ISOENZYMES

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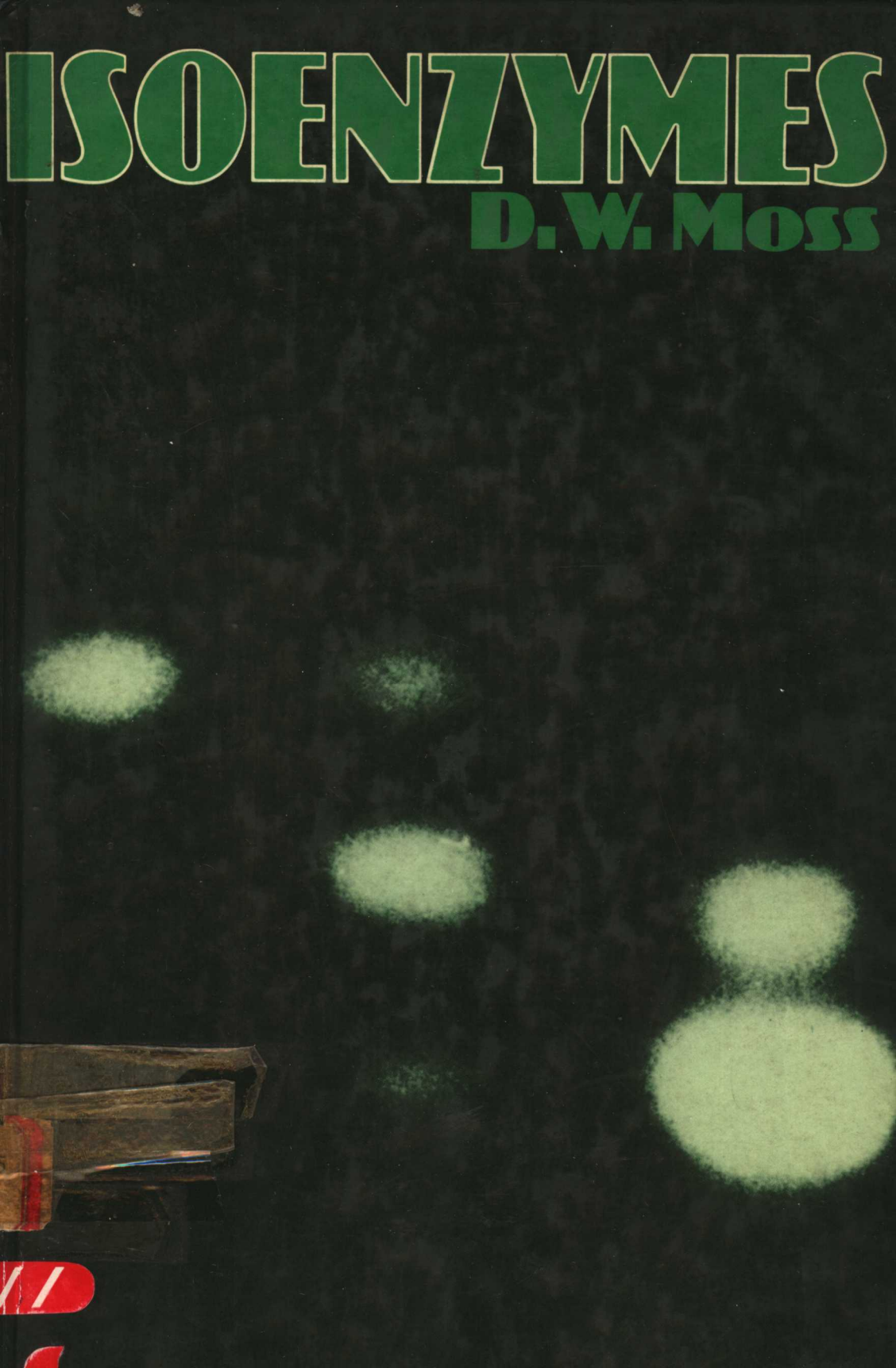

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Preface

The increased interest in multiple forms of enzymes that began with the application of new methods of fractionation to preparations of enzymes and other proteins some 25 years ago led quickly to an appreciation that the existence of enzymes in multiple forms, or isoenzymes, is a general phenomenon. The results of pioneering studies and those which followed in the early years of isoenzyme research consisted, not surprisingly, mainly of descriptions of the existence and characteristics of heterogeneity in various enzyme systems. Summaries of these results were provided in books such as J.H. Wilkinson's *Isoenzymes*, the first edition of which appeared in 1965. Some clearer ideas of the nature of the phenomena had become apparent by the time that the second edition of *Isoenzymes* was called for in 1970, and a limited use of the word isoenzymes itself, to describe only certain of the various categories of enzyme multiplicity then recognized, was already being proposed. Nevertheless, a largely enzyme-by-enzyme organization of the contents of the book was still appropriate.

Considerable advances, both experimental and conceptual, were made in isoenzyme research in the 1970s, and in 1977 Professor Wilkinson suggested to the present author that these should be taken into account in a joint revision of *Isoenzymes*. Professor Wilkinson's untimely death put an end to this project and the present book is therefore the work of a single author. Those who were familiar with Henry Wilkinson's work in clinical enzymology will appreciate the extent of the loss thus sustained.

It has seemed appropriate in writing this book to discard a solely phenomenological approach and to try instead to bring out those generalizations concerning the occurrence, nature, properties and, where possible, functions of multiple forms of enzymes which seem to be

justified by the results of research. These are illustrated by examples drawn almost entirely from animal, and especially human, enzyme systems on the basis of the author's greater familiarity with them. Readers whose interests lie mainly in the biochemistry of plants and micro-organisms may be disappointed by the limited attention that these categories of living matter have received; nevertheless, I hope that such readers will find some general principles of interest to them. The title *Isoenzymes* has been retained without qualification for this reason. The term 'isoenzymes' is also still widely used in an operational sense to describe any multiple forms of an enzyme, whatever their origins. This has provided a further reason for retaining the simple title, although descriptions of multiple forms of enzymes which do not fall within the current formal definition of isoenzymes are included in the book.

I thank those authors, editors and publishers indicated in the text who have given permission for the use of illustrations. I am greatly indebted to my collaborators for their part in my own experimental work on multiple forms of enzymes, and all authors will appreciate my debt to Mrs Brenda Salvage who prepared the typescript.

Donald Moss
July 1981

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1 Multiple Forms of Enzymes and the Emergence of the Isoenzyme Concept

The virtually limitless spectrum of chemical reactions catalysed by enzymes – far wider than the range of reactions influenced by inorganic or synthetic catalysts – was recognized early in the history of enzymology to be due to the existence of an almost equally wide range of enzymes, each with a characteristic specificity. In the third edition of his textbook of chemistry, published in 1837, J.J. Berzelius considered two alternatives: that a few enzymes with wide specificity might be responsible for this great range of catalytic ability, or that many specific enzymes might exist. He regarded the latter possibility as more likely (Dixon, 1971). Although the association of a uniquely-specific enzyme with each catalysed reaction could not be made, in view of the discovery of enzymes specific for particular chemical groups or reactions, classification of enzymes in functional terms became, and has remained, the most useful and practicable system. For equally valid reasons, the main effort in the systematic study of enzymes became concentrated on factors which influence the rate of the catalysed reaction, since this approach offered the best prospect of understanding the nature of the catalytic process and its functional significance. The possession of a particular type of catalytic ability thus became the primary consideration in the selection of enzymes for the study of aspects of catalysis, as it did in enzyme classification.

The early history of enzymology provides many examples of differences of properties between functionally-similar enzymes from different sources, and as early as 1895 Emil Fischer had noted the need to specify the origin of an enzyme when describing its properties. As well as differences between analogous enzymes from such dissimilar but frequently-used sources as yeast and mammalian tissues (e.g. yeast and liver alcohol dehydrogenases) differences between enzymes with similar

catalytic actions from various tissues of a single species were also recognized before 1950, as for example in the case of non-specific acid phosphatases from human prostatic and other tissues. However, successful attempts to demonstrate differences between enzymes of wide distribution in human tissues were few before this date, so that the weight of opinion was against the existence of organ-specific enzyme variants.

Even when several studies of multiple forms of an enzyme had accumulated, authors continued to find it necessary to go to considerable lengths to anticipate objections that their observations were the result of artefacts of the experimental techniques employed, or of ill-defined phenomena such as aggregation or association of a single enzyme with other components. For example, in discussing these possibilities in relation to their own, and earlier, results on the heterogeneity of horseradish peroxidase, including seasonal variations in the relative amounts of different components and differences in their reactivity towards various substrates, Jermyn and Thomas (1954) note that 'the existence of multiple components in naturally occurring enzymes is far from being generally accepted'. The viewpoint of classical enzymology towards analogous enzymes from different sources was expressed in the first edition of the authoritative monograph by Dixon and Webb (1958) in the words:

> 'It is a remarkable fact that in general the catalytic properties, specificity, activity, affinities, etc., of a given enzyme vary little with the source. Although there may be slight physical differences in a given enzyme when it is produced by different cells they are usually unimportant, and the enzyme remains essentially the same enzyme'.

Concentration on functional rather than structural aspects of enzymes was reinforced at first by uncertainties about the chemical nature of enzymes and later, when the protein nature of enzymes was accepted, by the absence of methods for the isolation and analysis of proteins. Some early experimental studies on protein structure, such as those made possible by the ultracentrifuge, seemed to encourage speculation that proteins would prove to have repeating structural elements in common, and that the possibilities for structural variations between molecules would consequently be limited (Fruton, 1979). However, elucidation of the amino acid sequences of proteins in increasing numbers from the mid-1950s onwards demonstrated their individuality and disposed of theories of protein structure which predicted the repetition of common structural elements at the primary level. Studies of the characteristics of enzymic catalysis had by this time established the concept of the active centre, a

relatively small region of the molecule at which attachment of the substrate takes place. Therefore, the possession of identical active centres could be expected to endow analogous enzyme molecules from different sources with their common catalytic properties, while allowing scope for variations in other properties through structural differences in catalytically-inactive regions of their molecules.

In some respects the recognition that the identity of each polypeptide chain is determined by its specific amino acid sequence, and therefore that not only this primary structure but also the three-dimensional secondary and tertiary structures which follow from it are characteristic of a particular protein, may appear to impose new restrictions on the possibility of structural variation between functionally-similar proteins. Furthermore, investigations of the relationship between structure and function (e.g., in the case of haemoglobin) have drawn attention to the functional importance of structural features distant from the primary substrate- or ligand-binding site, seeming further to reduce the extent to which protein structures can differ while retaining an overall similarity of function. However, this latter consideration itself adds a new dimension of interest to the search for variant forms of enzymes and other biologically-active proteins, since it increases the likelihood that the structural differences between them will be associated with functional differences, the nature and significance of which would not be apparent from the study of a single molecular species.

The emergence of a generalized concept of the existence of enzymes in multiple forms was dependent on the development of means for the separation and characterization of closely similar protein molecules, through which the prevalence of such multiple forms came to be recognized.

Although analytical techniques such as moving-boundary electrophoresis had brought to light the heterogeneity of certain purified enzymes, e.g. of crystalline lactate dehydrogenase from beef heart (Neilands, 1952), the improvement of separative methods based on differences in net molecular charge was responsible for the great increase in interest in enzyme heterogeneity from the middle 1950s onwards. Chromatography on substituted-cellulose ion-exchange materials was used in some early studies of the multiple forms of lactate dehydrogenase – an enzyme with a central position in the development of the isoenzyme concept – and this is still an important preparative technique in isoenzyme studies. However, the demonstration of the widespread occurrence of enzymes in multiple forms is due mainly to the application of techniques of zone elec-

trophoresis, especially with starch gel as the supporting medium, and with the adaptation of histochemical methods to visualize the separated enzyme zones *in situ* (Hunter and Markert, 1957). The 'zymogram' technique, as it has been called, has remained the most useful single experimental method in studying the multiple forms of enzymes, especially in the detection of enzyme heterogeneity in tissue-extracts or blood serum for clinical purposes or when screening for enzyme polymorphisms in human or animal populations. Its importance can be gauged by the fact that electrophoretic mobility has become the most widely accepted property by which the multiple forms of an individual enzyme are designated, with components being assigned serial numbers in order of decreasing anodal mobility.

Besides the technique of zone electrophoresis, however, a wide range of methods for the separation and characterization of enzymes is regularly brought into use, comprising various forms of chromatography, electrophoresis and electro-focusing, studies of kinetic and immunological properties, selective inactivation by various agents, and structural analyses of differing degrees of completeness, with the ultimate aim of defining the differences between multiple enzyme forms in molecular terms (Moss, 1979).

The first generally accepted descriptive term for the existence of different molecular forms of proteins with the same enzymatic specificity was introduced by Markert and Møller (1959), who coined the word *isozymes* to describe this phenomenon. As is often the case with new coinages, the derivation of the word aroused some controversy, some authors preferring the spelling *isoenzymes,* and as a result both forms have survived and are used interchangeably.

When first introduced, the term isozymes (or isoenzymes) was not restricted to multiple forms of an enzyme existing within a particular biological context, e.g. a single species, but alternative or more restricted applications have also been suggested. Augustinsson (1961) proposed that multiple enzyme forms should be regarded as isoenzymes only when the differences between them involved little or no variation in the combination of enzyme and substrate. While this emphasizes the concept of an invariate active centre with the possibility of some variation in structure in other molecular regions, multiple forms of an enzyme which exhibit significant differences in their catalytic properties are now regarded as being particularly interesting. Isoenzymes have also been regarded as multiple forms of enzymes having a common tissue of origin, with the term 'heteroenzymes' suggested for the more general case of catalytically-

similar enzymes found in different organs or species (Wieland and Pfleiderer, 1962). However, these attempts at more restricted definitions have not received wide acceptance.

The earliest uses of the term isoenzymes were also without implications as to the reasons for the existence of the multiple enzyme forms so described, although the problems posed for genetics by the multiplicity of proteins with a common activity were soon recognized (Markert and Møller, 1959). However, as the nature of some multiple forms of enzymes became clearer through genetic and structural studies, it became possible to define isoenzymes in terms of their genetic origins. According to the current recommendations of the Commission on Biological Nomenclature of IUPAC-IUB (1977), isoenzymes are defined as multiple molecular forms of an enzyme occurring within a single species, as a result of the presence of more than one structural gene. The multiple genes may be due to the presence of multiple gene loci or of multiple alleles. (The term 'allelozymes' is also used to denote isoenzymes deriving from allelic genes). Also included in this definition of isoenzymes are those multiple forms of enzymes which arise by the association of protein subunits that are themselves products of distinct structural genes.

Variant forms of enzymes which originate by post-genetic modifications of a single polypeptide chain, as in the conversion of inactive precursors of proteolytic enzymes to their active forms, are not regarded as isoenzymes, nor are the covalently-modified (e.g. phosphorylated or dephosphorylated) or conformationally-different forms in which certain enzymes may exist, and through which regulation of their activities is effected. Changes such as these and the enzyme forms with a more or less transient existence to which they give rise are not considered in this book. However, other stable multiple forms of enzymes which do not appear to be of genetic origin will be described, although in many cases their nature and significance are imperfectly understood.

An analogy can be drawn between the periodic table of the elements, drawn up originally on the basis of similarity of properties of elements in the same group, and the present systematic list of enzymes first proposed by the Enzyme Commission of the International Union of Biochemistry, which classifies enzymes according to the nature of the reaction which they catalyse*. In the way that isotopes of an element with different nuclear structures but common properties share the same position in the periodic table, isoenzymes catalysing the same reaction are subsumed

* Enzymes are referred to by their trivial names in the text of this book. Their corresponding Enzyme Commission numbers are given in the index.

under the same identifying number in the Enzyme Commission's list. However, the analogy is not an exact one. Isotopes of a given element all possess identical arrangements of their outer electron shells and consequently are identical in their chemical properties. Members of a particular set of isoenzymes are generally not completely identical in their catalytic properties, except in the nature of the reaction which they catalyse, and the extent of such functional differences gives rise to disagreement in some cases as to where a distinction should be drawn between sets of isoenzymes on the one hand, and groups of distinct but similar enzymes on the other.

The formal definition of isoenzymes now current, with the distinct genetic origins of multiple forms of enzymes as its basis, avoids the problem of specifying the degree of functional similarity which is to be expected in deciding whether the multiple forms in question should be classed as isoenzymes or not. Such difficulties are generally resolved by usage. Thus, although various proteolytic enzymes, such as trypsin and chymotrypsin, are functionally similar and are clearly of distinct genetic origins, they are not regarded as isoenzymes, or even as multiple enzyme forms, but as distinct enzymes, because of the otherwise marked differences between them. Similarly, non-specific acid and alkaline phosphatases display considerable similarities with regard to substrate specificity, but these two classes of enzymes are also not considered to be isoenzymic.

In some instances the conventions by which similar enzymes are regarded as distinct and are assigned individual numbers in the list of enzymes derive from the dates and circumstances of their discovery. For forty years a distinction has been drawn between enzymes capable of hydrolysing esters of choline, the acetylcholinesterase ('true' cholinesterase) characteristic of nervous tissue and the cholinesterase ('pseudo' cholinesterase) of serum, on the basis of their different but overlapping substrate specificities, although in other catalytic properties these enzymes are closely similar. Their independent genetic origins were demonstrated by the discovery of inherited variants of serum cholinesterase soon after the introduction of suxamethonium into anaesthetic practice in 1949. The relationships between these catalytically-similar but genetically-distinct forms therefore fall within the scope of the current definition of isoenzymes and it seems likely that, had their discovery and characterization taken place in more recent years, they might have been regarded as such, rather than as separate enzymes with consecutive numbers in the Enzyme Commission's list, as at present.

However, opinion is by no means unanimous on the isoenzymic status of more recently recognized enzymes with similar but not identical catalytic properties. Hexokinases which convert glucose to glucose-6-phosphate are widely distributed in mammalian tissues, and multiple forms of these enzymes are generally considered to be isoenzymes. A kinase present in the liver of some species is distinguished from this group by its more restricted substrate specificity and its higher Michaelis constant for glucose. This enzyme, referred to as glucokinase, has been given a separate identifying number, although many workers consider it to be a member of the hexokinase isoenzyme system (Purich *et al.*, 1973). Particularly difficult problems of classification arise with enzymes such as the non-specific esterases, which exist in numerous multiple forms in many species. Individual forms can be distinguished on the basis of their relative specificites for various synthetic substrates, but groups of such esterases are in some cases encoded by structural genes which are closely linked on a single chromosome, suggesting the common evolutionary origin thought to be characteristic of isoenzymes.

Just as in older studies the absence of a general awareness of the existence of variants of a single enzyme caused each discovery of heterogeneity of a particular catalytic property (for example, in a tissue extract) to be seen as evidence for the existence of distinct and unrelated enzymes, current acceptance of the isoenzyme concept may predispose enzymologists to group together under this description catalytic activities, which on closer examination, are indeed found to be manifestations of the presence of distinct enzymes. An example of this tendency is provided by a minor component of tryosine aminotransferase activity found in the cytoplasm of rat liver. At first regarded as an isoenzyme of the main tyrosine aminotransferase of this tissue, subsequent investigation showed the minor activity to be due to aspartate aminotransferase, an enzyme with quite distinct properties (Spencer and Gelehrter, 1974).

In spite of problems of definition, the concepts embodied in terms such as 'isoenzymes', or even the less restrictive 'multiple molecular forms of enzymes', are valuable in directing attention to features of enzyme evolution, structure and function from which significant generalizations can be inferred. Some of the generalizations which have already emerged as a result of the stimulus given to enzyme research by the isoenzyme concept are outlined in the following chapters.

2 Origins and Structures of Multiple Forms of Enzymes

The definition of isoenzymes as the products of distinct structural genes implies that those multiple enzyme forms which fall within its scope will differ to a greater or lesser extent in their amino acid sequences. In turn, these differences in primary structure will also entail greater or lesser differences in the higher levels of protein structure. The interpretation of the differences between isoenzymes in structural terms is well advanced in several cases. However, the origins of other categories of enzyme heterogeneity, and therefore the differences in structure existing within them, are in general much less clearly understood.

ORIGINS OF ISOENZYMES

The groups of genes which determine the structures of families of isoenzymes can represent several different phenomena: the existence of multiple gene loci, the occurrence as the result of mutation of pairs of unlike genes (alleles) at the same locus, or the modification of the structures or expression of genes in somatic cells, e.g. as an accompaniment of malignant transformation (Fig. 2.1).

Isoenzymes which are the products of allelic genes are distributed in the population according to the laws of Mendelian inheritance, and these hereditary patterns identify the nature of their genetic origins. Multiple forms of enzymes resulting from the existence of multiple gene loci have become disseminated throughout the whole species during the course of evolution, so that all individuals typically possess the same complement of isoenzymes. Consequently the genetic origins, and therefore the isoenzymic status of the multiple forms, cannot be readily inferred by comparing their patterns of occurrence. In some cases however, allelic