

CHAPTER I

Introduction: General properties of
oxidation enzymes

There are three fundamental problems in connection with cellular oxidations, viz. which substances are oxidized, how are they oxidized and how is the energy so derived utilized by the cell? Unfortunately our knowledge of how energy liberated in oxidation processes is transformed into other forms of energy, such as osmotic, electrical, etc., is very meagre. Biochemists have so far concerned themselves principally with the problems of intermediary metabolism and mechanism of reaction, and for lack of experimental methods have been obliged to shelve the problem of energy transmission.

The mechanisms of cellular oxidation are the main concern of this book. The greater part of our knowledge of mechanism is derived from experiments on a few animal tissues, special yeasts and bacteria. The question may well be raised, to what extent is the biochemist justified in reasoning from one cell to another? May we assume that mechanisms which hold for specific yeasts and bacteria apply as well to moulds, fungi, higher plants, and bacteria in general? There is no unequivocal answer as yet to this question. Cells of every description have some chemical equipment in common. For example, catalase, cytochrome, riboflavin and the pyridine nucleotides are found in bacteria, plants and animal tissues. It is tempting to interpret these elements of universality as proof that the mechanisms of oxidation are more or less similar for all types of cell. There is, however, little to be gained by such an over-simplification. Practically nothing is known about the oxidative mechanisms of the vast majority of cells. It remains for future experimentation to decide whether oxidations in different types of cells are merely variations on the same theme.

Enzymic catalysis is the motif of all cellular oxidations. The oxidation of foodstuffs or metabolites is not a process which occurs spontaneously; it requires the presence of highly specific

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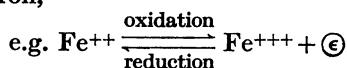
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protein molecules known as enzymes. For example, alanine in aqueous solution at 38° is extremely resistant to oxidation. However, in presence of a specific enzyme extracted from liver or kidney, alanine is rapidly oxidized by molecular oxygen. The enzyme is said to catalyse the oxidation of alanine. The properties of these enzymes will be considered in detail in later sections and for the moment the question of how enzymes work will be deferred. It is by virtue of the intracellular catalysts that the cell is able to carry out oxidation of foodstuffs with extraordinary velocities at low temperatures, and without the use of vigorous oxidizing agents.

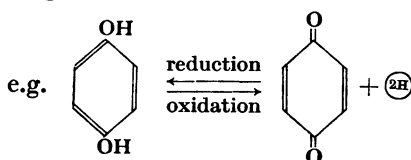
There is no universal oxidizing agent for all cells. Molecular oxygen is the ultimate agent in all aerobic organisms. However, anaerobic organisms perform a multitude of oxidations in the complete absence of oxygen, and even aerobic organisms are not entirely dependent upon oxygen as the oxidizing agent. The study of the mechanisms of biological oxidations has elucidated the existence of oxidizing agents other than oxygen. The details of any cellular oxidation-reduction process will be found to depend upon the particular oxidizing agent which comes into play.

We may assume that the oxidation of any substance must involve one of the following four types of chemical change (or combinations thereof):

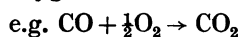
- (1) Loss of electron,



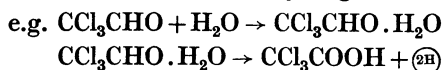
- (2) Loss of hydrogen,



- (3) Gain of oxygen,



- (4) Addition of water with loss of hydrogen,



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The oxidizing agent is referred to as an electron acceptor in case (1) and as a hydrogen acceptor in case (2). Needless to say, neither a free electron nor atomic hydrogen is liberated in solution; the transfer is made directly to the appropriate acceptors. The circles around the symbols for the electron and atomic hydrogen indicate this transfer. In oxidations involving gain of oxygen either molecular oxygen or some oxide can act as the oxidizing agent. Case (4) is essentially a combination of cases (2) and (3).

In the present state of our knowledge it is very often a matter of convention whether a particular reaction shall be considered as one involving a loss of electrons or a loss of hydrogen. The net result is the same, although the postulated mechanisms are different. The oxidation of many organic compounds entails a simultaneous transfer of a hydrogen atom and an electron. In cellular oxidations we are dealing with all these four types of oxido-reductions, though mechanisms involving a transfer of hydrogen are most common.

The reduction of substances involves the converse processes to those of oxidation, i.e. gain of electrons, gain of hydrogen or loss of oxygen. Oxidation and reduction are simultaneous, mutually dependent processes. The problem of how foodstuffs are oxidized is therefore inextricable from the problem of how oxidizing agents are reduced.

Before proceeding to the systematic study of biological oxidations a survey of the properties and characteristics of the amino-acid oxidase will be made. It is not that this enzyme is unusually important, but rather that no proper appreciation of biological oxidations is possible without some familiarity with the properties of oxidation enzymes. The general properties of the amino-acid oxidase may be assumed to parallel those of other oxidation enzymes.

The enzyme is easily prepared from the kidneys of ox, pig or sheep by the following method⁽¹⁾. The kidney is finely minced and mixed with 7 vol. of acetone. The mixture is stirred and filtered. The residue is again mixed with acetone and filtered off. The almost dry residue is collected, spread in a thin layer and allowed to dry at room temperature. The dry powder is rubbed

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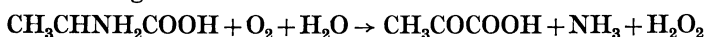
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up with *ca.* 20 vol. of buffer pH9 and the mixture is centrifuged. The somewhat opalescent supernatant fluid contains the active enzyme.

A mixture of *d*(-)-alanine and the enzyme absorbs molecular oxygen. Chemical analysis shows that coincident with the oxygen uptake alanine disappears whilst pyruvic acid and NH_3 appear. The following are the stoichiometric relations:

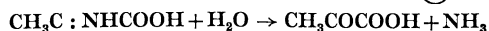
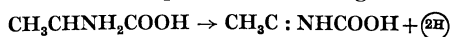


There is a transfer of two atoms of hydrogen from alanine to oxygen,* the former being oxidized to pyruvic acid and NH_3 , the latter being reduced to H_2O_2 . If the enzyme is destroyed by boiling the solution for a few seconds no reaction between alanine and oxygen takes place. It is clear that the enzyme catalyses a reaction which is not spontaneous.

The catalytic power of the amino-acid oxidase is not confined to *d*(-)-alanine. Many other α -amino acids of the same optical series, i.e. of the *d*-configuration, are also oxidized by oxygen in presence of the enzyme(1). However, the oxidation of simple amines, α -amino acids of the *l* series, diamines, fatty acids and any other group of organic compounds, is not catalysed by the enzyme. Thus the amino-acid oxidase is specific not for a particular amino acid but for the group of α -amino acids stereochemically related to *d*(-)-lactic acid, i.e. the non-naturally occurring isomer. Not all oxidation enzymes show *group* specificity. The malic enzyme of animal tissues, for example, catalyses only the oxidation of *l*(-)-malic acid. This enzyme by contrast shows specificity for an individual *compound*. In general, oxidation enzymes exhibit a high degree of specificity for their substrates, i.e. the substances whose oxidation is catalysed.

The speed of oxidation of alanine by molecular oxygen in presence of a given amount of enzyme and a fixed partial pressure

* The oxidation of alanine proceeds in two stages:



The first and catalytic stage is the dehydrogenation of alanine to the corresponding imino acid. The second and spontaneous stage is the hydrolysis of the imino acid to the corresponding ketonic acid with liberation of NH_3 .

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of oxygen gas depends for a given temperature upon two factors: (1) the hydrogen-ion concentration and (2) the concentration of alanine. Fig. 1 shows how the rate of oxidation is affected by the *pH*. Below *pH* 7 and above *pH* 10 the rate falls off sharply to zero. The maximum activity is shown at *ca.* *pH* 9. Little importance can be attached to the maximum *pH*, since the value depends on the particular buffer system employed. The *pH* curve with one buffer system rarely parallels that of another. But regardless of which buffer system is employed no activity is found at *pH* values lower than 6 or higher than 11. It is not entirely

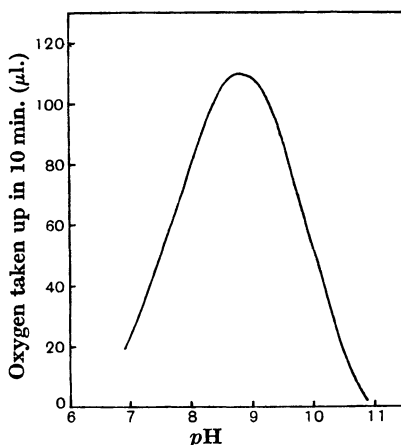


Fig. 1. *pH* curve of the *d*-amino-acid oxidase. Substrate *dl*-alanine. (Krebs [1935]. *Biochem. J.* **29**, 1620.)

clear why activity disappears in these ranges of hydrogen-ion concentration. No doubt destruction of the enzyme plays some role. But other factors such as the ionization of the enzyme and the substrate are probably concerned. Fig. 2 shows how the rate of oxidation depends on the concentration of alanine. The rectangular hyperbola relation is quite typical for enzymes in general. The substrate concentration giving half the maximum velocity (i.e. the Michaelis constant *K_m*) obtained from the curve is $5 \times 10^{-3} M$. The value of the *K_m* varies from enzyme to enzyme. The equilibrium of compound formation between enzyme and substrate determines the value of the Michaelis constant.

The velocity of oxidation of alanine is directly proportional to the concentration of enzyme. Certain enzymes exhibit no measurable activity below a critical dilution of the enzyme. This phenomenon is usually attributed to a rapid rate of destruction of the enzyme in the course of its catalytic function. If the rate of destruction is high and the amount of enzyme is small, complete destruction of the enzyme sets in before any measurable oxidation takes place.

The rate of oxidation of alanine by oxygen in presence of the enzyme is linear only for about 20 min. and then gradually falls

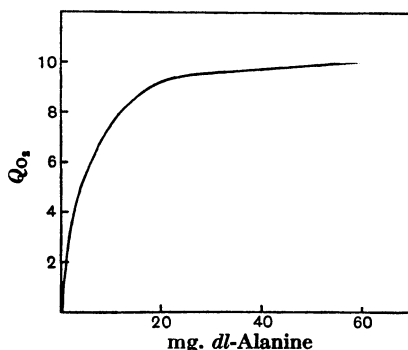


Fig. 2. Relationship between rate of oxidation by amino-acid oxidase and concentration of *dl*-alanine. (Keilin & Hartree [1936]. *Proc. Roy. Soc. B*, 119, 114.)

off. This is quite a general phenomenon among oxidation enzymes. It is possible to rule out such factors as inhibition of the reaction by the reaction products or mass-action equilibria. Experiment shows that in the main the falling off in rate is due to the destruction of the enzyme. There is little information available as to why oxidation enzymes in particular exhibit such fragility in the course of their catalytic function in reconstructed systems, and it would be of interest to know the lifetime of an enzyme in the intact cell.

The amino-acid oxidase can be reversibly resolved into a colourless protein and a yellow substance of comparatively low molecular weight, which we shall refer to as the prosthetic group (2). Neither portion alone can catalyse the oxidation of

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alanine. The catalysis requires the simultaneous presence of the two components. Fig. 3 shows how the rate of oxidation of alanine depends upon the concentration of the yellow prosthetic group (flavinadenine dinucleotide) for a given amount of the colourless protein. It is obvious from the curve that the protein forms a compound with the prosthetic group in a definite stoichiometric proportion and that the protein-prosthetic group compound dissociates under the conditions of the experiment. Dissociation is indicated by the fact that the asymptotic maximum activity value is not reached abruptly. In neutral or

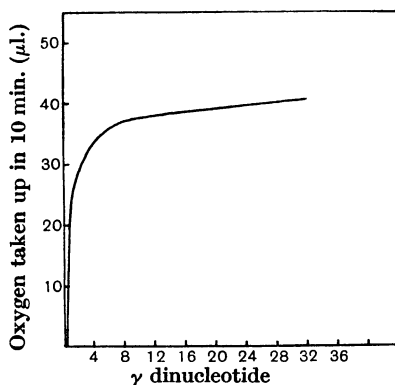


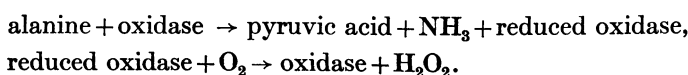
Fig. 3. Relationship between rate of oxidation of alanine by amino-acid oxidase and concentration of flavinadenine dinucleotide. (Warburg & Christian [1938]. *Biochem. Z.* **298**, 150.)

slightly alkaline solutions the equilibrium is in favour of dissociation.

Both components of the amino-acid oxidase are highly specific. The protein cannot be replaced by any other protein. Similarly, the flavinadenine dinucleotide prosthetic group cannot be replaced by compounds very similar in chemical structure, such as riboflavinphosphate or adenylic acid(2). It is therefore obvious that both components of the amino-acid oxidase are essential for catalytic activity.

A concentrated solution of the highly purified oxidase is yellow in colour. On addition of alanine to the oxidase solution the colour is bleached(3). On shaking vigorously with air the colour

is restored. The colour of the oxidase is due entirely to the flavin moiety. It is well known that reducing agents bleach the colour of flavin solutions and that reduced flavins are rapidly oxidized by molecular oxygen with production of H_2O_2 . The oxidation of alanine by molecular oxygen may now be described by the following equations:



The oxidase catalyses the reaction by undergoing a cycle of reduction by alanine and oxidation by molecular oxygen. An interesting corollary is that the oxygen is not concerned in the oxidation of alanine but is required only to reoxidize the reduced oxidase. The actual oxidation of alanine is the prerogative of the oxidase.

The knowledge of the cyclical changes which the oxidase undergoes simplifies, but by no means solves, the problem of the mechanism of the catalysis. Why does the flavin-prosthetic group when in combination with the specific protein enjoy the unique property of oxidizing alanine? What is the role of the specific protein? In the present state of our knowledge no complete explanation is possible. The following facts, however, point the way. Families of oxidation enzymes are known which have in common the same prosthetic group. Each member of such families differs from all the others in its specific protein and in the nature of its catalysis. In other words the specific protein portion determines the nature of the catalysis. From this fact it follows that the protein must be structurally constituted so as to exert some special influence on the substrate. What is the nature of this influence? Consider the case of the succinic enzyme which catalyses the oxidation of succinic acid ($\text{COOHCH}_2\text{CH}_2\text{COOH}$) to fumaric acid. Malonic acid ($\text{COOHCH}_2\text{COOH}$), which is the lower homologue of the dicarboxylic acid series, is not attacked by the enzyme. The enzymic oxidation of succinic acid is strongly inhibited by low concentrations of malonic acid (4) and can be completely abolished by high concentrations. Such striking inhibitory effects are not produced by any other carboxylic acid yet tested. The following explanation is given for this

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phenomenon of competitive inhibition. The actual process of oxidation is preceded by the formation of a substrate-enzyme compound. A special group in the protein molecule (usually referred to as the active group) is concerned in this linkage. To return to the example of the succinic enzyme, the “active” group is capable of combining not only with succinic acid but also with the homologous compound malonic acid. But whereas succinic acid undergoes oxidation malonic acid remains unchanged. The inhibition by malonic acid is therefore explicable in terms of a competition between malonic acid and succinic acid for the active group of the enzyme. This phenomenon of competitive inhibition is general for all oxidation enzymes as yet tested and is the best available proof for the assumption that the substrate combines with the enzyme, and that a special group of the enzyme is concerned in this combination.

The next problem is to explain why the substrate undergoes oxidation when combined with the enzyme. On that point experiment is silent though theory is eloquent. It is stated that the enzyme “activates” the substrate or that a “strain” is imposed on the substrate molecule. These are terms of ignorance and merely express the experimental fact that the substrate undergoes an oxidation in presence of the enzyme which it does not undergo spontaneously. The nature of activation or straining of the substrate molecule is the spearhead of present research in biological oxidations.

The description of the flavinadenine dinucleotide portion of the amino-acid oxidase as the prosthetic group does not imply that it is the only catalytic portion of the molecule, and that the protein is merely ballast. In some cases, as in that of the amino-acid oxidase, the prosthetic group undergoes a cycle of oxidation and reduction. In other cases, as in that of peroxidase, the prosthetic group undergoes another type of cyclical change. By prosthetic group we shall mean the nucleus of comparatively low molecular weight with which the specific protein is combined or is in association. This nucleus may be flavin dinucleotide, Fe porphyrin, copper, pyridine dinucleotide or thiamine.

The classification of oxidation enzymes may be based (1) on the chemical nature of the substrates, (2) on the components of

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the complete oxidation systems, and (3) on the chemical nature of the prosthetic group. From the point of view of ease of presentation the third basis of classification seems most satisfactory and will be used in this book whenever possible. In Chapters II–VI we shall consider oxidation enzymes with known prosthetic groups. Chapters VII–IX deal with enzymes whose prosthetic groups if any are still unknown.

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