as competitive inhibitors in distinction from previously mentioned agents whose action is non-competitive. Creatine phosphokinase requires Mg^{++}, Mn^{++} or Ca^{++} for maximum activity and is inhibited by Zn^{++}, manganese ions activate 5-nucleotidase, nickel ions inhibit. Increasing the concentration of the required ion, or removal of the competing inhibitor ion by chelating agents such as EDTA or cysteine, reactivates the enzyme.

Substances with structures similar to specific substrates may be bound to the enzyme and hence block the active site. A true equilibrium exists in these circumstances and increasing the concentration of substrate decreases the inhibition. The classical example of this type of competitive inhibition is that of succinate dehydrogenase by malonate.



Optical enantiomorphs may also sometimes inhibit in this manner.

Antivitamins—that is, substances closely related to the vitamins but unable to perform their functions, are a special class of competitive inhibitor, competing at the level of synthesis of prosthetic group or coenzyme.

Finally, there are antienzymes. It is assumed that these are possessed by intestinal parasites to prevent their destruction by the digestive enzymes. However, since they are proteins, enzymes are capable of inducing the formation of specific antibodies. A lack of response in immunological cross-reactions has established species differences in otherwise apparently identical enzymes.

Nomenclature and Classification

The name enzyme, which means 'in yeast', was suggested by Kuhne in 1878 as a generic term. For individual enzymes, Duclaux in 1898 proposed that the last three letters of the name 'diastase', which had come to be used as a general term, be suffixed to a root indicating the substrate or nature of the reaction catalysed. This system proved satisfactory until recent times when it became apparent that an international authority must devise and govern a systematic scheme of classification and nomenclature for the ever-increasing number of known enzymes. This formidable task has now been accomplished, and the *Report of the Commission on Enzymes of the International Union of Biochemistry* (1961) sets out fully and clearly the recommendations of this body. It is to be hoped most sincerely that these recommendations will be generally adopted in their entirety and thus bring order and coherence to this rapidly expanding field of knowledge.

The report recommends that nomenclature of enzymes be based on substrate and type of reaction catalysed. In many instances this makes for an unwieldy name, so that following the practice of other branches of science, a systematic name for precise identification and a trivial or working name for general use are proposed. The latter is in most cases the name currently employed. The use of abbreviations for enzymes, e.g. MDH for malate dehydrogenase, is not approved.

The Commission proposes a system which provides a classification of enzymes by type of reaction and also a scheme for numbering enzymes. Six main classes are proposed (Table 1.1): Oxidoreductases, Transferases, Hydrolases, Lyases, Isomerases and Ligases (synthetases). Lyases remove groups from their substrates leaving double bonds, or add groups to double bonds; ligases couple together two molecules of substrate with simultaneous breakdown of adenosine triphosphate or a similar triphosphate.

A code number of four integers separated by points is given to each

[*Continued p. 12*

TABLE 1.1

The Classification and Numbering of Enzymes

Appendix D of the *Report of the Commission on Enzymes of the International Union of Biochemistry* (Pergamon Press, 1961).

1. Oxidoreductases

1.1 Acting on the CH—OH groups of donors

1.1.1 With NAD or NADP as acceptor
1.1.2 With a cytochrome as an acceptor
1.1.3 With O_2 as acceptor
1.1.99 With other acceptors

1.2 Acting on the aldehyde or keto-group of donors

1.2.1 With NAD or NADP as acceptor
1.2.2 With a cytochrome as an acceptor
1.2.3 With O_2 as acceptor
1.2.4 With lipoate as acceptor
1.2.99 With other acceptors

1.3 Acting on the CH—CH group of donors

1.3.1 With NAD or NADP as acceptor
1.3.2 With a cytochrome as an acceptor
1.3.3 With O_2 as acceptor
1.3.99 With other acceptors

1.4 Acting on the CH—NH_2 group of donors

1.4.1 With NAD or NADP as acceptor
1.4.3 With O_2 as acceptor

1.5 Acting on the C—NH group of donors

1.5.1 With NAD or NADP as acceptor
1.5.3 With O_2 as acceptor

1.6 Acting on $NADH_2$ or $NADPH_2$ as donor

1.6.1 With NAD or NADP as acceptor
1.6.2 With a cytochrome as an acceptor
1.6.4 With a disulphide compound as acceptor
1.6.5 With a quinone or related compound as acceptor
1.6.6 With a nitrogenous group as acceptor
1.6.99 With other acceptors

1.7 Acting on other nitrogenous compounds as donors

1.7.3 With O_2 as acceptor
1.7.99 With other acceptors

1.8 Acting on sulphur groups of donors

1.8.1 With NAD or NADP as acceptor
1.8.3 With O_2 as acceptor
1.8.4 With a disulphide compound as acceptor
1.8.5 With a quinone or related compound as acceptor
1.8.6 With a nitrogenous group as acceptor

1.9 Acting on haem groups of donors

1.9.3 With O_2 as acceptor
1.9.6 With a nitrogenous group as acceptor

1.10 Acting on diphenols and related substances as donors

1.10.3 With O_2 as acceptor

1.11 Acting on H_2O_2 as acceptor
1.98 Enzymes using H_2 as reductant
1.99 Other enzymes using O_2 as oxidant

1.99.1 Hydroxylases
1.99.2 Oxygenases

2. Transferases

2.1 Transferring one-carbon groups

2.1.1 Methyltransferases
2.1.2 Hydroxymethyl-, formyl- and related transferases
2.1.3 Carboxyl- and carbamoyltransferases

2.2 Transferring aldehydic or ketonic residues
2.3 Acyltransferases

2.3.1 Acyltransferases
2.3.2 Aminoacyltransferases

2.4 Glycosyltransferases

2.4.1 Hexosyltransferases
2.4.2 Pentosyltransferases

2.5 Transferring alkyl or related groups
2.6 Transferring nitrogenous groups

2.6.1 Aminotransferases
2.6.2 Amininotransferases
2.6.3 Oximinotransferases

2.7 Transferring phosphorus-containing groups

2.7.1 Phosphotransferases with an alcohol group as acceptor
2.7.2 Phosphotransferases with a carboxyl group as acceptor

2.7.3 Phosphotransferases with a nitrogenous group as acceptor
2.7.4 Phosphotransferases with a phospho-group as acceptor
2.7.5 Phosphotransferases, apparently intramolecular
2.7.6 Pyrophosphotransferases
2.7.7 Nucleotidyltransferases
2.7.8 Transferases for other substituted phospho-groups

2.8 Transferring sulphur-containing groups

2.8.1 Sulphurtransferases
2.8.2 Sulphotransferases
2.8.3 CoA-transferases

3. Hydrolases

3.1 Acting on ester bonds

3.1.1 Carboxylic ester hydrolases
3.1.2 Thiolester hydrolases
3.1.3 Phosphoric monoester hydrolases
3.1.4 Phosphoric diester hydrolases
3.1.5 Triphosphoric monoester hydrolases
3.1.6 Sulphuric ester hydrolases

3.2 Acting on glycosyl compounds

3.2.1 Glycoside hydrolases
3.2.2 Hydrolysing N-glycosyl compounds
3.2.3 Hydrolysing S-glycosyl compounds

3.3 Acting on ether bonds

3.3.1 Thioether hydrolases

3.4 Acting on peptide bonds (peptide hydrolases)

3.4.1 α-aminopeptide aminoacidohydrolases
3.4.2 α-carboxypeptide aminoacidohyrolases
3.4.3 Dipeptide hydrolases
3.4.4 Iminopeptide hydrolases
3.4.5 Prolinepeptide hydrolases
3.4.6 Peptide peptidohydrolases

3.5 Acting on C—N bonds other than peptide bonds

3.5.1 In linear amides
3.5.2 In cyclic amides
3.5.3 In linear amidines
3.5.4 In cylic amidines
3.5.99 In other compounds

3.6 Acting on acid-anhydride bonds

3.6.1 In phosphoryl-containing anhydrides

3.7 Acting on C—C bonds

3.7.1 In ketonic substances

3.8 Acting on halide bonds

3.8.1 In C-halide compounds
3.8.2 In P-halide compounds

3.9 Acting on P—N bonds

4. Lyases

4.1 Carbon-carbon lyases

4.1.1 Carboxy-lyases
4.1.2 Aldehyde-lyases
4.1.3 Ketoacid-lyases

4.2 Carbon-oxygen lyases

4.2.1 Hydro-lyases
4.2.99 Other Carbon-oxygen lyases

4.3 Carbon-nitrogen lyases

4.3.1 Ammonia-lyases
4.3.2 Amidine-lyases

4.4 Carbon-sulphur lyases
4.5 Carbon-halide lyases

5. Isomerases

5.1 Racemases and epimerases

5.1.1 Acting on aminoacids and derivatives
5.1.2 Acting on hydroxyacids and derivatives
5.1.3 Acting on carbohydrates and derivatives

5.2 Cis-trans isomerases
5.3 Intramolecular oxidoreductases

5.3.1 Interconverting aldoses and ketoses
5.3.2 Interconverting keto- and enol- groups
5.3.3 Transposing C=C bonds

5.4 Intramolecular transferases

5.4.1 Transferring acyl groups
5.4.2 Transferring phosphoryl groups
5.4.99 Transferring other groups

5.5 Intramolecular lyases