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Review

An odyssey with oxygen

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Prologue

In 1942, I graduated from Osaka University School of Medicine and almost immediately enlisted in the Japanese navy as a medical officer. When the War ended in 1945, I returned home to Osaka, where I found that the second largest city in Japan, having a population of about four million, had been almost completely destroyed by bombing and that the scenery, once green and beautiful, had become desperately dreary. The house where my parents and I had been living during the pre-war years had been completely wiped out without a trace. I had no place to live. Food was scarce and rationed, and the future looked gloomy. I had just turned 25 years and stood at the crossroad of my life, wondering if I should become a clinical doctor like my father and brothers or should go into basic research.

Fortunately, Professor Tenji Taniguchi, my former mentor, offered me a position as a junior faculty member at the Department of Bacteriology, Osaka University, where I was able to start my career as a research scientist. However, the living conditions were poor, and research facilities were inadequate; and the supply of commodities, such as gas, electricity, and even water was sparse, let alone chemicals and experimental animals. Although the morale of my colleagues was high, we were obliged to spend most of our time reading journals and books in the library or participating in seminars.

Tryst with tryptophan

One day, I was quite unexpectedly visited by Dr. Yashiro Kotake, Emeritus Professor and one of the most famous biochemists in Japan before World War II. In 1925, he and his colleagues elucidated the major metabolic pathways of tryptophan in mammals, and discovered and identified kynurenine as the key intermediate. Thereafter they showed this intermediate to be converted to kynurenic acid, anthranilic acid or xanthurenic acid and excreted in the urine (Fig. 1).

He gave me several grams of L-tryptophan that he and his colleagues had prepared from casein by tryptic digestion and told me to use it for my research, since he had already retired and had no more use for this precious compound. Needless to say, I was delighted and very grateful to him for his valuable gift, but, frankly speaking, did not know what to do with it. About that time we received news that the Rockefeller Foundation had contributed back issues of major biomedical journals that had been published during the war to Tokyo University and I decided to travel to Tokyo to look through these journals. We were all impressed and astounded by the progress made by the American scientists even during war time. I was particularly intrigued by the paper by G.S. Mirick describing soil microorganisms that produced adaptive enzymes capable of metabolizing various aromatic compounds such as *o*- and *p*-aminobenzoate, tryptophan, and so forth [1]. Although Dr. Kotake's life's work on tryptophan metabolism had been carried out in animals, because I was working in the Department of Bacteriology, I immediately decided to try Mirick's approach. Upon returning to Osaka, I went out to the yard behind the laboratory, obtained several samples of soil from the scorched ground and mixed them with a trace amount of tryptophan and tap water in test tubes, and waited. It was such a simple and undoubtedly the least expensive experiment I ever performed throughout my career! After a few days, I was able to observe some cloudiness in the supernatant indicating the growth of soil bacteria that could utilize tryptophan as their sole source of carbon and nitrogen. By repeating the subculture process several times, I isolated in pure form a strain of *Pseudomonas* sp. In these bacteria, I discovered that tryptophan was degraded via kynurenine to anthranilic acid, exactly the same sequence of reactions Professor

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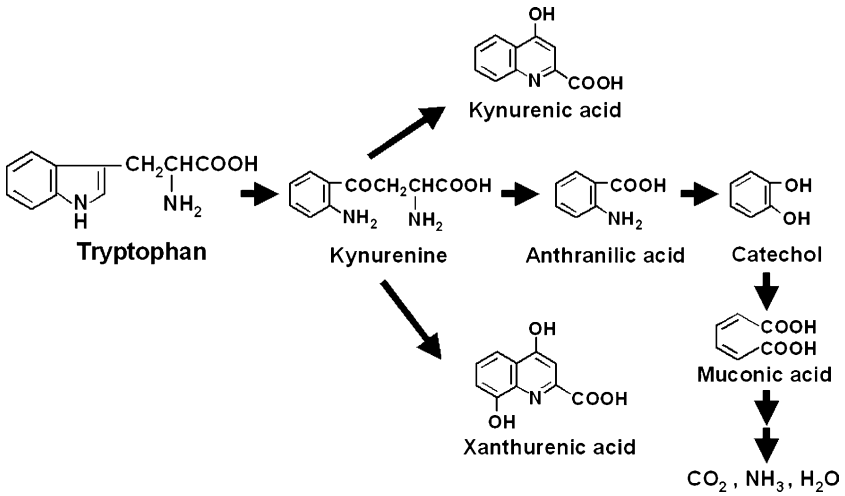

Fig. 1. Major metabolic pathways of tryptophan.

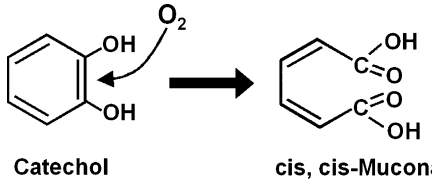

Fig. 2. Enzymic conversion of catechol to *cis*, *cis*-muconate.

Kotake had shown to occur in animals many years ago. However, I was startled to find also that in this bacterium, anthranilate was further degraded to catechol and ultimately to CO_2, NH_3, and H_2O (Fig. 1). In order to elucidate this new metabolic pathway of tryptophan, I then attempted to extract and purify the enzymes involved in it. At that time, the methods for extraction of enzymes from bacterial cells had not been worked out; and after trying several rather primitive methods of extraction, I was able to solubilize only one enzyme in a cell-free form. The partially purified enzyme catalyzed the oxidative cleavage of catechol to produce *cis*, *cis*-muconic acid (Fig. 2).

Because the Warburg manometer (respirometer) was the only modern scientific instrument in the laboratory, I used it to quantify the amount of atmospheric oxygen consumed and demonstrated a stoichiometric relation, namely, one mole of molecular oxygen was consumed while one mole of catechol was converted to one mole of *cis*, *cis*-muconic acid. The product of the reaction was extracted with ether from the acidified reaction mixture and identified [2]. However, one crucial question as to the role of molecular oxygen in this catalytic mechanism remained unsolved at that time.

The role of oxygen

The fundamental mechanisms underlying the metabolism of molecular oxygen in tissue respiration have long been one of the most important subjects in biochemistry, ever since Lavoisier started the study of biological oxidation processes some 200 years ago. In 1932, Professor Heinrich Wieland, a German Nobel laureate in chemistry, authored a book entitled "On the Mechanism of Oxidation" [3], in which he proposed his famous "dehydrogenation theory." According to this theory, the principal focus in the field of respiratory physiology should be the elucidation of pathways in which electrons or hydrogen atoms are transferred from a substrate through various carriers to molecular oxygen. The enzymes catalyzing these reactions were termed "dehydrogenases"; and in cases in which molecular oxygen served as the ultimate acceptor, forming H_2O or H_2O_2, the enzymes were referred to as "oxidases." However, molecular oxygen would never be incorporated into the substrate according to Wieland. When Wieland envisaged the overall reaction as the addition of oxygen, he considered the oxygen atoms to be always derived from water molecules and not from atmosphere oxygen, as exemplified by the conversion of aldehydes to acids. Thus, according to the central dogma of Wieland's dehydrogenation theory, the direct incorporation of molecular oxygen to a substrate was completely ruled out as being a part of biological oxidation mechanisms.

Upon close examination of this enzyme, I found its enzymatic properties to be somewhat different from those of hitherto known oxidases and dehydrogenases described in the literature at that time. For example, (1) The partially purified enzyme was colorless and did not appear to contain heme, flavin or any other cofactors; (2) methylene blue and other dyes, and coenzymes could not substitute for molecular oxygen; (3) orthobenzoquinone appeared not to be involved as a dissociable intermediate, because aniline did not inhibit the enzyme activity; (4) the enzyme activity was not inhibited by KCN, NaN_3 or CO but was inhibited completely by 10^{-3} M $AgNO_3$. These and other lines of evidence indicated that this enzyme was not a typical oxidase or dehydrogenase and suggested that molecular oxygen may be directly incorporated into the substrate

catechol. Other properties of this enzyme as well as the unique oxidative cleavage of the aromatic structure indicated this enzyme to be a novel one different from phenolase and other similar enzymes involved in the metabolism of numerous phenolic compounds usually found in plants, mushrooms, insects, and so forth. We therefore proposed to name it "pyrocatechase" [2]. It was a non-heme iron protein and had a molecular weight of approximately 90,000 Da.

However, when I spoke to my mentors and colleagues at Osaka University, most of them were skeptical of my interpretation, because at that time, the dehydrogenation theory of Wieland was an undisputable central dogma in the textbooks of biochemistry and enzymology. If I had proposed the direct addition of molecular oxygen to a substrate in my paper, it would not have been accepted by any reputable journal. In fact, in many textbooks published later, pyrocatechase long remained an orphan enzyme being classified as miscellaneous or unclassified in a corner of the category of oxidoreductases.

When I presented this work at the first Annual Meeting of the Japanese Biochemical Society after the war in April 1949 at Kyoto University, Professor Kotake applauded our work and mentioned that his mentor in Germany, Professor Max Yaffe of the University of Königsberg, had fed benzene to dogs, isolated muconic acid from the urine, and proposed that pyrocatechase or a similar type of enzyme might be present in mammals. I was also happy to have received numerous questions and discussions concerning the nature of this new type of oxidative enzyme, some people having agreed with my interpretation, although most people still appeared not to be convinced.

In 1949, another unexpected event, like when Dr. Kotake came to visit me, occurred. I received an invitation from Professor David E. Green, the director of the Enzyme Institute of the University of Wisconsin, Madison, Wisconsin in the USA, to spend a year in his laboratory as a William Waterman fellow of enzyme research. There, after having read a symposium on Respiratory Enzymes published in 1942 by the University of Wisconsin Press [4] and Respiratory Enzymes edited by H.A. Lardy [5], I became all the more convinced that pyrocatechase catalyzes direct addition of molecular oxygen to the substrate catechol and that this enzyme is a hitherto unknown new type of oxidoreductase. However, direct evidence to support my interpretation was still unavailable at that time. After spending some time in Berkeley, Bethesda, and St. Louis, I was appointed chief of the Section on Toxicology of the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health in Bethesda in 1954.

The ^{18}O experiment

While reorganizing the research programs at the start of the new appointment, I decided to examine the possibility that pyrocatehase, my first love, could incorporate isotopically labeled molecular oxygen $^{18}O_2$ into the substrate, catechol. $^{18}O_2$ was not commercially available at that time; and so I wrote a letter to Dr. David Samuel, who was the head of the isotope division of the Weizman Institute in Israel, asking for his help. He kindly supplied me with some precious $H_2{}^{18}O$, from which I was able to generate $^{18}O_2$ by an electrolytic procedure. In collaboration with Dr. M. Katagiri, an excellent enzymologist from Osaka, and Dr. S. Rothberg, who was skillful in mass-spectrometry, I was able to carry out the crucial experiment in which catechol and the enzyme were incubated in two flasks, one containing $^{18}O_2$ and $H_2{}^{16}O$, and the other, $^{16}O_2$ and $H_2{}^{18}O$. The product, muconic acid, was then isolated, crystallized, and examined for ^{18}O content by mass spectrometry. Almost 100% of the ^{18}O in the product was found to have been derived from air, while almost none of it was from $H_2{}^{18}O$ [6]. Thus, it took me almost 5 years until I was able to prove my hypothesis by indisputable experimental evidence.

As this new class of oxidative reactions, oxygen fixation, was catalyzed by a novel group of enzymes that required a new terminology, these enzymes were later designated as "oxygenases." Concurrently and independently, Howard S. Mason and colleagues at the University of Oregon showed that phenolase purified from a mushroom incorporated one atom of molecular oxygen into a substrate such as phenols while the other atom of molecular oxygen was reduced to H_2O, and proposed to name such enzymes "mixed function" oxidases [7]. We named the latter type of enzymes "monooxygenases," in contrast to "dioxygenases," in which case both atoms of oxygen are incorporated into a substrate molecule, as in the case of pyrocatechase.

At first, these oxygenase-catalyzed reactions were generally thought to be rather unusual, being limited to only primitive living organisms such as soil bacteria or mushrooms. However, it soon became apparent that oxygenases are ubiquitously distributed not only in microbes but also in plants and animals, and play important roles in the biosynthesis and metabolism of numerous physiologically important compounds as well as in the degradation of various synthetic compounds including drugs, carcinogens, and so forth. In contrast, oxidases and dehydrogenases are mainly, if not exclusively, involved in energy metabolism. Fig. 3 shows a metabolic map of tryptophan in which red oxygen atoms indicate the molecular oxygen incorporated into substrate by various specific oxygenases [8]. It serves to illustrate not only the ubiquitous presence of oxygenase-catalyzed reactions in this physiologically important metabolic pathway but also shows the presence of numerous novel enzymes and metabolic reactions initiated by various specific oxygenases such as indoleamine dioxygenase, poly- and mono-ADP ribosylation reactions, NAD biosynthesis, and so forth.

50 years since then

The discovery of oxygenases initiated an explosion of research on oxygen fixation reactions and oxygenases that has continued over the last 50 years. A vast amount of

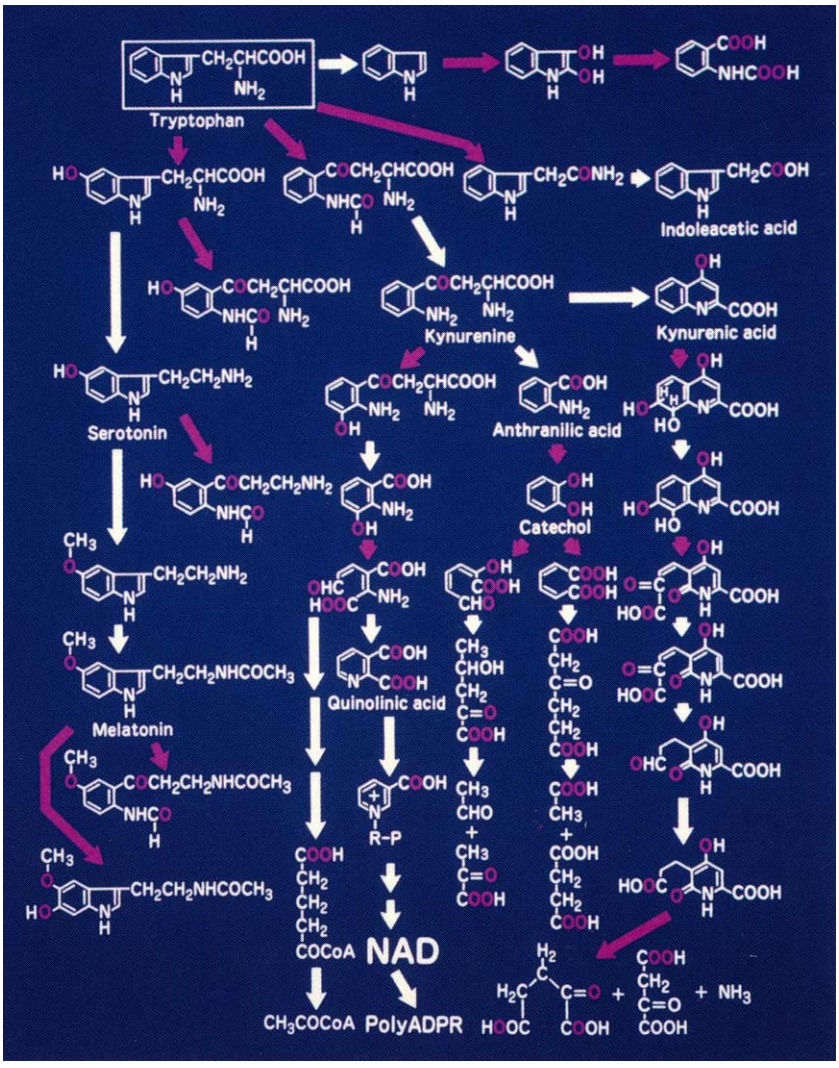

Fig. 3. A metabolic map of tryptophan. The red arrows indicate oxygenase-catalyzed reactions. The red oxygen atoms have been incorporated into these substrates each by specific oxygenases.

progress has been made in this field of research, which progress has had an enormous impact on not only physicochemical science but also nearly all fields of medical, biological, and even environmental health sciences. The stupendous past accomplishments in this field are summarized in several monographs and proceedings of the symposia listed in the reference section [9–16]. I am so gratified to see that this field is further expanding into the realms of the unknown, as exemplified by the collection of excellent papers and reviews in this special issue. It is my fervent hope that this present publication will excite many young scientists to dare to seek after Truth, even when current dogma may point in another direction.

Acknowledgments

This minireview or memoir is dedicated to all my former mentors, colleagues, collaborators, and friends who have encouraged, guided, and helped me during the past 60 years since I started as a rookie scientist soon after the Second World War. Their names are too numerous to mention here; but if I am allowed to single out just a few, I am most indebted to Professor Tenji Taniguchi, who instilled in me the courage and gave me the opportunity to start my career in that most difficult post-war period; to Professor Masami Suda, who was my first mentor and collaborator and whose constant encouragement and guidance kept my work on tryptophan metabolism going under those formidable conditions; and to Professor Arthur Kornberg, who taught me how to be a critical and creative scientist. Last but not least, I express my deepest gratitude to the co-guest editors of this special issue, Drs. M.J. Coon, R.W. Estabrook, L. Que, Jr., and S. Yamamoto, without whose help and hard work it would not have been possible to carry out this difficult task.

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