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TRYPTOPHAN, OXYGEN, AND SLEEP

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PROLOGUE

My father, Jitsuzo Hayaishi, was born in 1882 in Miyazu, a small town about 50 miles north of Kyoto where he practiced medicine for some years. He must have been a very courageous and ambitious person to go to America and start his medical training all over again. He took a boat to California, crossed an unimaginably enormous expanse of land by train, and ended his journey in Baltimore, where he was admitted to the University of Maryland School of Medicine. After he received his M.D. and had passed the National Board Examinations, he returned to California to practice medicine. Soon he met and married my mother, Mitsu Uchida, who was visiting her brother in San Francisco, and settled in Stockton, where I was born in 1920. A

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year later my father decided to go to Berlin to study immunology for two years at the Robert Koch Institute. We returned to Japan in 1923, settling in Osaka, where my father spent the rest of his life practicing medicine. A conscientious doctor, devoted to clinical practice, he was always respected and trusted by his patients. His only hobby was reading the new medical journals and books. Perhaps his sincere attitude toward medical science and the scholarly atmosphere at home were what led me to a career in medicine and basic research.

I graduated from the Osaka University School of Medicine in 1942 during World War II, and served as a medical officer in the Japanese Navy for three years. With the war's end in 1945, I returned to Osaka to find that the city had been almost totally demolished. The home of my parents and family had completely vanished. Food was scarce, and supplies of most commodities, gas, electricity, and even water, were extremely limited. I then thought of joining my parents in their hometown, Miyazu, and helping my father in his medical practice, as he was then already 64 years old. But my father had decided to return to Osaka to open a small hospital and to look after patients during this time of food shortages and very poor hygienic conditions. Inspired by his courage and foresight, I decided to remain in Osaka. Had I chosen the practice of medicine, as had many of my classmates, I would have been economically very comfortable even in those difficult times.

Instead, perhaps because I inherited my father's adventurous spirit, I was rash enough to undertake laboratory work and joined the Department of Bacteriology, located in the Institute of Microbial Diseases, at the Osaka University School of Medicine. I was very fortunate to be surrounded by many excellent and ambitious young colleagues, including Teishiro Seki, Masami Suda, and Tsunehisa Amano. Despite the disastrous events during and after World War II, everyone in the department was eager to read the new journals and catch up with the latest developments in the United States and Europe. Morale was high and people were enthusiastic. Reading *Bacterial Metabolism* by Marjory Stephenson and *Dynamic Aspects of Biochemistry* by Ernest Baldwin impressed and inspired me and aroused my interest in biochemistry.

TRYST WITH TRYPTOPHAN

Osaka University was a center of biochemical research in Japan, and Yashiro Kotake and his associates were well known for their research on tryptophan metabolism. Kotake was responsible for the isolation and characterization of kynurenine, the key intermediate of tryptophan metabolism in both mammals and microorganisms. He kindly provided us with several grams

of tryptophan, kynurenine, and other related chemicals, and encouraged us to use these valuable compounds for our experiments. At that time, research funds were extremely limited, facilities were poor, chemicals were scarce, and there were no laboratory animals. To overcome some of these difficulties, I decided to use microorganisms. So I went to the backyard of the Institute, took some soil samples, mixed them with tryptophan and water in a test tube, and waited. It was a very simple experiment, but after a few days, I observed some cloudiness in the supernatant. Thus, by this enrichment culture technique, I was able to isolate several strains of soil bacteria that could grow on tryptophan as their sole source of carbon and nitrogen.

It was already known then, mainly through the efforts of Kotake and his associates, that in mammals tryptophan was degraded through kynurenine to kynurenic acid, anthranilic acid, and xanthurenic acid as the end metabolites. However, in my soil isolates, I was able to demonstrate in collaboration with Masami Suda, Yoshiharu Oda, and others that in microorganisms anthranilic acid was further metabolized to catechol, then to muconic acid, and ultimately oxidized to CO_2, ammonia, and H_2O (Figure 1). 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 (1). It appeared to me that this was the first clear-cut demonstration of the enzymatic cleavage of the benzene ring, namely, the enzymatic conversion of an aromatic into an aliphatic compound. This enzyme was a nonheme iron protein with a molecular weight of approximately 90,000. 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 (Figure 2). I chose this name because several catechol oxidases had already been reported at that time, but these enzymes seemed to be grossly different from my pyrocatechase. They did not cleave the aromatic structure of catechol and they were copper proteins rather than nonheme iron proteins. The catalytic mechanism of pyrocatechase remained unclarified for some

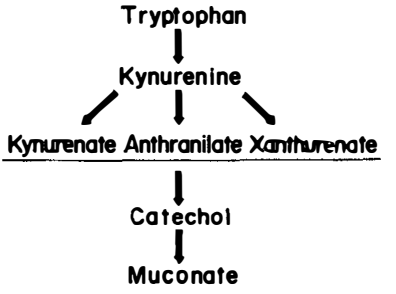

Figure 1 Major metabolic pathways of tryptophan(~1950)

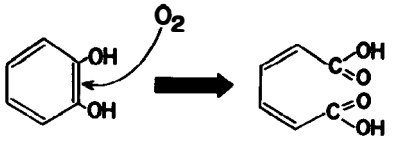

Figure 2 Pyrocatechase-catalyzed reaction

years. In fact, in the *Enzymologie* textbook edited by Otto Hoffman-Ostenhof and published in 1954, pyrocatechase was described as "not yet classified" in the category of oxidoreductases (2).

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 might be surmised by Lavoisier's principles of biological oxidation, formulated in the 18th century. He postulated the addition of oxygen atoms to the substrate in oxidations and the removal of oxygen from the oxide in reductions. I was startled to learn, however, that at the start of the present century, the enzymatic incorporation of molecular oxygen into substrates had been ruled out by Heinrich Wieland, the 1927 Nobel laureate in chemistry. In 1932, he wrote a book *On the Mechanism of Oxidation,* which was then considered the "bible" of biological oxidation (3).

In his famous dehydrogenation theory, he excluded direct addition of the oxygen molecule to the substrate in a biological oxidation system. According to this theory, biological oxidation processes entail the transfer of hydrogen atoms, or their equivalent, from the substrate molecule to an appropriate acceptor, such as a coenzyme or one of various dyes. Oxygen molecules may, in some instances, serve as immediate electron acceptors, and enzymes that catalyze such reactions are termed "oxidases." Thus, according to Wieland, molecular oxygen accepts hydrogen atoms and is reduced to water or hydrogen peroxide. However, it is never incorporated into the substrate, as had been proposed by Lavoisier and his contemporaries some 200 years earlier. According to Wieland, when the overall reaction appears to be the addition of oxygen, the oxygen atoms are always derived from a water molecule rather than molecular (or atmospheric) oxygen. For example, when aldehydes are converted to acids, they are hydrated first and then dehydrogenated. Whereas the sum appeared to be the addition of oxygen to the substrate aldehyde to form acid, this oxygen atom, it was postulated, is really derived from water rather than from molecular oxygen.

Wieland thought the direct addition of molecular oxygen to a substrate to be 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. Furthermore, orthobenzoquinone, the dehydrogenated product of catechol, did not serve as an intermediate in the overall reaction. Unfortunately, it was not possible to carry out experiments to prove my hypothesis, because the facilities were not available then in Japan. About this time, I married Takiko Satani in 1946, and we welcomed the arrival of our daughter Mariko in 1947.

PILGRIMAGE TO THE UNITED STATES

One day early in the summer of 1949, I received a letter from David E. Green inviting me to join his group as a postdoctoral fellow. I knew that Green had spent may years in Cambridge, England and had just returned to the United States to become Director of the Enzyme Institute at the University of Wisconsin in Madison. I was delighted, but hesitated to accept the offer, because Japan was still an occupied country and I feared that hostility against the Japanese might still exist among the American people. My starting salary as an instructor at Osaka University was 60 yen a month (approximately 17 US cents, not dollars!), whereas the William Waterman Fellowship in Enzyme Chemistry that Green had arranged for me was $250 a month, a 1500-fold difference! Furthermore, research facilities and working conditions were presumably much better in the United States. After pondering over Green's letter for some time and discussing this matter with my father and friends, I finally accepted the offer.

Shortly thereafter, I received an airplane ticket from Green and boarded a luxurious double-decker, four-engine Boeing B-377 from Tokyo airport. I felt as if I was going up to heaven from hell. It took almost 36 hours to fly from Tokyo to San Francisco, with three stops for refueling. I changed planes in San Francisco, then Chicago, and finally arrived at the Madison airport. The time was mid-November, 1949. With a heavy snowfall in progress, awaiting me were two young Americans, Bernard Katchman and Ephraim Kaplan. I felt extremely fortunate to have the opportunity to become good friends with Bernie and Eph, the kindest and most considerate human beings I had ever met. In spite of the language barrier and the ethnic and religious difference, they treated me like a brother, teaching me things in the lab and instructing me in the matters of daily life. Without their help, I would almost certainly have been lonesome, desperate, and homesick.

Soon I was able to adjust to and enjoy the American way of life in this beautiful university town and made many friends, among them Philip and Muriel Feigelson, Henry Lardy, Philip Cohen, and Takeru Higuchi. Yet I was uneasy because my experiments were contradicting Green's cyclophorase hypothesis. At the Federation meetings in April of 1950, I heard a talk by Arthur Kornberg and was greatly impressed by his presentation.

It was not a long lecture, only 10 minutes, but I felt as if I were hit by a bolt of lightening. I wanted very much to work under his guidance. Arthur kindly suggested that I apply for an National Institutes of Health (NIH) fellowship, but I could not wait for the final decision and left Madison in September to spend four months with Roger Stanier at the University of California at Berkeley. There again I was very fortunate to meet a number of outstanding scientists, including C. B. van Niel, H. A. Barker, Michael Doudoroff, and William Hassid, all helpful, kind, and welcoming. During my stay in Roger's laboratory, we both worked very hard and were able to publish six papers on tryptophan metabolism in bacteria, which appeared in the *Journal of Biological Chemistry, Journal of Bacteriology,* and *Science* (4–9). It was undoubtedly one of the most productive periods of my life.

In December 1950, my wife and daughter joined me and we moved from California to Bethesda, Maryland to join Arthur's group at the National Institute of Arthritis and Metabolic Diseases. Again I was very lucky, since this was the beginning of the so-called "Golden Age" at the NIH. In the Enzyme Section, which was headed by Arthur, a number of young active scientists were working very hard, including Bernard Horecker and Leon Heppel. Every day, we had a luncheon seminar, which was also attended by Herbert Tabor and Alan Mehler. Sometimes Earl and Thressa Stadtman, Chris Anfinsen, Alton Meister, Sidney Udenfriend, Bernhard Witkop, and other outstanding scientists joined us. It was a stimulating and friendly atmosphere, and because of my friendship with Arthur, I felt confident and comfortable.

For two years at the NIH, I worked with Arthur on bacterial degradation of uracil and characterized the intermediate products, malonyl CoA and acetyl CoA (10, 11). Feodor Lynen came to visit me at the NIH on several occasions, because of his interest in acetyl CoA. This was the beginning of a long association with Fitzy, not only scientifically rewarding, but also cemented by a most heart-warming friendship.

About two years after my arrival in Bethesda, Arthur decided to move to St. Louis to assume the chairmanship of the Department of Microbiology at the Washington University School of Medicine. He asked me to come with him as an assistant professor, his first academic appointment. Again I hesitated somewhat. As it was only my third year in the United States, it seemed an almost overwhelming task for me to teach medical students in English and at the same time help Arthur renovate the department and reorganize the teaching schedule. After a long talk with Arthur, I finally accepted the offer and moved to St. Louis in late 1952.

I spent the summer of 1954 as a visiting scientist at the NIH, collaborating with Herbert Tabor, who had isolated a metabolite of histamine from rat urine but was having some difficulty with its identification. From the

elementary analysis, it appeared to be a conjugate of ribose and imidazoleacetic acid, yet it resisted acid and alkaline hydrolysis. From Herman Kalckar I obtained a partially purified riboside hydrolase of *Lactobacillus*. As predicted, the compound Herb had isolated from urine was unequivocally identified as the riboside of imidazoleacetic acid (12, 13). With an offer to be Chief of the Section on Toxicology in the Laboratory of Pharmacology and Toxicology at the National Institute of Arthritis and Metabolic Diseases, I decided to accept and return to Bethesda in late 1954.

DISCOVERY OF OXYGENASE

In reorganizing the research program of the Section on Toxicology, I immediately thought of pyrocatechase, my first love. I decided to explore the possibility that pyrocatechase could incorporate isotopically labeled molecular oxygen into catechol. When I spoke about this idea to several friends at the NIH, almost everyone was skeptical. After all, dehydrogenation was the dominant theory in the field of biological oxidation. The hottest topic in this field at that time was oxidative phosphorylation, which had been studied by many big names, including Severo Ochoa, Ephraim Racker, William Slater, Britton Chance, and Albert Lehninger.

Nevertheless, I was determined to carry out this crucial experiment. Again I was lucky, because I was able to collaborate with Simon Rothberg, a skillful mass spectroscopist at the NIH, and Masayuki Katagiri, an excellent enzymologist from Japan. I used the heavy isotope, ^{18}O, present either as molecular oxygen or as water. Since $H_2{}^{18}O$ was not commercially available in the United States at that time, I had to write to David Samuel at the Weizmann Institute in Israel, who kindly supplied me with a highly concentrated preparation of $H_2{}^{18}O$. The product, muconic acid, was isolated and analyzed for its ^{18}O content by mass spectrometry. Were pyrocatechase a dehydrogenase or an oxidase, the incorporated oxygen should, according to Wieland's theory, have been derived from water. Contrary to that theory and the generally held belief, our results clearly demonstrated that the incorporated oxygen was derived exclusively from molecular oxygen.

Despite our clear-cut results, our data were at first brushed off by our peers and greeted with skepticism. As I was completing a manuscript and about to mail it to the *Journal of Biological Chemistry,* Alan Mehler told me that Howard Mason and his associates in Oregon had just demonstrated that a mushroom phenolase complex could catalyze the incorporation of one atom of molecular oxygen into 3,4-dimethylphenol to form 4,5-dimethylcatechol. Mason's paper (14), along with my communication to the *Journal of the American Chemical Society* (15), appeared in 1955 as "Letters to the Editor." These findings, along with subsequent work by Konrad Bloch and

others, established that "oxygen fixation" did indeed occur in biological systems, and the concept of "biological oxygenation" was introduced. In essence, we had revived the old concept of Lavoisier, and I proposed that we call this new type of oxidative enzyme an "oxygenase." In principle oxygenases can be classified into two categories: dioxygenases—which incorporate two atoms of molecular oxygen per substrate molecule, as exemplified by pyrocatechase—and monooxygenases. The latter incorporate one atom of oxygen per substrate molecule, the other atom being reduced to water at the expense of a reducing equivalent such as NADH or NADPH; tryptophan 5-monooxygenase, which produces 5-hydroxytryptophan from tryptophan, is a typical example.

In 1956, I was asked to organize the first symposium on oxygenases at the American Chemical Society meeting in Atlantic City, and in this capacity I invited Mason, Bloch, Mika Hayano, and others. By then, biological oxygenation was generally accepted, and the new field of oxygenases had emerged. In 1957, two major things happened to me. One was a visit from Hoffman-Ostenhof from Vienna, who was planning the International Congress of Biochemistry in that city in 1958. Recognizing the chemical and physiological significance of oxygenases, he asked me to organize and chair a colloquium at the Congress. The other remarkable event was a visit from Kikuo Ogiu, Chairman of the Department of Pharmacology of the Kyoto University Faculty of Medicine. He conveyed an invitation from Ko Hirasawa, Dean of the Faculty of Medicine, to come to Kyoto to be the Chairman and Professor of the Department of Medical Chemistry. This department of biochemistry, the second oldest in Japan, had been founded in 1899 by Torasaburo Araki, the last student of Felix Hoppe-Seyler. It was considered the most presitigious center for biochemistry in Japan. Such an appointment, to a 37-year-old graduate of Osaka University, was unprecedented in Japanese academic circles, and I felt very much honored and flattered. It was the most crucial decision I was called on to make in my scientific career, and I finally decided to return to Japan.

When I was about to leave Bethesda, I met Jesse Greenstein of the National Cancer Institute, one of the pioneers in the biochemistry of cancer. Although he knew me only casually, he urged me to apply for an NIH research grant and kindly volunteered to write a strong recommendation letter. Encouraged by his suggestion, I applied and was fortunate to receive substantial NIH support for a number of years after my return to Japan. This funding, together with other grants from the Jane Coffin Child's Memorial Fund, the Rockefeller Foundation, and various Japanese government agencies, especially the Ministry of Education, Science and Culture, enabled me to rebuild and refurnish the old department at Kyoto University and to continue my work with many young scientists.

KYOTO UNIVERSITY, MY SECOND ALMA MATER

When I moved to Kyoto in February 1958, the first graduate student who applied to join me was Yasutomi Nishizuka, in later years famous for his work on protein kinase C. Soon the Department of Medical Chemistry was deluged with applicants from all over Japan. These postdoctoral fellows and graduate students were all highly motivated, bright, and hard working. The most important thing I had to teach them was to be creative researchers rather than encyclopedic scholars. Although my salary as the Chairman and Professor of one of the most prestigious departments in the medical school in Japan was about one thirteenth (7.7%) of what I earned at the NIH, the work was rewarding and spirits were high.

In the summer of that year, I attended the Fourth International Congress of Biochemistry in Vienna and chaired the Colloquium on Oxygenases. After the Congress, I spent a month touring Europe with Masami Suda, visiting many biochemistry laboratories in England, Germany, France, Denmark, Sweden, Italy, and Switzerland, meeting many scientists whom I had known only from the literature and correspondence. When I visited the Karolinska Institute in Stockholm, Sune Bergström, who had recently returned from Lund to chair the Department of Biochemistry, greeted me warmly and showed me around the Institute. He told me about his pioneering work on prostaglandins, which revealed their versatile and fascinating biological functions, and suggested the involvement of oxygenase reactions in their biosynthesis. This was my first exposure to prostaglandins and the start of a long association with Bergström, Bengt Samuelsson, Sten Orrenius, Peter Reichard, and their colleagues at the Karolinska Institute, and Lars Ernster of Stockholm University. I was also greeted very warmly by Otto Warburg in Berlin, who was happy to hear that tryptophan pyrrolase was a hemoprotein dioxygenase and to discuss the nature of so-called "active oxygen."

Tryptophan pyrrolase cleaves the indole ring of tryptophan, and was shown in 1957 to incorporate two atoms of molecular oxygen into the tryptophan substrate to form formylkynurenine (Figure 3). To reflect this finding, it was renamed tryptophan 2,3-dioxygenase. This enzyme is a hemoprotein, and we later demonstrated that the enzyme-substrate-oxygen complex is the obligatory intermediate in the reaction. Various forms of chemically active oxygen have been reported, including singlet oxygen, superoxide, and hydroxyl radicals, but these do not seem to be involved as dissociable intermediates per se 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 resides

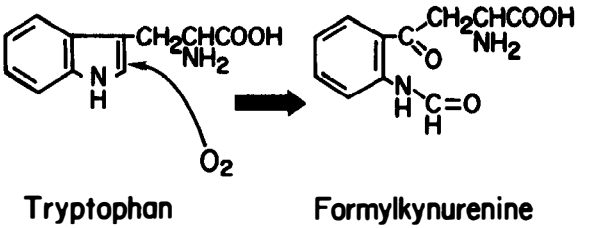

Figure 3 Tryptophan pyrrolase (2,3-dioxygenase)

in the ternary complex of enzyme heme:oxygen:substrate. The session was chaired by John T. Edsall, who, I recall, made very warm and encouraging remarks at the end of my presentation. This prediction was amply substantiated by the subsequent work of Yuzuru Ishimura and others in my laboratory with tryptophan dioxygenase and later by Fusao Hirata with indoleamine dioxygenase. Furthermore, such a ternary complex was later demonstrated to be an intermediate in the P450-catalyzed monooxygenase reaction studied by Ron Estabrook and associates and also by Irwin Gunsalus and coworkers.

At first, these oxygenase reactions were generally thought to be rather unusual and limited to primitive organisms, such as soil bacteria and mushrooms. It took several years before the universal existence of oxygenases was confirmed in many laboratories (16, 17). During the next decade, a number of oxygenases were isolated from animals, plants, and microorganisms (18), and some of them were crystallized by Mitsuhiro Nozaki, Hitoshi Fujisawa, Shozo Yamamoto, and others in my laboratory (19). These studies elucidated numerous metabolic pathways of physiological importance and demonstrated the ubiquitous presence of oxygenase reactions. For example, the two most important metabolic pathways for tryptophan in mammals, one leading to formation of pyridine nucleotide coenzymes and poly(ADP-ribose), and the other to formation of various indoleamines, are initiated by two well-known oxygenases, tryptophan 2,3-dioxygenase and tryptophan 5-monooxygenase, respectively.

In the 1940s, Roger Stanier isolated several strains of soil microorganisms that metabolized tryptophan via kynurenic acid. When I was in his laboratory in 1950, the pathway through anthranilate and catechol was referred to as the "aromatic pathway," while the pathway through kynurenic acid was referred to as the "quinolinic pathway." After returning to Kyoto in 1958, I carried out detailed studies on both pathways in collaboration with many researchers, including Shigeru Kuno, Yasutomi Nishizuka, Shosaku Numa, Arata Ichiyama, Hitoshi Fujisawa, Siroh Senoh, Yutaka Kojima, and others.

A large number of intermediates and enzymes responsible for the steps in these pathways were isolated and characterized.

We then proceeded to investigate the metabolic pathways of tryptophan in mammalian liver. Two pathways were delineated, one leading to the biosynthesis of NAD, and the other, the glutarate pathway, leading to the complete combustion of the carbon skeleton of tryptophan. Again, intermediates were isolated and characterized, and the enzymes responsible for each step were purified in collaboration with Nishizuka, Numa, Senoh, Ichiyama, Atsushi and Teruko Nakazawa, Hiroshi Okamoto, Takashi Murachi, and others.

DISCOVERY OF POLY- AND MONO(ADP-RIBOSYL)ATION

During the course of these studies, we were able, in collaboration with Nishizuka, Tasuku Honjo and Kunihiro Ueda, and subsequently with Hiroto Okayama, Yutaka Shizuta, and Ronald Reeder, to demonstrate that NAD, the well-known coenzyme of many dehydrogenases, can serve as an ADP-ribosyl donor in a unique type of covalent modification of proteins. In the presence of the appropriate enzyme, either a single ADP-ribosyl moiety can be covalently attached to an acceptor protein, or in some instances, this moiety can be 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, Factor 2 was inactivated and protein synthesis was inhibited. The reaction, catalyzed by diphtheria toxin, accounts for its toxicity. The first demonstration of this unique covalent modification of proteins had thus been made (20). Later studies in other laboratories have shown that other bacterial toxins, such as pertussis, botulinum, and cholera, catalyze a similar reaction. The biosynthesis and the structure of poly(ADP-ribose) were investigated independently in three laboratories: Paul Mandel's laboratory in Strassbourg, Takashi Sugimura's laboratory in Tokyo, and ours in Kyoto (21). It has been almost 30 years since the discovery of this unique and interesting polymer in the nucleus of mammalian cells, but its true physiological function remains unknown; several lines of circumstantial evidence indicate its involvement in DNA repair, in development, and in carcinogenesis (22).

TRYPTOPHAN REVISITED

With regard to other aspects of tryptophan metabolism, we discovered and studied two new enzymes during the period from 1967 to 1983: indoleamine 2,3-dioxygenase (IDO), and tryptophan side-chain α,β-oxidase (TSO). In

1937, Kotake and Noboru Ito had reported that rabbits fed D-tryptophan excreted D-kynurenine in their urine. In as much as liver tryptophan pyrrolase (TPO) is specific for the L-isomer, these authors postulated the existence of another enzyme for cleavage of the pyrrole moiety of D-tryprophan. In 1963, Kiyoshi Higuchi, Kuno, and I isolated and purified a new enzyme from rabbit intestine that catalyzed such a reaction. In contrast to TPO, which was found only in liver, this new type of TPO was found in many tissues other than the liver. The liver enzyme is specific for L-tryptophan, whereas the intestinal enzyme acts on both L- and D-tryptophan, as well as on L- and D-5-hydroxytryptophan and several indoleamines. The intestinal enzyme has been therefore referred to as indoleamine 2,3-dioxygenase.

Interestingly, IDO was shown to require and utilize the superoxide anion, O_2^-, a univalently reduced form of oxygen, in addition to molecular oxygen. The evidence for participation of the superoxide anion in the IDO-catalyzed reaction can be 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^- or by enzymatic generation of O_2^- by the xanthine oxidase system. Secondly, scavengers of O_2^-, such as superoxide dismutase or tyron, inhibit the reaction. Finally, the heavy oxygen isotope, ^{18}O, is incorporated into the product of the reaction when carried out in the presence of $K_2{}^{18}O$.

In 1979, in collaboration with Ryotaro Yoshida, Yoshihiro Urade, and coworkers, we demonstrated that IDO activity in mouse lung increased more than 100-fold over the basal level in response to infection with a virus such as influenza (23). When mice were exposed to the virus, the specific activity of IDO in mouse lung started to increase after a 5-day lag period and peaked within 10 days; the activity then gradually decreased and returned to normal levels in about 3 weeks. When this complex response was studied in greater detail by Yoshida and coworkers, we could show that IDO activity in human lung slices was induced by γ-interferon, while α- and β-interferons were hardly active. Further studies in my laboratory, together with those in the laboratories of Ryo Kido, Elmer Pfefferkorn, and others, revealed that when virus, bacteria, or parasites invade host tissues, interferons are generated and IDO activity is elevated. It is also well known that O_2^- is generated by granulocytes under these conditions. As a consequence, tryptophan, an essential amino acid, is degraded by IDO and O_2^-. and its depletion inhibits the growth of viruses, bacteria, parasites, and tumor cells.

Our preliminary studies with IDO, the participation of O_2^-, and the possible role of IDO in inflammatory processes were first announced at the First International Study Group for Tryptophan Research (ISTRY) meeting in Padova, Italy in 1974, organized by the late Luigi Musajo, Graziella Allegri, and their associates. Presumably, in recognition of this effort, I received in

1988 the degree of Honoris Causa from the University of Padova, the second oldest university in Europe.

The other new enzyme, tryptophan side-chain α,β-oxidase (TSO), isolated and purified from *Pseudomonas,* catalyzes a novel tryptophan degradation (24). In collaboration with Katsuji Takai, Shu Narumiya, Flora Zavala, and others, we showed that TSO catalyzes the oxidation of the side chain of tryptophan, forming one equivalent each of 3-indoleglycolaldehyde, ammonia, and CO_2. TSO shows a broad substrate specifity and oxidizes not only L- and D-tryptophan, but also a variety of indole deriatives, such as indole ethanol and methanol. Interestingly, a number of peptides containing tryptophan also serve as substrates for TSO. The product of enzymatic oxidation by TSO of a tryptophan residue was identified to be α,β-didehydrotryptophan. Because many peptides (e.g. somatostatin, ACTH, glucagon, LHRF) and proteins are substrates for such a modification, TSO has proved to be a useful method for modification of tryptophan residues in peptide chemistry.

ACTIVE RETIREMENT

In 1983, I retired from Kyoto University, as is mandatory at the age of 63. During my 25-year tenure, the second Department of Medical Chemistry was created in 1968, and Shosaku Numa was installed to head this department. He had been a protégé of Feodor Lynen, who came often to Japan and visited us in Kyoto. Being the son-in-law of Wieland, he was always appreciative of my discovery and subsequent work on oxygenases. In 1976, at the Tenth International Congress of Biochemistry held in Hamburg, I presided over the General Assembly of the International Union of Biochemistry (IUB), and recommended that Lynen become the President-Elect. Shockingly, he succumbed soon thereafter to an aortic aneurysm. A colorful, kind, and gregarious person, an exemplar of Bavarian "Gemütlichkeit," he earned the respect and affection of everyone he met. On the occasion of a visit to his laboratory in Münich, I had met Harland Wood, who was spending a sabbatical leave there, and started another long and wonderful friendship, which was tragically lost with Harland's death in 1992.

During my time at Kyoto University, I had been responsible for the guidance of more than 500 staff members, postdoctoral fellows, graduate students, and visiting scientists from all over Japan and numerous foreign countries. Furthermore, concurrent with my appointment at Kyoto University, I served as Professor at the Department of Biochemistry of Osaka University School of Medicine (1961–1963), and also as Professor at the Department of Physiological Chemistry and Nutrition, Faculty of Medicine of the University of Tokyo (1970–1974). Through these appointments, I

had the pleasure of meeting many outstanding scientists and greatly enjoyed the association with a large number of able young students in both universities.

Upon the occasion of my retirement, David E. Green sent me this greeting.

> The fortunate ones are thrice blessed. The first blessing is to have a profession that can lead to organized discoveries. The second blessing is to share these discoveries with many colleagues. The third blessing is to make a discovery that improves the quality of life.
>
> Most scientists have the first blessing. A few come close to the second blessing. A rare few, who are among our greatest scientists, receive all three blessings. Osamu Hayaishi is among the handful in the world that have reached these three great honors. We all honor him for this triumph of triumphs.

I doubt that I deserved then or now such august compliments, but I do consider myself extremely fortunate to have lived and participated in a most exciting era of biochemistry and to have shared the excitement with so many friends and with young, able collaborators from all over the world.

In 1983, I was appointed President of the Osaka Medical College at Takatsuki City near Osaka. At about the same time, the Research Development Corporation of Japan (JRDC), a subsidiary of the Science and Technology Agency of the Japanese government, appointed me to be leader of the Hayaishi Bioinformation Transfer Project. This project, part of Exploratory Research for Advanced Technology (ERATO), was initiated by the Japanese government in 1981 to foster the creation of advanced technologies and promote future interdisciplinary scientific activities. With the generous funding from ERATO, I was able to organize a team of some 30 scientists and to launch a new project investigating the metabolism and functions of prostaglandins and related compounds in the central nervous system of mammals. With this I found an unexpected opportunity for new directions in my research career.

SABBATICAL LEAVES

Although most Japanese universities lack a sabbatical system, professors are usually allowed some leave provided that the teaching and administrative responsibilities are assumed by others in the department. During my tenure at Kyoto University, I spent six months in the Laboratory of Clinical Biochemistry at the National Heart Institute with Sidney Udenfriend in 1962. While in Udenfriend's laboratory, I was able to associate with many excellent scientists, including Julius Axelrod, Bernhard Witkop, and Herbert Weissbach. Herb and I discovered an ADP-stimulated threonine deaminase from *Clostridium tetanomorphum,* one of the first examples of a positive feedback regulation. On my way home to Japan, I visited Jacques Monod and Francois

Jacob at the Pasteur Institute in Paris. When they heard my seminar, they both exclaimed "allostery," the term that they had just coined for the negative feedback phenomenon.

In 1968, the late Sidney Colowick and Victor Najjar invited me to spend three months as a visiting professor in the microbiology department at Vanderbilt University School of Medicine in Nashville, supported by a special grant from the NIH. At the same time, similar appointments were held by Stanford Moore in the biochemistry department and by Paul Greengard in the pharmacology department. Greengard, Colowick, and I were able to show the reversibility of the adenylate cyclase reaction utilizing the crystalline enzyme preparation that Masaharu Hirata and I had purified from *Brevibacterium* in Kyoto. The results clearly indicated the "high-energy" bond nature of the cyclic phosphodiester linkage (25). During that three-month stay in the "southern capital," we made many new friends—Earl Sutherland, Stanley Cohen, Oscar Touster, Rollo and Jane Park, Leon Cunningham, Tadashi Inagami, and Michio Ui—and thoroughly enjoyed their hospitality. It was also a wonderful opportunity to learn about the southern states, which, according to Colowick, is the best way to become acquainted with and appreciate US history and tradition.

In 1973, when the Ninth International Congress of Biochemistry was held in Stockholm, I was nominated President of the IUB. Partly because I have always considered myself a bench biochemist and ignorant of international affairs and partly because of the language problem, I was hesitant at first to undertake the appointment. I was eventually persuaded by Harland Wood, who telephoned me several times at the NIH during my stays there as a Fogarty Scholar (1972–1974). It was during this period in Bethesda, where Takiko and I spent two to three months in each of three summers, that I would write or edit books, organize meetings, and do some collaborative work with scientists both inside and outside the NIH. This was a wonderful way to recharge batteries, learn new things, and make many new friends.

As the successor to Hugo Theorell to preside over the IUB, I worked hard to make the organization more prosperous, active, and meaningful to every biochemist in the world. I was very fortunate to have had many excellent helpers and advisors, above all, Harland Wood, William Whelan, the hard-working and excellent general secretary, and Kunio Yagi. I felt very much honored that the IUB decided to create an Osamu Hayaishi lectureship supported by an endowment from Keizo Saji, President of the Suntory Company, a long-time friend. The first lecture of this series was presented by Jeremy Knowles at the Twelfth International Congress of Biochemistry in Perth in 1982.

In 1987, the Osaka Bioscience Institute was created by the City of Osaka in commemoration of its centennial anniversary, and I was appointed as the

director of this new institute, where I have been able to continue my work on prostaglandins in the central nervous system (CNS).

THE MYSTERY OF SLEEP

Prostaglandin (PG) was discovered in semen by Raphael Kurzrok and Charles Lieb (with the technical assistance of Sarah Ratner!) in 1930, but the unique structure of this family of compounds was not elucidated until the early 1960s by Bergström and coworkers. During the next 30 years, research in the prostaglandin field progressed exponentially. Various PGs were shown to be ubiquitously distributed throughout almost all types of cells in mammalian organs and tissues and to play vital roles in the regulation of a variety of physiological functions and pathological conditions, such as contraction and relaxation of smooth muscles, aggregation and disaggregation of blood platelets, inflammation, and pain. In 1982, the Nobel Prize in Medicine or Physiology was awarded to three pioneers in this area: Bergström, Samuelsson, and John Vane. However, the abundance, metabolism, and function of PGs in the CNS was poorly understood. To quote from the *Biochemical Basis of Neuropharmacology,* "The mammalian CNS contains predominantly the PGF series, with small amounts of a PGE in some cases. None of the available evidence indicates that prostaglandins act as typical CNS neurotransmitters" (26).

In the late 1970s, several groups, including my own, reported the presence of a relatively large amount of PGD_2 in the brains of rats and other mammals, including humans. Until that time, PGD_2 had long been considered a minor and biologically inactive prostanoid or perhaps even as a non-enzymatic decomposition product of PGH_2. However, these publications indicated that PGD_2 is unique among the PGs in being present in relatively high concentrations in the mammalian brain. Subsequently, Takao Shimizu, Kikuko Watanabe, Urade, and others found that PGD_2 is actively synthesized and metabolized by specific enzymes in both neurons and glial cells.

To determine the neural functions of PGD_2, Yasuyoshi and Yumiko Watanabe studied the intracerebral distribution of its binding protein, the putative receptor, by autoradiography with [^{3}H]PGD_2 combined with computer-assisted image processing and color coding. The binding protein for PGD_2 was located mainly in the gray matter, namely the neuron-rich areas. It was highly concentrated in specific regions, such as the olfactory bulb, cingulate cortex, occipital cortex, hippocampus, hypothalamus, and preoptic area, indicative of PGD_2 involvement in certain specific neural functions.

The preoptic area has long been proposed as a center of sleep and temperature regulation on the basis of neuroanatomical and electrophysiological experiments. However, the biochemical mechanisms involved in the

induction of sleep had never been elucidated. While studying the effect of PGD_2 on brain temperature, Ryuji Ueno and I, in collaboration with the late Teruo Nakayama and Youzou Ishikawa of Osaka University, happened to observe that when saline was injected into the preoptic area of control rats under conditions of partial sleep deprivation, the rats remained awake most of the time. However, when a few nanomols of PGD_2 were micro-injected into the preoptic areas, the wakefulness period decreased almost 50% and the amount of sleep increased more than fivefold. The site of action was confined to the preeoptic area, whereas a PGD_2 injection into other areas (e.g. posterior hypothalamus, cerebral cortex, locus ceruleus, thalamus) had no significant effect. The response was dose-dependent and specific for PGD_2, indicating that PGD_2 may be a natural or physiological sleep-promoting substance in the rat brain (27).

Sleep is one of the most important and yet least understood biological phenomena of the brain. Although we spend almost one-third of our lifetime sleeping and repeat the sleep-wake cycle every day and night, the biochemical mechanism of sleep-wake regulation has thus far eluded us. During the past several decades, an intensive search for endogenous sleep-regulating substances has been carried out in laboratories throughout the world, but the results have not been convincing. Having decided to focus our research on the mystery of sleep, I consulted Yasuji Katsuki, who kindly introduced me to Shojiro Inoué of the Tokyo Medical and Dental University. With his help, we employed the more sophisticated continuous infusion sleep bioassay system, originally developed by Inoué and Kazuki Honda. Eventually we could show that PGD_2 and E_2 are probably two of the major endogenous sleep-regulating substances. PGD_2 induces sleep and PGE_2 promotes wakefulness in the rat, dog, and monkey, and likely in humans as well. The site of PGD_2 action appears to be localized to the sleep center in or near the preoptic area. PGE_2 action is on the wake center in or near the posterior hypothalamus. The REM sleep center in the brain stem also appears to be under the control of PGD_2 and E_2, although further experiments are required to pinpoint the exact sites of their actions (28, 29).

Recently, we discovered that inorganic quadrivalent selenium compounds are potent, specific, and noncompetitive inhibitors of brain prostaglandin D synthase, with a K_i value of about 10 μM. In contrast, hexavalent compounds and organic selenium compounds are not inhibitory at all. Other enzymes in the arachidonate cascade system are not inhibited. This inhibition can be reversed by sulfhydryl compounds, such as glutathione or dithiothreitol (DTT). When selenium chloride was slowly infused into the third ventricle of a rat, sleep was inhibited in a time- and dose-dependent manner. After about two hours, both slow-wave sleep and REM sleep were almost fully inhibited and

the rat was completely and continuously awake (30). The effect was reversible: When the infusion was interrupted, sleep was restored. Furthermore, the effect was reversed by sulfhydryl compounds such as DTT and reduced glutathione, as in the case of in vitro experiments. These results clearly show that PGD_2 is involved in the regulation of physiological sleep and that PGD synthase is the key enzyme in control of natural sleep.

In 1990, when I presented a lecture on our work on sleep at the Royal Society in London, I met Victor Pentreath of the University of Salford, United Kingdom, who told me that the sleep seen in African sleeping sickness patients is probably caused by PGD_2. He and colleagues determined the amount of PGD_2 and PGE_2 and also $IL_1\alpha$, another putative sleep substance in the cerebrospinal fluid (CSF) in sleeping sickness patients. The amount of PGD_2 was specifically, significantly, and progressively elevated in the CSF, indicating that sleep in the terminal stage of these patients is caused mainly, if not exclusively, by PGD_2 (31). Roberts and coworkers reported that patients with systemic mastocytosis enter into deep sleep after the episodic production of large amounts of PGD_2 by their mast cells (32). All told, these results indicate that PGD_2 induces sleep in humans under certain pathological conditions.

As regards the waking effect of PGE_2, in order to find out if PGE_2 is indeed involved in the regulation of the sleep-wake cycle, Hitoshi Matsumura assessed the effect of AH 6809, a PGE_2 antagonist in the smooth muscle and pain systems. If PGE_2 induces wakefulness or inhibits sleep under physiological conditions, such a PGE_2 antagonist should counteract the effect of endogenous PGE_2; that is, it should increase the amount of sleep or decrease the time of wakefulness. When AH 6809 was infused slowly into the third ventricle of a rat during the night, the amount of slow-wave sleep was increased by 22% over the control, and REM sleep by 89%. These data clearly indicate that PGE_2 is involved in the maintenance of the waking state under physiological conditions (33).

In 1988, while visiting at Stanford University, I was introduced to William Dement, Director of the Sleep Disorder Center, who maintained a large colony of narcoleptic dogs. In collaboration with Dement and his colleagues, Seiji Nishino and I were able to demonstrate that the treatment of cataplexy in these narcoleptic dogs with PGE_2 or PGE_2 methyl ester induced a dose-dependent reduction of this manifestation of pathological REM sleep.

Narcolepsy is a genetic disorder of sleep, characterized by an uncontrollable desire for sleep. This sudden attack of sleep is usually triggered by emotions such as anger, joy, or even appetite. When such a dog sees his favorite food or female, he almost immediately undergoes paralysis and sleeps. To study this phenomenon more quantitatively, Dement and coworkers developed a unique biological assay system called the "food-elicited

cataplexy" test. In this test, the elapsed time to finish eating 12 pieces of food and the number of attacks of cataplexy during this period are determined. When the PGE_2 methyl ester, which penetrates the blood-brain barrier much more easily than PGE_2, was injected intravenously, the number of attacks as well as the total elapsed time decreased significantly compared with the control dog, indicating that PGE_2 inhibits sleep or induces wakefulness in a narcoleptic dog. In principle, therefore, PGE_2 and its derivatives could be a potentially useful drug for prevention or treatment in narcoleptic patients (34).

During the past 10 years or so, I have again been fortunate to be associated with and helped by a large number of established leading scientists in the sleep research field, colleagues who have been extremely helpful and encouraging to me, an amateur sleep researcher. Among them I am especially grateful to Shojiro Inoué, Michel Jouvet, William Dement, Michael Chase, Victor Pentreath, and Alexander Borbély for their collaboration, hospitality, and kindnesses.

Recently, the molecular biological approach to the sleep problem has yielded several unexpected findings that may lead to an unusual new hypothesis about the mechanism of sleep regulation. PGD synthase catalyzes the isomerization of PGH_2 to PGD_2 (Figure 4). This enzyme is a monomeric protein with a molecular weight of approximately 26,000. In order to study the properties and the regulatory mechanisms of PGD synthase, and possibly to find a new specific inhibitor of this enzyme, Urade and I purified the enzyme from human and rat brains, isolated cDNAs encoding PGD synthase, and determined the nucleotide and amino-acid sequences of the rat brain enzyme and two isozymes of the human enzyme. A computer-assisted homology search in databases of protein primary structures showed PGD synthase to be a member of the lipocalin superfamily (35), small secretory proteins that bind and transport small lipophilic molecules widely distributed in the animal kingdom (e.g. mammals, birds, reptiles, insects, and crustacea). The only known exception is PGD synthase, which is an enzyme rather than a lipid transporter. The gene structure of the members of this family has also been delineated, and that of the rat enzyme was remarkably analogous to the gene structures of several other members of this family, in the number and sizes of exons and phase of splicing of introns. Based on these results, a phylogenetic tree of the lipocalin superfamily was constructed by Hiroyuki Toh of the Protein Engineering Research Institute, Osaka.

Because of the high evolutionary divergence of the lipocalin superfamily, homology of the amino-acid sequences is rather weak. Yet, the tertiary structure is well conserved, forming a remarkably similar β-barrel, as revealed by X-ray crystallographic studies on retinal-binding protein, β-lactoglobulin,

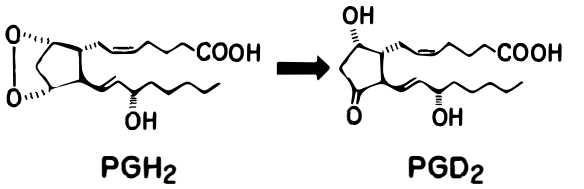

Figure 4 Enzymatic conversion of PGH_2 to PGD_2 by PGD synthase

and insecticyanin. The deduced tertiary structure of the brain PGD synthase indicates that the free SH group of cysteine at residue 65 is unique to the human and rat PGD synthases in the lipocalin family. The residue is located in the hydrophobic pocket and is thought to be in the active site of the enzyme. As mentioned earlier, inorganic quadrivalent selenium compounds are potent, specific, and noncompetitive inhibitors of brain PGD synthase, presumably interacting with this free sulfhydryl group.

The autoradiograms obtained after in situ hybridization with ^{35}S-labeled antisense RNA for rat brain PGD synthase showed that the mRNA for the enzyme was intensely expressed in the choroid plexus and the leptomeninges throughout the brain, and in the spinal cord of the adult rat. Positive signals indicating mRNA encoding the enzyme were also observed in oligodendrocytes, but hardly detected in neurons throughout the CNS (36).

Immunohistochemical studies with monoclonal and polyclonal antibodies against the enzyme revealed immunoreactivity in the leptomeninges and choroid plexus. The immunoreactivity for the enzyme was also found in oligodendrocytes, confirming our previously reported data, but these positive cells were diffusely and sporadically distributed in various parts of the brain. Immunoreactivity was occasionally observed in somata and dendrites of stellate neurons, apical dendrites of pyramidal neurons, and microglia-like cells in the superficial part of the cortex. However, no positive signals for the mRNA of the enzyme were detected in these cells.

The PGD synthase activity (nmol/min/mg of protein) was remarkably high in the isolated leptomeninges (14.2) and choroid plexus (7.0) of the adult rat brain, as compared with the activity in the whole brain (2.9).

During the past 10 years, we have shown that PGD_2 is the major endogenous sleep-regulating substance in the brains of rats, monkeys, and probably humans, and that its site of action is in or near the preoptic area. However, the major site of synthesis of PGD_2 has not yet been clearly demonstrated. In the study described above, PGD synthase was shown to

be predominantly expressed in the leptomeninges, choroid plexus, and, to a lesser extent, in oligodendrocytes in the adult rat brain. PGD synthase-like immunoreactivity was also demonstrated mainly in these tissues and cells. Furthermore, the enzyme activity was shown to be localized in these tissues. The experimental evidence therefore strongly indicates that PGD synthase is present mainly, if not exclusively, in choroid plexus, leptomeninges, and oligodendrocytes, and that PGD_2 is produced mostly in these tissues and cells.

Earlier, we characterized PGD synthase as a membrane-associated enzyme, because the enzyme is N-glycosylated, has a putative signal sequence, and by immuno-electron microscopy is located on the rough-surfaced endoplasmic reticulum and outer nuclear membrane of oligodendrocytes. These characteristics are also shared by various secretory proteins. According to Kikuko Watanabe, rat CSF contains a significant PGD synthase activity (200–300 nmol/min/mg protein). Hoffmann et al (37) recently demonstrated that the protein termed β-trace, a major constituent of human CSF, shows a high degree of homology with the rat and human PGD synthases, indicating that β-trace is structurally identical to PGD synthase. Furthermore, Achen et al (38) recently demonstrated that the major protein secreted by amphibian choroid plexus has the highest homology (41% identity and 84% similarity) with the rat and human PGD synthase among lipocalins so far identified.

As mentioned earlier, immunoreactivity for PGD synthase is sometimes observed in certain neurons and microglia-like cells in the superficial part of the cortex, although the mRNA for the enzyme is not detected in these cells. This difference might be due to the greater ease of detection of immunoreactivity, despite the use of a highly sensitive method for in situ hybridization. Alternatively, these results may be interpreted to mean that the enzyme was originally produced in the cells of leptomeninges and choroid plexus, and possibly oligodendrocytes, and then secreted and transported through the CSF to the neurons.

Several lines of experimental evidence presented by us (36) and others pose intriguing and provocative hypotheses that need to be critically evaluated. Are the major sites of production of PGD_2, the leptomeninges, choroid plexus, and oligodendrocytes? If so, how is the process controlled? Alternatively, the PGD synthase may be secreted and transported to some other sites, possibly glial cells and neurons, where the supply of its substrate is presumably more abundant. Although the choroid plexus and leptomeninges have long been believed to play a role in the mechanical support and chemical homeostasis of the brain, a more active function as a neuroendocrine pathway for communication within the CNS has also been suggested recently. For example, the choroid plexus and walls of the ventricles contain

serotonergic-binding proteins (39–41), and glucose utilization increases in the choroid plexus during slow-wave sleep (42). The presence of the sleep-promoting substance(s) in the CSF has been shown by a number of investigators (for a review, see 43), and suggests a humoral, rather than a neural, mechanism for sleep regulation. In their pioneering work on "sleep substances," Rene Legendre and Henri Pieron in 1907 deprived adult dogs of total sleep for 150 to 293 hours, took their CSF, blood, or serum, injected it into normal dogs, and showed that sleep-promoting substances accumulated in the CSF or serum under these conditions (44). Independently, Kuniomi Ishimori in Japan made similar observations with dogs in 1909 (45). Subscquently, several other authors also reported the presence of a sleep-promoting substance(s) in the CSF and urine, and some of them were chemically identified. However, their site of synthesis has not been clearly delineated. Our results (36) may provide some experimental basis for a rather provocative hypothesis that PGD_2 is not a typical and classical neurotransmitter but rather a "neurohormone" that circulates through the CSF in the ventricles, subarachnoidal space, and superior sagittal sinus. Alternatively, the PGD synthase itself may serve as a transporter or a carrier protein for PGD_2. Or else, PGD synthase may relocate into other parts of the brain tissue and produce PGD_2 locally.

EPILOGUE

Life is interesting because it is so unpredictable. During the past 50 years, my research interests have wandered about to several places, mostly guided by serendipity though sometimes by the sagacity of experience. Now I have come to my final destination, sleep. (I hope that readers of this essay have stayed awake to this point.) I am determined to continue my effort to challenge and solve this formidable problem.

I have always been fortunate to be associated with so many outstanding, kind, and helpful people, both scientists and nonscientists, whose names are too numerous to mention here, but to whom I express my deepest gratitude. Finally, I dedicate this article to Takiko, for her many years of faithful support and dcvotion, and for providing a happy family life with our daughter, Mariko, Masashi Akizuki, her husband, and their sons, Masato and Shuji. I am deeply indebted to Dr. Arthur Kornberg for critical and thorough reading of this manuscript and numerous helpful comments. I also thank Drs. L. Frye, F. I. Tsuji, and S. Sri Kantha for help in the preparation of this manuscript, and Miss Junko Kawahara for secretarial assistance.

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