KEITH J. LAIDLER

THE CHEMICAL KINETICS OF ENZYME ACTION

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BY

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PREFACE

A VERY considerable amount of work is now being carried out on the various aspects of enzyme reactions; some of it deals with the structures of enzyme molecules, some with the kinetics and mechanisms of enzyme action. Enzymes have a special appeal because their study lies in the borderland between the biological and the physical sciences, and anyone working with them must have some knowledge of each field. The amount of labour required to acquire even a working knowledge of both sides of the problem is very great, and this book has been prepared with the idea that it might be helpful to collect together some of the main results and concepts that have been developed on the physical side.

An author writing on a subject that is advancing rapidly has to face the situation that certain parts of his book will necessarily be out of date in a fairly short time, perhaps even before the book is published. In view of this fact I decided to place much greater emphasis on general principles, and less on the details of individual enzyme systems. Many of these principles have survived more or less intact for some time, although they are all in a constant state of development, and it seems likely that some, at least, will prove to have been well founded. As far as our knowledge of individual enzyme systems is concerned, however, it seems more probable that great changes in our ideas will follow, probably within a very few years.

The first eight chapters are mainly concerned with the more general aspects of enzyme action. Chapters II, III, and IV, for example, deal with basic kinetic laws, and the principles and methods discussed in them will probably remain valid for some time to come. Chapters V, VI, and VII are of a more speculative nature, in that they involve deductions about mechanisms made on the basis of kinetic and other studies. Chapter VIII, dealing with effects of hydrostatic pressure, describes a subject that is still in its infancy, and which may be expected to develop considerably within the next few years.

Four chapters, Chapters IX to XII, are concerned with individual enzyme systems. No attempt has been made to be exhaustive, but merely to describe some of the main features of a small group of enzymes. The principle I have adopted in writing these chapters has been to select a few enzymes on which enough work has been done to make one feel that some of the main features of their action has been elucidated: in all cases, however, it is possible that further work will throw an altogether different light on the situation. This principle of selection has caused the omission of certain enzymes, notably the carbohydrases, which have not yet been

investigated in sufficient detail, and for which it has seemed to me that a review would at the present time be premature. (This is mainly due to the fact that the carbohydrases have not yet been prepared in pure form.) My own research interests have so-far been mainly in the hydrolytic enzymes, and those working on the other types of enzymes will undoubtedly feel that the treatment of Chapters XI and XII is very sketchy. There are several excellent reviews of these enzymes, and I did, indeed, consider omitting these chapters altogether and confining the book to hydrolytic enzyme action; this, however, seemed undesirable in view of the fact that many of the basic principles discussed in the earlier chapters owe much to what has been learnt about non-hydrolytic enzymes.

Another aspect which workers in the field will consider to have been inadequately treated is that concerning the effects of ions on enzyme action. This matter is only dealt with very briefly and the discussion is scattered throughout the book; a consolidated chapter treating this subject as a whole would have been desirable. However, after much consideration I decided that knowledge of this field has advanced so little (in spite of the many excellent efforts that have been made) that such a chapter would necessarily have too ephemeral a value to be worth undertaking. Perhaps within a few years the task will be worth while.

I owe to many colleagues my thanks for the help they have given me, both directly and indirectly, in the writing of this book. My association and collaboration with Dr. M. F. Morales of the U.S. Naval Medical Research Institute has contributed very markedly to the development of my ideas on the subject of enzyme kinetics and mechanisms. Several friends have been kind enough to read, and to criticize in detail, early drafts of parts of the book on which they themselves are expert: they are Professor R. Lumry of the University of Minnesota and Professor W. J. Kauzmann of Princeton (denaturation); Professor R. F. Beers of M.I.T. and Professors Britton Chance and P. George of the University of Pennsylvania (catalase and peroxidase); Professor I. B. Wilson of Columbia (cholinesterase); Professor L. Ouellet of the University of Ottawa (ATP-ase). Dr. B. R. Stein and Mr. E. K. Gill of the University of Ottawa have helped me greatly with the proof-reading, and my wife has rendered great assistance with the preparation of the indexes. Finally, but not least in importance, to my present and former students working in this field I am grateful for what they have taught me.

K. J. L.

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CHAPTER I

THE CHEMICAL CHARACTERISTICS OF ENZYMES

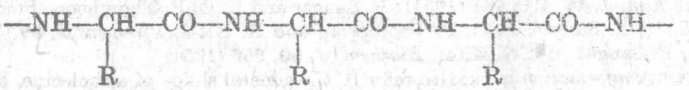
THE enzymes are important and essential components of biological systems, their function being to catalyse the chemical reactions that are essential to life. Without the efficient aid of the enzymes these chemical processes would occur at greatly diminished rates, or not at all.

This book is concerned mainly with the kinetics of the reactions catalysed by enzymes, and with mechanisms by which the reactions occur. The general properties of enzymes have been discussed in a number of books and review articles.† In the present chapter will be given only a very brief account of those characteristics of enzymes about which some knowledge is necessary for an understanding of the kinetic aspects of the subject. The main topics that will be considered are the chemical nature of enzymes, some qualitative features of enzyme-catalysed reactions, and the nature of the active centres of the enzyme molecules.

The Chemical Nature of Enzymes‡

A number of enzymes have now been prepared in highly purified form and their chemical properties studied in some detail. In 1926 Sumner made a very important advance when he succeeded in crystallizing the enzyme urease and in showing that it is a pure protein. Since that time many other enzymes have been prepared in pure form and have been shown either to be pure proteins or to be largely protein in nature. Most proteins are soluble in water, in dilute salt solutions, and in dilute solutions of alcohol in water. They are insoluble in water containing high proportions of alcohol, are salted out of aqueous solutions by neutral salts, and are precipitated by reagents such as picric acid and phosphotungstic acid. These properties are typical of the enzymes.

The essential structural unit in the proteins is a long chain arising from the condensation of a number of α-amino acid residues. These residues are held together by peptide (—CO—NH—) linkages, and the structure may be represented schematically as:



† For detailed treatment of enzymes see J. B. Sumner and K. Myrbäck (ed.), *The Enzymes*, Academic Press, New York (1951). For a more concise discussion see K. J. Laidler, *Introduction to the Chemistry of Enzymes*, McGraw-Hill Book Co., New York (1954).

‡ For a detailed treatment of the general properties of the proteins see H. Neurath and K. Bailey (ed.), *The Proteins*, Academic Press, New York (1954).

Such structures are known as peptide, or polypeptide, chains. The groups represented as R in the above formulation may be hydrogen (if the amino acid is glycine) or may be one of a number of groups corresponding to the various amino acids. There are usually a considerable number of different amino acids within a single protein molecule, and these amino acids are almost invariably in the L-configuration. The nature and proportions of the amino acids in a protein can be determined by carrying out complete hydrolysis and analysing for the various amino acids. The question of the order in which the amino acids are arranged is, however, a much more difficult one to settle, and this problem is being actively attacked at the present time.†

Another matter of considerable interest is the exact conformation‡ taken up by the chain of amino acids. This will be considered in some detail in Chapter XIII, but certain brief comments will be made now. There are two main classes of proteins, the fibrous and the globular; in the former the individual molecules are long and thin, while in the latter they have more spherical shapes. In the fibrous proteins it seems certain that the polypeptide chains are arranged in the form of a fairly tight straight spiral, probably with about four amino acid residues in each turn of the spiral (see Fig. 76 in Chapter XIII, p. 347). In the globular proteins these spirals also exist but each spiral is folded (see Fig. 78 in Chapter XIII, p. 350), being held in this conformation by various kinds of chemical bonds. Many important properties of the proteins depend on the exact manner in which the polypeptide chains are arranged within themselves and with respect to one another.

In particular, the ability of an enzyme to catalyse a chemical reaction may depend in a very critical way on the conformation of the peptide chain. It is found that enzymes show their maximum catalytic effect when they are freshly extracted from the biological material. On standing, and especially under the action of heat, of acids and bases, and of various other substances such as urea, they show a continuous diminution of catalytic activity. This process is known as the *inactivation* of the enzyme, and is found to be a special aspect of a more general type of process that is observed with all

† For recent work of this kind on the protein insulin see especially F. Sanger and H. Tuppy, *Biochem. J.* **49**, 463, 481 (1951); F. Sanger and E. O. P. Thompson, *Biochem. J.* **53**, 353–66 (1953); F. Sanger, E. O. P. Thompson, and R. Kitai, *Biochem. J.* **59**, 509 (1954); H. Brown, F. Sanger, and R. Kitai, *Biochem. J.* **60**, 556 (1954).

‡ The term *conformation* is used to refer to the general shape of a molecule, and is to be distinguished from *configuration*, which refers to the relative arrangements of the atoms or groups in a molecule. The conformations of a given molecule, also referred to as rotational isomers, are converted into one another merely by rotation about single bonds, and without the breaking and making of major bonds: in some instances, however, as with proteins, it is permissible to make and break hydrogen bonds and other weak bonds in passing from one conformation to another. For a recent review of the significance of conformations in organic chemistry, see D. H. R. Barton and R. C. Cookson, *Quart. Revs.* **10**, 44 (1956).

proteins and is known as *denaturation*. Essentially denaturation is due to a change in the conformation of the protein. The kinetics and mechanisms of protein denaturations are discussed in some detail in Chapter XIII.

Many of the characteristic properties of the enzymes arise from the very large sizes of the molecules. Owing to the low rates of diffusion processes the enzymes do not readily move from one place to another in a biological system, and in particular they do not generally pass through the cell membranes.

Other characteristic properties of the enzymes (and of other proteins) arise from their electrical nature. The R side-chains of the proteins frequently contain ionizing groups, such as —COOH, $—NH_2$, phenolic —OH, etc. Under suitable conditions of acidity these groups ionize to give charged groups:

$$—COOH + H_2O \rightleftharpoons —COO^- + H_3O^+ \quad (pK \approx 4)$$

$$—NH_2 + H_2O \rightleftharpoons —NH_3^+ + OH^- \quad (pK \approx 9)$$

$$—OH + H_2O \rightleftharpoons —O^- + H_3O^+ \quad (pK \approx 10).$$

In fairly neutral solutions, such as usually exist in physiological systems, many of the ionizing side groups exist in the charged forms, so that as a result enzyme molecules may contain a considerable number of positively and negatively charged groups. There is much evidence, to be considered later, that the existence of these ionizing groups has much to do with the catalytic activity of the enzymes. Moreover, as will be discussed in detail in Chapter V, the remarkable variations of catalytic activity with pH are to be correlated with changes of ionization of certain of these groups. Another point of some interest is that the charged groups of proteins tend to bind foreign ions (such as those of metals) and that the binding of such ions is sometimes closely related to enzymatic action.

Another effect arising from the ionization of protein molecules is that migration may occur in an electric field; this phenomenon is known as *electrophoresis*. In sufficiently acid solution the carboxyl groups in the protein will be in the undissociated —COOH form, whereas the amino groups will be in the charged $—NH_3^+$ form; the molecule will therefore have a net positive charge and will migrate towards the cathode if a potential is applied. Conversely, at low acidity the groups will be present as $—COO^-$ and $—NH_2$, and the molecule, having a net negative charge, will migrate towards the anode. At some intermediate pH the number of positive groups will be equal to the number of negative ones; the molecule will then have no net charge, and there will be no movement in a field. The pH at which there is no migration is known as the *isoelectric point* and is a characteristic property of a protein.

Prosthetic Groups

Certain enzymes, like pepsin and urease, are found on detailed analysis to be pure proteins. Others, however, have been found to consist of a non-protein part in addition to the protein. In such cases the protein part of the enzyme is known as the *apoenzyme*, and the non-protein part as the *prosthetic* group. The enzyme as a whole, consisting of the apoenzyme and the prosthetic group, is known as the *holoenzyme*. Sometimes the prosthetic group is easily separated from the apoenzyme, in which case it is sometimes known as a *coenzyme*. An example is the enzyme lactic dehydrogenase (Chapter XI) which is easily split into its apoenzyme and a substance known as diphosphopyridine nucleotide (DPN) or as Coenzyme I; for enzymatic activity the presence of this coenzyme is essential. In the case of other enzymes it is found that ions are attached to the protein and that removal of these causes loss of enzymatic activity; such ions are usually known as *activators* rather than as coenzymes. In the above cases an easy way of separating the protein from the prosthetic group is by dialysis; the prosthetic group passes readily through the membrane while the apoenzyme remains behind.

In other enzymes the apoenzyme is firmly bound to the prosthetic group and cannot be separated from it by dialysis or by other simple means. This is the case with the enzyme catalase (Chapter XII), which consists of a protein very firmly bound to haematin, its prosthetic group. In some enzymes the apoenzyme is very firmly bound to metal that cannot easily be removed. It appears that the distinction between the coenzymes and the prosthetic groups that are firmly bound is simply one of degree, the function of the enzyme apparently not being related to the strength of binding to the apoenzyme.

Enzymes as Catalysts

The essential characteristic of a catalyst† is that it influences the rate of chemical reaction but is not itself used up during the process and can in ideal cases be recovered at the end of the reaction. This does not, of course, imply that the catalysts act by virtue of some external effect which they exert, and that they do not themselves enter into reaction: on the contrary it is well established that in all types of catalysis the catalyst forms some kind of complex with the substrate (i.e. the reacting substance) and that this complex finally breaks down into the products of reaction and the catalyst. It is frequently found that the rates of catalysed reactions are directly proportional to the concentration of the catalyst; in the case of reactions that do not occur at an appreciable rate in the absence of catalyst this means

† For a detailed treatment of catalysts of various kinds see P. H. Emmett (ed.), *Catalysis*, Reinhold, New York, vol. 1 (1954), vol. 2 (1955), vol. 3 (1956), vol. 4 (1957), vol. 5 (1957).

that a plot of rate against catalyst concentration is simply a straight line passing through the origin.

It is a characteristic property of catalysts that they catalyse reactions to the same degree in forward and reverse directions. The following simplified argument (which is not to be taken as a rigorous proof) gives an indication of why this is so. Consider the chemical equilibrium: $A+B \rightleftharpoons C+D$. The rate of this reaction may under certain conditions be given by:

$$v_1 = k_1[A][B], \tag{1}$$

where k_1 is the rate constant and [A] and [B] the concentration of the two reactants. The rate from right to left is similarly:

$$v_{-1} = k_{-1}[C][D]. \tag{2}$$

At equilibrium these rates must be equal, so that

$$k_1[A][B] = k_{-1}[C][D], \tag{3}$$

whence

$$\frac{[C][D]}{[A][B]} = \frac{k_1}{k_{-1}} = K, \tag{4}$$

where K, equal to k_1/k_{-1}, is the equilibrium constant for the reaction. Since the catalyst does not enter into the over-all reaction it cannot have any effect on the extent to which the reaction occurs, i.e. it cannot affect K. If on the addition of a catalyst the rate v_1 is increased by a certain factor, k_1 must be increased by the same factor, and it therefore follows that k_{-1} and v_{-1} are also increased by the same factor.

In all of the above respects the enzymes exhibit the typical properties of catalysts. In some ways, however, they show unusual characteristics which are not found with other types of catalysts. Among these are those properties, like sensitivity to heat and extremes of pH, in which they show the typical behaviour of proteins. Another way in which they are unusual is that on a molecular basis they are remarkably effective in comparison with other types of catalysts. A very crude but sometimes useful way of giving an indication of the efficiency of a catalyst is in terms of its *turnover number*, which is defined as the number of molecules that are caused to react in one minute by one molecule of catalyst. Under certain conditions the enzyme catalase has a turnover number of about 5,000,000 and this is higher by many powers of ten than that shown by other catalysts for the decomposition of hydrogen peroxide. This is rather an extreme case, but in every instance it appears that the natural enzyme has a higher turnover number than has any other catalyst.

Specificity

Another respect in which enzymes have unusual catalytic properties is in their specificity. Various different types of effects have been observed, of which the following are particularly important:

1. *Absolute specificity.* When an enzyme will bring about reaction in only a single substrate it is said to exhibit absolute specificity. A well-known example is the enzyme urease, which catalyses the hydrolysis of urea, H_2NCONH_2, to ammonia and carbon dioxide, but has no action on any other substance (including, for example, the closely analogous methyl urea, $CH_3NHCONH_2$). Succinic dehydrogenase is also absolutely specific with respect to the oxidation of succinic acid. Absolute specificity with respect to coenzyme is frequently encountered.

2. *Group specificity.* A lower degree of specificity is shown by certain enzymes that act upon a number of substrates but which have definite requirements with respect to the types of atomic groupings that must be present in these molecules. The enzyme pepsin, for example, will hydrolyse certain peptide linkages but requires among other things that an aromatic group be present in a certain position with respect to the peptide linkage; the precise requirements are outlined later (Chapter IX). Most of the proteolytic enzymes show such group specificity.

3. *Reaction specificity.* The lowest degree of specificity is shown by enzymes that catalyse a certain type of reaction irrespective of what groups are present in the neighbourhood of the critical linkages in the substrate. For example, the enzymes known as lipases will catalyse the hydrolysis of any organic ester (including the lipids). There are usually differences in rates for the different substrates; some lipases, for example, act more rapidly on the esters of short-chain acids than on those of long-chain acids, while others do just the reverse. Group and absolute specificity may indeed be regarded as special cases of reaction specificity, certain substrates reacting with zero velocity.

4. *Stereochemical specificity.* It is frequently found that an enzyme will catalyse the reaction of only one stereochemical form of the substrate; for example, the proteolytic enzymes usually only act on peptides that are made up of amino acids in the L-forms. Similarly, lactic dehydrogenase catalyses the oxidation of L-lactic acid, but not that of the D-form.

Much work still remains to be done on the question of the molecular explanation of specificity, and full understanding will only come when more knowledge has developed about the exact structures of enzyme molecules. Some of the ideas that have been put forward are discussed in Chapter VI, and here it may simply be noted that the exact fitting together of enzyme and substrate molecules may be essential for reaction. The replacement of one group by another may affect this fit in various ways; a group may simply, by virtue of its shape and size, get in the way and prevent the intimate contact of the vital parts of the enzyme and substrate molecules; or it may bring about a change in the general conformation of the substrate molecule.

Classification of Enzymes

Since there are very many different enzymes it is convenient to discuss them with reference to some type of classification. Various methods of classification are possible and none is completely satisfactory; those most useful from the standpoint of kinetics and mechanism are in terms of the types of reaction that the enzymes catalyse. No complete classification will be given here, since this has been done in a number of places elsewhere.† It is, however, convenient to give some kind of breakdown, and this will be limited largely to those enzymes that are specifically treated in later parts of the book. A great many enzymes, although of considerable biological importance and general interest, have received very little study from the standpoint of kinetics and mechanism, and are therefore at the most discussed very incidentally in the present book.

The main types of enzymes on which kinetic studies have been made are (1) the hydrolytic enzymes, (2) the oxidative enzymes, (3) the adding (and splitting) enzymes, and (4) the transferring enzymes. These may be discussed further as follows:

1. The *hydrolytic enzymes* catalyse reactions of the type:

$$AB+H_2O \rightleftharpoons AOH+BH.$$

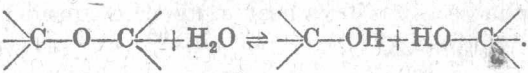
The main types are:

(*a*) The *proteolytic enzymes*, which catalyse the hydrolysis of peptide linkages:

$$-CO-NH-+H_2O \rightleftharpoons -COOH+H_2N-.$$

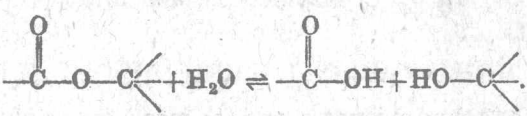
Examples are pepsin, trypsin, chymotrypsin, and carboxypeptidase, which act upon proteins and certain synthetic peptides. The kinetics of the reactions of these four enzymes have been investigated in some detail, and an account is given in Chapter IX.

(*b*) The *carbohydrases*, which catalyse the hydrolysis of the glucosidic linkage, which occurs in the di- and polysaccharides and various other substances:



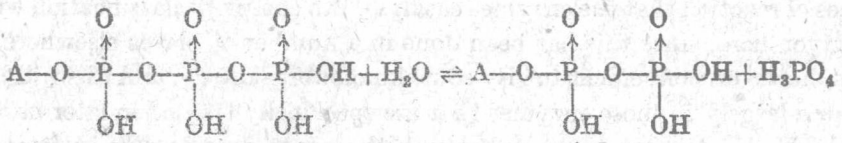
An example is sucrase, which catalyses the hydrolysis of sucrose to glucose and fructose. A few kinetic studies have been made on certain aspects of these enzymes, but since the information is fragmentary they are referred to only briefly in various parts of the book.

(*c*) The *esterases*, which hydrolyse esters to the acid and alcohol:



† See, for example, O. Hoffmann-Ostenhof, *Adv. Enzymol.* 14, 219 (1953).

Examples are the *lipases*, which hydrolyse the fats, and the *phosphatases*, which hydrolyse phosphate esters. One phosphatase which is of great biological importance is adenosinetriphosphatase (ATP-ase) or myosin, which catalyses the hydrolysis of adenosinetriphosphate into adenosinediphosphate;

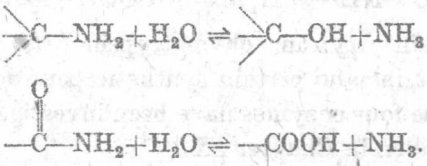


(A = adenosine). An esterase of great biological importance is cholinesterase, which catalyses the hydrolysis of acetylcholine into choline and acetic acid:

$$CH_3COOCH_2CH_2N^+(CH_3)_2+H_2O \rightleftharpoons CH_3COOH+HOCH_2CH_2N^+(CH_3)_3.$$

Mainly on account of their biological significance (with respect to muscle and nerve, respectively) ATP-ase and cholinesterase have been studied from the kinetic standpoint in very considerable detail; they are discussed in Chapter X. Other esterases have, however, received scant attention from this point of view, partly owing to difficulties in purifying and characterizing them.

(*d*) The *deaminases* and *deamidases* catalyse the hydrolysis of amines and amides with the liberation of ammonia:



An important example is urease, which catalyses the hydrolysis of urea:

$$H_2NCONH_2+H_2O \rightleftharpoons CO_2+2NH_3.$$

The kinetics of this reaction have been studied in considerable detail and are discussed in Chapter X.

2. The *oxidative enzymes* are concerned with oxidations of various types:

(*a*) The *dehydrogenases* catalyse the removal of two hydrogen atoms from a substrate molecule and transfer them to the coenzyme (which in some cases is built into the protein molecule). Lactic dehydrogenase, for example, brings about the oxidation of lactic acid to pyruvic acid, the two hydrogen atoms being taken up by Coenzyme I, which must be present for this enzyme to function:

$$CH_3CHOHCOOH+Co\text{-}I \rightleftharpoons CH_3COCOOH+H_2Co\text{-}I.$$

The kinetics of the dehydrogenases, and the mechanisms of their action, are discussed in Chapter XI.

(*b*) The *oxidases* bring about oxidation processes in which hydrogen is transferred directly to molecular oxygen. These enzymes are also treated briefly in Chapter XI.

3. The *adding enzymes* catalyse simple addition reactions of the type:

$$A+B \rightleftharpoons AB.$$

They may also be referred to as *splitting* enzymes since they also catalyse these reactions from right to left. An example is fumarase, which catalyses the interconversion of fumaric and malic acids:

$$HOOC.CH{=}CH.COOH+H_2O \rightleftharpoons HOOC.CH_2.CHOH.COOH.$$

Another example is carbonic anhydrase, which catalyses the splitting of carbonic acid and the combination of carbon dioxide and water:

$$H_2CO_3 \rightleftharpoons H_2O+CO_2.$$

4. The *transferring enzymes* catalyse reactions in which groups are interchanged between two molecules:

$$AB+CD \rightleftharpoons AC+BD.$$

An important example, the kinetics of which have been studied in great detail (see Chapter XII), is peroxidase, which catalyses the transfer of oxygen from hydrogen peroxide to suitable acceptors:

$$H_2O_2+AH_2 \rightleftharpoons 2H_2O+A.$$

The Active Centres of Enzymes

During the course of the various investigations that have been made on enzyme systems it has become increasingly apparent that the catalytic properties are not primarily properties of the enzyme molecules as a whole, but depend upon the existence of certain relatively small areas on the enzyme molecules. These small areas, believed to account for the main effects, are conveniently referred to as *active centres*, or *active sites*. It is at these active centres that the enzymes combine with the substrates and at which chemical reaction occurs. These active centres are, at least in some cases, structures of some complexity, and contain a number of different chemical groups arranged in such a manner as to accommodate the substrate. Some of these groups combine with the substrate, by hydrogen bonding or in other ways.

This view of the nature of enzyme action is in marked contrast to earlier ideas, according to which a large fraction of the surface is catalytically active. Now that a number of pure enzymes have been prepared, it has been possible to deduce on the basis of direct evidence, to be outlined later, that in many cases only a small fraction of the surface is active. With some enzymes, in fact, the evidence is that on each molecule there is only a single active centre, its area corresponding to a diameter of probably only a few

Ångströms. Other enzyme molecules have been found to have two active centres, and some have more, although in such cases there is evidence that the enzyme molecules may be associated.

It follows from this that it may be possible, in certain cases, to break enzyme molecules down into smaller fragments, some of which retain enzymatic activity. The search for small molecules that have powerful enzymatic activity is, indeed, an important and interesting aspect of enzyme

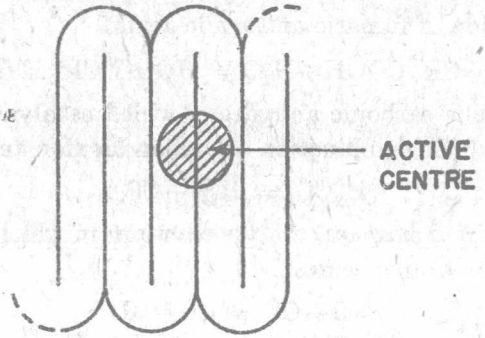


FIG. 1. Schematic diagram of a folded polypeptide chain, the active centre of the enzyme being shown to extend over two parts of the chain

research, and will undoubtedly contribute much to our understanding of enzymatic mechanisms. Perlmann† in particular has succeeded in breaking down pepsin molecules into fragments some of which, although small enough to pass through dialysis membranes, are enzymatically active.

In emphasizing the importance of active centres one should not completely discount the importance of the rest of the molecule. The fact that enzymes suffer a loss of activity when comparatively small changes are brought about in their structures suggests very strongly that active centres may depend for their existence on a particular type of folding in the protein molecule. This situation is represented schematically in Fig. 1. In this figure the active centre is shown as having components in two different chains of the protein molecule. When the protein undergoes denaturation the different components of the active centre come apart, with loss of activity. In Perlmann's protein fragments it is to be presumed that in some way the folding remains much the same in the critical regions in spite of a general decomposition of the molecule. It may be mentioned that some support for this composite view of active centres is provided by the fact (cf. Chapter XIII) that substrates frequently protect enzymes from deactivation; this they may do by holding the chains together in their original folds.

A good deal of evidence has now accumulated regarding the detailed

† G. Perlmann, *Nature*, 173, 406 (1954).

nature of active centres in individual enzymes, and about the number of active centres per enzyme molecule. Some of this evidence will be presented later in this book when particular enzymes are discussed, but it may be useful at this stage to describe some of the general methods that have been employed, and to summarize the main results.

Of the methods for determining the number of active centres per enzyme molecule, some are of general application, whereas some can only be applied in special cases. The more general methods usually involve a study of the number of molecules of an activator or an inhibitor that can be attached to an enzyme molecule, the work being accompanied by measurements of the way in which the enzyme activity is affected by the attachment. These methods sometimes give upper limits to the number of centres, since it is possible for inhibitors and activators to become attached at sites that are not concerned in reaction. The question of how much inhibitor or activator is attached can be determined in several ways. In some cases these substances are very firmly bound to the enzyme so that it is possible to isolate and purify a definite enzyme-activator or enzyme-inhibitor complex and study its composition. The inhibitor di-*iso*propylfluorophosphate (DFP), for example, forms very stable compounds with chymotrypsin and other enzymes, and in several instances these compounds have been found to contain one inhibitor residue to each enzyme molecule.† Zinc also forms a stable complex with carboxypeptidase, and maximum activity has been found to be associated with the attachment of one zinc atom to each protein molecule.‡

In other cases the complex formed between enzyme and activator or inhibitor undergoes dissociation too readily to permit an analysis to be made of its composition, and the nature of the attachment must then be studied using less direct methods. The enzyme may be allowed to come into contact with inhibitor or activator at a known total concentration, and the enzyme then separated from the solution by ultracentrifugation or equilibrium dialysis.§ From the composition of the resulting solution it is possible to calculate how the inhibitor or activator is bound to the enzyme. This may be repeated over a range of concentration and by suitable extrapolation procedures one can then determine the extent of binding that corresponds to saturation of the enzyme. An example of the use of this method is the work of Doherty and Vaslow,‖ using α-chymotrypsin. In this investigation, which is described in more detail on pp. 241–3, they found that complete inhibition was achieved when 1 mole of inhibitor combined with 22,000

† A. K. Balls and E. F. Nutting, *Adv. Enzymol.* 13, 321 (1952).

‡ B. L. Vallee and H. Neurath, *J. Biol. Chem.* 217, 253 (1955).

§ Cf. I. M. Klotz, F. M. Walker, and R. B. Pivan, *J. Amer. Chem. Soc.* 68, 1486 (1946).

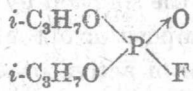
‖ D. G. Doherty and F. Vaslow, *J. Amer. Chem. Soc.* 74, 931 (1952); cf. also M. W. Loewus and D. R. Briggs, *J. Biol. Chem.* 199, 857 (1952).

grammes of enzyme. Since the molecular weight of α-chymotrypsin is close to this value it follows that there is one active centre to each molecule of this enzyme. This method can be used equally well with activators; the procedure would then be to determine how much activator is bound when maximum activation is achieved.

A second procedure, applicable only to enzymes that catalyse reaction between two different substrates or between a substrate and a coenzyme, involves studying the combination between the enzyme and only one of these substances. For example, if an enzyme catalyses reaction between A and B, the enzyme can be caused to combine with A, or with B, without the occurrence of any reaction. Using the equilibrium dialysis technique it is then possible to determine the maximum amount of A that will combine with the enzyme. The number of moles of A that will combine with one mole of enzyme is then the number of active centres of the type that are responsible for the binding of A; similar work can then be done with B. Such methods cannot in general be used with enzymes that act upon a single substrate, because the binding of the substrate is accompanied by reaction. In favourable cases, however, the method might be used; the chemical change would have to be very slow compared with the binding.

Various methods are available for learning something of the chemical nature of active centres of enzymes. A valuable method involves the analysis of velocity-pH curves for enzyme systems, and is discussed in some detail in Chapter V with reference to the available experimental data. The essence of the method is that the pK's of the ionizing groups in the active centres can be determined, and from the values of these reliable guesses can sometimes be made about the nature of the groups.

A second group of methods involves the use of irreversible inhibitors, and chemical analysis of the enzyme-inhibitor compounds formed. Most of the work of this kind has been done with di-*iso*propylfluorophosphate (DFP),



and has been carried out by Balls, Jansen, Jang, Nutting, and co-workers.† This substance is a very powerful inhibitor of such enzymes as chymotrypsin and cholinesterase and in each case the enzyme-inhibitor compound formed contains a phosphorus atom. By breaking the molecule down in a controlled manner it is possible to determine with what grouping in the enzyme molecule this fluorine atom is combined. Unfortunately the situation is complicated by the fact that apparently shifts take place during the

† For reviews see A. K. Balls and E. F. Nutting, *Adv. Enzymol.* **13**, 321 (1952); B. J. Jandorf, H. O. Michel, N. K. Schaffer, R. Egan, and W. H. Summerson, *Discuss. Faraday Soc.* **20**, 134 (1955).