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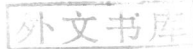
OSAMU HAYAISHI

# OXYGENASES

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# OXYGENASES

*Edited by*

OSAMU HAYAISHI

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## PREFACE

Oxygen is the most abundant element on the earth and is certainly one of the most important elements for life. Therefore, it has been a popular subject among biochemists since Lavoisier initiated the study of biological oxidative processes some 200 years ago. Nevertheless, very little is understood about how oxygen molecules are metabolized in the living organisms, because the principal developments in the field of biological oxidation have concerned the elucidation of pathways in which electrons are transferred from substrate through various carriers to molecular oxygen. The enzymes that catalyze dehydrogenation of primary substrates have been designated "dehydrogenases." In cases in which molecular oxygen serves as the immediate electron acceptor forming water or hydrogen peroxide, the enzymes have been called "oxidases." In 1955 evidence was obtained in several laboratories that oxygen may also play a more direct role in substrate oxidation. In a diverse group of reactions, including hydroxylations, cleavage of aromatic rings, and cyclization of steroids, the mechanism involves the direct addition of oxygen to the substrate. These reactions are catalyzed by specific enzymes which we proposed to name "oxygenases."

During the last several years, oxygenases have been found to be distributed ubiquitously in animals, plants, and microorganisms and to play important roles in the metabolism of not only various aromatic and cyclic compounds but also of aliphatic compounds.

The purpose of this book is to summarize our present knowledge of these enzymes and to further discuss and speculate on various possibilities as to the mechanism of this new type of enzymic reaction at a molecular level. In addition, attempts have been made to incorporate some recent developments concerning oxygen-carrying proteins, such as hemoglobin, myoglobin, hemerythrin, and hemocyanin. It is hoped that discussions on the mode of action of these oxygen-carrying proteins and cytochrome oxidases may contribute a great deal to the understanding of oxygen metabolism in general.

I would like to express my appreciation to my colleagues, past and present, who have collaborated with me during the last seven years on the

project of oxygenases, namely Drs. S. Rothberg, M. Katagiri, A. H. Mehler, H. Tabor, W. B. Sutton, Y. Saito, B. Witkop, A. Patchett, S. Kuno, M. Tashiro, H. Taniuchi, H. Yamada, S. Senoh, T. Tokuyama, S. Sakan, K. Horibata, K. Tsukada, N. Itada, Y. Kojima, N. Kanetsuna, Y. Nishizuka, M. Nakajima and S. Hatanaka.

My special thanks are due to Dr. I. P. Crawford who has been helpful in the preparation of manuscripts and in editorial work. It is also a pleasure to express my sincere appreciation to my former colleagues, Dr. M. Suda, for his interest and many useful discussions and Dr. A. Kornberg and Dr. S. M. Rosenthal, whose encouragement and generous support were the driving force in initiating and continuing this project. Most of the experimental data from my laboratories at Bethesda and Kyoto, presented in this book, were supported by financial aids from the National Institutes of Health, the Rockefeller Foundation, the Jane Coffin Child's Memorial Fund, and the Ministry of Education of Japan, to which my heartfelt gratitude is expressed. Finally, but not least, thanks are extended to the authors and to the editorial staff of Academic Press for their cooperation and to Dr. Yasutomi Nishizuka who has done very thorough work in the preparation of the subject index.

*June 1962*

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## CHAPTER 1

# History and Scope

OSAMU HAYAISHI

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## I. Introduction

The investigation of biological oxidation was begun by Lavoisier about two hundred years ago. Since then, the mechanism by which organic substances are oxidized by living organisms has remained one of the most important and colorful topics in biological science. Lavoisier and his contemporaries defined the term "oxidation" as the addition of oxygen atoms to a substrate,  $S$ , and the opposite process, that of reduction, was regarded as the removal of oxygen from an oxide (Eq. 1).



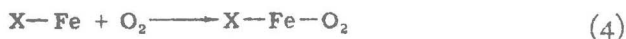
When enzymological techniques were applied to this problem, it soon became apparent that living organisms contain a number of

enzymes which catalyze the oxidation of various biological compounds; these were designated "oxydases" (Bertrand, 1896). The early workers generally presumed that oxygen molecules were affected and modified by oxydases in such a way that the stable oxygen molecules were activated, and then combined with substrates. The nature of this so-called "activated oxygen," however, was not elucidated for many years, although organic peroxides and ozonides were postulated by a number of investigators in this field. At the turn of the twentieth century, Bach and his co-workers proposed that oxygen reacted with an acceptor, A, in the primary reaction to produce an organic peroxide, which then reacted with a substrate, S, to form an oxide (Bach and Chodat, 1903).

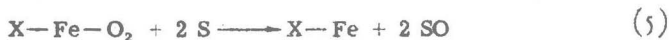


The enzymes which catalyzed reactions (2) and (3) were named "oxygenases" and "peroxidases," respectively. This interpretation, however, failed to gain general acceptance and was eventually abandoned chiefly because very little experimental evidence was available for the formation of organic peroxides during general oxidative processes.

About 20 years later, Otto Warburg proposed a theory of cell respiration which has had considerable influence on the students of biological oxidation over the years. He believed that the essential process in cell respiration was the activation of oxygen and that this activation was catalyzed by iron-containing compounds (Warburg, 1949). Free iron itself was inactive but a compound related to hematin was claimed to be implicated as catalyst and was designated as "the respiratory enzyme" (*das Atmungsferment*). In many ways his theory was reminiscent of that of Bach, since Warburg assumed that the primary reaction in cell respiration is the reaction between molecular oxygen and iron, as follows:



The oxidation of the organic molecule then follows according to Eq. (5).



## II. Dehydrogenases, Oxidases, and Oxygenases

This role of oxygen molecules per se in biological oxidation processes was vigorously challenged and questioned upon the discovery by Schardinger of an enzyme in milk which catalyzed the conversion of aldehydes to acids in the presence of methylene blue but in the complete absence of oxygen. In this case, the oxidation of aldehydes was accompanied by the concomitant reduction of methylene blue under anaerobic conditions. This finding prompted Wieland to investigate the nature of biological oxidation processes and to propose a generalized mechanism by which many oxidations could proceed in the complete absence of oxygen. According to this scheme, the essential characteristic of biological oxidation processes is the removal or transfer of electrons from the substrate molecule to an appropriate acceptor (Eq. 6).



This "dehydrogenation" theory gained experimental support through ingenious experiments by Thunberg, and in the last several decades numerous dehydrogenases have been isolated, purified, and crystallized from animal and plant tissues as well as from microorganisms. Pyridine nucleotides, flavin nucleotides, and cytochromes have been found to act as electron acceptors for various dehydrogenases. In the occasional case when oxygen molecules serve as the immediate electron acceptor, the enzymes have been called *oxidases*.

Classical oxidases can be divided into two categories. In the first category, the enzyme catalyzes the transfer of two electrons to one molecule of oxygen forming hydrogen peroxide as shown in Eq. (7). Some flavin-containing enzymes, such as D-amino acid oxidase, glucose oxidase, xanthine oxidase, and so forth, belong to this category of enzymes.



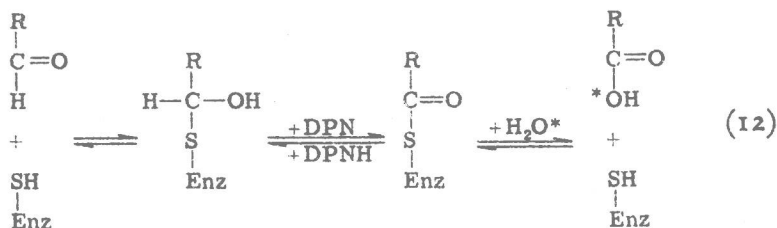
In the second group, two electrons are transferred to a half molecule or one atom of oxygen to produce water as a product (Eq. 8). In this case the bond between the two oxygen atoms must be cleaved, whereas in the first case the bond between the two oxygen atoms may be preserved. Cytochrome oxidase and ascorbic acid oxidase are examples of the second class of oxidases.



In many biological reactions in which the over-all reaction can be formulated as an addition of oxygen, it has been generally assumed that hydration or hydrolysis is involved, and that the oxygen atoms are derived from the water molecule rather than from atmospheric oxygen. An example of this mechanism is shown in Eqs. (9) and (10), where the substrate is hydrated in the primary reaction, and then dehydrogenation occurs in the second reaction. The sum (Eq. 11) is an addition of oxygen to the substrate, but the oxygen is derived from the water molecule rather than from atmospheric oxygen.



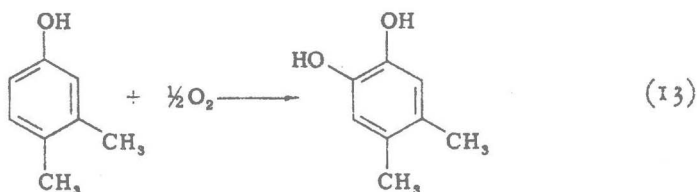
An example of an oxidation in which the oxygen atom is derived from the water molecule is the enzymic transformation of aldehydes to acids catalyzed by aldehyde dehydrogenases. In this case acyl thioesters formed in the oxidation step are converted to acids by hydrolysis (Eq. 12).



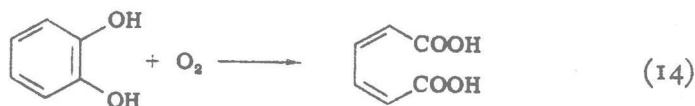
In 1932, H. Wieland made the following statement in his famous book, *On the Mechanism of Oxidation*. "Limiting ourselves to the chief energy-supplying foods, we have in this class carbohydrates, amino acids, the higher fatty acids, and glycerol. There is no known example among them of an unsaturated compound in the case of which it is necessary to assume direct addition of oxygen, that is, additive oxidation." This statement was generally accepted until, in 1955, direct utilization of atmospheric oxygen was discovered by several investigators through the use of a heavy oxygen isotope,  $O^{18}$ .

In 1955, Mason and his collaborators, through the use of  $O_2^{18}$  and  $H_2O^{18}$ , found that during the oxidation of 3,4-dimethylphenol to 4,5-dimethylcatechol catalyzed by a phenolase complex, the oxygen

atom incorporated into the substrate molecule was derived exclusively from molecular oxygen, not from the oxygen of water molecules (Eq. 13). This finding was in drastic contrast with the previously held concept of biological oxidation that oxygen could act only as an ultimate electron acceptor, and that all the oxygen atoms incorporated into substrates are derived from the oxygen atoms of water.



Subsequently Hayaishi and associates (1955), using  $\text{O}_2^{18}$  and  $\text{H}_2\text{O}^{18}$ , found that the two atoms of oxygen inserted into catechol by the action of pyrocatechase were both derived exclusively from atmospheric oxygen (Eq. 14).



These two reactions may be schematically represented by Eqs. (15) and (16).



The over-all reaction in Eq. (16) may be visualized as the addition of both atoms of oxygen to a molecule of substrate S. It was soon realized that in the case shown in Eq. (15), one of the oxygen atoms is incorporated into a substrate molecule and the other atom is reduced to  $\text{H}_2\text{O}$  in the presence of an appropriate electron donor, such as DPNH, TPNH, tetrahydrofolic acid, or ascorbic acid (Eq. 17) (Hayaishi *et al.*, 1954).



These two types of reactions both involve "oxygen fixation" into a substrate molecule, and therefore they are different from the classical

oxidase reactions shown in Eqs. (7) and (8). They are similar to the oxygenation reactions known to occur by chemical or photochemical processes, and we proposed a new term "oxygenase" to designate the enzymes which catalyze these reactions (Hayaishi and co-workers, 1956).

### III. Methodology

The mass spectrometer has been a valuable tool in the past in metabolic studies in which stable isotopes such as deuterium, carbon-13, nitrogen-15, and oxygen-18 have been used as tracers.

The use of oxygen-18 in the form of water has found extensive application in studies of enzyme reaction mechanisms, oxidative phosphorylation, and so forth. The pioneering studies by Cohn, Koshland, and Boyer, however, have dealt mainly with the analysis of oxygen-18 in  $\text{H}_2\text{O}$ , phosphate, or  $\text{CO}_2$ . The recent development of new techniques to be used with this instrument in stable isotope studies has opened up new areas for investigation which may otherwise have remained dormant.

It has always been recognized that major progress in the oxygen-18 methodology would be made when it would be possible to convert stoichiometrically all the oxygen atoms of any organic compound to  $\text{CO}_2$ . A step in this direction was the work of Roberts (1938) and Mears (1938) who found that oxygen-18 localized in a carboxyl group could be exchanged catalytically with  $\text{H}_2\text{O}$ . Thus amino acids enriched with oxygen-18 could be analyzed experimentally, although the process was rather time-consuming and lacking in precision.

In 1949, Bentley and Neuberger made use of gaseous oxygen-18 for their studies of the mechanism of action of glucose oxidase (notatin). They showed that the oxygen atoms of hydrogen peroxide produced by the enzymic oxidation of glucose were derived exclusively from molecular oxygen and not from the oxygen atoms of water molecules.

In 1953, Dorfman, then a graduate student in the laboratory of Von Doering at Columbia University, found that he could successfully combine the techniques of Unterzaucher and Schultze, and made the first major breakthrough in converting organic compounds stoichiometrically to  $\text{CO}_2$ . His method consisted of pyrolysis of the organic compound in a nitrogen stream and the conversion of all the oxygen in the compound to CO by passage through carbon heated to  $1340^\circ\text{C}$ . The scrubbed CO was then oxidized to  $\text{CO}_2$  by passage through an  $\text{I}_2\text{O}_5$  column heated to  $125^\circ\text{C}$ . This method suffers in being time-consuming and in requiring at least 10-20 mg. of a purified sample.

The technique of oxygen analysis employed currently by many investigators is that of Rittenberg and Ponticorvo or its modification. These will be described in detail in Chapter 2.

In order to demonstrate the enzymic fixation of the atoms of molecular oxygen into various substrates, the reaction products are isolated from two parallel incubation mixtures differing only in that oxygen-18 is present as a component of water in one case and as atmospheric oxygen in the other. The percentage of oxygen-18 incorporation in the product is calculated by comparison of observed  $O^{18}$  enrichment with the theoretical maximum which would be attained if all the oxygen atoms inserted into the molecule were derived from the isotopic source of the medium. Corrections are, of course, made for any nonisotopic oxygen atoms present in the substrate molecule prior to the reaction. A brief example of this type of experiment is provided by the following. Pyruvatechase was incubated with its substrate, catechol, in the presence of  $O_2^{18}$  and  $H_2O^{16}$  in one case and  $O_2^{16}$  and  $H_2O^{18}$  in another. The incubation was carried out in a flask shown in Fig. 1. The atmosphere

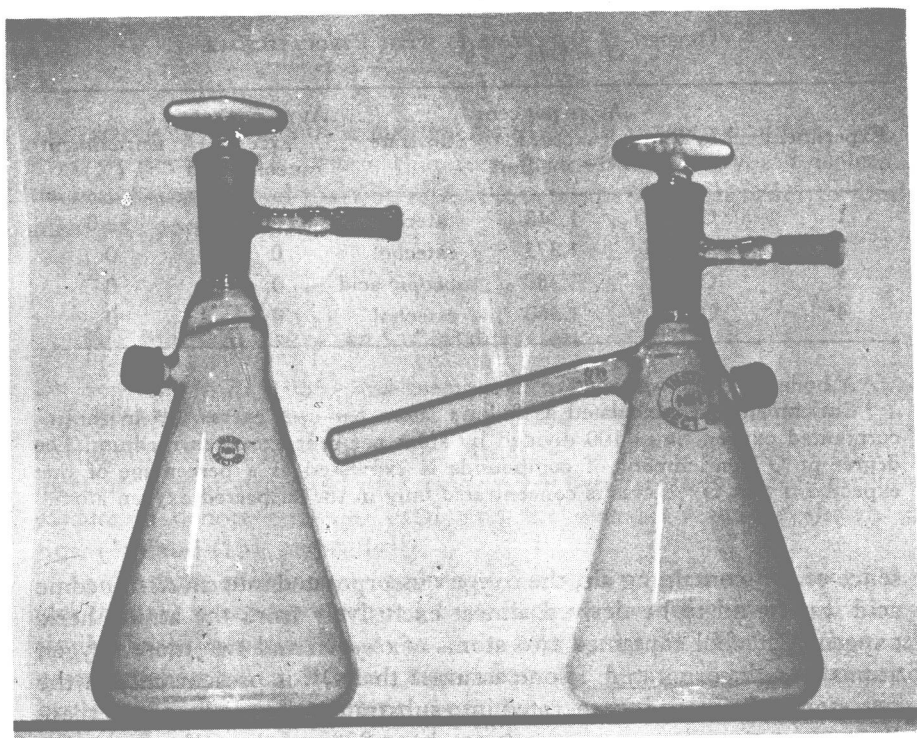


FIG. 1.



was replaced by the  $O^{18}$ -containing air through the high vacuum stopcock on the top. Then the degassed enzyme preparation was introduced through the rubber vaccine cap to initiate the reaction. Aliquots were removed through the same rubber stopper in order to follow the progress of the reaction. After the reaction was over, the product, *cis, cis*-muconic acid in this case, was isolated, purified, and crystallized, and the  $O^{18}$  content was determined by the above procedure. As a parallel experiment, the reaction was carried out exactly the same way except that  $O^{18}$ -enriched water,  $H_2O^{18}$ , and normal air were used instead of  $O^{18}$ -containing air and normal water as in the first case. In addition, similar experiments were carried out with *cis, cis*-muconic acid as a substrate and also with the boiled enzyme in order to exclude the possibility that the  $O^{18}$  incorporation was due to an exchange reaction at the product level or to a nonenzymic reaction.

As can be seen from Table I, when the reaction was run in the pre-

TABLE I  
OXYGEN-18 EXPERIMENTS WITH PYROCATECHASE

Experiment	Medium	Atom per cent excess in the medium	Substrate	Atom per cent excess in muconic acid	Enrichment <sup>b</sup> (%)
1	$O_2^{18}$	1.343	catechol	0.611	92.0
2	$H_2O^{18}$	1.373	catechol	0	0
3	$O_2^{18}$	1.380	muconic acid	0	0
4 <sup>a</sup>	$O_2^{18}$	1.360	catechol	0	0

<sup>a</sup> A boiled enzyme was used in Experiment 4.

<sup>b</sup> Enrichment was calculated as follows: atom per cent excess  $O^{18}$  in the incorporated oxygen times 100 divided by atom per cent excess in medium. The degree of  $O^{18}$  enrichment of compounds is expressed as a percentage of that expected if that  $O^{18}$  excess is concentrated only in the suspected oxygen atom.

sence of  $O^{18}$ -containing air, the oxygen incorporated into *cis, cis*-muconic acid was found to be derived almost exclusively from the atmospheric oxygen. Catechol contained two atoms of oxygen and two more oxygen atoms were incorporated. If one assumes that  $O^{18}$  is present only in the two atoms of oxygen incorporated into substrate,  $O^{18}$  enrichment in these two atoms of oxygen corresponds to about 92% of the  $O^{18}$  enrichment of the atmospheric oxygen used in these experiments. A small loss of