From Oxygenase to Sleep

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It All Started from a Persimmon Seed

I graduated from Osaka University School of Medicine in 1942 at the age of 22 and then served in the Japanese Navy as a medical officer until the end of the Second World War. On a dreary cold afternoon in the late autumn of 1945, I returned home to Osaka, where I saw that the entire city had been almost completely destroyed by air raids and that my family's house had disappeared without a trace. Under the circumstances, I decided to join my parents, who had evacuated to their rural home village of Ejiri, perhaps to help my father with his medical practice. Before leaving Osaka, I visited my former mentor, Professor Tenji Taniguchi, to tell him of my plans. He listened to me attentively and then asked me if I knew the old Japanese saying, "Take a seed of persimmon rather than a ball of rice." I was puzzled and speechless for a moment. In those days, food was scarce, and rice was rationed. A ball of white rice would be a feast! On the other hand, the seed of a persimmon would grow into a tree in 10 or 20 years and then bear plenty of flowers and fruits, which would in turn yield numerous new seeds that would grow to produce yet more trees. Standing at a crossroad of my life at the age of 25, I was faced with the following question: "Should I become a clinical doctor and practice medicine like my father, or should I become a research scientist like Professor Taniguchi?" After pondering over the Professor's remarks and discussing the situation with my father for several days, I finally decided to join Professor Taniguchi's department and started my career as a rookie microbiologist in the Department of Bacteriology, Osaka University, my alma mater. However, it was obviously not a rational decision. Believe it or not, my starting salary was only 60 yen per month, equivalent to 20 cents, not even one dollar a month! Life was miserable and so were the conditions in the laboratory. Research funds were almost non-existent, and even if we had had money, there were no sources of chemicals, experimental animals, or other needed commodities. The facilities were outdated, and the supply of electricity, gas, and even water was limited, so it looked almost impossible to start any experimental research.

One day, I was visited quite unexpectedly by Dr. Yashiro Kotake, Professor Emeritus of Osaka University and a world-renowned biochemist in prewar Japan. He gave me several grams of tryptophan as a gift and encouraged me to use this amino acid for my research. I was very grateful to him but, frankly, did not know what to do with this precious material. At that time, tryptophan metabolism had already been investigated by numerous biochemists, including Kotake and co-workers. Tryptophan had been shown to be metabolized to kynurenic acid, anthranilic acid, and xanthurenic acid and secreted into the urine of rats and other mammals, including humans. Kotake was famous for this discovery and for the identification of kynurenine, the key intermediate of tryptophan metabolism. In 1934–1935, he wrote two solicited review articles on this subject in the Annual Review of Biochemistry (Volumes 3 and 4). Because Kotake's lifework had been carried out with the animal systems and because of the poor existing conditions of our laboratory, I decided to try microorganisms. I went out into the back yard of the University building, took a spoonful of scorched soil, mixed it with a trace amount of Kotake's tryptophan and water in a test tube, and simply waited. Several days passed, and then a faint white cloudiness appeared in the supernatant in the test tube containing tryptophan, whereas nothing happened in the test tube without the amino acid. This cloudiness slowly increased after several days, and by transferring the
contents to another test tube containing tryptophan, I was eventually able to isolate a strain of soil bacteria that could grow with tryptophan as its sole source of carbon and nitrogen. This microorganism was later identified as a member of the genus *Pseudomonas*. I then found rather serendipitously that in this microorganism, unlike in mammals, anthranilate was further converted to catechol and then to muconic acid, which eventually decomposed completely to CO$_2$, NH$_3$, and H$_2$O. Encouraged and excited by this somewhat unexpected discovery, I then tried to extract enzymes from this microorganism to study the details of this new metabolic pathway of tryptophan. The only enzyme that I was able to solubilize from the dried cells of *Pseudomonas* was the one that catalyzed the oxidative cleavage of the benzene ring of catechol, yielding cis,cis-muconic acid as the reaction product (Fig. 1) (1).

This enzyme had two unique and important features that had not been described before. 1) Unlike many other enzymes found in the past that metabolize phenolic compounds, it cleaved and opened the aromatic structure and produced an aliphatic compound. 2) Molecular oxygen could not be replaced by any other oxidant, electron, or hydrogen acceptors such as dyes, including methylene blue, dichloroindophenol, and so forth, or by coenzymes such as heme, FAD, NAD, etc. Various other properties of this enzyme appeared to be different from those of classical dehydrogenases and oxidases. A stoichiometric utilization of 1 mol of oxygen was demonstrated by the use of the Warburg respirometer, and so I naively assumed that molecular oxygen had been incorporated into the substrate, catechol. However, I was startled to learn later that the direct addition of oxygen had been completely ruled out in biological oxidation processes according to Professor D. E. Green and asked him for suggestions and comments on my "enzymic oxygenation" hypothesis. I had never met him before, but I was aware that he had spent many years at Cambridge University, England, where Sir Frederick Hopkins had discovered tryptophan and investigated its metabolism and function, and that Green had recently returned to the United States to become the director of the newly built Enzyme Institute at the University of Wisconsin in Madison. I did not hear from him for several weeks and almost forgot about it. One day in the early spring of 1949, again fate directed a turn in my path. I received a letter from Green; he did not say much about my manuscript but instead politely invited me to come to the Enzyme Institute and offered me a William Waterman Fellowship in Enzyme Chemistry. I was pleasantly surprised. However, Japan was still an occupied country, and I was not sure if it would be safe for me in the United States and if I could possibly live comfortably in a foreign country that had been an enemy until a few years before. Indeed, most of my friends and colleagues cautioned me to wait several years until the situation had improved. After pondering over Green’s letter for several days and again discussing the situation with my father, I finally decided to take a chance. I accepted his invitation.

**Trip to the United States**

In November 1949, Green sent me an airplane ticket, and I departed from Haneda Airport in Tokyo on a double-decker four-engine Boeing B-377, which was a luxury in those days. After changing flights several times, I finally
landed at the Madison airport in the middle of a snow flurry and was greeted by two postdoctoral fellows from Green’s lab, Bernard Katchman and Ephraim Kaplan, both of whom later became my lifelong and best friends. In fact, they were the kindest and most considerate human beings I had ever met and treated me like a real brother, not only in the lab, acquainting me with various new methods and materials and jargon, but also in daily life, teaching me English, sometimes even Yiddish jokes, and introducing me to a Jewish delicatessen, which I liked very much. Thanks to their warm and generous hospitality and kindness, I was quickly able to adjust to the new environment despite a language barrier and the ethnic and religious differences and to enjoy the American way of life in this beautiful and peaceful university town. Soon I made many friends such as Philip Cohen, Henry Lardy, Van Potter, Takeru Higuchi, and others. Henry Lardy was especially encouraging and helpful to me and was interested in my “oxygenation hypothesis.” He suggested that I should read several books and reviews that were pertinent and useful when I took up this problem several years later (4, 5).

In the meantime, I started to work on Green’s cyclophorase hypothesis under his guidance and soon became aware that many of my own and other collaborators’ experiments did not support his thesis. In fact, his cyclophorase hypothesis became controversial in the biochemical society at large. In April 1950, I attended the Federation meetings at Atlantic City and by chance listened to the paper given by Arthur Kornberg. It was a short report in the General Session lasting perhaps only 10 or 12 min, but I was so impressed and inspired by his presentation that I asked Bernard Katchman, who was sitting next to me, about Arthur. He told me that Arthur was a rising young star in biochemistry in the United States and advised me that if I wanted to remain in America another year or so, I should apply to work with a young mentor like Arthur. A few weeks later, Arthur was invited to Madison for a lecture, and I was able to meet him in person. Boldly, I asked him if he could take me into his lab as a postdoctoral fellow. He was very receptive to this idea and suggested that I should apply for a National Institutes of Health (NIH) fellowship, which I immediately did according to his instructions.

While I was waiting to hear from NIH about my application for a fellowship, I received a telephone call from Professor Roger Stanier of the University of California; he invited me, with some urgency in his voice, to come to his laboratory for a collaborative project on tryptophan metabolism. He had previously and independently used the enrichment culture technique and isolated a pseudo-monad from soil, but his strain degraded tryptophan via kynurenic acid instead of anthranilic acid. He proposed to isolate and purify all the enzymes involved in these two different pathways, which he named the “quinolinic” and “aromatic” pathways, respectively. Because his proposal was a very interesting and challenging one and was closely related to my previous experience in Osaka, I accepted his offer; so with Dr. Green’s consent, I resigned my post at the Enzyme Institute, bid farewell to Bernie and Eph, and moved to Roger’s lab in the Life Science Building at Berkeley at the end of August 1950. There again, I was fortunate to become acquainted with many outstanding scientists such as C. B. van Niel, H. A. Barker, Michael Doudoroff, William Hassid, and others, all of whom were kind, helpful, and welcoming. In Roger’s lab, we both worked very hard by day, exhausting almost all available methods for extracting enzymes from bacterial cells described in the textbooks. In fact, Roger stated, “I have never worked so hard before and after” (6). Almost 3 months had passed without any sign of progress. Then one day I met H. A. Barker in the corridor and started casually chatting with him about my problem. He suggested that I try an old method described by Mirick some years ago in which alumina powder and bacterial cells are mixed and ground in a mortar with a pestle (7). We were at first a bit skeptical because it sounded too simple and primitive; after all, the more sophisticated methods we had tried thus far had been uniformly unsuccessful. As a last resort, we tried it anyway. Lo and behold, it worked! We were able to extract almost all enzymes from the cell body and to partially purify them for further characterization. During the next month or so, Roger and I worked day and night and were able to publish a series of six papers over a span of about a month, which appeared in reputable journals such as the Journal of Bacteriology, Journal of Biological Chemistry, and Science (8–13). It was indeed the most productive period in my career, at least quantitatively speaking (6).

**National Institutes of Health**

Time had passed so quickly, and in December 1950, Arthur telephoned me and told me that my application had been approved. Takiko, my wife, and Mariko, my daughter, who was about 3 years old at the time, both of whom were living in Japan, joined me in Berkeley, and we then moved to Bethesda, MD. Bethesda was a very nice attractive residential area in the suburb of Washington, D.C., and we were all very happy there. Arthur was the Chief of the Enzyme Section, NIAMD, but unlike Green’s lab, his was a small one, and Arthur was doing his own experiments with Bill Pricer, a senior technician. I was the
only postdoctoral fellow working in his group. Bernard Horecker and Leon Heppel were the other senior members in the section, and they worked independently of Arthur. Arthur introduced me to nucleic acid and phospholipid chemistry and metabolism, and we worked together on the bacterial degradation of uracil using the enrichment culture technique. I also introduced the use of microbial enzymes to explore the metabolism of histidine, histamine, urocanic acid, etc., in collaboration with Herb Tabor and Alan Mehler. These studies were reviewed in “Special Techniques for Bacterial Enzymes: Enrichment Culture and Adaptive Enzymes” in Methods in Enzymology (14). Every day from noon to 1:00 p.m., we attended the so-called luncheon seminar. Every one in his lab took turns in alphabetical order and gave a seminar on a selected paper of his choice. This lunch seminar, which was also attended by people from other sections and departments, including Herb and Celia Tabor, Terry and Earl Stadtman, Alan Mehler, Bruce Ames, Jesse Rabinowitz, Hans Klenow, and others, had one important characteristic. In many seminars and journal clubs that I had attended previously, the speakers normally reported facts and described the results and conclusions, almost like a copying machine. In contrast, this luncheon seminar in Arthur’s lab was devoted to the discussions and criticisms of every detail of the strategy of the paper; it was a sort of exercise in maneuvers instead of just one in learning new results and facts. This unique training course became legendary and a tradition in many other laboratories, especially in those of the Kornberg school. For example, when I moved back to Japan in 1958, this type of luncheon seminar became a famous event and was nicknamed the “Hayaishi gymnasium or training school” at Kyoto, Osaka, and Tokyo Universities, where I served concurrently as the Chairman of their Departments of Biochemistry.

Two years passed quickly, and one day Arthur told me that he had decided to move to St. Louis as Chairman and Professor of the Department of Microbiology at Washington University School of Medicine, one of the leading medical schools in the United States, and that he wanted me to come with him as an assistant professor. I felt very flattered and honored but was not able to accept his offer immediately because I had been in the United States only 3 years as a postdoctoral fellow and had no confidence in myself regarding teaching students and handling administrative duties and other chores in English. However, Arthur was enthusiastic and persuasive, and after talking things over with friends and my wife, Takiko, we finally decided to accept Arthur’s offer and moved to St. Louis in late 1952.

Life in St. Louis was also quite pleasant. I met many outstanding scientists there, including Carl and Gert Cori, Martin Kamen, Stanley Cohen, Oliver Lowry, and others, all of whom were kind and helpful. As expected, however, I had to spend much of my time teaching and working on grant applications and other chores, all in English. Thus, my productivity in the lab slowed down to some extent. Summer in St. Louis was notoriously hot and humid, so we took a short vacation in the Rockies in the summer of 1954. When we came back to St. Louis, I unexpectedly received a phone call from Dr. Sanford Rosenthal of NIH, who asked me if I would be interested in assuming the position of Chief of the Toxicology Section at NIAMD, NIH, in Bethesda. I felt very flattered and honored. After all, it was only my 5th year in the United States, I was only 34 years old, and this was an offer of a position equivalent to one that Arthur had occupied only 2 years ago. I thanked Dr. Rosenthal for thinking of me but told him that I was not a toxicologist by training and that I did not think I was the best qualified candidate for this position. Dr. Rosenthal laughed and told me that he was well aware that I had started as a microbiologist but that I was now a full-fledged biochemist-molecular biologist. He said that NIH wanted someone who could change the old-fashioned toxicology laboratory into a modern biochemical and molecular biological toxicology section. Furthermore, he assured me complete freedom in choosing the subject and approach of my research program if I accepted this position. Arthur congratulated me and encouraged me to accept this offer, so once again, we were on the move. In December 1954, my family and I returned to Bethesda, which meant my fourth move during my 5-year stay in the United States!

Discovery of Enzymic Fixation of Molecular Oxygen: Oxygenases

As I began to reorganize the research program of the Toxicology Section, I decided to investigate the mechanism of the pyrocatechase reaction because such a reaction might be involved in the detoxification of various drugs and poisonous compounds. I remembered that when I presented my previous work on pyrocatechase at the First Annual Meeting of the Japanese Biochemical Society after World War II in April 1949 at Kyoto University, Professor Yashiro Kotake, who had given me tryptophan as a gift several years before, mentioned that when he was a postdoctoral fellow at the University of Königsburg, Professor Max Yaffe, his mentor, had fed benzene to a dog and isolated muconic acid from the urine. Kotake suggested that such a pyrocatechase-like enzyme might also be present in mammals. Because $^{18}$O, a heavy isotope of oxygen, was not commercially available at that time, I
wrote to Dr. David Samuel, head of the Isotope Department at the Weizmann Institute of Science in Israel, who kindly provided me with concentrated \( \text{H}_2\text{^{18}O} \). We generated \( \text{O}_2\text{^{18}} \) by electrolysis and carried out the crucial but simple experiments. The results were clear-cut and demonstrated unequivocally that the oxygen atoms incorporated into the product of the reaction, \( i.e. \text{cis},\text{cis}-\text{muconic acid}, \) had been derived almost exclusively from molecular oxygen, not from oxygen in water molecules (15). A full account of these results, together with those of another set of experiments with \( ^{18}\text{O} \) and \( \text{H}_2\text{^{18}O} \) conducted using tryptophan and tryptophan pyrrolase, was then published subsequently (16, 17) and reviewed recently in Classics of the Journal of Biological Chemistry by Kresge, Simoni, and Hill (18).

These results therefore clearly established that, contrary to the central dogma proposed by H. Wieland, oxygen fixation reactions do indeed occur in biological systems. Concurrently and independently, H. S. Mason and co-workers reported that mushroom phenolase incorporated one atom of molecular oxygen into the substrate, whereas the other atom was reduced to \( \text{H}_2\text{O} \) (19). In 1956, I organized the first symposium on oxygenases at the American Chemical Society meeting in Atlantic City and proposed to name this novel group of enzymes that catalyze the incorporation of molecular oxygen into their substrate, \( \text{viz.} \) oxygen fixative reactions, as “oxygenases.” In 1957, Professor Otto Hoffmann-Ostenhof of the University of Vienna came to visit me at my NIH office and invited me to organize and chair the first international colloquium on oxygenases at the 4th Congress of the International Union of Biochemistry (IUB), which was to be held in Vienna in 1958. It was my first association with the IUB, and I gladly accepted his invitation. In late 1957, Kyoto University School of Medicine decided to appoint me as Chairman and Professor of the Department of Medical Chemistry, one of the oldest and most prestigious centers of biochemistry in major Japanese universities at that time. In the same year, I was invited to attend the First International Meeting on Enzyme Chemistry held in Tokyo and Osaka as a delegate of the American Biochemical Society. After the meeting, I was able to spend several more weeks in Japan and had a chance to talk to a number of leading biochemists, my old colleagues, and friends and also met numerous young investigators, all of whom enthusiastically asked me to come back to Japan. After having returned to Bethesda and talking it over and over again with Takiko and my now 11-year-old daughter, Mariko, I finally made up my mind to accept the offer from Kyoto, and so we went back to Japan in February 1958.

Return to Japan

In the summer of that year, I went to Vienna to attend the 4th IUB Congress and chaired and presented a lecture in the Colloquium on Oxygenases, the first international meeting for this novel respiratory enzyme. After the Congress, I was invited to a number of universities and institutes in Europe and spent almost a month touring, lecturing, and meeting famous biochemists whose names had been known to me only from their publications and correspondence. I also learned a lot by making many new friends such as Sune Bergström, Bengt Samuelsson, and their associates, who told me about prostaglandins, and we discussed the oxygenase nature of prostaglandin synthase. In 1962, Oxygenases, the first comprehensive treatise on this subject, was published by Academic Press (20). In 1964, I presented a plenary lecture entitled “Oxygenases” at the 4th IUB Congress in New York, which was chaired by Professor John T. Edsall, then Chief Editor of the Journal of Biological Chemistry. I still remember his kind compliments and warm words of encouragement after my talk. Needless to say, I was deeply touched.

When I started to work in Kyoto in February 1958, Yasutomi Nishizuka applied to join me as my first graduate student. He later became one of the most famous biochemists in Japan after his discovery of protein kinase C. Soon the department was filled with young and ambitious postdoctoral fellows, graduate students, and visiting scientists from all over Japan as well as from abroad. All these people were bright, highly motivated, and hard working, and all were eager to learn dynamic biochemistry and enzymology, which I had learned from my experience during the past 10 years in the United States, especially from Arthur Kornberg. The time spent with Arthur, the first 2 years as a postdoctoral fellow in Bethesda and the subsequent 2 years as an assistant professor in St. Louis, provided valuable and unforgettable experiences for me, and our friendship lasted the rest of his life. Arthur Kornberg passed away in October 2007.

However, the economical conditions in Japan were still lagging far behind. The exchange rate was ~400 yen/dollar compared with the present rate of 105 yen/dollar. My salary as the youngest professor of Kyoto University was less than 8% of my salary at NIH. Fortunately, the Japanese Government had made a special effort to provide me with a large number of grants, and many foundations and pharmaceutical companies offered their generous support in the form of startup grants. In addition, NIH provided a substantial amount of money in the form of a research grant, and the Jane Coffin Memorial Fund, Rockefeller Foundation, China Medical Board, and several pharma-
obic companies in the United States contributed significant amounts of money as well, not only for research but also for rebuilding and remodeling the old buildings and even some for constructing a new building for radioactive experiments and a library. At first, oxygenase-catalyzed reactions were generally thought to be rather unusual and suggested to be limited to primitive forms of life such as soil bacteria and mushrooms. However, subsequent work in my Kyoto laboratory and experiments by others all over the world revealed that oxygenases are found ubiquitously in animals, plants, and microorganisms and play important roles not only in the biosynthesis and degradation of natural compounds but also in the degradation of synthetic compounds such as drugs, insecticides, chemicals, toxins, and so forth, as exemplified by cytochrome P450, now often described as the most versatile biological catalyst known (21). In contrast, oxidases and dehydrogenases are mainly, if not exclusively, involved in energy metabolism. Fig. 2 shows a metabolic map of tryptophan, in which red oxygen atoms indicate the molecular oxygen incorporated into substrate by various specific oxygenases (22). It illustrates the ubiquitous presence of oxygenase-catalyzed reactions in this physiologically important metabolic pathway and also shows the presence of numerous novel enzymes and metabolic reactions initiated by various specific oxygenases. For example,
we were able to demonstrate the presence in cell nuclei of a new macromolecule, poly(ADP-ribose), which is synthesized from NAD and is found covalently attached to histones and other nuclear proteins (23). Poly(ADP-ribose) is now known to participate in DNA repair, apoptosis, and chromatin stabilization. These studies were extended to elucidate the mode of action of diphtheria toxin. We discovered that diphtheria toxin catalyzed the ADP-ribosylation of aminoacyltransferase II, and this finding provided the molecular basis for the understanding of the toxicity of this toxin (24). This was the first demonstration that a bacterial toxin is an enzyme. Subsequently, toxins derived from cholera, botulinum, and pertussis were shown to catalyze similar ADP-ribosylation of various G-proteins, and these enzymes have been used extensively to reveal the signal transduction mechanisms regulated by these G-proteins. Another important discovery in relation to tryptophan metabolism was the induction of indoleamine 2,3-dioxygenase (IDO) by interferon (25), which results in depletion of tryptophan. This induction leads to inhibition of the growth of viruses and tumors. This contention was confirmed by a subsequent independent publication by Pfefferkorn, who used γ-interferon and Toxoplasma-infected fibroblasts (26). More recently, the induction of this oxygenase and the resulting depletion of tryptophan have been proposed as a mechanism for preventing embryos from being rejected by the maternal immune mechanism (27, 28).

IDO catalyzes the formation of formylkynurenine from tryptophan, the reaction of which is essentially identical to that of tryptophan pyrrolylase or tryptophan 2,3-dioxygenase, but requires and utilizes superoxide anion O2 for its activity (29). Tryptophan pyrrolylase is found only in liver cells, whereas IDO is distributed ubiquitously in almost all other types of cells except liver.

Since the original discovery of oxygenases, we have found and characterized more than 30 oxygenases; crystallized four of them; and conducted extensive studies concerning their structure, catalytic mechanisms, and physiological functions. These results have been summarized and reviewed in a number of monographs and proceedings (20, 30–35). In December 2005, the Biochemical and Biophysical Research Communication published a special issue entitled “Celebrating 50 Years of Oxygenase” (36); 91 authors with their coauthors contributed excellent articles describing their most recent achievements in this field. In April 2006, a Takeda Science Foundation Symposium commemorating the 50th anniversary of the discovery of oxygenase was held in Kyoto and was attended by more than 200 participants from all over the world. Another commemorative symposium was held in June 2006 as part of the 20th Congress of the International Union of Biochemistry and Molecular Biology, which was also held in Kyoto. It was gratifying to me to see how the persimmon seeds I had planted in the scorched land of Osaka had grown into a huge forest bearing numerous beautiful flowers and delicious fruits after all these years. I am also humbled by the fact that my discovery of oxygenases has created a new vista in the field of bioenergetics and respiratory physiology and has triggered a research explosion that has had an enormous impact on nearly all fields of medical, biological, and physiochemical sciences as well as on environmental health sciences (37).

From Oxygenases to Sleep

In addition to these studies on amino acid metabolism, our work extended to oxygenases involved in the metabolism of other biologically important compounds such as prostaglandins, vitamin A, carcinogenic hydrocarbons, and aromatic compounds. Prostaglandins (PGs) are so-called local hormones that are produced from arachidonic acid by the action of PG synthase or cyclooxygenase, which incorporates four atoms of molecular oxygen simultaneously per molecule of substrate. These compounds are ubiquitously distributed in virtually all mammalian tissues and organs and exhibit numerous and diverse biological effects on a wide variety of physiological and pathological activities. However, relatively little was known about PGs in the mammalian brain until the late 1970s. In 1982, we showed PGD2 to be the most abundant prostanoid in the brains of rats and other mammals, including humans (38), and subsequently demonstrated that it is produced in the brain from the substrate PGH2 by the action of the enzyme PGD synthase (39). Because PGD2 had long been considered as a minor and biologically inactive prostanoid, our findings suggested that it might be a unique constituent of the brain and might have some important and possibly specific function in this organ. Soon we discovered that PGD2 induced sleep when microinjected into the brains of rats. When PGD2 was infused continuously into the third ventricle of a freely moving rat during the night, both slow-wave sleep (SWS) and rapid eye movement (REM) sleep increased significantly during the infusion. The effect was specific to PGD2 and dose-dependent. As little as several femtomoles of PGD2/s was effective in inducing excess sleep. Most important, the sleep induced by PGD was indistinguishable from physiological sleep as judged by electrophysiological and behavioral criteria, clearly indicating that PGD2 is the endogenous and physiological sleep-promoting substance in the brain, viz. a sleep hormone (40). These
findings formed my last publication from Kyoto University, and in that year, 1983, I retired at the mandatory retirement age of 63. I was then appointed as President of Osaka Medical College in Takatsuki City, ~50 km west of Kyoto. The Exploratory Research for Advanced Technology (ERATO), a subsidiary of the Ministry of Science and Technology, provided me with a special grant-in-aid of ~1.5 billion yen (~15 million U.S. dollars) during the period of 1983–1988, which I used to continue to explore the mystery of sleep.

Because I am an enzymologist, we first purified the PGD synthase from the brains of rats and other mammals, finally crystallized it, and then delineated its tertiary structure. After an extensive search for an enzyme inhibitor, we found inorganic tetravalent (4+) selenium compounds to be potent, specific, and reversible inhibitors of the brain PGD synthase (41). When selenium chloride was infused into the third ventricle of a rat during the day, both SWS and REM sleep were time- and dose-dependently inhibited. After ~2 h from the start of the infusion, both forms of sleep were almost completely inhibited, and the effect was reversible. When the infusion was interrupted, sleep was restored. Furthermore, the inhibition was reversed by the simultaneous infusion of SH compounds such as dithiorthreitol and reduced GSH, as in the case of the in vitro enzyme activity. These results clearly showed that PGD synthase is a key enzyme in sleep regulation under physiological conditions (42).

In 1987, I was appointed as the first director of the Osaka Bioscience Institute, where I further continued my project on sleep after having resigned from Osaka Medical College in 1989. The details of the molecular mechanisms involved in sleep–wake regulation by PGD₂ and PGE₂ were worked out during the next 20 years or so in my laboratory and in collaboration with numerous other laboratories in Japan, the United States (especially Saper’s group at Harvard), and in Europe (especially Jouvet and Luppi’s group in Lyon as well as Borbély and Tobler’s group in Zurich). These mechanisms are briefly summarized below and have been detailed in several recent reviews (43–47).

Lipocalin-type PGD synthase (L-PGDS), the key enzyme in sleep induction, is present mainly, if not exclusively, in the membrane system surrounding the brain, viz. the arachnoid membrane and choroid plexus, although a small amount of enzyme protein is also detectable in oligodendrocytes in the brain parenchyma, as evidenced by the data obtained by in situ hybridization, immunohistochemical staining for PGDS protein, and direct determination of enzyme activity in isolated tissues. Once produced, PGD₂ is secreted into the cerebrospinal fluid and circulates in the ventricular and subarachnoidal space. In contrast, the PGD type 1 receptor is localized exclusively in a small area on the ventrostral surface of the basal forebrain (48). PGD₂ circulating in the cerebrospinal fluid then binds to this receptor. The signal initiated by the binding of PGD₂ to its specific receptor is transmitted across the pia membrane and through the brain parenchyma to the ventrolateral preoptic area, a sleep center where so-called sleep neurons are activated as evidenced by the increase in the number of c-Fos-positive neurons there. This transduction is mediated via adenosine by the A₂A adenosine receptor. The ventrolateral preoptic area projects to the tuberomammillary nucleus (TMN), a wake center located in the posterior hypothalamus, through GABAergic and galaninergic neurons and sends inhibitory signals to down-regulate the wake neurons. Thus, sleep is induced by up-regulation of the sleep neurons and at the same time by down-regulation of the wake neurons by a flip-flop mechanism (Fig. 3) (49, 50).

Thus, our studies revealed the presence of “a previously unrecognized signal transduction mechanism in the central nervous system of mammals.” The mechanism underlying the sleep regulation by PGD₂ is somewhat reminiscent of the signal transduction mechanisms of hormones, cytokines, and neurotransmitters at the cellular level, viz. PGD₂ is bound to its receptor on the surface of the meningeal cells, followed by transduction via adenosine. In that sense, adenosine may be envisaged as a second messenger in this system and constitutes “a link between humoral and neural regulation of sleep.”

To substantiate these conclusions and to further elucidate the exact role of the PGD₂ system in the whole live animal, we generated knock-out (KO) mice for L-PGDS. These mice are supposedly unable to produce PGD₂ and therefore might be predicted to be unable to sleep. Contrary to this expectation, however, the KO mice were viable and appeared to be quite healthy and to grow, breed, and even sleep normally.

The circadian profiles of non-REM (NREM) and REM sleep of wild-type (WT) and L-PGDS KO mice appeared to be essentially identical under macroscopic examination, and there was no major difference between them in the daily amounts of sleep and wakefulness. The same results were also obtained for PGD receptor (DPR) KO mice. These findings apparently contradicted the previous pharmacological experimental results described above, viz. that the PGDS inhibitor and DPR antagonist can promptly and effectively block sleep by intracerebroventricular infusion. However, such a lack of effect on phenotypes is not uncommon in KO mice for neurotransmitters,
peptides, and so forth that are reportedly involved in sleep or arousal regulation. For example, mice lacking histidine decarboxylase or histamine H_1 receptors exhibited a circadian rhythm almost identical to that shown by WT mice, even though each of these abolished systems is thought to regulate wakefulness. Taken together, these experimental results were interpreted to mean that because sleep is essential for life, the sleep regulatory system is composed of a complicated network with built-in redundancies; therefore, a deficiency caused by removing a gene in one system may be effectively compensated by other systems during early ontogenic development. To obtain more convincing evidence that would fully justify such an interpretation, it was necessary to demonstrate conclusively that the inhibitory effect of SeCl_4 on sleep was due exclusively and specifically to the inhibition of PGDS activity and not to some nonspecific or general toxic effect of selenium.

The selenium-induced inhibition of sleep in rats was confirmed in mice by administering an intraperitoneal bolus injection of SeCl_4 to WT mice at 11:00 in the morning. In good agreement with the previous experiments in rats, both NREM and REM sleep were inhibited promptly, effectively, and almost completely after ~1 h, and this inhibitory effect lasted ~5–6 h under these conditions. When L-PGDS KO mice were used instead of the WT mice, no inhibition was observed at all, clearly indicating that the sleep inhibition by SeCl_4 was due to its specific inhibitory effect on L-PGDS and not to some other nonspecific toxic effect. When SeCl_4 was administered to L-PGDS/hematopoietic PGDS double KO mice under the same experimental conditions, neither the inhibition of sleep nor the increase in sleep during the night was observed. SeCl_4 had no effect at all on the sleep of L-PGDS/hematopoietic PGDS double KO mice. These results clearly show that SeCl_4 is not toxic to sleep per se but inhibits sleep by inhibiting the endogenous production of PGD_2 by PGDS (51). The sleep of DPR KO mice was also not inhibited significantly by selenium, indicating that the PGD_2 system in these mutant mice was not functioning and had probably been replaced by some other system during embryonic development.

These mice were subjected to sleep deprivation to determine whether PGD_2 is also involved in the homeostatic regulation of sleep. As mentioned above, the circadian profiles of the sleep-wake patterns of the WT and PGDS KO mice appeared to be essentially identical under macroscopic examination. However, when the WT mice were subjected to sleep deprivation for 6 h immediately before the onset of their wake period, a pronounced rebound was observed in NREM sleep, whereas little, if any, rebound occurred in NREM sleep in the PGDS KO mice. The total amount of NREM sleep rebound exceeded >60 min in the WT mice as well as in histamine H_1 receptor KO mice and prostaglandin EP_1 receptor KO mice, which were used as controls. On the other hand, sleep rebound was almost nonexistent in PGDS, DPR, and A_2A adenosine receptor KO mice, thus indicating that PGDS plays a crucial role in the homeostatic regulation of NREM sleep (52). Transgenic mice overexpressing the human PGDS gene generated in my laboratory were also tested. Again, the circadian profiles of sleep-wake patterns of the WT and genetically engineered mice were essentially identical under macroscopic examination. However, gross
phenotypic changes were observed under certain specific conditions such as extraneous physical and chemical stimuli. These results also supported the notion that the PGDS gene is one of the genes involved in the control of homeostatic as well as circadian regulation under physiological conditions (53).

PGD$_2$ and PGE$_2$ are positional stereoisomers and have been shown to sometimes exhibit opposite biological effects. For example, PGD$_2$ lowers the body temperature, whereas PGE$_2$ increases it, and PGE$_2$ stimulates the secretion of luteinizing hormone-releasing hormone, but PGD$_2$ suppresses it. Also, PGD$_2$ decreases the transmucosal potential difference in rat colon mucosal membrane, whereas PGE$_2$ increases it. PGE$_2$ is produced in the brains of various mammals, including humans, and exerts an awakening effect on rats and monkeys after administration to the brain. When AH6809 (GlaxoSmithKline), a PGE$_2$ antagonist in the smooth muscle and pain systems, was infused into the third ventricle of a rat during the night, the amount of wakefulness was significantly and dose-dependently decreased with a concomitant increase in the amounts of both REM and NREM sleep. These results clearly indicate that PGE$_2$ is involved in the maintenance of the arousal state under physiological conditions (54). A number of physiological and pathological activities are known to be centrally evoked by PGE$_2$, and thus, it is possible that the PGE$_2$-induced arousal state might be a secondary effect of these activities, especially hyperthermia. However, these possible indirect causal relationships have now been completely ruled out by a number of experiments. Furthermore, when perfused through a microdialysis probe in the TMN, PGE$_2$ significantly increased histamine release and synthesis. Among the agonists of the four distinct subtypes of PGE$_2$ receptors (EP$_1$–EP$_4$) tested, only the EP$_4$ receptor agonist (ONO-AE1-329) mimicked the excitatory effect of PGE$_2$. In situ hybridization revealed that EP$_4$ receptor mRNA was expressed in the histaminergic neurons of the TMN region. Furthermore, only perfusion of the TMN with the EP$_4$ receptor agonist induced wakefulness. These findings indicate that PGE$_2$ induces wakefulness through activation of the histaminergic system via EP$_4$ receptors (Fig. 3) (47).

Sleep is perhaps one of the most important and yet least understood of the physiological functions of the brain. Although we repeat sleep-wake cycles every day and night and spend almost one-third of our precious lifetime sleeping in bed or somewhere else, we are not able to answer even the most simple questions about sleep. “What is sleep?,” “why do we need to sleep?,” and most important, “where and how are sleep and arousal regulated and controlled?” Thus, sleep remains one of the greatest mysteries in medical and biological sciences in this new century. In the meantime, the number of patients suffering from sleep disorders has been recently increasing exponentially and now exceeds >30% of the total population in most countries. More than 88 different sleep disorders or sleep-related diseases have been described and classified in textbooks on sleep, but in most instances, the etiologies are not clearly understood simply because basic sleep science is still in its infancy. I sincerely hope that our studies on the sleep and wake hormones, PGD$_2$ and PGE$_2$, respectively, will contribute to a better understanding of this formidable problem and may eventually provide a valid and useful approach for the development of safe drugs for the rational treatment of sleep disorders.

Epilogue

In this paper, I have tried to describe my own scientific journey, which I consider would be of some interest and merit to the readers of this Journal, with the topics selected mostly from >60 years of my own research. My personal and family history, anecdotes, various episodes in my scientific career, etc., have been described previously (55–58).

I dedicate this article to Arthur Kornberg, my mentor and dearest friend. Throughout my lifetime, I have been extremely fortunate to have had countless excellent teachers, mentors, colleagues, students, friends, and members of my family who have contributed positively to my life, and to them, I express my deepest gratitude.

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