

REVIEW ARTICLE

The discovery of aequorin and green fluorescent protein

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We discovered aequorin and green fluorescent protein (GFP) in 1961 from the same species of jellyfish (Shimomura *et al.*, 1962). Our target was a luminescent substance, aequorin, and GFP was isolated as a by-product of aequorin owing to its bright conspicuous fluorescence. Both are unusual proteins but they had no particular importance when we first reported them. Their importance became apparent in the course of studies, and now, 40 years after their discovery, they are well known and widely used, aequorin as a calcium probe and GFP as a marker protein.

In the characterization of these proteins, information obtained from the bioluminescence of the ostracod *Cypridina* played an extremely important role. Without the information gained on *Cypridina* luminescence, the characterization of aequorin would not have been possible. Because I had studied the bioluminescence of *Cypridina* before I worked on aequorin, I would like to begin my story with my encounter with *Cypridina*.

It was the spring of 1955. I was working as a teaching assistant at the Pharmacy School of Nagasaki University, and it was my fourth year in the role. My supervisor, Professor Shungo Yasunaga, was interested in my education and wanted me to broaden my knowledge. He kindly gave me a leave of absence for one year, and arranged for me to work at the laboratory of Professor Yoshimasa Hirata, at Nagoya University, as a visiting researcher. Professor Hirata was an expert in the chemistry of natural products. At my first meeting with Professor Hirata, he showed me dried *Cypridina* stored in a large vacuum desiccator and asked me to purify the luciferin and crystallize it.

Cypridina is a tiny egg-shaped crustacean ostracod only 2–3 mm in length (illustrated here in Fig. 1), and is abundant in shallow seas around Japan and south-east Asia. At night, the ostracod swims along and, upon encountering a predator, ejects luciferin and luciferase into the water, producing a cloud of blue luminescence. The animal then swims away into the darkness of the surrounding water. The luminescence is emitted by an enzyme reaction, commonly known as the luciferin–luciferase reaction, in which the reaction of luciferin with oxygen to produce light and oxyluciferin is catalysed by the enzyme luciferase. When *Cypridina* is dried, the specimen

keeps its luminescence property almost permanently, and it will emit light again by simply wetting it with water. Because of this property, the Japanese military collected a large quantity of this organism during the World War II for intended use as a low-intensity light source.

Professor Hirata's objective was the structural determination of *Cypridina* luciferin. For that purpose, pure luciferin was essential, and crystallization was the only practical means to confirm purity at that time. The luciferin of *Cypridina* had been studied at Newton Harvey's laboratory at Princeton University for almost 30 years at the time, but no information on the chemical nature of the luciferin had been gained. Professor Hirata was fully aware that *Cypridina* luciferin is an extremely unstable substance, and it is rapidly oxidized in air. He mentioned to me that he could not give this project to any of his students because it was too risky as a degree subject owing to the extraordinary instability of luciferin, and therefore the difficulty of the project and its slim chances of success.

I made several small-scale tests of luciferin extraction, to determine how unstable the compound was and also to estimate the amount of luciferin in dried *Cypridina*. The luciferin content was indeed very low, probably about 10 p.p.m., and in solution it was highly sensitive to air, being oxidized and destroyed even with a trace of contaminating oxygen. Therefore, extraction had to be carried out in an atmosphere in which oxygen was completely absent. Nitrogen gas and argon contain trace amounts of oxygen that are difficult to remove, and therefore they are not suitable. Despite its risk of explosion, the only method was to use hydrogen gas that had been purified by passing it through a red-heated copper catalyst to remove oxygen. I planned to extract 500 g of dried *Cypridina* with methanol in the complete absence of air, at a temperature lower than 40 °C, to obtain 2 or 3 mg of purified luciferin for crystallization. The 500 g of *Cypridina* contained about half a million organisms, and it was 10 times the amount used at Princeton University. Professor Hirata agreed to my plan, and his glass blower constructed an over-sized soxhlet apparatus for me.

I extracted luciferin in hydrogen under reduced pressure using this apparatus (Fig. 2), purified the extract in an atmosphere of nitrogen and then tried to crystallize the purified luciferin. I tried all combinations of solvents and salts I could think of, but all my efforts ended with the creation of amorphous

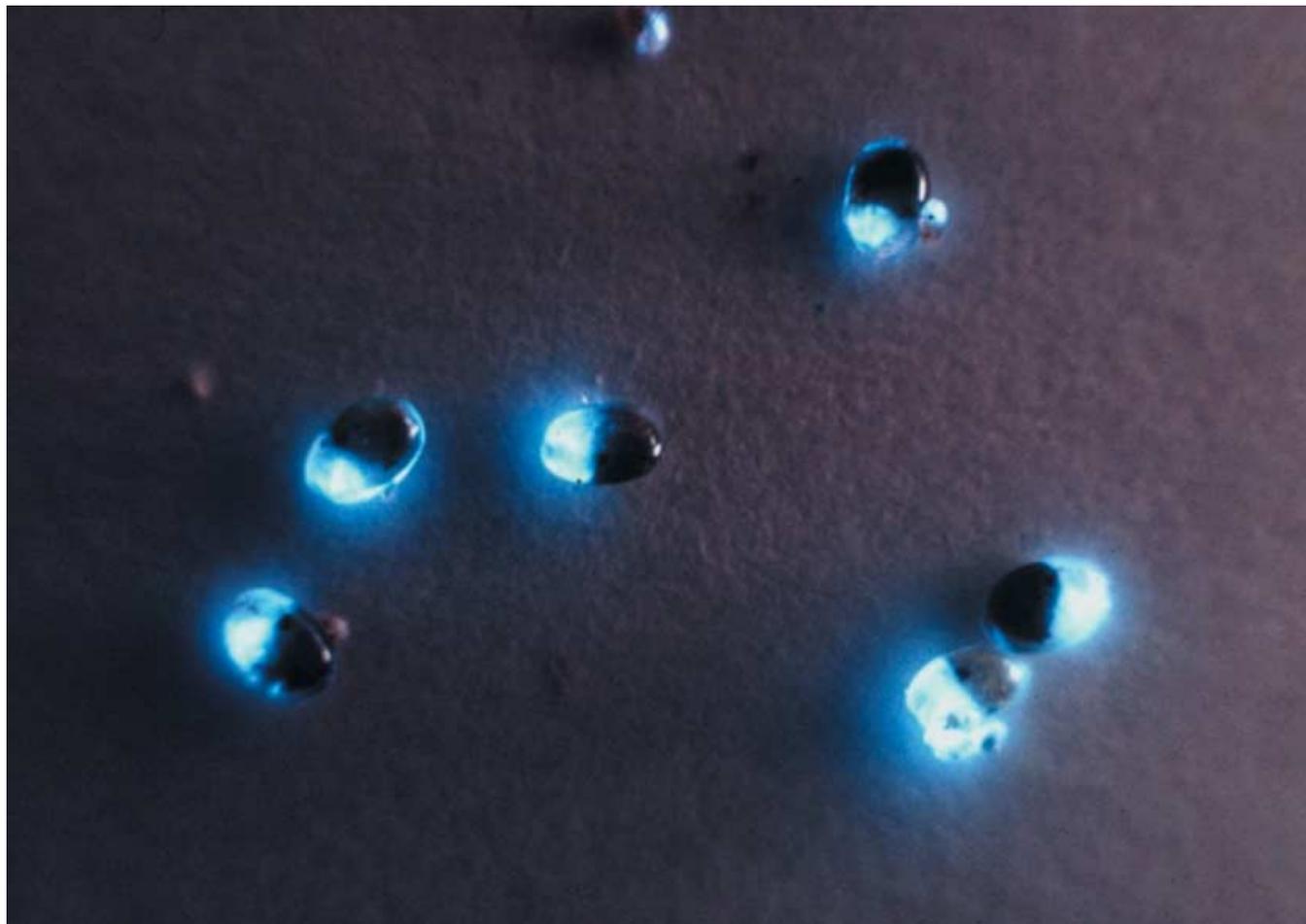


Fig. 1. The ostracod *Cypridina*. Photo by Toshio Goto.

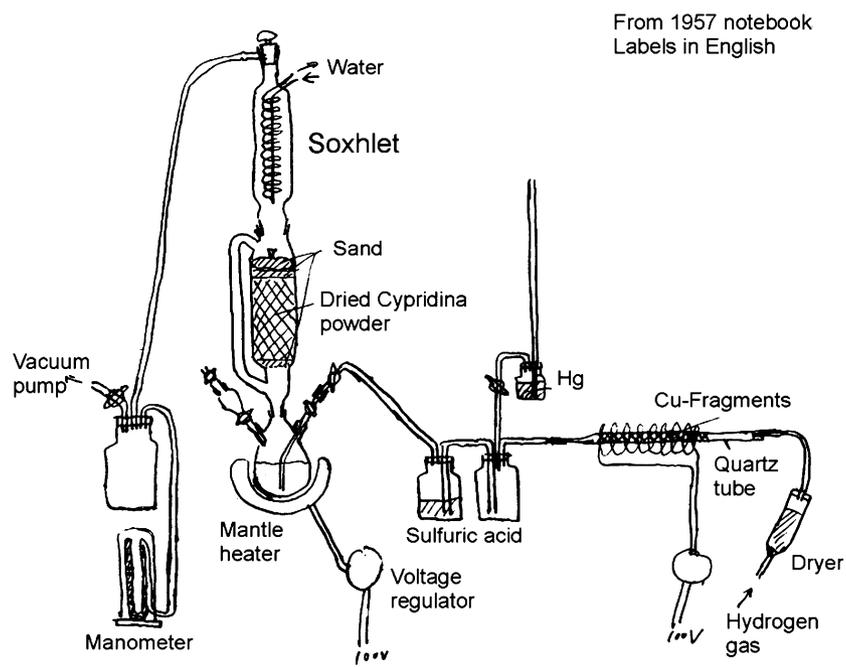


Fig. 2. Our apparatus for extracting luciferin in hydrogen under reduced pressure.

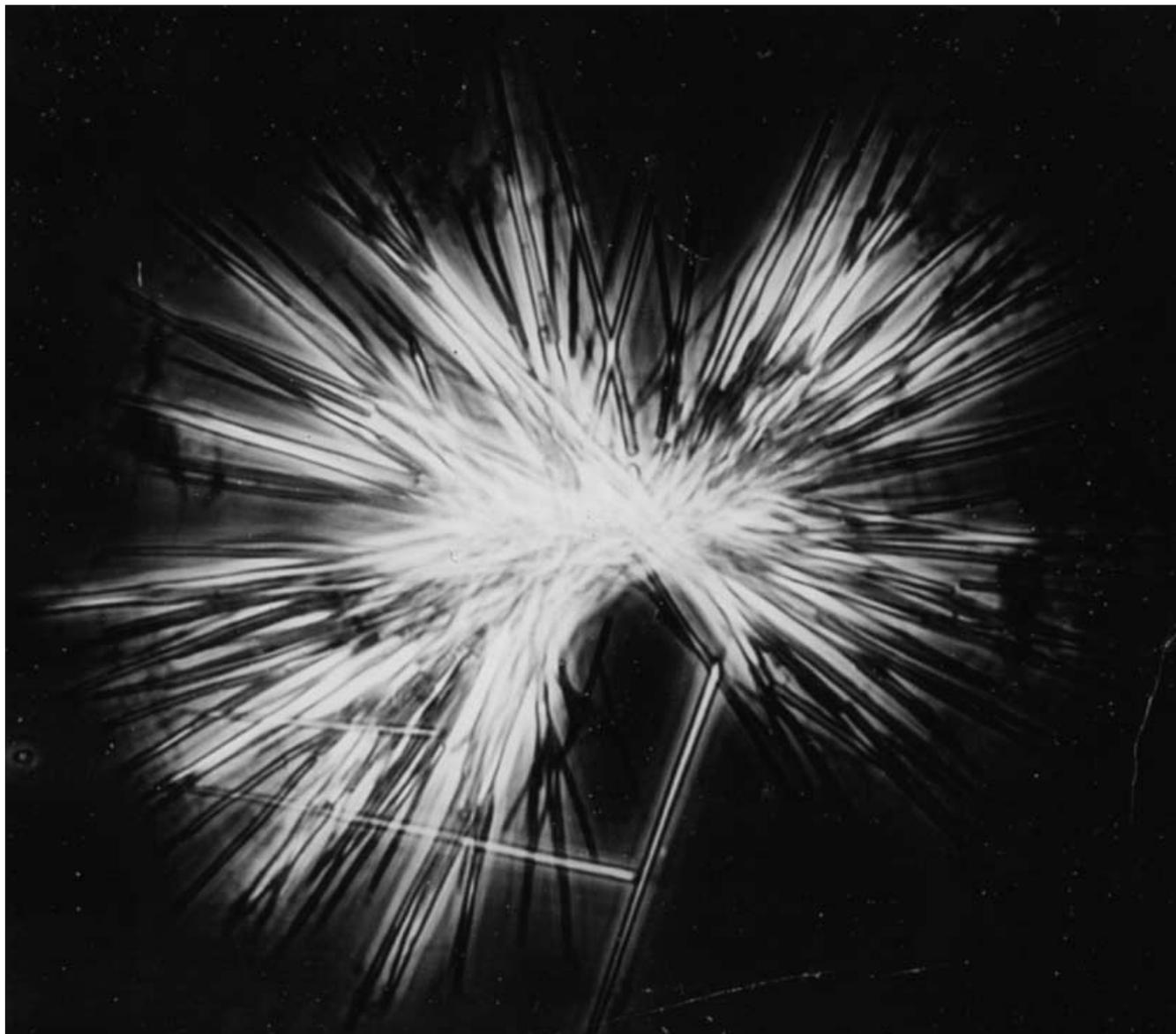


Fig. 3. Crystallized luciferin.

precipitates, and any leftover luciferin became useless by oxidation by the next day. I therefore had to extract and purify fresh batches of luciferin for my further efforts of crystallization. The whole experimental process involved day-and-night work for 7 days. I wasted six or seven batches of purified luciferin in unsuccessful efforts until finally I saw the first crystals of *Cypridina* luciferin on one cold morning of February 1956; however, this occurred in an unexpected way.

On the previous night, I had some leftover purified luciferin after my crystallization attempts. Because I could not think of any further ideas to improve the crystallization, I decided to use the material for amino acid analysis. Thus, I added an equal amount of concentrated hydrochloric acid to the luciferin solution. The colour of the solution instantly changed

from yellow to dark red. Because it was late at night, I went home without heating the sample. The next morning, I saw that the solution had discoloured and become a light orange colour, with a small amount of dark precipitate at the bottom of the test tube. Under the microscope, the precipitate appeared as fine red needle-like crystals, as illustrated in Fig. 3. The result indicated that the luciferin – unusually – could be crystallized in a high concentration of hydrochloric acid. I achieved this unexpected result 10 months after I had begun the crystallization experiments.

This successful crystallization (Shimomura *et al.*, 1957) made it possible to investigate the chemical structure of *Cypridina* luciferin and the mechanism of its luminescence reaction. The complete structures of *Cypridina* luciferin and its oxidation

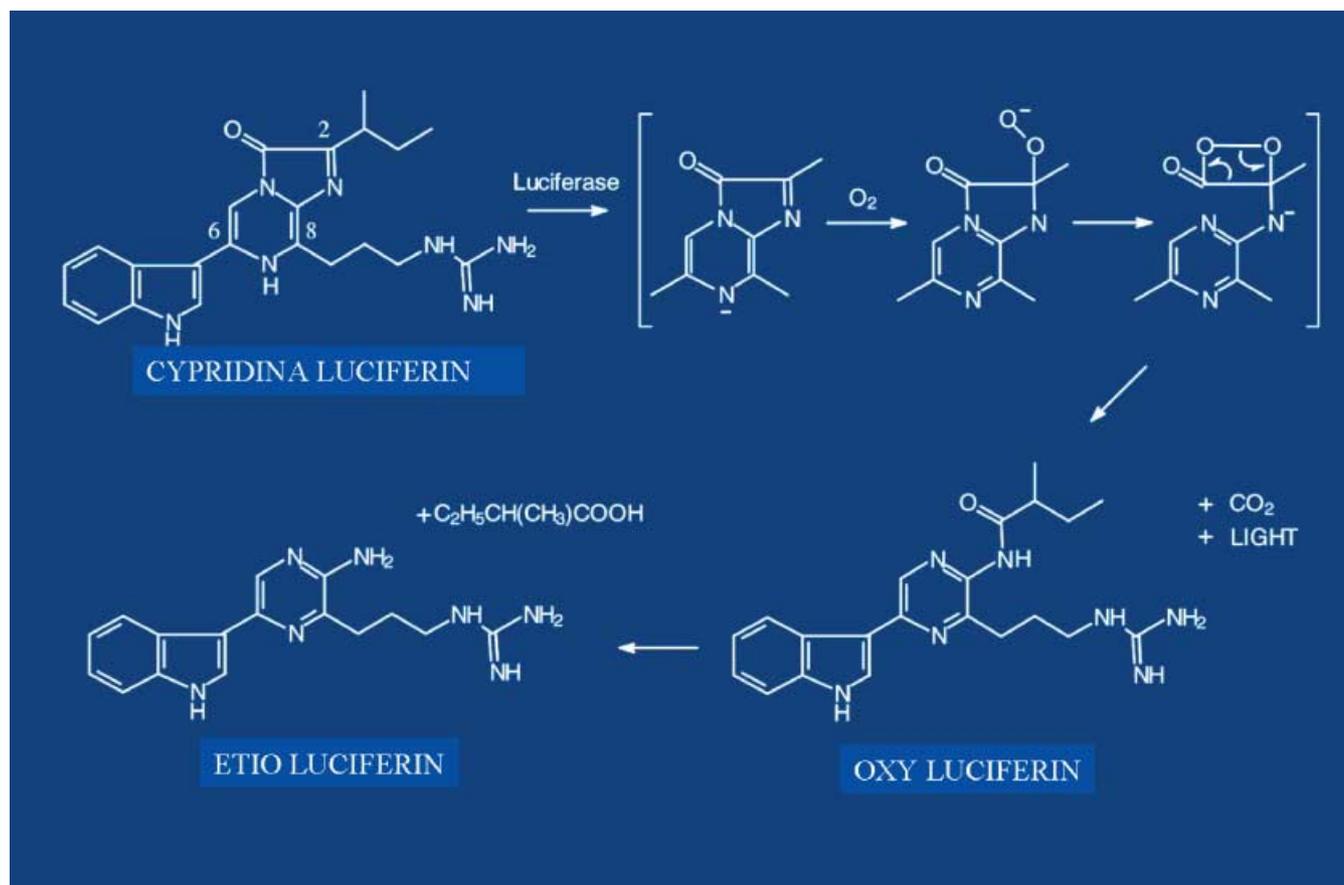


Fig. 4. The luminescence reaction of *Cypridina* luciferin.

process were elucidated 10 years later (Kishi *et al.*, 1966; McCapra & Chang, 1967; Stone, 1968).

The luminescence reaction of *Cypridina* luciferin is illustrated in Fig. 4. Luciferin (Fig. 4, upper left) contains an imidazopyrazinone skeleton. In the presence of luciferase and oxygen, it forms an intermediate peroxide at position 2, followed by its cyclization into a dioxetanone ring. The dioxetanone instantly decomposes into oxyluciferin and CO₂ accompanied by the emission of light. Oxyluciferin contains a 2-aminopyrazine skeleton in its acylated form, and this compound is slowly hydrolysed into etioluciferin containing a 2-aminopyrazine skeleton. It was these structures that made it possible for us to determine the structure of the aequorin chromophore in the early 1970s.

Sadly, in 1959 my boss Dr Yasunaga died of cancer. In the same year, I received an invitation from Dr Frank Johnson of Princeton University to join his laboratory as a research associate. He offered me travel expenses, but I chose to apply for a Fulbright Travel Grant, because in that way I would be better prepared to go to America. Fulbright grants at that time were very well managed by the U.S. State Department and were meticulously planned. I was given a 4-week course in basic English conversation and a 3-day orientation prior to my

departure. In August 1960, I was among the Fulbright recipients onboard *Hikawa-maru* leaving Yokohama for Seattle. At the departure, a thousand well-wishers jammed the pier to see us off with coloured tapes. After 13 days of comfortable voyage to Seattle and then 3 nights of travel by rail across the continent, I arrived at Princeton with great excitement.

Shortly after my arrival, Dr Johnson asked if I would be interested in studying the bioluminescence of the jellyfish *Aequorea*. He showed me a small vial containing white powder, and said that it was a freeze-dried extract of the jellyfish that would emit light if moistened with water. We went into a darkroom and tested it. The powder, however, did not emit any light. Despite this unsuccessful demonstration, I was quite impressed by Dr Johnson's description of the brilliant luminescence of the live jellyfish and the great abundance of specimens at Friday Harbor in the State of Washington. I agreed to study the jellyfish.

In the early summer of 1961, we travelled from Princeton to Friday Harbor. Dr Johnson purchased a new station wagon for the trip and Dr Johnson's assistant Yo Saiga and my wife accompanied us. The car was fully loaded with equipment, chemicals and luggage for four. At the time, the interstate highway was not yet complete and we drove through Chicago



Fig. 5(a). The jellyfish *Aequorea* and its light-emitting organs.

and Minneapolis and then took U.S. route 2 all the way to the west coast. Dr Johnson was the only driver and he drove for 12 h every day. After 7 days of driving, we arrived at Anacortes and then took the ferry to Friday Harbor. The director of Friday Harbor Laboratory at the time was Dr Robert Fernald. He assigned us to Laboratory 1, and we shared a room with three other scientists, one of whom was Dixy Lee Ray, who later became governor of the State of Washington. She was always accompanied by a dog, her well-known trademark, and although the lab was in a sanctuary barred to dogs, she circumvented this by declaring that the animal was her assistant.

The jellyfish *Aequorea* was highly abundant at Friday Harbor. It is shaped like a hemispherical umbrella, measuring 7–10 cm in diameter, as seen in Fig. 5(a,b). A constant stream of floating jellyfish passed along the side of the lab dock every morning and evening, riding with the current caused by the tide. Sometimes they were extremely abundant, covering the surface of the water. We carefully scooped up the jellyfish using a shallow dip-net. The light-emitting organs are located along the edge of the umbrella (Fig. 5c); the luminescence image shown in Fig. 5(d) illustrates their exact position. Thus, the margin of the umbrella

containing light-emitting organs can be cut off with a pair of scissors, making a strip of 2–3 mm wide that we termed a 'ring'. When the rings obtained from 20 or 30 jellyfish were squeezed through a rayon gauze, a turbid liquid was obtained, which we called the 'squeezeate'. The squeezeate contained granular light-emitting organs, but it was only very dimly luminescent. However, the luminescence significantly increased when the squeezeate was added to water and the granules were cytolysed.

We tried to extract luminescent substances, luciferin and luciferase, from the squeezeate by various methods, but all our efforts failed. We ran out of ideas after only a few days of work. I was convinced that the cause of our trouble was the luciferin–luciferase hypothesis that dominated our thinking. I suggested to Dr Johnson that we should forget the idea of extracting luciferin and luciferase and, instead, try to isolate the luminescent substance regardless of what it might be. However, I was unable to convince him. He did not support my plan because it had neither theoretical or experimental backing. Because of the disagreement on experimental procedure, I started to work alone at one side of a table, while, on the other side, Dr Johnson and his assistant continued their

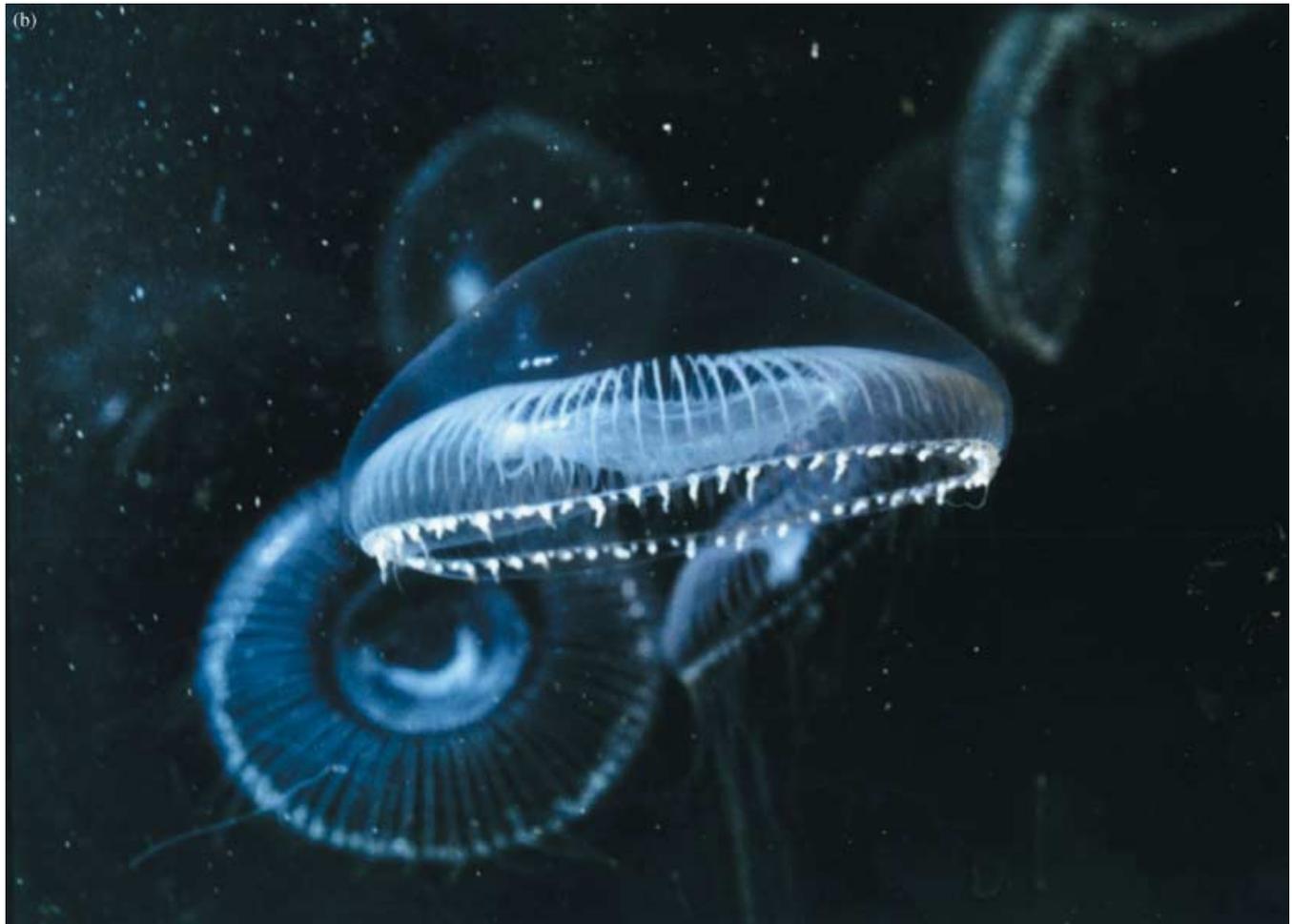


Fig. 5(b). *Continued.*

efforts to extract luciferin by grinding luminous tissues with sand. It was an awkward situation.

The principle in the isolation of bioluminescent substances is simple, but it is not always easy in practice. To avoid the loss of luminescence activity, the luminescent substance is extracted from the tissue under conditions that reversibly inhibit luminescence, or that cause a selective inactivation or removal of a co-factor necessary for light emission. For example, in the case of a luciferin–luciferase system, the luciferin is usually extracted with methanol or boiling water, which inhibits luminescence by inactivating the luciferase. The luciferase itself can then be obtained from cold aqueous extract after the luciferin has been exhausted by spontaneous luminescence reactions. If a co-factor is involved in light emission, its exhaustion causes luminescence to stop, as in the case of the co-factor ATP in firefly bioluminescence.

In the case of *Aequorea*, however, I could not find any condition that would reversibly inhibit luminescence. I tried known enzymatic co-factors, such as ATP, FMN and NADH, but none showed any activation effect, indicating that they were not

involved. I tried various enzyme inhibitors and they either had no effect or completely destroyed the ability of the material to luminesce. I became conceptually exhausted and could not come up with one single further idea.

I spent the next several days soul-searching, trying to imagine the reaction that might occur in luminescing jellyfish and searching for a way to extract the luminescent principle. I often meditated on the problem on a drifting rowing boat under clear summer skies. Friday Harbor in summer at that time was quiet and peaceful, quite different from the present-day, when it is saturated with busy pleasure boats and noisy seaplanes. Meditation afloat was safe but if I fell asleep the boat was carried away by the tide so that I had to row for a long time to get back to the laboratory.

One afternoon, a thought suddenly struck me, a thought so simple that I should have had it much sooner: even if a luciferin–luciferase system is not involved in the jellyfish luminescence, an enzyme or protein is probably involved in the luminescence reaction. If so, the activity of this enzyme or protein can probably be altered by a pH change. There might

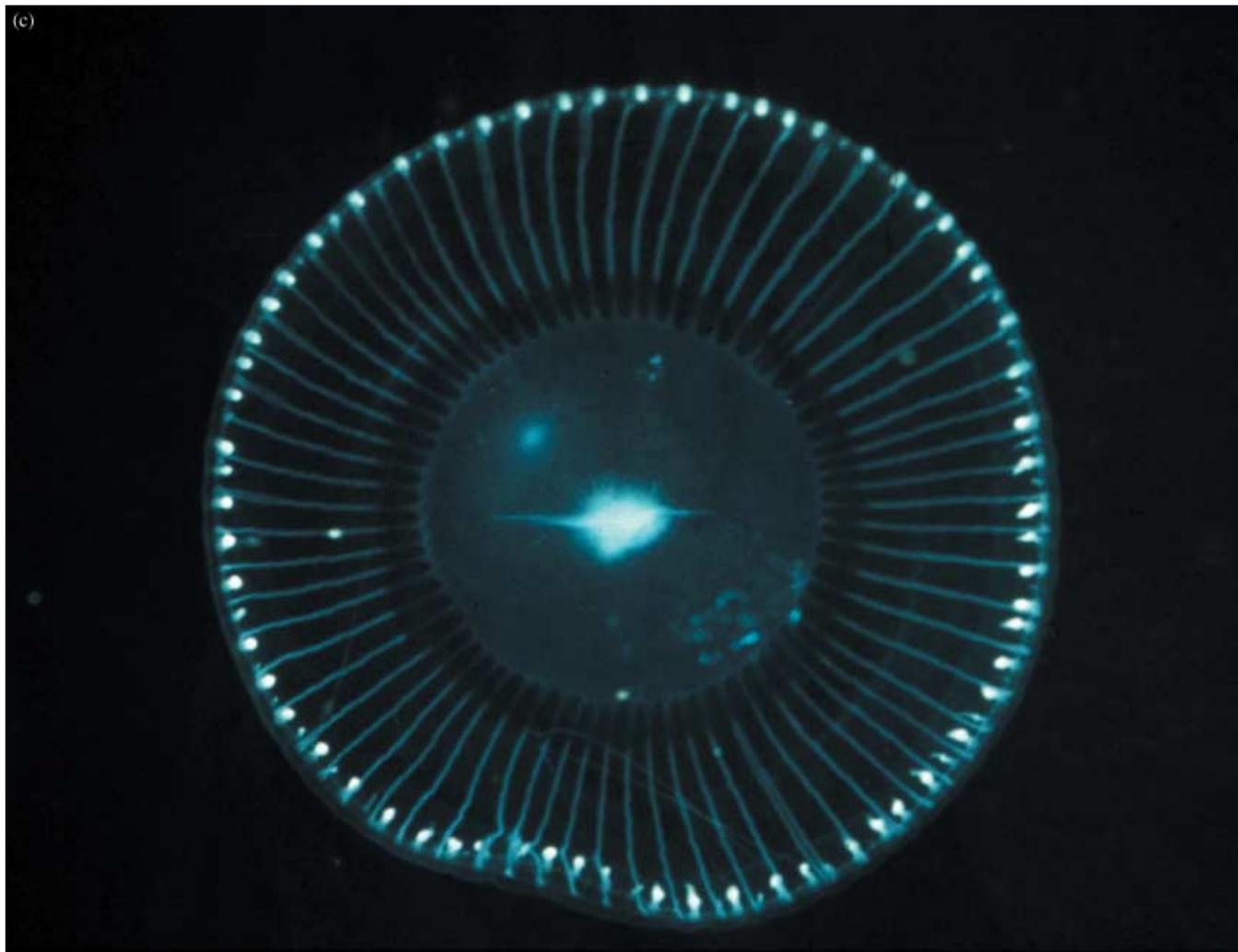


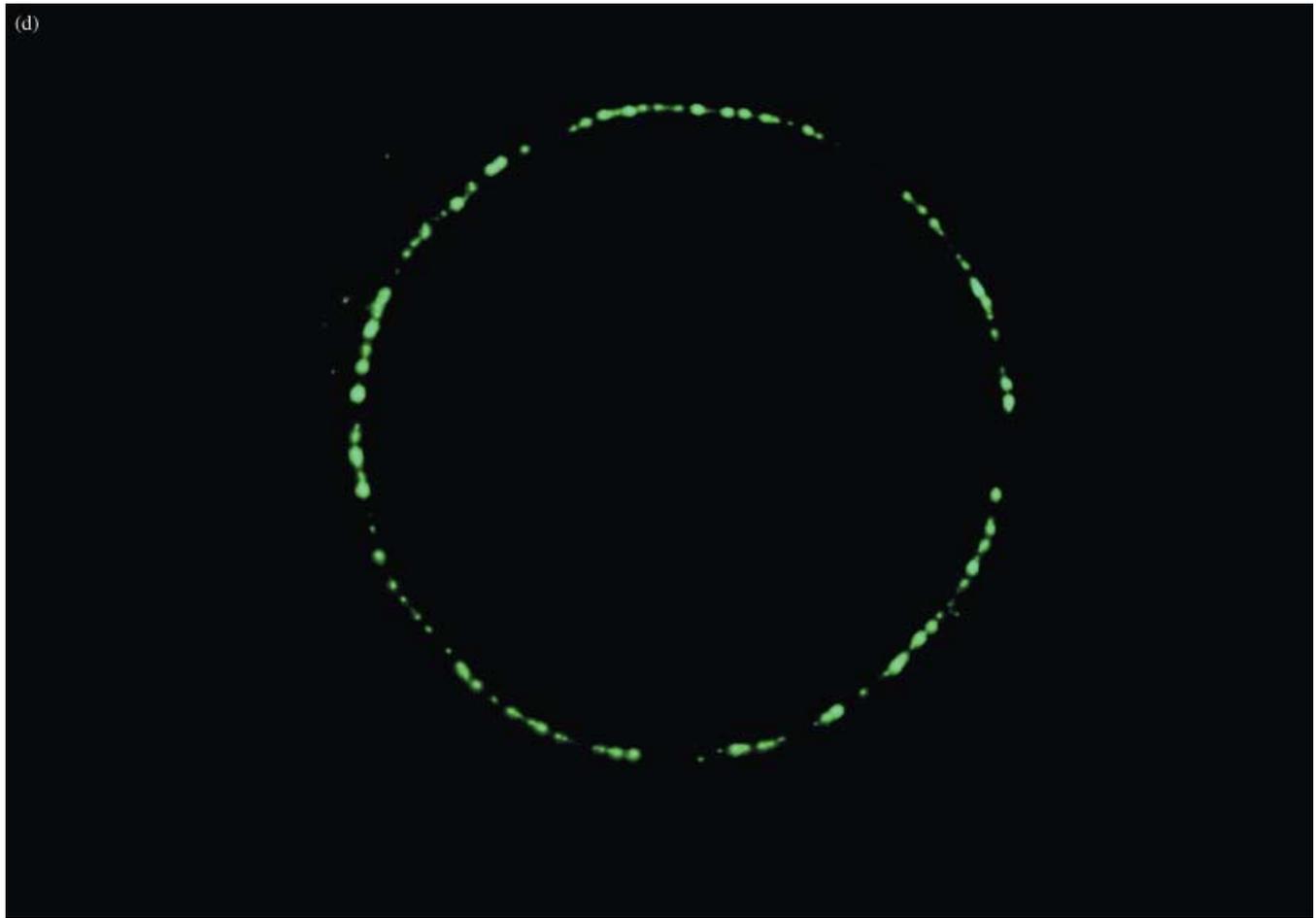
Fig. 5(c). *Continued.*

be a certain degree of acidity at which an enzyme or protein is reversibly inactivated. I immediately went back to the lab, made a squeezeate, and tested a small portion of it with acetate buffer solutions of various acidities. I found that the squeezeate was luminous at pH 6 and pH 5, but not at pH 4. I filtered the rest of squeezeate, and mixed the solid part containing granular light-emitting organs with pH 4 buffer. After 2–3 min, when the cells were cytolysed, I filtered the mixture. The filtrate, now free of cells and debris, was almost without luminescence, but it regained its luminescence when neutralized with a small amount of sodium bicarbonate. The experiment showed that the luminescence substance was reversibly inhibited at pH 4, and it was extracted into the solution, indicating that I had succeeded in extracting the bioluminescence substance, at least in principle.

A surprise came very shortly afterwards. I threw the solution I had just tested into a sink, and the inside of the sink lit up with a bright blue flash. As the overflow from an aquarium

was flowing into the sink, I thought that it was the presence of the seawater that had caused the luminescence. So I mixed a small amount of seawater with the extract: it gave explosively strong luminescence. As the composition of seawater is known, I easily worked out that the activator was Ca^{2+} . I called Dr Johnson and repeated the experiment for him. The discovery of Ca^{2+} as the activator in turn suggested that EDTA should serve as a better inhibitor of luminescence than acidification. It was the first important breakthrough in the study of aequorin.

We devised a simple procedure to extract the luminescence substance from the jellyfish using EDTA (Fig. 6). Our next task was to catch and process as many jellyfish as possible. We collected jellyfish from 6 a.m. to 8 a.m., then after a quick breakfast we would cut rings from the jellyfish until noon. We devoted all afternoon to the extraction. After dinner, we again collected jellyfish from 7 p.m. to 9 p.m., and the catch was kept in an aquarium for the next day. We soon found that the bottleneck in the operation was the step of cutting rings with

Fig. 5(d). *Continued.*

scissors, which is a delicate and very slow process. Even after considerable practice, it took more than 1 min to cut one jellyfish ring, and 3 h work by four people could not produce more than 500 rings. To increase productivity, we hired several highschool students, trained them and paid them 2 cents for each ring they cut. We also decided to buy jellyfish from local children, paying a penny per jellyfish. However, just when our operation was in full swing, the jellyfish suddenly vanished from the area. We therefore extracted only about 10 000 specimens of *Aequorea* in the summer of 1961.

We returned to Princeton with the jellyfish extract packed in dry-ice, and then began purification of the light-emitting principle by numerous repetitions of column chromatography. The purification was completed in early 1962, with a total yield of 5 mg of protein, of which only 1 mg was highly purified. During the purification of aequorin, another protein with a bright green fluorescence was separated and also purified. It was later named GFP. The luminescent substance was a protein with a molecular weight of about 20 000, and it emitted blue light when a trace of Ca^{2+} was added, even in the absence of oxygen. We named the protein 'aequorin' (Shimomura *et al.*, 1962).

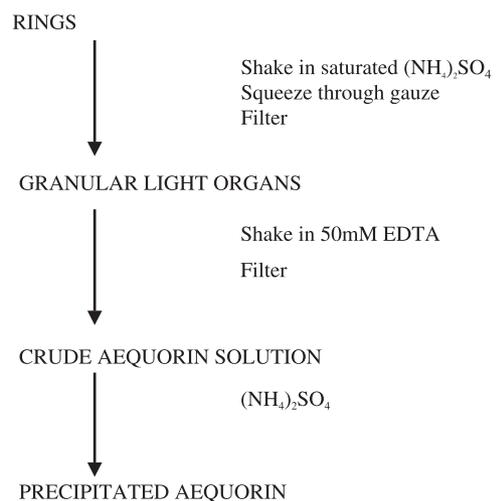
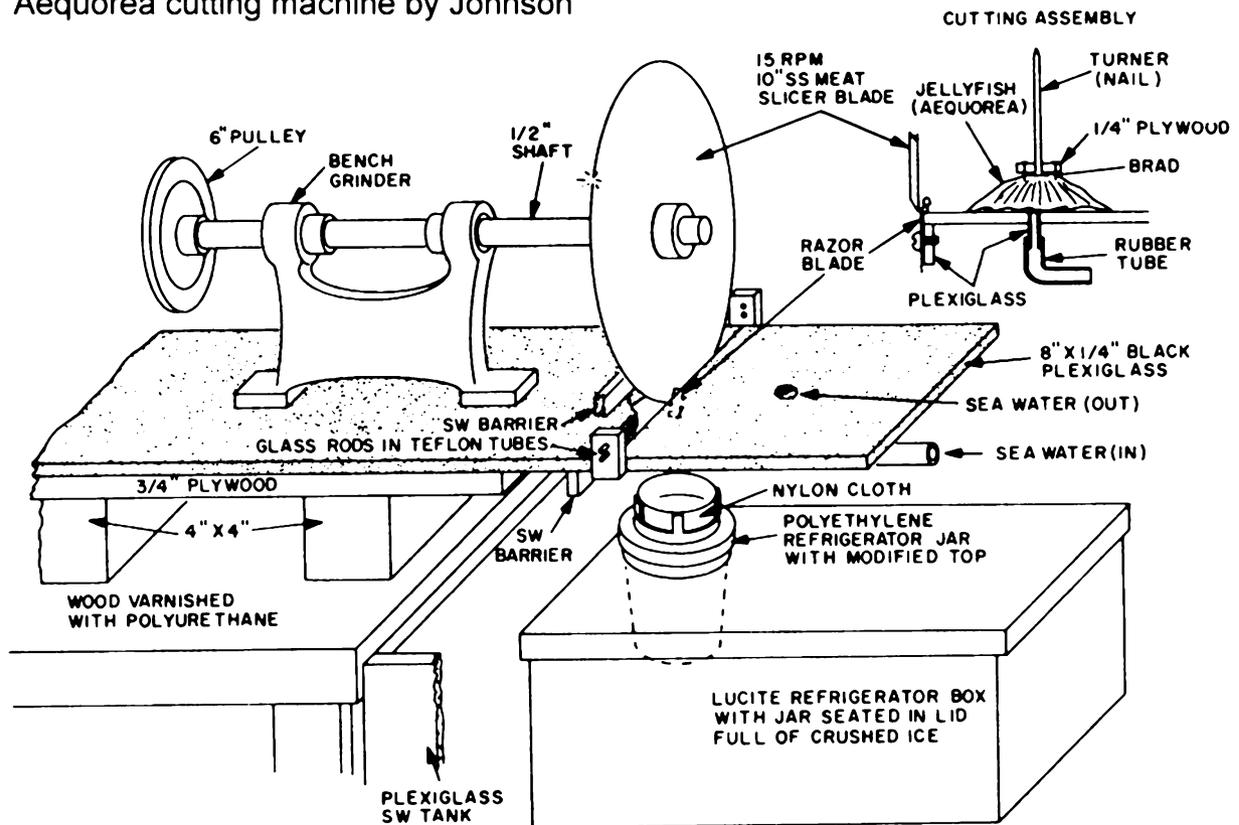


Fig. 6. Our method to extract the luminescence substance from *Aequorea* using EDTA.

Aequorea cutting machine by Johnson



1969

Fig. 7. Our apparatus for cutting the light-emitting rings from *Aequorea*. F. H. Johnson, Naval Research Reviews, February 1970, 16–23 (Office of Naval Research, Dept. of Navy, Washington, DC).

Aequorin is an extraordinary protein that stores a large amount of energy and releases the energy when calcium is added, resembling a charged battery that releases its charged energy by a short circuit. We were greatly interested in studying the mechanism of its luminescence reaction. However, every attempt to extract the chromophore of aequorin resulted in the self-destruction of the chromophore by an intramolecular reaction. We therefore decided to postpone further study on aequorin.

Five years later, Ridgway & Ashley (1967) reported the first biological application of aequorin. They observed transient Ca^{2+} signals in single muscle fibres of barnacle with microinjected aequorin. The importance of aequorin became obvious and we decided to resume our study. However, there was no way to extract the native chromophore. Therefore, we aimed at a fragment of the chromophore that is formed when aequorin is denatured with urea in the presence of 2-mercaptethanol. This compound was blue-fluorescent and named AF-350 based on its absorption maximum at 350 nm (Shimomura & Johnson, 1969).

To obtain 1 mg of AF-350, 100–200 mg of pure aequorin was required, which would require about 2.5 tons of jellyfish or 50 000 animals. To process 50 000 jellyfish in one summer,

we would need to collect, cut and extract 3000 jellyfish each day, allowing for days of bad weather and poor fishing. It was a workload that could not be accomplished by collecting jellyfish at the lab dock and cutting rings with scissors at a rate of one per minute. To solve the problem, Dr Johnson constructed two jellyfish cutting machines, as illustrated in Fig. 7. The machine had a rotating meat slicer blade; jellyfish were placed on the cutting platform and, using the jellyfish turner, the jellyfish was rotated against the slicer blade by hand. The ring was cleanly cut and dropped into a receiving jar. With these machines we could cut 1200 rings in an hour. We also exploited new fishing grounds at the town dock to obtain more jellyfish and buckets filled with jellyfish were transported to the lab usually by car and sometimes by Boston whaler.

After five years of hard work and with hundreds of thousands of jellyfish, the chemical structure of AF-350 was finally determined (Shimomura & Johnson, 1972). The result was surprising: AF-350 and *Cypridina* etioluciferin have the same 2-aminopyrazine skeleton, as illustrated in Fig. 8. The structure of AF-350 (Fig. 8c) contained the skeleton of a 2-aminopyrazine, the skeleton identical with that which exists in *Cypridina* oxyluciferin (Fig. 8b) and etioluciferin (Fig. 8d),

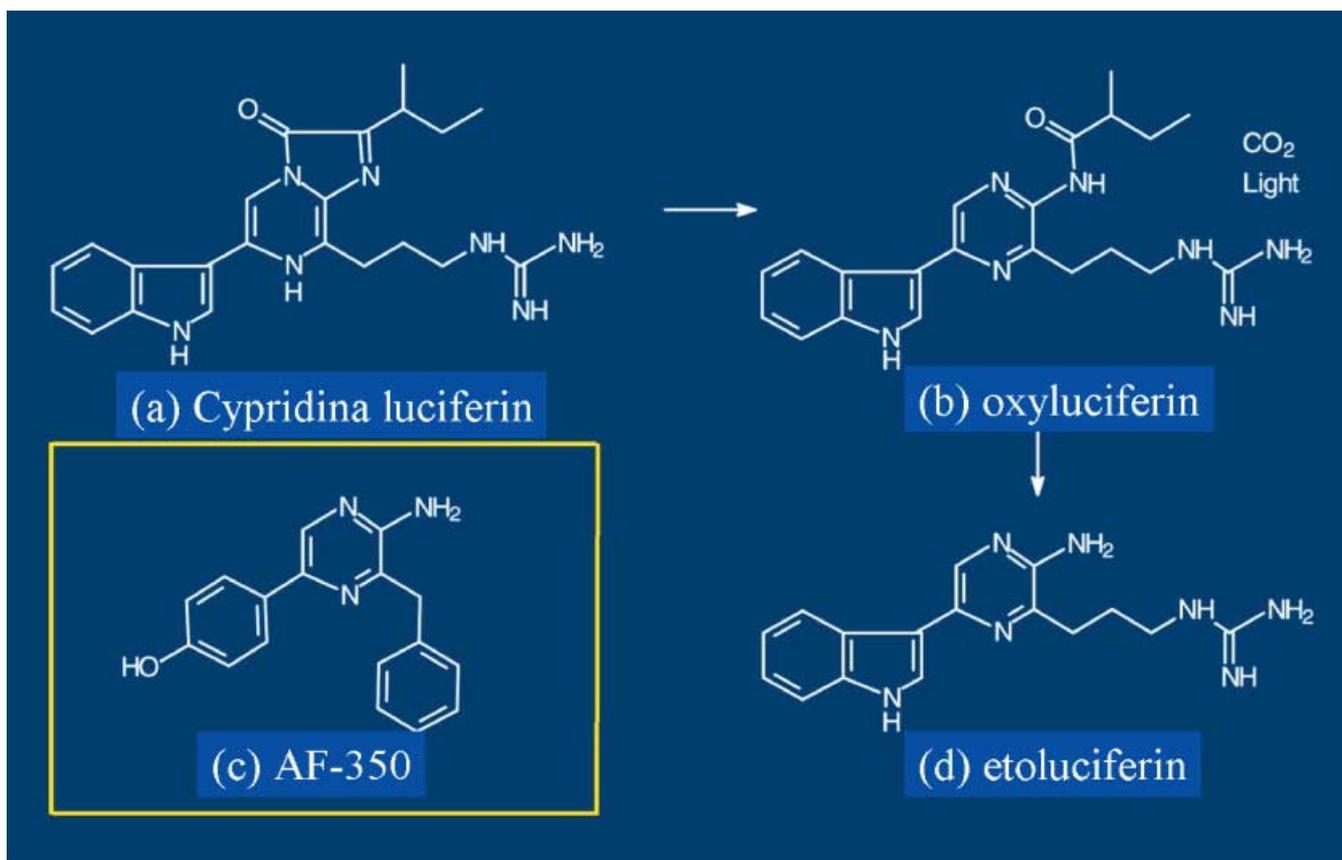


Fig. 8. The chemical structure of AF-350.

although the side chains are different. The resemblance suggested a close relationship between the luminescence systems of *Aequorea* and *Cypridina*. Thus, we looked for a compound that corresponds to *Cypridina* oxyluciferin containing acylated 2-aminopyrazine in *Aequorea*. We found it in the spent solution of luminesced aequorin (Shimomura & Johnson, 1973). It was AF-350 acylated with *p*-hydroxyphenylacetyl group; this compound was later named 'coelenteramide' (Shimomura & Johnson, 1975a).

The result pointed to the involvement of a hypothetical compound coelenterazine as the possible chromophore of aequorin (Shimomura *et al.*, 1974) and this was a pivotal step in our research on aequorin. In the absence of the information on *Cypridina* luminescence, we could not have found the structure of the aequorin chromophore. Although coelenterazine was a hypothetical substance when the structure was first presented, the actual substance was soon isolated from the luminous squid *Watasenia* and chemically synthesized by Shoji Inoue and his collaborators (Inoue *et al.*, 1975). Presently, coelenterazine is known as the luciferin of many kinds of luminous organisms (Shimomura *et al.*, 1980; Campbell & Herring, 1990), and it can be also found in various non-luminous organisms, such as sardine and herring (Shimomura, 1987), possibly as a result of a food-chain relationship.

In 1975, we found that apoaequorin in the spent solution of aequorin could be regenerated into original aequorin by treating it with coelenterazine in the presence of oxygen (Shimomura & Johnson, 1975b). This finding proved that aequorin contains a coelenterazine moiety. In 1978, based on various properties and reactions of aequorin, we proposed that aequorin contains coelenterazine-2-peroxide (Shimomura & Johnson, 1978), which was confirmed later by carbon-13 NMR spectrometry (Musicki *et al.*, 1986). This discovery provided us with a rough structural image of the aequorin molecule.

The cloning and expression of apoaequorin cDNA were accomplished between 1985 and 1987 by two groups (Inouye *et al.*, 1985, 1986; Prasher *et al.*, 1985) and when it became possible to produce recombinant aequorin in living cells the supply of natural aequorin became unnecessary. Mysteriously, two or three years later, the once abundant *Aequorea* around Friday Harbor suddenly and almost completely disappeared from the area. Since then, it has been difficult to collect even a few specimens of *Aequorea*. If this had happened 20 years sooner we would never have been able to solve the puzzle of aequorin luminescence.

In the late 1980s, we prepared more than 30 kinds of modified forms of aequorins by replacing the coelenterazine moiety of aequorin with various analogues of coelenterazine

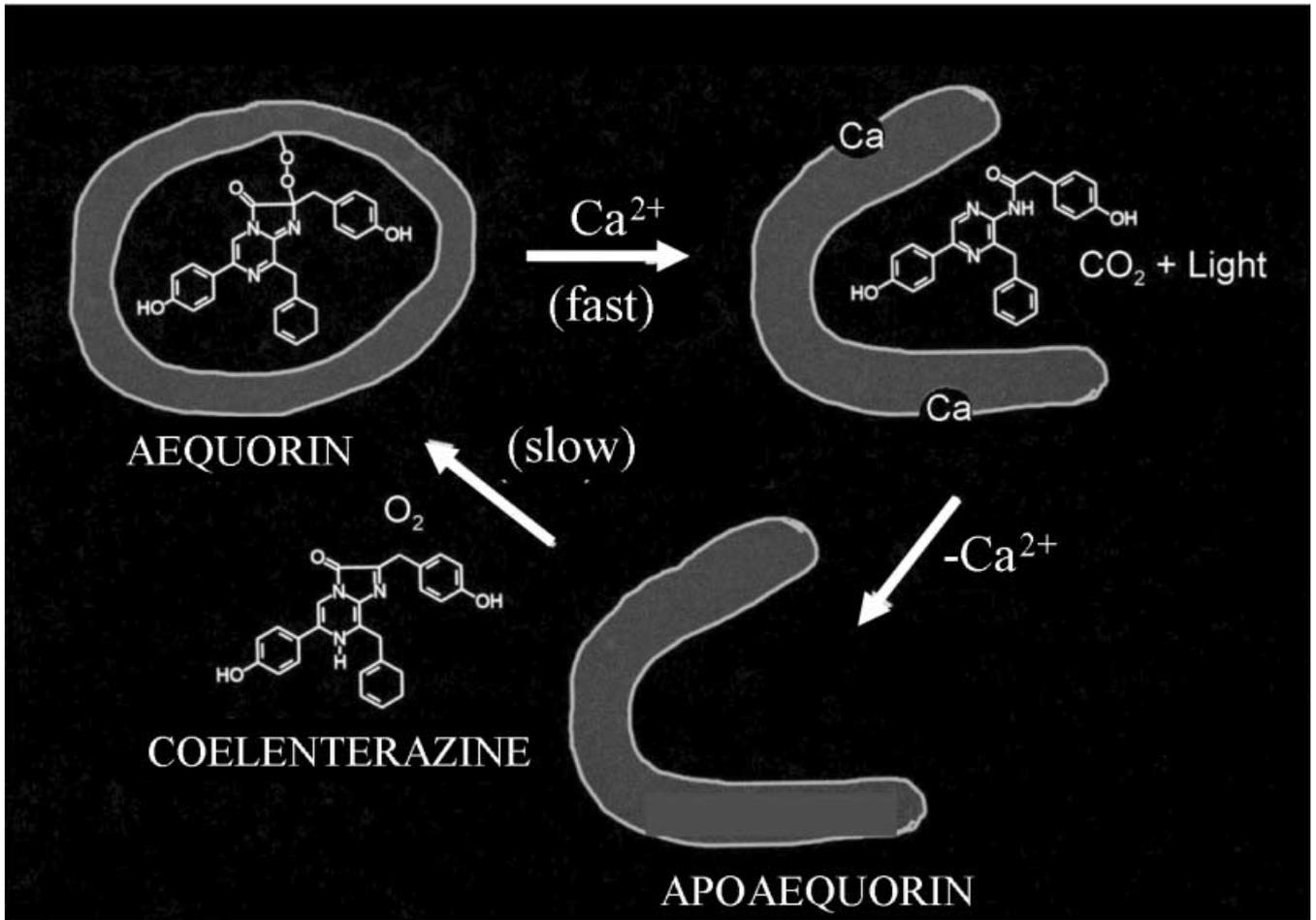


Fig. 9. The reactions involving aequorin.

(Shimomura *et al.*, 1988, 1989, 1993). Those aequorins, termed semisynthetic aequorins, have different calcium sensitivities. During the same period, I learned that attempts at X-ray crystallography of aequorin were in progress at three different laboratories. I waited for their results, but none appeared and so in 1997 we decided to take up the X-ray project ourselves and organized a team. We first developed a new technique to produce high-purity recombinant aequorin (Shimomura & Inouye, 1999) and then the X-ray structure of aequorin was finally determined in 2000 (Head *et al.*, 2000). The X-ray structure revealed that aequorin is a globular protein. The coelenterazine moiety is shielded in the central cavity of the protein and the outside molecules have no access to the coelenterazine moiety. The peroxide group of the coelenterazine moiety is stabilized by hydrogen bonds to the protein residues that include Tyrosine 184. Figure 9 illustrates the overall aequorin reactions. Binding of two Ca^{2+} ions to aequorin causes conformational changes of the protein part of aequorin, resulting in the opening of the protein and the decomposition of coelenterazine peroxide into coelenteramide and CO_2 ,

accompanied by emission of light. Apoaequorin can be regenerated into aequorin with coelenterazine and oxygen.

With respect to GFP, Davenport and Nicol first described green fluorescence of the light organs of *Aequorea* in 1955 (Davenport & Nicol, 1955). In 1961, we found during the purification of aequorin that the green fluorescent substance in *Aequorea* is a protein (Shimomura *et al.*, 1962). We called the protein 'green protein' because its precipitated form is green under room light. Hastings & Morin (1969), however, called it 'green fluorescent protein', and I thought the name was more appropriate than 'green protein'. Morin & Hastings (1971) suggested that the emission of green luminescence from *Aequorea* involves a Förster-type energy transfer from aequorin to GFP. In 1974, we purified GFP completely and crystallized it (Morize *et al.*, 1974) (Fig. 10).

In 1979, we elucidated the structure of the GFP chromophore (Shimomura, 1979). In this work, we first denatured GFP by heat then digested the denatured GFP with papain. From the digest, we isolated and purified a fragment containing the chromophore. At the moment I saw the absorption

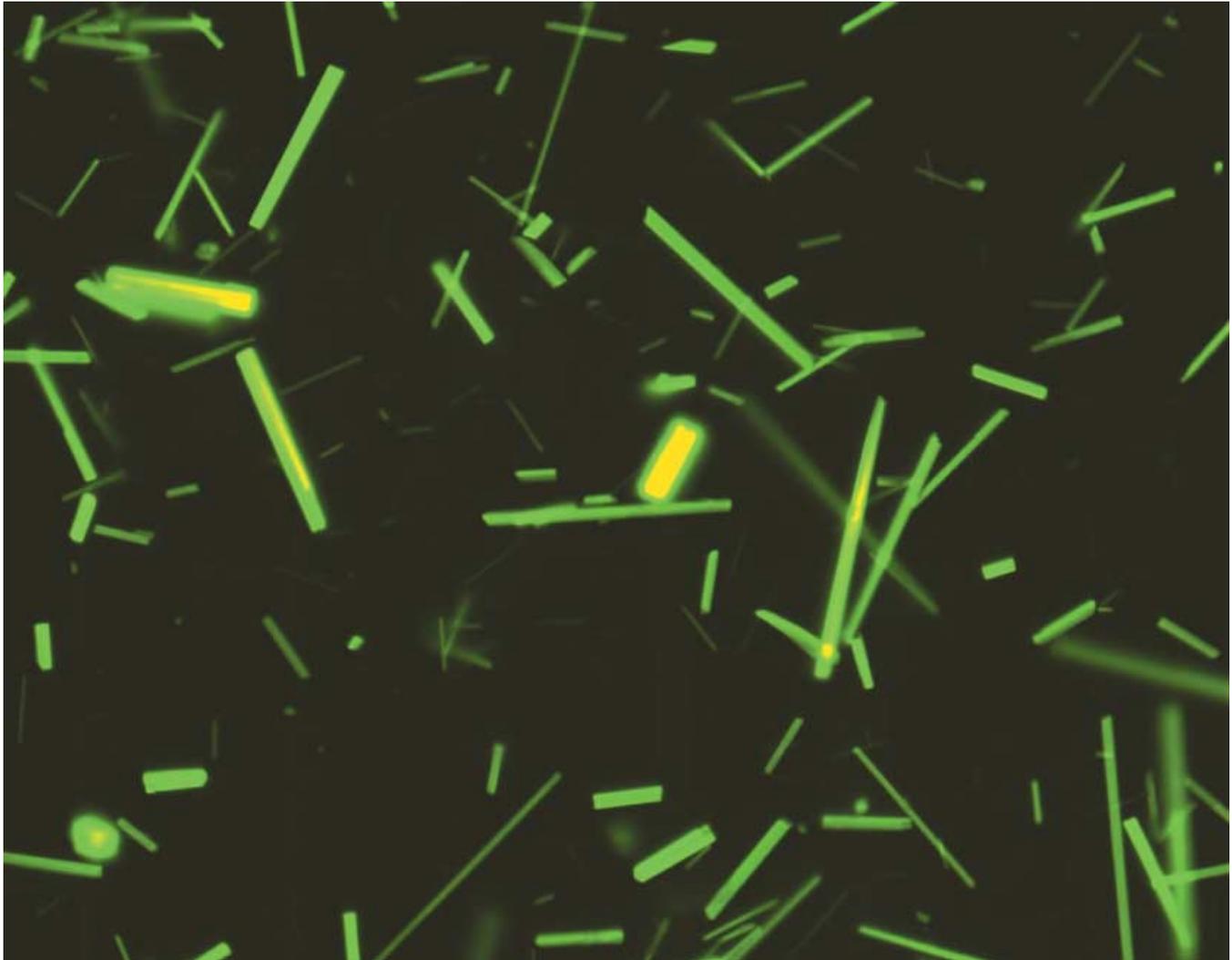


Fig. 10. Crystallized GFP. Photo by Shinya Inouye.

spectrum of the purified chromophore, I recognized its resemblance to the spectrum of an imidazolone that I had synthesized during my study of *Cypridina* luciferin in the late 1950s (Shimomura & Eguchi, 1960). Acid hydrolysis of the chromophore produced *p*-hydroxybenzaldehyde, and several amino acids suggested the presence of *p*-hydroxybenzal group. Therefore, I synthesized a second compound, the spectral characteristics of which satisfactorily matched those of the GFP chromophore. From this, I deduced the structure of the GFP chromophore. In the same year, I learned that Dr Bill Ward was studying GFP and I decided to discontinue my work on GFP to concentrate on my studies of bioluminescence.

Since then, the structure of GFP chromophore has been verified by Cody *et al.* (1993). The X-ray structure of GFP was solved in 1996 by two different groups (Ormo *et al.*, 1996; Yang *et al.*, 1996). cDNA of *Aequorea* GFP was cloned by Prasher *et al.* (1992) and expressed in living organisms by Chalfie *et al.* (1994) and also by Inouye & Tsuji (1994). The

successful expression of GFP has established the basis of using GFP as a marker protein for gene expression, resulting in the present widespread popularity of GFP. The usefulness of GFP has been further enhanced by the discovery of red-fluorescent GFP-like proteins in anthozoan corals (Matz *et al.*, 1999) and also by various improvements and developments. I am, as an original co-discoverer of this protein, very happy to see the growing usefulness of GFP.

In retrospect, my work on *Aequorea* was helped by many people in various ways and I was certainly fortunate. My three mentors played decisive roles in my life and research. In 1955, Dr Yasunaga sent me to Professor Hirata's lab in Nagoya, giving me a career as a scientist instead of a pharmacist. Professor Hirata gave me the subject of *Cypridina* luciferin to study. My first research work, the crystallization of *Cypridina* luciferin, was very difficult, and I succeeded only after 10 months of very hard effort. Even if my success was accidental, it gave me self-confidence, and a feeling of 'if it is not impossible, then I

can do it'. Then in 1961, Dr Frank Johnson gave me the subject of *Aequorea* to study, and in this bioluminescence system, the information on *Cypridina* luminescence was essential in determining the chromophore structure of aequorin. It was a lucky coincidence that the *Cypridina* project had come first. I believe, however, that my self-belief supported me in solving the difficult problems involved in the study of aequorin. Based on my experience, I think it is important not to give up when a difficult problem is encountered in research. Overcoming a difficulty may reward us with a self-belief that could be more valuable than the solution to the problem.

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