NRI SYMPOSIA ON MODERN BIOLOGY

MOLECULAR MECHANISMS OF ENZYME ACTION

Edited by
YASUYUKI OGURA
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PREFACE

In April 1965 the Nomura Securities Company, in celebration of its fortieth anniversary, founded the Nomura Research Institute of Technology and Economics (NRI). The aim of the institute was to provide independent, objective and impartial research covering wide range of disciplines in the human, social and natural sciences to government and industry on a contract basis. The life sciences have been emphasized as an area in great need of laboratory study since the need to solve the practical problems presented by increasing population and rapidly deteriorating environment, the need to achieve better control of diseases, and many other demands are being made of the knowledge of the life processes.

The NRI plans to hold symposia in order to provide better understandings of the life processes particularly in connection with new and progressive comprehensive areas of the biological disciplines. The mechanism of enzyme action was selected as the topic of the first symposium because of its importance in clarifying life processes at molecular level. It is by the catalytic actions of enzymes that living organisms, including human beings, are "alive," performing growth and proliferation and responding in coordination with changes in ambient conditions. Enzymes are, in addition, also being utilized for practical purposes, such as use as drugs for treating diseases, in diagnostics, food processing, and industrial catalysis. Moreover, knowledge of the mechanism of enzyme action is indispensable not only for practical applications but also for solving many other problems directly related to human life.

We were commissioned to organize the first NRI Symposium on Modern Biology held in 1966, and the occasion coincided with the retirement of Professor Yasuyuki Ogura of the University of Tokyo. It was decided that the symposium be held in his honor considering the great contributions he has made to enzymology.

Professor Ogura was born in 1910 and graduated from the Tokyo Imperial University (now the University of Tokyo), Faculty of Science, Department of Botany in 1935. Following graduation he attended the graduate school of the University of Tokyo under Professor Hiroshi Tamiya, and received his Ph. D. in 1951. He spent two years, from 1953 to 1955, in the United States working on enzymes with Professor Briton Chance at the Johnson Foundation Laboratory. He became a professor in the Department of Biochemistry, Faculty of Science, the University of Tokyo in 1960. Professor Ogura has devoted much of his time to studying reaction mechanism of enzymes and has become a leader in the field. Many of his disciples are spread throughout the world and are actively contributing to the field of enzymology.

The symposium was honored by having an opening lecture given by Professor Hiroshi Tamiya on the history of the study of oxidoreductive enzymes in Japan, which was the main object of Professor Ogura's investigation. The program included many topics on oxidation-reduction enzymes, which is the research area specialized in by Professor Ogura, as well as several topics on hydrolyzing enzymes.

It is to our great satisfaction that the symposium, which presented the state of enzyme study in Japan, was such a success. We hope that the symposium results will contribute to both further progress in science and the solving of practical problems.

We would like to extend thanks to all those helped to make the symposium success, in particular, to Professor Tamiya who gave help and advice during all stages of the symposium, to all the speakers who so kindly responded to our requests, and to Dr. Takahisa Ohta and his associate who made arrangements for the symposium to proceed smoothly. We also indebted to Professor Tamiya for his great efforts in reviewing the draft of this book. Finally, we wish to express our appreciation to the University of Tokyo Press for their helpful cooperation in the publication of this volume.

Yuji Tonomura
Takao Nakamura
Hiroyuki Matsumiya

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PREFATORY NOTE

EARLY DAYS (1883–1930) OF THE STUDY OF OXIDOREDUCTIVE ENZYMES IN JAPAN

Hiroshi Tamiya

It is a profound pleasure for me to have been asked to make an opening address at this meeting held in honor of Professor Yasuyuki Ogura, with whom I worked for many years in the field of enzymology especially in relation to biological oxidoreductive processes.

As far as I know, the man who first conducted a study in the relevant field in Japan was Hikorokuro Yoshida (*1*). He published a paper entitled " Chemistry of lacquer (Urushi) " in the Journal of the Chemical Society (London) as early as in 1883. From what I have read, Yoshida's career is not clear except that his work was performed at the then newly established Chemical Laboratory of the Imperial Geological Survey. He pursued—in an astonishingly authentic Western fashion—a detailed chemical survey on the nature and behavior of the tree juice of Urushi (*Rhus vernicifera*) which had traditionally been used in China and the Far East as material for preparing world famous lacquer wares. From samples of raw Urushi juice, he separated a substance that was found to play an essential role in the lacquering process, and, based on the results of elementary analysis, he assigned to it a chemical formula $C_{14}H_{18}O_2$,

naming it " urushic acid " (it was, however, revealed by later workers to be a diphenol having the composition $C_{21}H_{34}O_2$ and renamed " urushiol "). The key event occurring in the lacquering process is the gradual hardening or " drying " of the spread Urushi juice accompanied by the darkening of its color. This process was explained by Yoshida as resulting from oxidation of urushic acid to a substance he called " oxy-urushic acid " which " polymerized " to form the resinous matter. On performing an elementary analysis of this reaction product, he concluded that in this oxidative process one molecule of urushic acid had taken up one atom of oxygen of air.

The point of primary importance from our point of view is that the reaction in question was proved by Yoshida to be caused by a catalytic agent, which was " albuminoid " in nature, being water-soluble but alcohol-insoluble and losing its activity (by being coagulated) at temperatures higher than 63°C. Moreover, he demonstrated that the optimum temperature for this catalytic agent was at around 20°C, and that it required the presence of air and sufficient moisture for its action. The need of moisture was a remarkable finding at that time, since the phenomenon of hardening of lacquer had been thought to be a process of drying. He proudly wrote that these findings " bear out the practical experience of our lacquer men, *viz.*, that lacquer dries best in the rainy season; it dries better in summer than in winter—a damp atmosphere of about 20–30°C being just the state of air during the rainy season of a year."

Thus, Yoshida was the pioneer in our enzymology and discoverer of one of the most important oxidative enzymes, laccase, as it was later called by G. Bertrand (*2*) of France. It is interesting to note that Yoshida did not use the term " enzyme " or " oxidase " in his paper; instead, he called the agent merely " diastase " or " diastatic matter " which meant the enzymes in general at that time.

The highly laudable, methodical adequacy and logical scrupulousness we see in the work of Yoshida are something that had been lacking in the conventional " science " of old Japan, and we cannot but think that he had some masterly instruction in performing his research.

Soon after the Meiji Restoration, several governmental colleges were established in Tokyo in 1877, and as their teaching staff a number of scholars were invited from Western countries, mostly from Germany and Britain for the fields of fundamental and applied natural sciences. Among these teachers, there was a British scientist named R. W. Atkinson

who taught analytical and applied chemistry at the College of Science. Although his name was not mentioned in Yoshida's paper, I presume he might have been Yoshida's instructor, since he was, most probably, the first to introduce knowledge about enzymes into this country. In a paper (*3*) published in 1881, he reported on his discovery of a strong diastatic enzyme in the mycelium of the "Koji"-mold, *Aspergillus oryzae*, which had been traditionally used in the process of fermentation of Japanese rice wine (sake), soy-sauce, and others.

During a period of almost 20 years after the publication of Yoshida's paper, there was no work done in Japan on the oxidoreductive enzymes until a scientist named Oscar Loew came from Germany to teach agricultural chemistry at the College of Agriculture. He was an outstanding plant biochemist whose name we still find in modern text books of plant physiology and biochemistry for his classical works on the mineral nutrition of higher plants. His sojourn in Japan was from 1893 to 1897 and from 1900 to 1907, and in 1901 he published a paper (*4*) on the hydrogen-peroxide decomposing enzyme contained in tobacco leaves, naming it "catalase." Among the students educated by him, there were some brilliant ones who later became professors of agricultural chemistry at the College of Agriculture. One of them, Keijiro Aso, performed a penetrating study on the oxidizing enzymes in higher plants (*5*). Using tissues of various plants (radish, potato, apple, bamboo, *etc.*), he investigated the properties of their oxidase(s)—with guaiac tincture or tetra-methyl-*p*-phenylenediamine as reagents—and peroxidase(s), with guaiacol, *p*-phenylenediamine or tetra-methyl-*p*-phenylenediamine as reagents. He demonstrated that the activities of these enzymes were all inhibited by hydrogen cyanide and phenylhydrazine. Using the juice obtained from a radish root, he attempted the fractionation of the enzymes, and found that both groups of the enzymes were precipitated when the juice was treated with ethylalcohol or saturated with ammonium sulphate. He also observed that on adding a small quantity of acetic acid to the juice the enzymes were freed from some impurities by precipitation of the latter.

Aso (*6*) also performed an interesting experiment making clear the cause of difference in color between black tea of Western countries and green tea of Japan. He showed that the blackening of tea leaves during the so-called "fermentation"-process—which, in fact, has nothing to do with the activities of microorganisms—was a result of the action of an

oxidase upon tannin contained in the leaves. In the processing of Japanese green tea, the newly harvested leaves are subjected, before drying, to steaming which kills the oxidase leaving the leaves maintaining their original green color.

Umetaro Suzuki who became later famous for his discovery of oryzanin, the anti-beriberi vitamin now called B_1, was also a student of Loew. He found (*7*) that the mulberry trees which were suffering from " dwarf-trouble " contained abnormally large amounts of oxidase and peroxidase in their leaves.

Toward the end of the 19th century, the Japanese government ceased its policy of inviting teachers from foreign countries, and the job of teaching young students was handed over to those who had been educated by the foreign teachers. At the same time, a number of promising young people were sent abroad to acquire first-hand knowledge of Western sciences. This was the first study-abroad period which lasted until the outbreak of World War I (1914). Among the works done by Japanese scientists during this period, we can find only a few that were concerned with oxido-reductive enzymes. Yamada (*8*) studied, in collaboration with A. Jodlbauer at Munich, the effects of ultraviolet and visible lights upon the action of peroxidase obtained from horseradish. Sano (*9*) studied at Würzburg, together with K. B. Lehmann, the tyrosinase contained in various plant tissues and bacteria. In the literature, their work is cited as being the first to report on the vulnerability of tyrosinase toward hydrogen cyanide.

During the period from 1910 to 1912 Keita Shibata went to Europe and studied plant physiology under W. Pfeffer at Leipzig and organic chemistry under M. Freund at Frankfurt am Main. Although he published no enzymological studies during this period, the work he performed at Pfeffer's laboratory was, as will be described later, of importance in relation to his later work on cytochrome. During his stay in Europe, his brother Yuji Shibata—who later became professor of inorganic chemistry at the University of Tokyo, and to whom we owe the introduction of spectroscopic techniques to chemistry in Japan—studied the chemistry of complex-salts at A. Werner's laboratory at Zürich. After returning to Japan, the brothers started (in 1916), in fruitful collaboration, a unique investigation of using metal-complex salts as enzyme models. They found that some complex-salts, especially those forming labile aquo-complexes, display oxidase-like actions upon various polyphenols. The large num-

ber of works done along this line by the Shibata brothers and their co-workers are summarized in a monograph (*10*) they published in 1936.

In 1913, M. Nagayo, then a new returnee from Germany and professor of pathology at the Medical College of the University of Tokyo, published a paper (*11*) reporting that in Germany the study of the " Nadi-reaction " (caused by the " Nadi-oxidase ") in various tissues and cells was attracting the attention of biologists and medical scientists. Stimulated by this paper, many of Nagayo's students began studies on this reaction from a histo-pathological standpoint. One of these students, S. Katsunuma, who performed most extensive investigations on this subject, summarized the results of his and his colleagues' studies in a monograph (*12*) published in Germany in 1924. From the enzymological point of view, the significance of Katsunuma's work lay in the following points.

It had already been generally agreed among scientists that the Nadi-reaction shown by raw tissues (without fixation with formalin) was a reaction caused by an oxidative enzyme, and that the enzyme might contain iron atom, since the reaction had been known to be inhibited by cyanide. To make clear the distribution of iron in various tissues and cells, Katsunuma performed vital staining of them using Quincke's reagent, *i.e.*, a solution of ammonium sulfide which, in the presence of iron, produces easily discernible ferro-sulfide. He demonstrated that the granules stained by this method coincided almost exactly with those showing the enzymic Nadi-reaction. He thus provided strong experimental evidence in favor of the iron-containing nature of the Nadi-oxidase which is now called cytochrome *c* oxidase. Much importance should be attached to the finding made by Katsunuma that the Nadi-positive granules were present in cytoplasm, leucocytes and so forth, but not in nuclei, which is in coincidence with our modern knowledge about the distribution of mitochondria.

Among the studies performed in Japan during the pre-World War I period, I should like to draw attention to that of E. Yamasaki (*13*) who worked at the laboratory of Kikunae Ikeda of the University of Tokyo. Ikeda was a professor of physical chemistry, whose name is now better known as the discoverer of the " essence of deliciousness " (monosodium glutamate). Ikeda motivated Yamasaki to study the kinetics of catalase reaction which, under certain conditions, did not follow the first-order rate law, as it was generally held at that time. With the assumption that, during the process of reaction, catalase underwent gradual inhibition by

its substrate (hydrogen peroxide) and product (oxygen), Yamasaki presented a kinetic formula which was shown to coincide well with experimental data.

During World War I (1914–1918), the Japanese scientific world was practically isolated from that of Western countries. With the beginning of the 1920s, a sort of study-abroad rush occurred, in view of the remarkable revival of science, especially in Europe. It was in 1920 that Thunberg in Sweden propounded his dehydrogenase theory, and several years later (1925), there appeared almost simultaneously two most important papers, one by Keilin on cytochrome and the other by Warburg on his Atmungsferment.

Among the countries in Europe, the one most swarmed with Japanese scientists (mostly medical) was Germany. Various kinds of oxidoreductive enzymes were investigated by them under the guidance of: O. Warburg at Berlin-Dahlem (Sakuma, *14*; Tanaka, *15*; Toda, *16*; working mostly on the role of iron in oxidoreductive reactions; Fujita, *17*, on the effect of carbon monoxide upon the respiration of leucocyte, *etc.*), M. Jacoby in Berlin (Tsuchihashi, *18*, on catalase), J. Wohlgemuth in Berlin (Koga, *19*; Maeda, *20*; Yamasaki, *21*; Hizume, *22*; Sugihara, *23*; Tateyama, *24*, on polyphenol oxidase), Th. Brugsch and H. Horsters in Berlin (Harada, *25*, on succino-dehydrogenase), C. Neuberg at Berlin-Dahlem (Kumagawa, *26*, on dehydrogenase activity of yeast), R. Willstätter at Munich (Suminokura, *27*, on laccase), *etc.*

Some went to Sweden or to England, *e.g.*, to H. v. Euler's institute at Stockholm (Nakamura, *28*, on catalase), F. G. Hopkins' institute at Cambridge (Kodama, *29*, *30*, on xanthine oxidase) and C. L. Evans' laboratory in London (Tsubura, *31*, on succino-dehydrogenase). U.S.A. was, at that time, not the country for the study of oxidoreductive enzymes; in the literature I could find only one Japanese, Ishikawa (*32*), who studied formico- and succino-dehydrogenases of bacteria in cooperation with A. J. Kendall at Chicago.

Unfortunately, the majority of these studies, except a few to be mentioned below, were scientifically of minor importance, having been pursued, in subordinate manners, under the guidance of their respective teachers. In fact, many of the scientists mentioned above did, after returning, little to contribute to the study of oxidoreductive enzymes in this country.

One of the exceptions may be the work of Kodama (*29*) performed at

Cambridge dealing with the xanthine dehydrogenase of milk. It was a pioneer work in measuring the redox-potential of the reaction system of oxidoreductive enzymes. Using a gold electrode, he showed that the reaction system of xanthine-dehydrogenase could become reactive to the electrode only in the presence of an oxidoreductive pigment such as methylene blue. By measuring the change of redox-potential of such a system, he followed the process of the electron-transport from the substrate to the pigment mediated by the enzyme.

Working with blood catalase at Jacoby's laboratory in Berlin, Tsuchihashi (*18*) succeeded in making the enzyme free from hemoglobin by shaking their mixture with alcohol and chloroform followed by adsorption to calcium phosphate. This procedure was used afterwards by many investigators working on blood catalase. It may be of interest to note here that Tsuchihashi—having observed that, with the advancement of the purification of the enzyme, there occurred a gradual decrease of nitrogen and protein contents in the preparation—stated that if the enzyme could be purified to a sufficient degree, it would become protein-free. Undoubtedly he was influenced by the concept of R. Willstätter who maintained at that time that the essential principle of enzymes was not necessarily protein. It was only 3 years later that Sumner succeeded in purifying urease in the form of a crystallized protein.

Fujita who studied at Warburg's institute wrote, after returning to Japan, a book (*33*, in Japanese) describing in detail the manometric technique of his German teacher. Although the technique had already been in use in some laboratories at that time, Fujita's book contributed much to the popularization of this method which was, and still is, indispensable in various fields of biochemistry and cell physiology.

Incited by, or independently of, the influences of the returnees from Europe, investigations on oxidoreductive enzymes in Japan became gradually active towards the middle of the 1920s. Kanda (*34*) of the Institute for Physical and Chemical Research performed a series of biochemical studies on the bioluminescence of a marine crustacean, *Cypridina*. He purified luciferin (the substrate of luciferase) to a considerable degree by salting it out from its solution with ammonium sulfate followed by precipitation with phosphotungstic acid.

An interesting finding was reported by Itano and Arakawa (*35*) in 1928, who investigated, at the Oh-hara Institute for Agricultural Research, the catalase contained in cells of a thermophilic cellulose-decomposing bac-

terium. The enzyme was found to display its activity even at temperatures as high as 80–100°C.

A long series of work (*36*; nearly 40 separate papers) entitled " Report of the peroxidase reaction " was published beginning in 1925 by A. Sato and his staff at the Medical School, Tohoku University. They made comparative studies on the peroxidase activities—using various measuring techniques—of leucocytes, milk, fetuses, *etc.* of rabbits, guinea pigs and men. From an enzymological point of view, however, their studies seemed to be of rather minor interest. Of the findings they reported, I cite here only one, namely that the peroxidase activity of rabbit milk became weaker than normal when the animal was made deficient of vitamin B_1, but restored its normal level on recovering from avitaminosis. Along a similar line, Yaoi (*37*) reported in 1928 that the methylene blue reducing power of pigeon muscle became weaker than normal when the animal was made deficient of vitamin B_1.

This retrospective account would not be regarded as being appropriate to its title unless it deals with the earlier activities of the Shibata school which later produced an array of brilliant works by E. Yakushiji, K. Okunuki, A. Takamiya, Y. Ogura and others on cytochromes, oxidases, dehydrogenases, catalase, *etc.*

The first paper which made Shibata's school widely known in the field of oxidoreductive enzymology was entitled " Untersuchungen über die Bedeutung des Cytochroms in der Physiologie der Zellatmung " by Shibata and Tamiya (*38*). The idea propounded in this paper, which was called the " theory of oxygenation of cytochromes " was, we must now admit, erroneous in some points, but contained certain material worthy of reappraisal from the angle of modern enzymology.

There had been a cogent background which led Shibata and Tamiya to their " oxygenation theory." In his studies on the metabolism of a mold *Aspergillus oryzae*, Tamiya (*39*) found that the mode of respiration of its mycelial mat—growing on the surface of culture solution, and respiring by virtue of its mycelia exposed to open air—was, in many respects, different from those of other aerobic microorganisms, such as yeast and bacteria, whose respiration takes place under conditions of submersion in the solution. It was demonstrated that the strength of the mycelial respiration was markedly dependent on the oxygen partial pressure, showing a maximum in the air containing about 70% O_2, whereas the respiration of submerged microorganisms has been known to be