XLII. THE ADDITION COMPOUND THEORY OF ENZYME ACTION.

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1. INTRODUCTION.

IN 1929 the writer, as a result of a study of three enzymes in *B. coli communis* which act on fumaric acid, propounded "the rather attractive hypothesis that one of the conditions for a reaction to occur at these enzymes is that all the substrates shall be combined there together—succinic acid and methylene blue for succinoxidase action, fumaric acid and ammonia with aspartase, and fumaric acid and water with fumarase" [Woolf, 1929]. Since that time, further experimental findings, coupled with a thorough study of the work of others, have led the writer to develop this hypothesis into a definite theory of enzyme action, which it is the object of this paper to describe.

According to the view about to be put forward, the meaning of the term substrate is widened to include all the molecules participating in the catalysed reaction, including, for example, water in a hydrolysis, and the methylene blue or other hydrogen acceptor in a dehydrogenase action. The addition compound theory may then be stated as follows. An enzyme is a definite chemical compound which is able to form an unstable addition compound with all its substrates, each at its own specific combining group in the enzyme molecule. The process of catalysis then consists of a series of tautomeric changes in the enzyme-substrate complex, as a result of which, in a certain proportion of cases, the complex is able to dissociate into free enzyme plus the products of the catalysed reaction.

The line of reasoning on which this theory is based falls naturally into two sections, first an examination of all the known data of enzyme kinetics, and the induction from them of general laws, followed by the deduction from these laws of the probable mechanism of the catalytic process. A complete presentation of the first section is obviously impossible in this place. The full evidence for the conclusions given below will be set out, however, in a review by the writer on "Enzyme Kinetics," which is now in course of preparation for *Biological Reviews*.

2. ENZYME KINETICS.

The enzyme-substrate compound.

The hypothesis that the enzyme and substrate unite stoichiometrically was first put forward explicitly by Michaelis and Menten [1913], who deduced from it the well-known equation

$$V = \frac{U \cdot [E] \cdot [S]}{K + [S]},$$

where $[E]$ and $[S]$ represent the concentrations of enzyme and substrate, V the velocity of the catalysed reaction, U the maximum velocity for unit enzyme concentration, and K , the "Michaelis constant," the concentration of the substrate at which half the maximum velocity is attained. The reciprocal of K is the apparent affinity of the enzyme for the substrate.

This equation was found by Michaelis and Menten to hold with great accuracy for the action of saccharase on sucrose, and has since been found to fit the results obtained with many other enzymes. The full accounts of the many applications of this equation which are given by Kuhn [1925] and Haldane [1930] leave no doubt that the hypothesis of a stoichiometric enzyme-substrate compound is in good accord with the facts of enzyme kinetics.

The p_H -activity curve.

Many enzymes give p_H -activity curves showing an optimum p_H about which the curve is more or less symmetrical. Several authors have interpreted these curves as being due to the ionisation of the enzyme as an ampholyte, the isoelectric or un-ionised form of the enzyme alone being catalytically active, activity being determined not by the charge on the colloidal enzyme complex as a whole, but solely by the state of ionisation of one definite acidic and one basic group in the enzyme molecule. This view is supported by a considerable body of evidence, which is of two types: that based upon the shape of the curve, and that drawn from the effects of salts on the reaction rate.

Quantitative deductions from the shape of the curve can only be made when it covers eight or more p_H units. On the ampholyte theory, the curve would fall away from the optimum in a Henderson-Hasselbalch curve if the affinity did not change with p_H . If the affinity falls as the p_H recedes from the optimum, the curve will be steeper than a true dissociation curve, to an extent depending on the change in affinity with the p_H . These theoretical predictions have been realised, for instance by Josephson [1925] for β -glucosidase, and for saccharase, which is discussed by Kuhn [1925] and Haldane [1930]. Evidence from the effects of salts is given in such studies as those of Myrbäck [1926, 1] on saccharase and [1926, 2] amylase, and by Mann and Woolf [1930] on fumarase. These authors found that salts affected the velocity of the catalysed reaction in just the way that would be expected if the salt, or one of its ions, combined with the enzyme and altered the p_K value of one

or both of the critical ionisable groups, and hence the proportion of enzyme molecules in the isoelectric or active form at any given p_H .

Now there are many enzymes which do not give a p_H -activity curve of the symmetrical type, as for example the dehydrogenases [Dixon and Thurlow, 1924; Quastel and Whetham, 1924; and unpublished observations in this laboratory] and catalase [Michaelis and Pechstein, 1913]. Though it does not seem to have been pointed out before, the ampholyte type of curve is not characteristic of all enzymes, but only of those of a particular class—the enzymes which catalyse reactions in which water is one of the substrates. The non-hydrating enzymes mentioned above give a curve which rises in the acid range until a maximum velocity is reached, which persists unchanged until the enzyme begins to be destroyed by excessive alkalinity. Such a curve suggests that the enzyme acts as an ionised acid or an un-ionised base, and this suggestion is borne out by the fact that Michaelis and Pechstein [1913] found that the p_H -activity curve of liver catalase is a very accurate Henderson-Hasselbalch curve, which is shifted by salts in a manner analogous to the p_K -shifts found for the hydrating enzymes.

The enzyme a chemical compound.

All the kinetic results indicated above suggest strongly that an enzyme is a definite chemical structure. Further reasons for this conclusion are given by Willstätter [1927] in a summary of his work on enzyme purification, and by Haldane [1930] on several other grounds.

3. THE ADDITION COMPOUND THEORY.

The activation of the substrate.

It is obvious that the substrate must be distorted or activated in some way at the enzyme in order that reaction may occur, and various mechanisms, such as the electric field of Quastel [1926], have been proposed to account for this activation. All such theories are compelled to assume a double mechanism at the enzyme, the activating mechanism and some chemical grouping which will bind the substrate while it is being activated and undergoing reaction [cf. Quastel and Wooldridge, 1927, 1, 2]. It seems to the writer that this double mechanism is an unnecessary assumption. When the substrate is bound at the enzyme, becoming part of a temporary enzyme-substrate complex, the very chemical forces which bind it must surely produce sufficient distortion in its molecule to make it abnormally reactive. Until the chemical nature of enzymes is discovered, the nature of these forces can only be the subject of more or less plausible speculation. It seems likely, however, that since substrates are often stable, saturated molecules—e.g. succinic acid—the union with the enzyme is of the nature of a multiple co-ordination compound or chelate ring, which, as shown by Sidgwick [1927], involves the polarisation of the molecules which are so linked. But whether this or some other

mechanism is the true one, it seems to the writer an economy of hypotheses to assume that the substrate-binding and substrate-activating processes are one and the same until evidence to the contrary is produced. This view is also put forward in a slightly different form by Haldane [1930]. It may be assumed, then, that when the enzyme and substrate are combined, the chemical structures of the molecules are so altered that the complex will readily undergo further tautomeric changes, which constitute the actual catalytic process. The nature of these changes is discussed below.

The mode of action of a hydrating enzyme.

A convenient illustrative enzyme is fumarase [*cf.* Woolf, 1929], which catalyses the reversible reaction

since the complication is absent of the hydrated product splitting into two molecules, and the forward and reverse reactions can be readily induced, so that the mechanism of both can be discussed. The kinetic results already considered show that the conversion of fumaric to malic acid can only occur at an enzyme molecule which is combined with fumaric acid and in the iso-electric form. It may therefore be assumed that for reaction to be effected there must be present at the enzyme a fumaric acid molecule, a hydrogen ion, and a hydroxyl ion. Now the reaction product, malic acid, is formed by the addition of these three bodies. It is therefore a natural step to assume that the enzyme can only act in the fully hydrated form because the hydrogen and hydroxyl ions are substrates for the reaction, as well as the fumaric acid.

An enzyme molecule can be pictured uniting with a molecule of fumaric acid. In the majority of cases, the substrate molecule will dissociate off unchanged. But while the substrate is at the enzyme, its structure is distorted, it is polarised by the chemical forces holding it there, in such a way that it is able to "accept" a hydrogen and a hydroxyl ion at the two carbon atoms of the structure —CH:CH— . If these two ions are also present at the enzyme at the same time, in a given proportion of cases the enzyme-fumaric-water addition compound will tautomerise so that the water ions leave the enzyme and add themselves across the double bond of the substrate, which will thus become free to dissociate off as malic acid. When this occurs, it is important to notice that the enzyme is left with neither a hydrogen nor a hydroxyl ion, *i.e.* in the zwitterion form.

There are thus four forms of the enzyme to consider—the dissociated acid, the dissociated base, the fully hydrated, and the zwitterion form. The last two together constitute the isoelectric form of the enzyme, which will be partitioned into hydrated and zwitterion forms in a constant ratio, which is independent of the p_{H} .

Consider now the reverse reaction. When a molecule of malic acid combines at the enzyme, it too will undergo polarisation so that the elements of

water become dissociable as ions. If the enzyme already has a hydrogen or a hydroxyl ion, it will be unable to take up water from the substrate, and reaction cannot occur. If however it is in the zwitterion form, in a proportion of cases water will leave the substrate and combine with the enzyme, the substrate then dissociating off as fumaric acid and leaving the enzyme fully hydrated. The equilibrium can therefore be represented

The mechanism here suggested thus conforms to the thermodynamic requirement that the reverse reaction in a reversible process shall pass through exactly the same stages, in the opposite order, as the forward process. The essential features of the process may be expressed as follows. The fumaroid skeleton, when combined with the enzyme, is so polarised that it acquires temporary acidic properties at one carbon atom and basic properties at the other. The complex thus becomes a dibasic acid and diacidic base, and the hydrogen and hydroxyl ions will partition themselves, statistically, between the acidic and basic groups of the enzyme and the substrate in a proportion determined by the relative affinity of the enzyme and the substrate for the water. It is obvious that from such a process a true thermodynamic equilibrium will be obtained.

In a system containing fumarase, fumaric acid and water there are the following forms of the enzyme to be considered:

Form 5 is catalytically active in the forward reaction and form 12 in the reverse direction. All these forms are in equilibrium with one another and with fumaric acid, malic acid and the ions of water in the medium, and the affinity of each ionic species of the enzyme for fumaric acid and malic acid will, in general, not be the same. The state of affairs is therefore rather complex. It is however amenable to treatment by the law of mass action, and is then found to lead quantitatively to the kinetic results which are actually found by experiment, and which are set out in Section 2 of this paper.

The above line of argument applies to any hydrating enzyme, with the complication that when the catalysed reaction is a hydrolysis, the reverse reaction involves two substrates, each of which must combine with the enzyme, with its own characteristic affinity.

The mode of action of a dehydrogenase.

Succinoxidase will be taken as an example, since it is the only dehydrogenase whose thermodynamic reversibility has been proved experimentally, and methylene blue will be considered to be the hydrogen acceptor. The catalysed reaction then consists of the transfer of two hydrogen atoms from the donator to the acceptor. Many considerations render it probable, however, that the actual act of oxidation consists in the transfer of two electrons to the dye from the donator, which has previously lost two hydrogen ions, the process being completed by the acquisition of two hydrogen ions by the charged acceptor. One must therefore visualise the donator being polarised by its combination with the enzyme so as to acquire acidic properties at the structure $-\text{CH}_2.\text{CH}_2-$, the enzyme, in a given proportion of cases, taking up the two temporarily ionisable hydrogens from the donator in the same way as fumarase takes up the temporarily ionisable hydrogen and hydroxyl from malic acid. In order to be able to do this, the enzyme must itself be an ionised acid, which is precisely the conclusion that is indicated by the shape of the p_{H} -activity curve.

There is good kinetic evidence that the donator must combine with the enzyme. Reasons were given by Woolf [1929] for supposing that the acceptor must also combine. These reasons are reinforced by the work of Bertho [1929], who found that quinone, acting as hydrogen acceptor with the dehydrogenases of certain lactic acid bacteria, gave a good Michaelis curve. Unpublished observations by the writer show that the same is true for methylene blue and other dyes acting as hydrogen acceptors with a variety of dehydrogenases. In order to be catalytically active, therefore, a dehydrogenase molecule must have combined at it a molecule of donator and a molecule of acceptor, and be itself dissociated as an acid. If DH_2 and D represent the reduced and oxidised forms of the donator, AH_2 and A the corresponding forms of the acceptor, E the enzyme and $*$ an electron, the sequence of changes may then be formulated as follows:

In this scheme the enzyme is represented as a dibasic acid, but the same general result would be obtained if it were taken as monobasic, though the formulation would be a little more lengthy. The doubly charged enzyme combines with both substrates. The donator is activated, and parts with two hydrogen ions to the enzyme. The two excess electrons are then transferred from the donator to the acceptor, this being the actual act of oxidation. The acceptor then acquires two hydrogen ions, and both substrates dissociate off in the altered condition, leaving the enzyme in its initial state. Since all the intermediate forms are in dynamic equilibrium, it is probable that only a small proportion of the enzyme-substrate complexes formed will be completely

transformed, and most will dissociate into enzyme and unchanged substrates. When the sequence of changes is read in the opposite order, it represents the reverse reaction—*e.g.* the reduction of fumaric to succinic acid by leuco-methylene blue. This mechanism, like that suggested for the hydrating enzymes, is therefore thermodynamically sound.

Similar considerations could be applied to other types of enzyme action, but these two examples suffice to indicate the scope and method of the theory. While it is claimed that the central idea is new, the writer wishes to acknowledge that he has freely drawn on the hypotheses of many previous workers, and adopted from them whatever elements seemed to him of value. It is claimed for this theory that it contradicts no known facts; that it gives a unitary explanation of the phenomena of enzyme kinetics, including p_H -activity curves, which no previous theory has professed to explain; that the mechanism of enzyme action proposed is a plausible one; and that it is a useful working hypothesis, in that it enables many quantitative predictions to be made, which can be tested by clear-cut experiments. It has certainly proved of extraordinary value as a working guide to the writer, and it is hoped to publish in the near future several enzyme studies suggested by the theory. The writer hopes that it may also prove of value to other workers in the field of enzyme action.

SUMMARY.

The theory is proposed that an enzyme is a definite chemical compound, which is able to form an addition compound with all its substrates, the actual process of catalysis consisting of a series of tautomeric changes in this enzyme-substrate complex, as a result of which, in a certain proportion of cases, it is able to dissociate into free enzyme and reaction products. This theory is shown to account in a quantitative manner for the observed effects of substrate concentration, p_H , and salts on the velocity of the catalysed reaction.

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