RECOLLECTIONS

My life with tryptophan—Never a dull moment¹

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I graduated from Osaka University School of Medicine in 1942 during the Second World War, and then served as a medical officer in the Japanese Navy for 3 years. With the end of the war in 1945, I returned to Osaka to find the entire city almost completely demolished. The home of my parents and family had completely vanished. Food was scarce and the supply of commodities, gas, electricity, and even water was extremely limited. Given these existing conditions, I still do not understand why I decided to go into basic research. If I had chosen the practice of medicine, as did many of my classmates, I would have been economically very comfortable even in those difficult times. Instead, I was rash enough to decide to work in the laboratory. Osaka University was one of the centers of biochemical research in Japan, and Professor Yashiro Kotake and his associates were well known for their research on tryptophan metabolism. Professor Kotake was responsible for the isolation and characterization of kynurenine, the key intermediate of tryptophan metabolism in both mammals and microorganisms. He kindly provided me with several grams of tryptophan, kynurenine, and other related chemicals, and encouraged

me to use these valuable compounds for my experiments. However, research funds were extremely limited, facilities were poor, chemicals were scarce, and there were no laboratory animals; therefore I decided to use microorganisms. I went out to the backyard of Osaka University, took some soil samples, and mixed them with tryptophan and water in a test tube, and waited. It was a very simple experiment, but after a few days, I was able to observe some cloudiness in the supernatant. Thus, by the enrichment culture technique, I was able to isolate several strains of soil bacteria that can grow on tryptophan as their sole source of carbon and nitrogen.

About that time, it was already known, mainly through the efforts of Kotake and associates, that in mammals, tryptophan was degraded through kynurenine to kynurenic acid, anthranilic acid, and xanthurenic acid as the end metabolites. However, in this soil microorganism, I was able to demonstrate in collaboration with Suda, Oda, and others that anthranilate was further metabolized to catechol and then to muconic acid, and ultimately oxidized to CO₂, ammonia, and H₂O. Encouraged by this unexpected discovery, I proceeded to isolate and purify the enzyme that catalyzed the oxidative cleavage of catechol to produce *cis,cis*-muconic acid as the reaction product. This enzyme was a non-heme iron protein and had a molecular weight of approximately 90,000 Da. Because two atoms of oxygen were consumed, I took it for granted that oxygen atoms were incorporated into the substrate and named the enzyme pyrocatechase instead of catechol oxidase. I chose this name because several catechol oxidases had already been reported at that time, but these enzymes seemed to be grossly different from pyrocatechase because they did not cleave the aromatic structure of catechol and they were copper proteins rather than non-heme iron proteins. However, the catalytic mechanism of pyrocatechase remained unclarified for some years. I recall with great pleasure the warm and encouraging comments of Professor Kotake when I presented these results at the first Annual Meeting of the Japanese Biochemical Society after the war, held in Kyoto in 1949.

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Being a very naive and inexperienced biochemist, I assumed that the oxygen consumed by the pyrocatechase-catalyzed reaction must have been incorporated into catechol, as depicted by Lavoisier in the 18th century. According to Lavoisier, the principle of biological oxidation was the addition of oxygen atoms to the substrate and the opposite process, that of reduction, was the removal of oxygen from the oxide. I was startled to learn, however, that at the beginning of the present century, the enzymatic incorporation of molecular oxygen into substrates was completely ruled out by Professor Heinrich Wieland, a German Nobel laureate. In 1922, Professor Wieland wrote a book entitled *On the Mechanisms of Oxidation*, which at that time was generally considered the bible of biological oxidation. In this book, he proposed the famous dehydrogenation theory and completely excluded the direct addition of the oxygen molecule to the substrate in the biological oxidation system. According to "the dehydrogenation theory" proposed by Wieland, the principle of biological oxidation processes is the transfer of hydrogen atoms, or their equivalent, from the substrate molecule to an appropriate acceptor such as coenzymes and various dyes. Oxygen molecules may, in some instances, serve as the immediate electron acceptor, then these enzymes are termed "oxidases." Thus, according to Professor Wieland, molecular oxygen accepts hydrogen atoms and is reduced to water or hydrogen peroxide. However, it is never incorporated into the substrate, as was originally proposed by Lavoisier and his contemporaries some 200 years ago. According to the dehydrogenation theory of Wieland, when the overall reaction is envisaged as the addition of oxygen, the oxygen atoms are always derived from the water molecule rather than from atmospheric oxygen. For example, when aldehydes are converted to acids, aldehydes are hydrated first and then dehydrogenation occurs so that the sum is the addition of oxygen to the substrate X to form XO, but this oxygen atom is derived from water rather than molecular oxygen. Therefore, according to Professor Wieland, the direct addition of molecular oxygen to a substrate was considered completely irrelevant to biological oxidation. Nevertheless, I considered the possibility that pyrocatechase may incorporate molecular oxygen into the substrate rather than dehydrogenate it, because oxygen could not be replaced by known coenzymes or electron acceptor dyes, and further, orthobenzoquinone, the dehydrogenated product of catechol, did not serve as the intermediate of the overall reaction. In 1955, I decided to explore the possibility that pyrocatechase could incorporate molecular oxygen into catechol. A set of experiments was performed in which a heavy oxygen isotope, ^{18}O (oxygen-18), was used in air in one flask, or in the form of water in the other. The product, muconic acid, was isolated and analyzed for its ^{18}O content by mass spectrometry. If pyrocatechase was indeed a dehydrogenase or an oxidase, the incorporated oxygen atoms should have been derived from water according to

Professor Wieland. However, contrary to the then generally held belief, our results clearly demonstrated that the oxygen atoms incorporated into the product molecules were derived exclusively from molecular oxygen and not from the oxygen of the water molecule. Despite our clear-cut results, our data were at first brushed off by our peers and met with skepticism. Concurrently and independently H.S. Mason and associates demonstrated that the phenolase complex of the mushroom incorporated one atom of molecular oxygen into a substrate, 3,4-dimethylphenol, to form 4,5-dimethylcatechol. These findings together with the subsequent work by K. Bloch and others established that "oxygen fixation" did occur in biological systems, and a new concept of "biological oxygenation" was introduced. In essence, this was the revival of the old concept of Lavoisier, and I proposed to term this new type of oxidative enzyme "oxygenases." In principle, oxygenases may be classified into two categories: dioxygenases, which incorporate two atoms of molecular oxygen per substrate molecule, as exemplified by pyrocatechase, and monooxygenases, which incorporate one atom of oxygen per substrate molecule; tryptophan 5-monooxygenase, which produces 5-hydroxytryptophan from tryptophan, as well as kynurenine 3-monooxygenase are typical examples of monooxygenases. These two monooxygenases play important roles in tryptophan metabolism and were later studied extensively by Ichiyama and Okamoto and their coworkers, respectively.

Tryptophan pyrrolase was shown in 1957 to incorporate two atoms of molecular oxygen into the substrate tryptophan to form formylkynurenine and therefore was renamed tryptophan 2,3-dioxygenase. This enzyme is a heme protein and we were able to demonstrate later that the enzyme-substrate-oxygen complex is the intermediate of this reaction. Various species of chemically active forms of oxygen have been reported, including singlet oxygen, superoxide, hydroxyl radicals, and so forth, but these do not seem to be involved per se as dissociable intermediates in most oxygenase-catalyzed reactions. In 1964, I presented a plenary lecture at the Sixth International Congress of Biochemistry in New York and proposed that the enzymatically activated form of oxygen in the tryptophan 2,3-dioxygenase-catalyzed reaction is the ternary complex of enzyme heme:oxygen:substrate. This prediction was amply substantiated by subsequent work by Ishimura and others in my laboratory with tryptophan dioxygenase and later with indoleamine dioxygenase. Furthermore, such a ternary complex was later demonstrated to be an intermediate in P-450-catalyzed monooxygenase reaction by Estabrook and associates and also by Gun-salus and associates.

At first, these oxygenase reactions were generally thought to be rather unusual and were limited to only primitive living organisms such as soil bacteria or mushrooms. It took several years before the ubiquitous existence of oxygenases was confirmed in many laboratories.

During the next decade, a number of oxygenases were isolated from animals, plants, and microorganisms, and some of them were crystallized by Nozaki, Fujisawa, Yamamoto, and others in my laboratory. These studies led to the elucidation of numerous physiologically important metabolic pathways and demonstrated the ubiquitous presence of oxygenase reactions. For example, Figure 1 shows the metabolic map of tryptophan. The pink arrows in this chart denote oxygenase reactions by which the pink oxygen atoms have been incorporated into these substrates. It is evident that a large number of oxygenases are involved in these important metabolic pathways. In fact, the two most important metabolic pathways of tryptophan in mammals, leading to the formation of pyridine nucleotide coenzymes and ultimately to poly(ADP-ribose) on one hand, and to that of various indoleamines on the other, are initiated by the two well-known oxygenases, tryptophan 2,3-dioxygenase and tryptophan 5-monooxygenase, respectively.

So far, I have presented my early work on tryptophan research that led me into oxygenase studies (Hayaishi, 1962, 1974; Nozaki et al., 1982; Yamamoto et al., 1991). Now I would like to discuss some other aspects of my tryptophan research. In the 1940s, Roger Stanier at the University of California isolated several strains of soil mi-

croorganism that metabolized tryptophan via kynurenine acid. In 1950, I spent about 4 months in his laboratory carrying out comparative studies on his and my strains of soil bacteria. The pathway through anthranilate and catechol was referred to as the "aromatic pathway," whereas the other pathway through kynurenine acid was referred to as the "quinolinic pathway." After returning to Japan in 1958, I carried out detailed studies at the Department of Medical Chemistry in the Faculty of Medicine of Kyoto University in collaboration with a large number of collaborators including Kuno, Nishizuka, Numa, Senoh, and others and a large variety of intermediates as well as enzymes responsible for each step were isolated and characterized.

We then proceeded to investigate the metabolic pathways of tryptophan in the mammalian liver. Two pathways, namely the NAD pathway leading to the biosynthesis of NAD and the glutarate pathway, which leads to the complete combustion of the carbon skeleton of tryptophan, were delineated. Various intermediates were isolated and characterized, and the enzymes responsible for each step were purified in collaboration with Nishizuka, Numa, Senoh, and others.

During the course of these studies, Nishizuka, Honjo, Ueda, and others in my laboratory were able to demonstrate that NAD, a well-known coenzyme of many dehydrogenases, can serve as an ADP-ribosyl donor and that in the presence of an appropriate enzyme and an acceptor protein, either mono ADP ribosylation of protein can be catalyzed or the ADP-ribosyl moiety can be covalently attached to acceptor proteins and then polymerized to poly ADP ribose. For example, in 1968, the ADP-ribosyl moiety of NAD was shown to be transferred to elongation factor 2. As a consequence, elongation factor 2 was inactivated and protein synthesis was inhibited. The reaction was catalyzed by diphtheria toxin, which explained the toxicity of this toxin. The first demonstration of this unique type of covalent modification of proteins had thus been made. Later studies in other laboratories have shown that other bacterial toxins such as pertussis toxin, botulinum toxin, cholera toxin, and others also catalyze a similar type of reaction (Hayaishi & Ueda, 1982).

Now I would like to turn to yet other aspects of tryptophan metabolism and describe two new enzymes that were discovered in my laboratory at Kyoto University during the period of 1967–1983. These two enzymes are indoleamine 2,3-dioxygenase, abbreviated as IDO, and tryptophan side-chain α, β -oxidase, or TSO. In 1937, Kottake and Ito reported that rabbits fed D-tryptophan excreted D-kynurenine in their urine. Because the liver enzyme tryptophan pyrrolase is specific for the L-isomer, these authors postulated the existence of another enzyme for cleavage of the pyrrole moiety of D-tryptophan. In 1963, we isolated, from rabbit intestine, a new enzyme that catalyzes such a reaction and purified it to apparent homogeneity. In contrast to TPO, which was found only

Fig. 1. Metabolic map of tryptophan and oxygenases. The pink arrows indicate oxygenase-catalyzed reactions. The pink oxygen atoms have been incorporated into these substrates each by specific oxygenases.

in the liver, this new type of tryptophan pyrrolase was found in many other tissues but not in the liver. The liver enzyme is specific for L-tryptophan, while the intestinal enzyme can act upon L- and D-tryptophan as well as on L- and D-5-hydroxytryptophan and several indoleamines. Therefore, this intestinal enzyme was referred to as indoleamine 2,3-dioxygenase. More interestingly, IDO was shown to require and utilize the superoxide anion, a univalently reduced molecular oxygen, in addition to molecular oxygen. The evidence for the participation of the superoxide anion in the IDO-catalyzed reaction is briefly summarized as follows. First, O_2^- is required and utilized by the enzyme. The O_2^- can be provided as the K salt of O_2^- , which is a stable salt of O_2^- , or by enzymic generation by the xanthine oxidase system. Second, scavengers of O_2^- , such as a superoxide dismutase, tyron, and so forth, inhibit the reaction. Third, the heavy oxygen isotope, $^{18}O_2$, is incorporated into the product of the reaction when the reaction is carried out in the presence of $K^{18}O_2$.

In 1979, in collaboration with Yoshida and coworkers, I was able to demonstrate that the IDO activity in mouse lung could be increased more than 100-fold over the basal level by virus infection such as that by influenza virus. When mice were exposed to influenza virus, the specific activity of IDO in the mouse lung started to increase after about a 5-day lag period and within about 10 days reached a peak; the activity then gradually decreased and returned to the normal level in about 3 weeks. This seemingly complicated observation was then studied in greater detail by Yoshida and coworkers; we were able to show that the IDO activity in human lung slices could be induced in vitro by γ -interferon. Recombinant glycosylated or nonglycosylated as well as natural γ -interferons were almost equally effective, whereas α and β interferons were hardly active. Further studies in my laboratory together with those in the laboratories of Professor Kido, Professor Pfefferkorn, and others revealed that when virus, bacteria, or parasites invade host tissues, interferons are generated and IDO activity is elevated. It is well known that O_2^- is generated by granulocytes under these conditions. As a consequence, tryptophan, an essential amino acid, is degraded and depleted by IDO and O_2^- ; and the growth of virus, bacteria, parasites, and tumor cells is thus inhibited. Our preliminary studies concerning IDO, the discovery of participation of O_2^- , and the possible

role of IDO in the inflammatory processes were first announced at the first ISTRY meeting held in Padova, Italy, in 1974, which was organized by the late Professor Musajo, Professor Allegri, and associates. I still remember with great pleasure Professor Musajo's kind remarks on our work on IDO and the hospitality of our Italian friends.

Now, I would like to discuss the other new enzyme, tryptophan side-chain α, β -oxidase or TSO, which was isolated and purified from *Pseudomonas* and which catalyzes a novel type of tryptophan degradative reaction. In collaboration with Takai and others, we showed that TSO catalyzes the oxidation of the side chain of tryptophan resulting in the formation of one equivalent each of 3-indole glycolaldehyde, ammonia, and CO_2 . TSO shows a broad substrate specificity and oxidizes not only L- and D-tryptophan but also a variety of indole derivatives such as indole ethanol, methanol, and so forth. Most interestingly, a number of peptides containing tryptophan are also found to serve as substrates for TSO. The product of the enzymatic oxidation by TSO of a tryptophan residue was identified to be α, β -didehydrotryptophan. Somatostatin, ACTH, glucagon, LHRF, and other peptides and proteins were subjected to such a modification; and TSO has proved to be a useful method for modification of tryptophan residues in peptide chemistry.

The above-mentioned results are only a brief summary of my work with tryptophan, which has been carried out with a large number of collaborators from Japan as well as from abroad, whose names are too numerous to mention here. Nonetheless, I express my deepest gratitude to all my collaborators, past and present, for their hard work, skillfully executed experiments, and above all for their warm friendship.

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