The Chemical Kinetics of Enzyme Action

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PETER S. BUNTING

SECOND EDITION

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AND

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Preface

THE field of enzyme kinetics has developed greatly since the first edition of this book appeared in 1958. As a result, the book has been completely rewritten. Several completely new topics—such as isotope effects and sigmoid kinetics— have been introduced. The present edition places somewhat greater emphasis on general principles than did the first; there are many reviews of individual enzyme systems, and it was felt that it would be more useful to develop the underlying theory.

We are grateful to many colleagues for help with the preparation of the present edition. Dr. W. E. Hornby of the University of St. Andrews has read much of the manuscript and has made many valuable suggestions. Several of our colleagues at the University of Ottawa—especially Mr. N. H. Hijazi and Mr. I. Hinberg— have also been very helpful in the same way.

Ottawa, 1972

K.J.L.
P.S.B.

List of Main Symbols Employed

THE conventional Michaelis constant is employed in the present edition, instead of its reciprocal as in the first edition; the symbol K_A has been employed for the Michaelis constant with respect to the substrate A. Similarly, for an inhibitor Q the *dissociation* constant of the enzyme-inhibitor complex EQ is represented by the symbol K_Q. The reciprocal quantities employed in the first edition have the great advantage of simplifying the form of the kinetic equations, but the conventional symbols seem to be too deeply entrenched for any change to be practicable.

A, B; [A], [B]	substrate; concentration of substrate } small letters, a, a_0 etc. are used in equations involving time-dependence
$[A]_0$, $[B]_0$	substrate concentration at zero time
[A]′	concentration of substrate inside a support
AQ	substrate-modifier addition complex;
[AQ]	concentration of complex
β	voidage of a column
C	reaction capacity
ε_r	dielectric constant
D	diffusion constant
D	denatured protein
e	base of natural logarithms
e	charge on the electron
E; [E]; {E}	uncombined enzyme; its concentration; total amount of enzyme
E	activation energy
E_0	activation energy at 0 K
$[E]_0$	total concentration of all forms of enzyme
$[E]_m$	concentration of enzyme in a support
EA; [EA]	enzyme-substrate complex; its concentration
EA′; [EA′]	second intermediate (e.g. acyl enzyme); its concentration
ΔE	change in internal energy
EQ; [EQ]	enzyme-modifier complex; its concentration
EAQ; [EAQ]	enzyme-substrate-modifier complex; its concentration
EA′Q; [EA′Q]	complex formed from EA′ and modifier; its concentration
F	function employed for supported enzymes
F, G	constants in transient-phase equations
$\Delta G^{\ominus}$	standard Gibbs-free-energy change
ΔG^{*}	free energy of activation
h	Planck's constant
H^+; $[H^+]$	hydrogen ion; concentration of hydrogen ions
$\Delta H^{\ominus}$	standard change in enthalpy
ΔH^{*}	enthalpy of activation
k, k_1, k_{-1}, k_2 etc.	rate constant; subscripts refer to numbered reactions
k	Boltzmann constant
k_0	second-order rate constant at low substrate concentrations
k_c	limiting rate constant at high substrate concentrations
$\tilde{k}_c$	pH-dependent k_c

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$K_1, K_2, \ldots$	equilibrium constant for reaction 1, 2, etc.
K_a, K_b	acid dissociation constants
$K_A, K_B, \ldots$	Michaelis constant for substrates A, B, ...
$\bar{K}_A, \bar{K}_B$	pH-dependent Michaelis constants
K_Q	dissociation constant for enzyme-modifier complex (for $EQ \rightleftharpoons E + Q$)
K_Q^i, K_Q^s	constants in general inhibition equation
K_Q^A	dissociation constant for a substrate-modifier complex ($AQ \rightleftharpoons A + Q$)
K_{AQ}	dissociation constant for enzyme-substrate-inhibitor complex ($EAQ \rightleftharpoons EA + Q$)
$K_{A'Q}$	dissociation constant for EA'Q ($EA'Q \rightleftharpoons EA' + Q$)
m, n	concentration of a reaction intermediate (e.g. EA, EA')
pK_a, pK_b	negative logarithm of acid dissociation constant for free enzyme
pK_a', pK_b'	negative logarithm of dissociation constant of enzyme-substrate complex
pK_a'', pK_b''	negative logarithm of acid dissociation constant of second intermediate (e.g. acyl enzyme)
pK_A	negative logarithm of Michaelis constant
$p\bar{K}_A$	negative logarithm of pH-dependent Michaelis constant
l	thickness of solid support
N; [N]	native protein; concentration of native protein
P	partition coefficient
P; [P]	protein; its concentration
P, Q, R, P', etc.	constants in transient-phase equations
ψ	electrical potential
q	partition function
Q; [Q]	modifier (activator or inhibitor); its concentration
Q	rate of flow
r	distance of approach of charge centres in bimolecular ionic reactions
R	gas constant
s	cross-sectional area
$\Delta S^{\ominus}$	standard entropy increase
$\Delta S^{\neq}$	entropy of activation
$\Delta S_{es}^{\neq}$	electrostatic contribution to entropy of activation
$\Delta S_{nes}^{\neq}$	nonelectrostatic contribution to entropy of activation
t	time
t^0	induction period
T	temperature (Kelvins)
τ	relaxation time
θ	fraction of solid consisting of pores
u	ionic strength
U; [U]	urea; its concentration
v, v_1, v_{-1}	velocity
v_0	initial velocity
$\Delta V^{\neq}$	volume of activation
V_A, V_B	limiting velocity, as A, B is varied

x	distance
x_e	concentration of X at equilibrium
X, Y; [X], [Y]	product of reaction; its concentration (small letters used for time dependence)
z, z_A, z_B...	ionic charge, in units of electronic charge (actual charges are z_Ae, z_Be, etc.)

The reference abbreviations employed in this book are those of the World List of Scientific Periodicals.

Contents

1. 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 the mechanisms by which the reactions occur. The general properties of enzymes have been discussed in a number of books and review articles[1]. 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 general nature of enzymes

A considerable number of enzymes have been prepared in highly purified form, and their chemical and physical properties studied in some detail. A very important advance was made in 1926 by Sumner[2], who was the first to crystallize an enzyme, urease, and to show that it is a pure protein. With the advances in techniques that have been made since then it has proved possible to prepare a number of enzymes in a high degree of purity. Sumner's procedure with urease was to extract jack bean meal with acetone, followed by crystallization from acetate buffer in the cold. In performing this extraction he utilized some of the basic properties of enzymes. The present section will outline some of these properties, with an indication as to how they have been utilized in the separation and purification of enzymes[3].

Most enzymes are soluble in water, in dilute salt solutions, and in dilute solutions of alcohol in water. They are, however, insoluble in water containing high proportions of alcohol or other organic solvents, and are salted out of solution by neutral salts, such as ammonium sulphate, in higher concentrations. They are precipitated out of solution by heavy metal ions such as lead and mercury, and also by changing the pH of a solution drastically, as by adding trichloracetic acid. These properties are often used to obtain crude preparations of enzymes as a first step in the purification process.

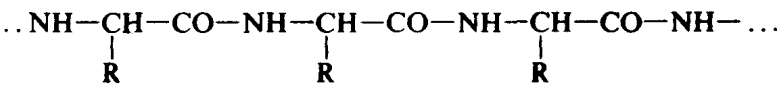
[1] See, for example, M. Dixon and E. C. Webb, *Enzymes*, Academic Press, New York, 1964; H. R. Mahler and E. H. Cordes, *Biological Chemistry*, Harper International, New York and Tokyo, 1966; R. E. Dickerson and I. Geis, *The Structure and Action of Proteins*, Harper and Row, New York, 1969.

[2] J. B. Sumner, *J. biol. Chem.*, **69**, 435 (1926); **70**, 97 (1926).

[3] For further details see J. Leggett Bailey, *Techniques in Protein Chemistry*, American Elsevier Publishing Co., New York, 1967.

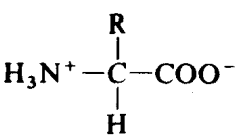
More recently-developed methods for purification include absorption. Proteins are absorbed to different degrees on materials such as silica and starch, and gels of these materials have been used for the separation of enzymes[1].

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



There is considerable variation between enzymes in the number of amino acids that are linked together in these polypeptide chains, the molecular weights ranging from a few thousands to several millions. This variation in size, and also in shape, has been used for the separation of enzymes and other proteins; in the ultracentrifuge, for example, molecules of different weights undergo sedimentation at different speeds. Size difference is also utilized in the molecular sieving (gel filtration) of proteins, a technique which provides good separation[2].

Other characteristic properties of the enzymes and of other proteins arise from their electrical nature. The amino acids themselves normally exist as zwitterions



at physiological pH values, but the charges due to these $-NH_2$ and $-COOH$ groups are lost on peptide bond formation; the groups at the ends of the chains are, however, free, and exist in the charged or uncharged forms according to the pH; typical pK values for these terminal groups are:

$$-COOH \rightleftharpoons -COO^- + H^+ \qquad pK \approx 4$$

$$-NH_3^+ \rightleftharpoons -NH_2 + H^+ \qquad pK \approx 9.$$

In addition, many of the R-side groups on the amino acids contain ionizing groups such as $-COOH$, $-NH_2$, and $-OH$. Table 1.1 lists the amino acids commonly found in proteins (including enzymes) and the following are the

[1] It is of interest that Sumner (*J. biol. Chem.*, **70**, 97 (1926)) was convinced that absorption could never be utilized for such separations.

[2] H. Determann, *Adv. Chromatogr.*, **8**, 3 (1969).

TABLE 1.1
Amino acids commonly found in enzymes

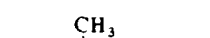
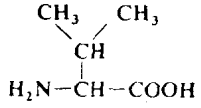
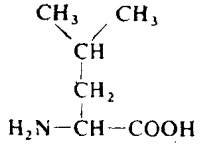
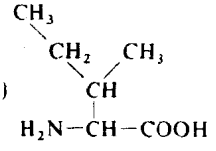
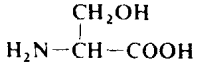
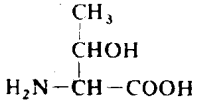
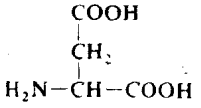
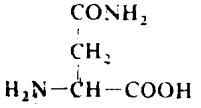
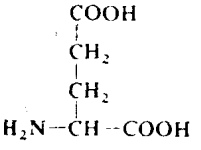
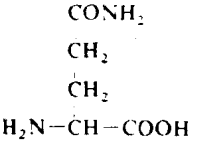
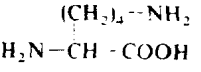
Name	Structure
Glycine (gly)	
Alanine (ala)	
Valine (val)	
Leucine (leu)	
Isoleucine (ile or ilu)	
Serine (ser)	
Threonine (thr)	
Aspartic acid (asp)	
Asparagine (asn)	
Glutamic acid (glu)	
Glutamine (gln)	
Lysine (lys)	

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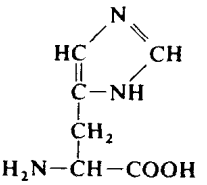
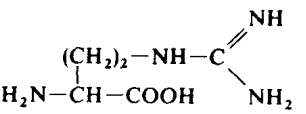
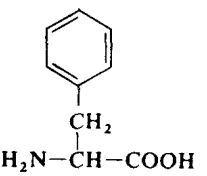
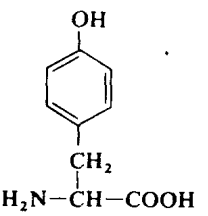
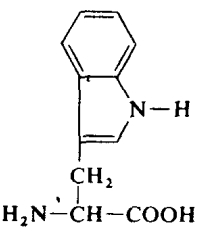
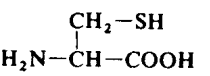
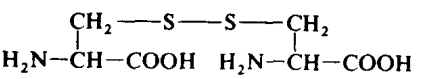
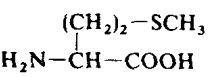
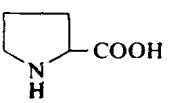
TABLE 1.1—continued

Name	Structure
Histidine (his)	
Arginine (arg)	
Phenylalanine (phe)	
Tyrosine (tyr)	
Tryptophan (try or trp)	
Cystein (cys)	
Cystine (cys-cys)	
Methionine (met)	
Proline (pro)	

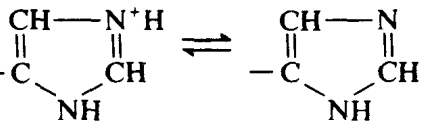
approximate pK values:

$$-COOH \rightleftharpoons -COO^- + H^+ \text{ (glu, asp)} \qquad pK \approx 4$$

$$-NH_3^+ \rightleftharpoons -NH_2 + H^+ \text{ (lys)} \qquad pK \approx 9$$

$$-OH \rightleftharpoons -O^- + H^+ \text{ (thr, ser, tyr)} \qquad pK \approx 10$$

$$-SH \rightleftharpoons S^- + H^+ \text{ (cys)} \qquad pK \approx 8$$



$pK \approx 7$

If sufficiently acid conditions prevail, the carboxyl, hydroxyl and sulphydryl groups will be in their neutral forms, while the amino and imidazole groups will be in their protonated (positively charged) forms; the molecule will therefore have a net positive charge and migrate towards the cathode if a potential is applied. Conversely, at low acidity there will be an overall negative charge, and the molecule will migrate towards the anode. At some intermediate pH value 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 an electric field; this pH at which there is no migration is known as the *isoelectric point*, and is a characteristic property of a protein.

This property of protein molecules has been exploited in isolating and identifying proteins, peptides and amino acids. Different species are readily separable by electrophoresis, owing to their different mobilities in an electric field. Two recent applications of this are electrofocussing[1], which involves the molecular sieve method combined with a pH gradient, and polyacrylamide electrophoresis[2], which combines molecular sieving with electrophoresis, without the pH gradient. These provide a sensitive method for separating proteins. Another technique involves the use of ion-exchange resins[3], which contain either positively-charged (such as $-NH_3^+$) or negatively-charged (such as $-SO_3^-$) groups; the amino acids or proteins attach themselves to these resins with different firmness depending on their overall charge, and this permits a separation.

There is much evidence, to be discussed later, that these ionizing groups on proteins have much to do with the catalytic activity of proteins. The remarkable variations of catalytic activity are to be correlated with changes of ionization of certain of these groups (see Chapter 5). Another point of importance 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 enzyme action.

[1] D. H. Leaback and A. C. Rutter, *Biochem. biophys. Res. Commun.*, 32, 447 (1968).
[2] B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121, 404 (1964); *Techniques in Biochemistry and Molecular Biology* (T. S. Work and E. Work, eds.), John Wiley and Sons, New York, 1969.
[3] P. B. Hamilton, *Adv. Chromatogr.*, 2, 3 (1966).

The structure of enzyme molecules

A detailed understanding of the catalytic function of an enzyme requires that as much information as possible be available about the structure of the enzyme molecule. There are several important aspects to this problem. In the first place, about 20 different amino acids are present in protein molecules, and it is necessary to know what amino acids are present, and in what number, in a given molecule. In addition, the sequence in which the amino acids occur along the polypeptide chain is important, since this determines the overall conformation† of the protein and this in turn has a great influence on the catalytic action. Finally, it is important to have direct information about the conformation of the molecule.

Amino-acid sequence

It is a comparatively simple matter, by the use of standard analytical techniques, to determine the proportion of the various amino acids in a protein molecule; if the molecular weight of the protein is known the number of the different amino acids in the molecule can then be determined. Automatic amino-acid analysers[1] are now available for solving this problem.

The determination of the sequence of amino acids in a protein chain is of much greater difficulty, in view of the very large number of units in even the smallest protein molecules. One of the smallest is insulin, which contains 51 amino acids and has a molecular weight of a little under 6000‡. The establishment of the amino-acid sequence in a protein molecule was first achieved for insulin in 1955 by Sanger. Since this pioneering work the sequences have been worked out for several other proteins, including the enzymes trypsin[2] (M.W. 23 800), chymotrypsin (M.W. 24 800), ribonuclease[3] (M.W. 12 600) and lysozyme[4] (M.W. 14 000). The methods employed are briefly as follows. Chains held together by disulphide (—S—S—) bonds are separated by oxidation or reduction of the bonds. These chains are then split into smaller peptide fragments, either by partial acid hydrolysis, by specific chemical cleavage[5] or by using one of the several proteolytic enzymes such

† 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 review of the significance of conformations in organic chemistry, see D. H. R. Barton and R. C. Cookson, *Quart. Revs.*, **10**, 44 (1956).

‡ Insulin usually exists in solutions as a dimer, of molecular weight about 12 000.

[1] See D. H. Spackman, W. H. Stein, and S. Moore, *Analyt. Chem.*, **30**, 1190 (1958).

[2] K. A. Walsh, D. L. Kauffman, D. S. V. S. Kumar, and H. Neurath, *Proc. natn. Acad. Sci. U.S.A.*, **51**, 301 (1964).

[3] R. R. Redfield and C. B. Anfinsen, *J. biol. Chem.*, **221**, 385 (1956); C. H. W. Hirs, S. Moore, and W. H. Stein, *ibid.*, **221**, 151 (1956).

[4] J. Jolles, J. Jauregui-Adell, and P. Jolles, *Biochim. biophys. Acta*, **71**, 488 (1963); R. E. Canfield, *J. biol. Chem.*, **238**, 2684, 2691, 2698 (1963).

[5] T. F. Spande, B. Witkop, Y. Degani, and A. Patchornik, *Adv. Protein Chem.*, **24**, 98 (1970).

as trypsin and chymotrypsin. Some easily identifiable group can then be caused to react with one part of the molecule; the chain can then be broken down and a study made to determine the amino acid to which the group has become attached. Ultimately, by ways such as this, and at the cost of much painstaking work, the entire sequence of amino acids in the original molecule can be established[1].

As an example, the amino-acid sequence determined for chymotrypsin is shown in Fig. 1.1. It is to be seen that there are three chains of amino acids, referred to as the A, B and C chains. These are held together by —S—S—

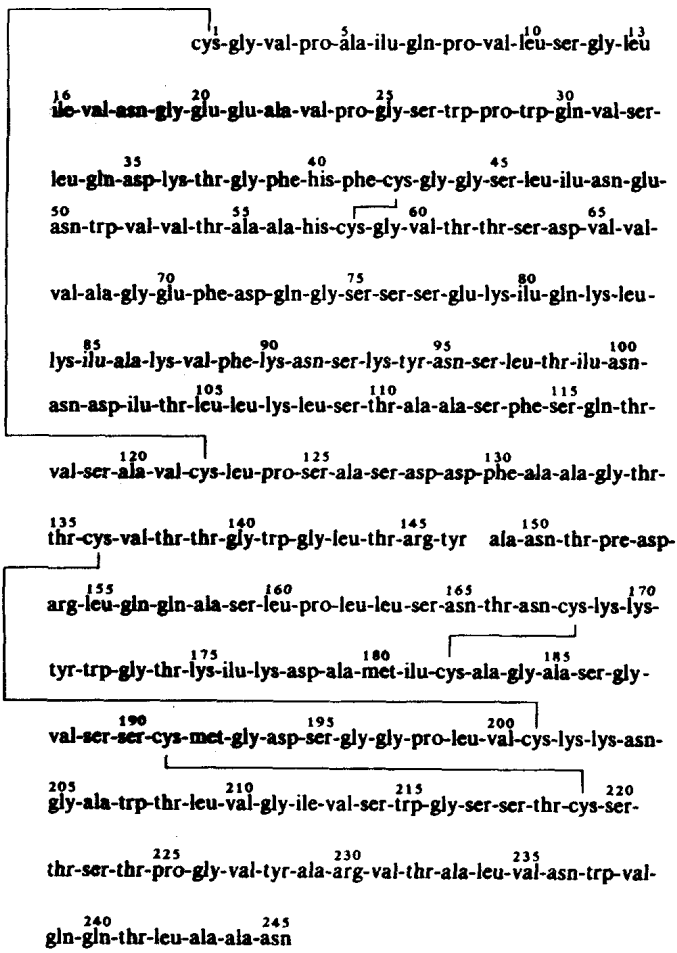


FIG. 1.1. The amino acid sequence in α-chymotrypsin (cf. Table 1.1 for list of amino acids). The numbering of amino acids is the sequence in the precursor chymotrypsinogen.

[1] For automated techniques see P. Edman and C. Begg, *Eur. J. Biochem.*, 1, 80 (1967); G. E. Van Lear and F. W. McLafferty, *A. Rev. Biochem.*, 38, 289 (1969).

linkages which are found in the amino acid cysteine (cf. Table 1.1). These —S—S— linkages play a very important role in protein structure, since they bring into close spatial proximity groups that might otherwise be far apart. This has a profound effect on the whole conformation, and on the biological activity.

At one time it was suspected that the amino acids in proteins are arranged in some regular and repeating pattern. Now that the sequence has been determined in a number of cases, however, it has become apparent that there is no evidence for such a conclusion. It has also been found that the sequence is the same for all samples of enzyme obtained from a given source.

Protein conformations

The properties of a protein cannot be understood entirely on the basis of the sequence of amino acids; they also depend very critically on the three-dimensional structure of the molecule. This three-dimensional structure determines the way in which the various side-groups on the molecule are brought into close proximity with one another, and this has an important effect on chemical and physical properties. In particular, if the protein is an enzyme, the relative positions of certain groups have a very important effect on the catalytic action.

Certain physical methods have been used for many years for gaining a very general idea of the overall shapes of protein molecules. These include the measurements of the viscosities of solutions, and of their light-scattering. These properties are very different for the molecules that are long and thin from what they are for molecules that have a more or less spherical form. By the use of such methods, proteins have been grouped into two main classes, the *fibrous* proteins, in which the molecules are fairly extended, and the *globular* proteins, which are very roughly spherical in shape.

Very much more detailed information about protein structure is provided by the technique of X-ray crystallography[1]. The reason that X-rays are the most suitable for work of this kind is that the detail with which an object can be observed, i.e. the resolving power of an instrument, depends in a fundamental way on the wavelength of the radiation employed; as a rough rule, no two objects can be seen separately if they are closer together than one-half the wavelength of the radiation used. The lengths of chemical bonds are between 0·1 and 0·2 nanometers (1 to 2 $\times$ 10^{-8} cm), so that one must use wavelengths not much longer than this; consequently, X-rays must be used. The employment of X-rays, however, introduces new difficulties, because no satisfactory way has yet been found to make lenses and mirrors that will focus X-rays. The way the technique has to be employed is to record and study the diffraction pattern that is produced when X-rays strike the material under

[1] For a discussion of basic principles of X-ray crystallography see G. Kartha, *Acct. chem. Res.*, **1**, 374 (1968). Many results for proteins are included in *Cold Spring Harb. Symp. quant. Biol.*, **36** (1972).

study. The analysis of an X-ray diffraction pattern is a matter of very considerable difficulty for a molecule as large as a protein, because of the very large number of interatomic distances that are involved. In 1953 Perutz[1] made a very important contribution to the analysis of X-ray patterns by his method of *isomorphous replacement*; this method depends upon the preparation and study of protein crystals into which heavy atoms, such as atoms of uranium, have been introduced without otherwise altering the crystal structure. Since that time a considerable number of protein structures have been worked out[2].

It is the electrons within the molecules that scatter the X-rays, so that the image calculated from the diffraction pattern reveals the distribution of electrons within the molecule. The usual procedure is to calculate, using high-speed computers, the electron density at a regular array of points, and to make the image visible by drawing contour lines through points of equal electron density. These contour maps can be drawn on clear plastic sheets, and a three-dimensional image can then be obtained by stacking the maps one above the other. The amount of detail that can be seen depends upon the resolving power of the effective microscope, and if this is sufficiently good the atoms appear as individual peaks in the image map. At lower resolutions, groups of unresolved atoms appear which can frequently be recognized.

There are a number of difficulties associated with work of this kind. The establishment of a protein structure by X-ray methods is an extremely time-consuming process, usually requiring several years before a conclusive result can be obtained. The enzyme must, of course, be available in pure crystalline form, and obtaining suitable crystals frequently presents considerable difficulty. The need to make an isomorphous replacement intensifies this problem, since the heavy-metal derivatives must be isomorphous with the native enzyme crystals. In addition, it is desirable that the heavy atom interacts with only one site in the enzyme, or at the most two. Obviously, the introduction of the heavy atom should not produce too much change in the conformation of the enzyme. A further difficulty is that one cannot be certain that the crystalline structure of the enzyme molecule is the same as its structure in solution. This problem can sometimes be overcome by the use of techniques available for the study of the three-dimensional conformations of molecules in solution; one of these is optical rotatory dispersion, which is referred to later (p. 13). Another approach that has been made is comparison of catalytic rates with the enzyme in solution compared with the wet crystal; for chymotrypsin[3] these rates have been found to be the same.

[1] D. W. Green, V. M. Ingram, and M. F. Perutz, *Proc. Roy. Soc.*, **A225**, 287 (1954); for a review see C. C. F. Blake, *Adv. Protein Chem.*, **23**, 59 (1968).

[2] For reviews see J. Kraut, *A. Rev. Biochem.*, **34**, 247 (1965); D. R. Davies, *A. Rev. Biochem.*, **36**, 321 (1967); L. Stryer, *A. Rev. Biochem.*, **37**, 25 (1968).

[3] G. L. Rossi and S. A. Bernhard, *J. molec. Biol.*, **49**, 85 (1970).

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