AN INTRODUCTION
TO
DIAGNOSTIC ENZYMOLOGY

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PREFACE

The science of enzymology has proved of great interest to biochemists during the past two or three decades, and the advances in our knowledge of life processes which have accrued have offered the prospect of improved methods of diagnosis. For a long time the digestive ferments were the only enzymes available for investigation in clinical laboratories, but recognition of the value of the serum phosphatases introduced in the 1930's suggested that the diagnostic potentialities of many other enzymes might profitably be explored. These interesting prospects, however, could not immediately be fulfilled largely owing to the lack of suitable technical procedures, but the discovery of the NAD- and NADP-dependent enzymes combined with the ready availability of the ultra-violet spectrophotometer has led to the inception of a wide range of new enzyme tests during the past seven or eight years.

The extensive use of these tests in hospital biochemistry has led to the accumulation of a vast amount of information, some of it rather contradictory. The present time is appropriate for an attempt to be made to assess this material in the light of experience gained in a routine clinical laboratory. The main object of the present monograph has been to review the diagnostic applications of enzyme determinations and, where possible, to suggest those most appropriate to a given clinical situation. Although primarily intended for clinicians, it is hoped that the book will prove useful to clinical biochemists, and to this end the methods employed have been critically reviewed and some of the more important procedures have been included in the appendix.

An outline of the main features of enzymic reactions is followed by a short discussion of the problems involved in applying their study in clinical practice. After a section on the digestive enzymes, the main classes of diagnostically important serum enzymes, i.e. phosphatases, transaminases, dehydrogenases, glycolytic enzymes and the cholinesterases, are considered. A chapter is devoted to recent advances in the study of some metabolic diseases. The final section of the book summarizes the factors governing the choice of enzyme tests in myocardial infarction and liver disease, and advances some speculations concerning the origin and fate of serum enzymes.

The therapeutic use of enzymes is considered to be outside the scope of the present volume and has therefore not been included.

After the manuscript had been submitted there became available the Report of the Commission on Enzymes of the International Union of Biochemistry, which makes a number of recommendations on enzyme nomenclature. At the risk of introducing possible inconsistencies, some of these have been incorporated at the proof stage and I should like to thank the printers for their forbearance. I am very grateful to my wife, Dorothy, not only for her patience while the book was being written, but also for typing the manuscript and assisting with proof reading.

I wish to thank the authors, whose names appear in the legends, and the following editors and publishers for permission to reproduce illustrations: The Editor, *Discussions of the Faraday Society* (Fig. 2); The Editorial Secretary, *Gut* (Figs. 13 and 14); The Editor, *British Medical Journal* (Figs. 15 and 50); The C. V. Mosby Company (Figs. 18 and 56); The Editor, *New England Journal of Medicine* (Fig. 21); The Athlone Press (Figs. 36 and 37); The American Association of Biological Chemists, Inc. (Fig. 40); The Editors, *Nature* (Fig. 41); The Williams and Wilkins Company (Fig. 45); The Editor, *Cancer* (Fig. 48); The Editor, *Annals of the New York Academy of Sciences* (Fig. 49); and S. Karger, Basle (Fig. 51).

I am greatly indebted to numerous clinical and laboratory colleagues for their advice and criticism. In particular I wish to thank Dr. J. Pryse-Davies, Dr. S. B. Rosalki, Dr. B. A. Elliott and Dr. D. T. Plummer who have collaborated in a number of investigations, the results of which are outlined in the following pages. I should also like to express my appreciation of the valuable criticisms of Professor E. J. King, Dr. H. T. Howat, Professor M. D. Milne, Dr. J. C. Thompson, Mr. H. Varley, Dr. A. B. Anderson, Mr. P. W. Perryman, Dr. I. M. P. Dawson, Dr. Joan Zilva, Dr. M. P. Tombs and Mr. K. B. Cooke. Finally, it is my pleasure to pay a sincere tribute to my chief, Professor N. F. Maclagan, who has not only read the entire manuscript, but has given unstinted help and encouragement throughout its preparation.

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CONTENTS

CHAPTER 1

THE NATURE OF ENZYMES

The life processes occurring in a single body cell consist of a series of interrelated complex reactions, many of which are consecutive in that the products of one reaction become the starting materials for another. Within the same cell many such processes occur simultaneously without any apparent mutual interference. The pattern of intracellular reactions is reproduced not only in the daughter cells produced by mitotic cell division but also in successive generations of the same species, the maintenance of which depends upon the continual reduplication of the same vital processes. The necessary control of all these highly complicated mechanisms is exerted by means of enzymes which are genetically transmitted.

Enzymes are catalysts, always highly specific for particular types of reaction, and frequently specific for individual reactants. Their activities are subject to the influence of many variables including the temperature and pH of their environment, the supply of reacting materials, the removal of reaction products and the presence of activators or inhibitors. The response of enzymic activity to changes in factors such as these is the means whereby the life of the cell, and hence of the whole organism, is maintained. Disease results when the control exerted by enzymes becomes deranged, no matter whether the initial lesion is caused by a virus or bacterial invasion, or by chemical or traumatic means. Some diseases such as galactosaemia and certain forms of anaemia are due to the congenital absence of certain enzymes.

Although fermentation processes had been known since the earliest times, the existence of enzymes was first recognized in 1833 when Payen and Persoz observed that the diastase of malt extract converted starch into sugar. Pepsin and trypsin were described a few years later by other workers, but during the second half of the nineteenth century investigations in enzymology were mainly concentrated upon the mechanism of fermentation.

Among the important discoveries of general application must be mentioned Pasteur's observation of the difference in the sensitivities of the optical isomers of tartaric acid towards fermentation, and the demonstration by Buchner that this process could be induced by cell-free yeast extracts. This connection with alcoholic fermentation led to the introduction of the word "ferment" which is still used in the German language, but elsewhere it has been almost completely superseded by the term "enzyme" first coined by Kühne in 1878 from ἐν ζύμῃ (in yeast) as a general term to include all such biochemical catalysts.

The substance upon which an enzyme acts is known as its "substrate". In 1894 Emil Fischer showed that the relation between an enzyme and its substrate may be highly specific and suggested that it resembled that between a lock and a key. The substrate molecule requires to have a spatial configuration such that it may become intimately bound to the enzyme surface forming an enzyme–substrate complex. This idea has been developed in recent years by the introduction of antimetabolites, compounds which are sufficiently closely related chemically to the normal substrate to compete with the latter in forming complexes with the enzyme. Such displacement of the normal substrate results in inactivation of the enzyme, and several useful drugs, e.g. the sulphonamides, 6-mercaptopurine, owe their activities to interference with enzyme action by this process of competitive inhibition.

ENZYME STRUCTURE

Little was known of the chemical structure of enzymes until 1926 when Sumner crystallized urease, the urea-splitting enzyme contained in the soya-bean. The pure material proved to be a protein, and since 1930, pepsin, trypsin and many other enzymes have been obtained in crystalline states and all have proved to be proteins. Some enzymes, such as the diagnostically important transaminases, are conjugated proteins in which the apo-enzyme, or protein moiety, is bound to a coenzyme, or prosthetic group. The readily water-soluble vitamins of the B group, e.g. pyridoxal phosphate, thiamine pyrophosphate (cocarboxylase), and riboflavin phosphate, are coenzymes and often may be removed from the apo-enzyme by prolonged dialysis. Such treatment leads to

inactivation of the enzyme, but replacement of the co-factor restores enzymic activity.

Enzymes have the physical and chemical properties characteristic of proteins, i.e. they are heat coagulable and are precipitated by trichloroacetic acid and other protein precipitants. Such procedures lead to denaturation and consequent inactivation, but precipitation with ammonium sulphate can usually be effected without loss of activity. Alcohol or acetone may also be used for the precipitation of enzymes, but at temperatures exceeding 4° dehydration and hence denaturation may result.

Apart from those associated with particulate matter, enzymes are generally soluble in water or dilute saline solutions. They form colloidal solutions and since they are of high molecular weight may be separated from diffusible matter by dialysis. Since a number of enzymes have been crystallized it has been possible to make fairly accurate estimations of their molecular weights by the techniques of sedimentation, diffusion and viscosity. The results cover a remarkably wide range, for while ribonuclease has a molecular weight of 12,700, L-glutamate dehydrogenase gives a figure of about 1,000,000. By comparing rates of diffusion it has been possible to obtain rough estimates of the molecular weights of a number of enzymes which have not yet been crystallized.

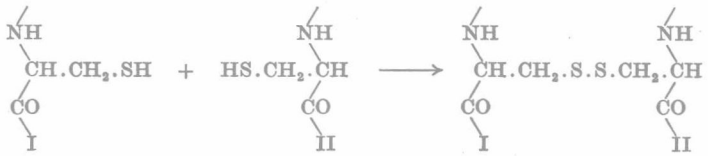
Since enzymes are proteins composed of amino-acids linked together in peptide chains, they are *zwitterions*, i.e. they possess positive and negative electrically charged groups in different parts of the same molecule. When the pH of the medium is adjusted to the point at which the net charge on the enzyme protein is minimal (the iso-ionic point), the solubility of the protein is also minimal and the enzyme may be precipitated along with other proteins of similar iso-ionic point. The possession of electrically charged groups has enabled enzyme-rich components to be separated from other protein components by electrophoresis. This process depends upon subjecting the protein solution which may be supported on a suitable medium, e.g. paper, starch gel, starch block, etc., to an electric potential applied across a suitable apparatus containing a buffer solution. Several examples are quoted in the following pages of enzymes being located with particular serum protein fractions by this means

Electrophoretic studies have shown that all the known

enzymes of blood serum are associated with the various globulin fractions (Fig. 1). This implies that these enzymes are also globulins and, indeed, there is some evidence in support of the view that many of the serum globulin components consist of enzymes.

Once an enzyme has been obtained in a pure crystalline form and its molecular weight determined, it is a comparatively straightforward analytical exercise to ascertain its amino-acid composition; but to determine the amino-acid sequence is an extremely complicated and difficult matter. Nevertheless, the techniques used by Sanger in his remarkable and brilliant study of the composition and amino-acid sequence in insulin have been applied to enzyme molecules and our knowledge of the structure of some of them is increasing. The peptide chains are generally believed to be bound as α-helices but little is known of how they are fitted into the enzyme molecule. Physical investigations have shown most enzymes to have globular molecules, and it is clear that the peptide chains must be coiled or folded in some way. During denaturation this coiling or folding is disturbed and enzyme activity is lost.

Two adjacent peptide chains or two different parts of a single folded chain may be held together by disulphide bridges in which cysteine residues are oxidized in the following manner:



Cysteine residue in peptide chain I

Cysteine residue in peptide chain II

Disulphide bridge linking peptide chains I and II

In a number of cases, terminal amino-acid groups or even short peptide chains may be hydrolysed from enzyme molecules without loss of activity, and in some enzymes, such as pepsin and trypsin, removal of such a chain promotes activation. In other cases removal of a single amino-acid may result in loss of activity. Studies with inhibitors and observations such as the above have given rise to the "active centre" concept to explain the means whereby the substrate is bound to the enzymic molecule. The high substrate specificity of many enzymes suggests that these

active centres are relatively complicated structures designed to secure a good "fit" with the normal substrate. Dixon (1955) has suggested that the active centre may straddle a number of adjacent peptide chains as shown diagrammatically in Fig. 2.

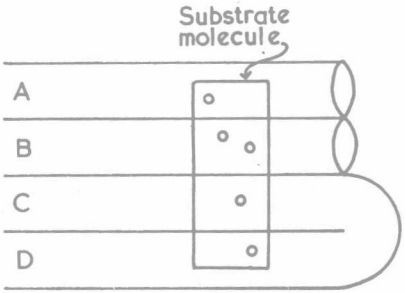


FIG. 2. Diagrammatic representation of a substrate molecule combined with an active centre of an enzyme. A, B, C, and D represent 4 adjacent folds of the peptide chains, each of which contributes to enzyme–substrate bonding. When the enzyme protein is distorted, the substrate molecule can no longer be accommodated (from Dixon, 1955).

Such an idea provides a convincing explanation of the loss of activity when the enzyme protein is subjected to distortion by physical or chemical means.

TERMINOLOGY AND CLASSIFICATION OF ENZYMES

The suggestion of Duclaux in 1898 that enzymes should be named after the substrate upon which they act coupled with the suffix "-ase" has been generally adopted, though certain of the digestive enzymes ending in "-in" have retained their original names. Thus the term "amylase" indicates an enzyme acting upon starch (amylum), and "sucrase", one acting upon sucrose. Further definition has proved necessary, sometimes to differentiate between two enzymes carrying out similar reactions but under different conditions, e.g. "acid phosphatase" and "alkaline phosphatase", and sometimes to indicate the nature of the chemical reaction, e.g. "glutamic oxaloacetic transaminase".

Enzymes may be classified into four main groups according to the nature of the reactions they catalyse, but each of these may be further subdivided as shown in Table 1. Some of the enzymes

listed could have been included in more than one group, thus alkaline phosphatase, for example, may, under certain conditions, catalyse the transfer of a phosphate group from one radical

TABLE 1

THE CLASSIFICATION OF THE DIAGNOSTICALLY IMPORTANT ENZYMES

I Enzymes which catalyse the addition or removal of water:
- (*a*) Hydrolysing enzymes (hydrolases), e.g.
  - Amylase
  - Pepsin
  - Trypsin
  - Lipase
  - Leucine aminopeptidase
  - Phosphatases, acid and alkaline
  - Cholinesterase
- (*b*) Hydrating enzymes (hydrases).

II Enzymes catalysing electron transfer, i.e. oxidation or reduction of substrate (oxidases, reductases, dehydrogenases), e.g.
- Glucose-6-phosphate dehydrogenase
- Lactate dehydrogenase
- *Iso*citrate dehydrogenase

III Enzymes transferring a radical from a donor to an acceptor molecule (transferases):
- (*a*) Phosphate radical transfer, e.g.
  - Phosphoglucomutase
- (*b*) Amino group transfer, e.g.
  - Glutamic oxaloacetic transaminase
  - Glutamic pyruvic transaminase
- (*c*) Transfer of carbohydrate residue, e.g.
  - Galactose-1-phosphate uridyl transferase

IV Enzymes which split or form $>C—C<$ bonds without group transfer (desmolases), e.g.
- Aldolase

V Miscellaneous enzymes, e.g.
- Phosphohexose isomerase

to another. Several other sub-groups of enzymes are known but have not been included because they have no known diagnostic value.

OCCURRENCE

While enzymes occur in all living cells, their distribution in human tissues varies considerably. Certain enzymes can only be found in a few tissues, thus pepsin occurs only (as its precursor,

pepsinogen) in cells of the gastric mucosa, and trypsin (also as a precursor, trypsinogen) in the acinar tissue of the pancreas. Many enzymes such as hexokinase are distributed widely throughout all tissues and consequently have little or no diagnostic value. The most important sites of production of the diagnostically useful enzymes are listed in Table 2, but this should not be regarded as exhaustive.

Many of the enzymes mentioned in Table 2 are intracellular

TABLE 2

DISTRIBUTION OF DIAGNOSTICALLY IMPORTANT ENZYMES

(?) indicates a rather uncertain or minor source which may cause difficulties of interpretation.

Enzyme	*Principal tissues in which enzyme occurs*
Acid phosphatase	Prostate, erythrocytes, (?) intestine
Alkaline phosphatase	Bone (osteoblasts), (?) liver, (?) intestine
Aldolase	Skeletal muscle, heart
Amylase	Saliva, pancreas
Cholinesterase (true)	Erythrocytes, brain, nervous tissue
Cholinesterase (pseudo)	Liver, serum
Galactose-1-phosphate uridyl transferase	Erythrocytes, liver
Glucose-6-phosphate dehydrogenase	Erythrocytes, spleen, liver, bone marrow
Glucose-6-phosphatase	Liver
Glutamic oxaloacetic transaminase	Heart, liver, skeletal muscle
Glutamic pyruvic transaminase	Liver
*Iso*citrate dehydrogenase	Liver
Lactate dehydrogenase	Heart, skeletal muscle, liver, kidney
Leucine aminopeptidase	Pancreas, liver
Malate dehydrogenase	Heart
Pepsin (-ogen)	Gastric mucosa
Phosphoglucomutase	Widely distributed
Phosphohexose *iso*merase	Widely distributed
Trypsin (-ogen)	Pancreas
Chymotrypsin (-ogen)	Pancreas

and consequently can only be released into the body fluids after the breakdown of the cell, thus after an episode of myocardial infarction the enzymes normally found in high concentration in heart muscle appear in excessive amounts in the blood plasma. Intracellular enzymes are often associated with subcellular particles: arginase, for example, occurs in the nucleus, succinate dehydrogenase is a mitochondrial enzyme, while others occur in microsomal preparations.

SUBSTRATES

Mention has already been made of the substrate specificity of a number of enzymes and of the means whereby this is attained. Of the enzymes listed in Tables 1 and 2 some, such as "true" or "specific" cholinesterase (better named acetylcholinesterase) which acts rapidly only upon acetylcholine, are highly specific in their substrate requirements whereas others are capable of acting upon a number of related compounds. "Pseudo" or "non-specific" cholinesterase is an example of the latter type for it hydrolyses several choline esters including butyryl- and propionyl-choline which are decomposed at a more rapid rate than acetylcholine. Lipase, too, is a relatively non-specific enzyme, for though its main physiological function is to saponify fats, it is capable of hydrolysing several other esters, and methods for determining its activity have been based upon its ability to hydrolyse tributyrin to glycerol and butyric acid. It behaves in many respects as a non-specific esterase, though it is specific in so far as its hydrolysing activity is confined to esters.

Enzymes which act upon a whole series of related compounds have certain specific structural requirements for their substrates. Esterases, for example, form enzyme–substrate complexes by attachment to their active centres of the carboalkoxyl groups of the ester, and when the enzyme is active against a number of related homologous esters it is clear that the hydrocarbon chain is not involved in the linkage.

The optical configuration of many compounds plays an important part in determining their susceptibility to enzyme attack. Thus malate dehydrogenase acts exclusively upon L-malate oxidizing it to oxaloacetate in the presence of nicotinamide-adenine dinucleotide (coenzyme I) (see p. 9). D-Malate is not affected and in the presence of the racemic form the L-stereoisomer alone is attacked, thus providing a method for the resolution of the DL form. A few cases are known in which the stereoisomer not attacked by the enzyme actually inhibits the reaction with the susceptible form. When a substrate with a symmetrical molecule is acted upon by a stereo-specific enzyme the product is optically active, thus L-glutamate dehydrogenase acts upon α-oxoglutarate to form L-glutamate. Some enzymes, however, are not stereo-specific and can act upon D and L forms with

equal facility and consequently may convert optical isomers into racemic forms. An example of an enzyme of this type is galactowaldenase which plays an important part in the metabolism of galactose (see Chapter 9).

Enzymes are usually specific also with respect to *cis* and *trans* isomerism, e.g. fumarase dehydrates malic acid to form the *trans* isomer, fumaric acid, only. Similarly in the reverse reaction, fumaric acid alone is hydrated and the *cis* isomer, maleic acid, is not attacked.

One of the most striking examples of substrate specificity concerns the enzyme, glucose oxidase, which almost exclusively oxidizes β-D-glucose to D-gluconic acid or its δ-lactone. α-D-Glucose and other sugars are oxidized either very slowly or not at all, and on only a few synthetic derivatives of D-glucose is there any significant effect. It might be noted in passing that this enzyme has provided a highly specific means for the determination of blood glucose.

COENZYMES AND ACTIVATORS

Diphosphopyridine Nucleotide and Triphosphopyridine Nucleotide (Coenzymes I and II)

Many enzymic reactions will not proceed unless a small concentration of a co-factor additional to the enzyme and substrate is present. Sometimes the co-factor is a simple metallic ion, such as Zn^{++} which activates lactate dehydrogenase or Mn^{++} required by *iso*citrate dehydrogenase and, in such cases, it is known as an "activator". The term "coenzyme" was originally applied to such an activator but is now restricted to a water-soluble dialysable organic compound in the absence of which an enzyme is inactive.

The first such substance to be detected was "cozymase" obtained by dialysis of yeast juice by Harden and Young in 1904. Complete removal of "cozymase" resulted in complete inactivation of the yeast extract which, however, became active again when treated with the co-factor. "Cozymase" has since been recognized as a co-factor in many other enzymic reactions, especially those involving reduction and oxidation, and was isolated in a pure state almost simultaneously by two inde-