Reference: *Biol. Bull.* **189:** 1–5. (August, 1995)

A Short Story of Aequorin

OSAMU SHIMOMURA

Marine Biological Laboratory, Woods Hole, Massachusetts 02543

Discovery of Aequorin

One day in the fall of 1960, shortly after my arrival at Princeton from Japan, Dr. Frank Johnson showed me a small jar containing a spoonful of white powder. He explained that the powder was a freeze dried "squeezate" made from the luminous jellyfish *Aequorea,* and that it would emit light when mixed with water. He asked me if I would be interested in studying the bioluminescence of this jellyfish. The powder did not emit any light when moistened. But I was quite impressed by Dr. Johnson's description of the brilliant luminescence of live jellyfish and the great abundance of specimens around Friday Harbor, Washington. So my response was a definite "yes." My experience in bioluminescence research at the time was meager and limited to only the luminescent system of the ostracod *Cypridina.* I imagined, vaguely, that the jellyfish would probably contain a kind of luciferin and a luciferase, possibly with one of the cofactors, such as ATP, FMN, or NADH, like the fireflies, luminous bacteria, and *Cypridina* that were known at that time.

In the early summer of 1961, we traveled from Princeton to Friday Harbor in Dr. Johnson's station wagon, which he had newly purchased for the excursion. The car was fully loaded with necessary equipment and chemicals, including a MacNichol integrating photometer of gigantic size (a two-foot cube), and four travelers (my wife and Yo Saiga, an assistant, came along) with all of their baggage on the roof. It took us seven days to the West Coast, through Chicago and Glacier National Park. Dr. Johnson was the only driver throughout the trip, driving 12 hours a day with an admirable toughness.

Upon arrival at the Friday Harbor Laboratories, we were welcomed by Dr. Robert Fernald, Director of the Lab. We set up our work space in Lab 1, a small building consisting of two rooms, and we started to work. There were three other scientists in the room, and one of them was Dr. Dixy Lee Ray, future governor of Washington State, who was always accompanied by a dog, her well-known trademark. The laboratory area was a sanctuary prohibited to common dogs, but she declared that the animal was her assistant, not a dog.

The jellyfish were abundant. 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. We carefully scooped up the jellyfish into buckets, one by one, using a shallow dip-net. The specimens of *Aequorea* are shaped like hemispherical umbrellas and are nearly transparent. An average specimen measures 3–4 inches in diameter and weighs about 50 g. The light organs—about 100 granules—are distributed evenly along the edge of the umbrella. Thus, the margin of the umbrella containing light organs could be easily cut off with a pair of scissors, yielding a thin strip called a "ring." When the rings obtained from 20–30 jellyfish were squeezed through a rayon gauze, a liquid called "squeezate" was obtained. The squeezate was only dimly luminescent, but when it was diluted with water, the luminescence increased significantly for a period of 5–10 minutes, as the granular light organs were cytolyzed.

We tried to extract luminescent substance from the squeezate by every thinkable method, but all failed; and we ran out of ideas after only a few days of work. Convinced that the cause of our failure 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 whatever it might be. I was, however, unable to convince him. He did not agree with my idea, which had neither theoretical backing nor experimental support. Because of the disagreement on the experimental procedure, I started to work alone at one side of a table while, on the other side, Dr. Johnson and his assistant Yo Saiga continued their efforts to extract a luciferin by grinding luminous tissues with sand. It was an awkward situation.

Received 23 March 1995; accepted 5 June 1995.

The basic principle of isolating a bioluminescent substance is to extract it from the tissue under conditions that reversibly inhibit luminescence, or that cause a selective inactivation, consumption, or removal of a component necessary for light emission. In the case of a luciferin-luciferase system, for example, the luciferin is usually extracted with methanol, which stops luminescence by inactivating the luciferase. And the luciferase can be obtained from an aqueous extract after the luciferin has been exhausted by several minutes of spontaneous luminescence reactions. If a cofactor is involved in light emission, its removal or exhaustion can cause a reversible inhibition of luminescence, as in the case of the firefly bioluminescence system that requires ATP as the cofactor.

In the case of the jellyfish *Aequorea,* however, the presumed lack of luciferin and luciferase severely limited the range of techniques usable for the extraction of the light-emitting principle. I did believe that jellyfish luminescence requires molecular oxygen like all other known bioluminescence systems (later proved to be incorrect!), but we had no other information about the luminescence system or cofactor requirements. In an effort to find a way to extract the luminescent principle, I tried to inhibit the luminescence of the squeezate by using the anaerobic conditions that were created by vacuum or by the addition of reducing agents, but nothing worked. The results forced me to assume that the jellyfish system, like that of *Cypridina,* requires a very low oxygen tension—a level less than that attainable in my evacuated container. Furthermore, all of the known enzymatic cofactors, such as ATP, FMN, and DPNH, showed no effect on the luminescence when added. Finally, the only recourse was to try various chemicals available in the stockroom of the Lab, with the hope that one would reversibly inhibit the luminescence. This was clearly an approach that relied entirely on good luck, and I was not surprised when all of my efforts failed. I was conceptually exhausted, and could not come up with one further idea.

I spent the next several days soul-searching, trying to imagine the reaction that occurs in luminescing jellyfish and searching for a way to extract the luminescent principle. I often meditated on a drifting rowboat under the clear summer sky. Friday Harbor in summer, at that time, was quiet and peaceful, differing from the present-day scene that is almost saturated with busy pleasure boats and noisy seaplanes. A rowboat always has the right of way over one with a motor, so nobody disturbed my drifting vessel; even large ferries saved me a wide berth. Thus, meditation afloat was safe, but if I fell asleep and the boat was carried away by the tidal current, then I had to row for a long time to get back to the Lab.

One afternoon on the boat, 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, another enzyme or protein is very probably involved directly in the light-emitting reaction. If so, the activity of this enzyme or protein can probably be altered by a pH change, at least to some extent. Indeed, there might be a certain level of acidity at which an enzyme or protein could be reversibly inactivated."

I immediately went back to the lab and made a squeezate. Then, I mixed a small portion of the squeezate with acidified water containing various amounts of acetic acid. The resultant mixtures at pH 6.0 and pH 5.0 were clearly luminous, but at pH 4.0, I saw no luminescence. I filtered off the liquid from the rest of squeezate and mixed the solid part, containing the granules of light organs, with water of pH 4.0. After the mixture was filtered, the filtrate, now free of cells and debris, was nearly dark, but it regained its luminescence upon neutralization with a small amount of sodium bicarbonate. Indeed, the experiment showed that the luminescent substance of the jellyfish can be extracted.

But my real surprise came in the next moment, when I added a small amount of seawater to the solution and saw that its light became explosively strong. The experiment showed that some component of seawater activates the luminescence. Because the composition of seawater is known, I quickly discovered that the activator is Ca^{2+}. The discovery of Ca^{2+} as the activator in turn suggested that EDTA should serve as a better inhibitor of luminescence than acidification during the extraction of the light-emitting principle. On the basis of these data, we devised a method of extracting the light-emitting principle.

With a workable procedure in hand for extracting the luminescent principle, our next task was to catch and process as many jellyfish as possible. We would collect jellyfish from 6 to 8 AM, 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 6:30 to 8:30 PM, and the catch was kept in an aquarium to be processed next day together with the catch of the next morning. We soon found that the bottleneck of the operation was the step of cutting rings with scissors, which is a delicate and very slow process. Even after considerable practice, it took more than 1 minute to cut one jellyfish ring, and 3 hours of work by four persons could not produce more than 500 rings. To increase productivity, we hired several high school girls, trained them, and paid them 2 cents for each ring they cut. We also decided to buy jellyfish from the kids of scientists living on the campus, paying a penny for each jellyfish. This job provided dual benefits, the fun of catching jellyfish and of earning money. I remember a 6- or 7-year-old girl who grossed more than 10 dollars in 2 days (probably with parental help). Unfortunately, just when our operation was in full swing, the jellyfish suddenly

vanished from the area. Thus, we extracted and processed only about 10,000 specimens of *Aequorea* that summer.

We returned to Princeton with the jellyfish extract packed in dry ice, and then began to purify the light-emitting principle from the extract by repeated chromatography on various kinds of large columns. It was a long process, and the utmost care was required to prevent the luminescence activity from being lost, which could be brought about by many causes. We completed the purification in early 1962, obtaining about 5 mg of the light-emitting principle. The substance was found to be a protein with a molecular weight about 20,000, and it emitted light when a trace of Ca^{2+} was added—whether in the presence or absence of oxygen, to our astonishment. We named the protein "aequorin" after the genus name of the jellyfish. Aequorin is an extraordinary protein containing a large amount of energy that can be released when calcium is added; thus it resembles a charged battery that releases the energy when short-circuited. The system was so unusual that some biochemistry professors expressed their skepticism. After 30 years of discovery, however, the importance of aequorin and its use as a calcium probe are firmly established in biochemistry and physiology. The word "aequorin" now can be found in various common dictionaries.

The Town Dock

In 1962, using the methods that had worked in the previous year, we obtained an additional amount of aequorin and began to study various aspects of the molecule, including its application in the measurement of calcium ions. We also wanted to know the mechanism of the luminescence reaction and the structure of the light-emitting chromophore. But our efforts to achieve these goals were soon blocked by an insuperable difficulty. When various methods were used to break down the molecules of aequorin, the first step of the reaction was always an intramolecular chemical change; so it was impossible to isolate intact chromophores. We therefore decided to postpone further study on the light-emitting mechanism.

In 1967, Ridgway and Ashley reported their observation, with the aid of microinjected aequorin, of transient Ca^{2+} signals in single muscle fibers of the barnacle. It was the first report on the use of aequorin in studying intracellular calcium, and it was soon followed by hundreds of papers. Because the importance of aequorin was now evident, we wanted to study the chemistry of the luminescence reaction. Although the structure of the native light-emitting chromophore seemed intractable, I thought that the structure of the chromophore after the luminescence reaction could be determined. For a structural study of the chromophore, I estimated that 100–200 mg of pure aequorin would be needed in a single experiment. About 50,000 jellyfish (2.5 tons) would be needed to produce this amount of aequorin. But to process 50,000 jellyfish in one summer, we would have to collect and cut at least 3000 of the animals each day, allowing for days of bad weather and poor fishing. This was a workload that could not be accomplished by collecting jellyfish at the lab dock and cutting ring with scissors at a rate of one ring per minute.

We resumed the jellyfish operation at Friday Harbor in the summer of 1967, not anticipating that it would continue for the next 20 years. To collect more jellyfish, we expanded our fishing ground beyond the lab dock, adding the Chevron dock (a small commercial pier), the town dock (public pier), and the shipyard (a covered boat storage), and we used a car to move around and to transport the buckets of jellyfish. When the current carried the stream of jellyfish far beyond the docks, we also used rowboats to collect jellyfish, a tricky activity that occasionally caused a collector to fall into very cold seawater. The Chevron dock was our favorite place during the first 2–3 years, because there was a part of it where a large number of jellyfish would stack up on an early morning tide. We had to be careful, however, not to make noise that might awaken sleeping people on the boats.

The town dock was very small—almost nonexistent—in the late '60s; but then it was rapidly expanded. By 1975, the dock had been extended far enough into the bay to intersect with the main jellyfish stream, and it then became a highly favorable spot for fishing. Indeed, the town dock with its large sign saying "Port of Friday Harbor" became our main fishing ground, and the collection became much easier than before. We harvested jellyfish every morning and evening. The collectors were usually my wife, our son and daughter, a couple of assistants, and me. Dr. and Mrs. Johnson also helped for the first several years. Because the jellyfish are nearly transparent in seawater, they cannot easily be seen with untrained eyes. Our children were only 3–4 years old when they began collecting jellyfish with specially made short nets; they had become as efficient as an average adult by the age of 8; and through high school they continued to be great helpers in my project.

Before beginning a collection, we filled buckets about half-full with seawater and placed them strategically along the edge of pier, then gathered jellyfish until the buckets were completely full. When a dense stream of animals was passing the dock, we could collect at a rate of 5–10 jellyfish per minute. When all the buckets were filled, we poured off some water to about 80% capacity, and then covered each bucket with a plastic bag to prevent seawater from spilling during transportation. The buckets—each crammed with about 100 jellyfish in very little water—were then packed into the trunk of a car (which could accommodate 12 buckets) and rushed to the lab. More

buckets were usually transported to the lab on a Boston Whaler by one of the assistants. Once at the lab, and before any rings were cut, the jellyfish were kept in aquaria to revive. In this manner, we were able to collect an average of 3000–4000 jellyfish each day at the town dock.

The town dock was very good for jellyfish fishing, but there were some problems. Often we found too many boats at the dockside; this decreased the open space where we could collect jellyfish. When the loading area, located halfway along the main dock, was fully occupied, we had to carry the heavy buckets of jellyfish all the way to our car, which would be parked more than 200 yards away. The biggest problem, however, was that there were too many boat people who asked us questions. "What are you doing?" "What are you collecting?" "How do you use them?" Almost every passerby felt obliged to ask us a question while we were busily collecting. Most people were satisfied by our simple reply: "These are for scientific research." Some people persisted until they had received a complete explanation of our research.

I cannot forget a funny exchange that took place one early morning. An old lady poked her head out from the window of a small boat, looked at the jellyfish on my net, and asked me, "How do you cook them?"

I answered, "We don't cook those jellyfish."

She gazed at me distastefully, "Do you eat them raw?" and her head disappeared.

"No! We don't eat them!" But my reply was too late.

The Jellyfish Factory

Cutting the jellyfish rings with scissors was impossibly slow; we could not produce the amount of aequorin that we needed using this technique. This problem was solved primarily by Dr. Johnson. He constructed the first model of a jellyfish-cutting machine in the summer of 1967; it was essentially a strip of wire screen that worked like a grater. An average jellyfish has about 100 light organs the size of poppy seeds located under the edge of its umbrella. By sliding the jellyfish over the screen, we hoped that the light organs would be scraped off the body and collected in a tray under the screen. We found, however, that the light organs were not scraped off by the wire screen. The next version of the cutting machine had a strip of coarse sandpaper over which seawater flowed slowly; the sandpaper was connected to one end of the first version. When jellyfish were slid down—first over the sandpaper, then the screen—most of the light organs were indeed scraped off. But the material accumulated in the tray contained an excessive amount of slime, and the quality of the material was much poorer than that of the hand-cut rings. Thus, the manufacture of a machine based on the principle of a grater was abandoned.

Dr. Johnson next purchased two circular meat-slicing blades (10″ diameter) at a local hardware store and began to build a cutting machine; this project took the next two summers to complete. The basic plan was to install a meat-slicing blade perpendicular to a black Lucite board, and with the blade slowly rotating, cut the ring off the jellyfish. The motor from a small laboratory shaker was used to rotate the blade. The jellyfish were rotated with a hand tool called a "peg," a small disk with several short nails on one side and a 2-inch-long, stick-shaped handle attached in the center on the other side. A jellyfish on the Lucite board was grasped by the nails of the disk and rotated by the stick, which was held between the index finger and thumb. The setup worked, at least in principle.

A number of improvements were made over the next two years. A razor blade was installed at the edge of the Lucite board; the razor blade and the rotating circular blade were in contact each other on their flat sides and the jellyfish was cut at the intersection of the two cutting edges. It made cutting so sharp and smooth that the jellyfish might not even feel that their rings were being cut off. To make the rotation of jellyfish easy, a seawater outlet was installed near the center of the board to lubricate its surface. An ice bath was installed to cool the ring reservoir; this prevented a loss of activity from the rings and also served as a preparation for the extraction process. In the summer of 1969, the quality of the machine-cut rings finally surpassed that of the hand-cut ones. We therefore set up two cutting machines and used them, thereafter, to cut all of the jellyfish.

With machines that could cut rings at 10 times the speed of a hand-cutter, and with a sufficient supply of jellyfish, our mode of operation had to be changed. We needed a large working space, and we also did not want to disturb other researchers with our messy, smelly, and noisy experimental processes. Fortunately, we were assigned to use the Gear Locker, a small, isolated building that had been used for storage in the past. Two large tanks installed outside the building were used for temporary storage of collected jellyfish.

Ring cutting was probably the most important step in determining the quality and yield of purified aequorin. Cutting too thick would increase the amount of impurities. Cutting too thin would decrease the yield because some of the light organs were cut through and destroyed. Therefore, we always assigned the best workers to do this job. Of the many excellent helpers we had in our jellyfish operation, I remember particularly three girls who worked for many summers and cut rings extremely skillfully and fast: Debby Nash, Liz Illg, and Laura Norris; the first was from the town and the other two were daughters of biology professors.

Our jellyfish cutting usually began at 11 AM. A counting person would put 80 jellyfish into each bucket, already half-full of seawater, and would then take the buckets to the cutters. Two cutters cut the jellyfish with the machines

that were installed side-by-side: the cutter would place a jellyfish onto the cutting board, quickly rotate it with a peg to spread out the edge of the umbrella where the light organs are located, and then—pushing the jellyfish to the cutting blade while simultaneously rotating the jellyfish quickly—cut off the rings, all in less than 5 seconds. The rings would fall automatically into the ice-cold reservoir, and the ringless jellyfish body was slid down into a waste bucket. These buckets, each filled with about 200 spent jellyfish, were carried to the nearest seashore about 50 yard away, called by us "jellyfish cliff," and dumped onto the rocks below. The heaps of jellyfish bodies, several thousands of them, were carried away by the next high tide.

The process of extracting aequorin from rings began at 2 PM; it was carried out by a team of two persons. The extraction was done in batches of 480 rings (*i.e.*, six buckets). The first person would drain the rings on a nylon gauze, then mix the drained rings with a cold EDTA solution saturated with ammonium sulfate. The rings shrank quickly and were also desensitized by the salt. They were cut with scissors into pieces 1–2 inches long, then stirred with a cake mixer for 10 minutes to dislodge the granular light organs from the tissue. The mixture was squeezed through a nylon gauze to remove the shrunken ring tissue, and then the turbid liquid obtained was filtered on a Buchner funnel using some Celite. The filter cake, containing the light organs, was given to the second person, who was responsible for the rest of the extraction process. The second person put the filter cake into a 2-liter flask containing cold EDTA solution (1 liter), then shook the flask vigorously to extract aequorin from the light organs into the EDTA solution. Finally, the mixture was filtered through a large Buchner funnel, and the filtrate containing aequorin was saturated with ammonium sulfate to precipitate the protein. The first person in the team would start a new batch of rings every 20 minutes, and the second person's work would also take 20 minutes. Thus, 3360 jellyfish rings could be extracted in about 2 hours and 40 minutes.

The precipitates of crude aequorin were purified at our laboratory in Princeton. The purification was done in several steps of column chromatography, mainly by Sephadex gel filtration and DEAE-cellulose chromatography, all at 0°C. It was indeed a lengthy, time-consuming process, notwithstanding the fact that aequorin should be purified as quickly as possible because it is constantly decomposing through spontaneous weak luminescence, even in the presence of a high concentration of EDTA. To purify an extract of 50,000 jellyfish, which contains a large amount of total protein, chromatography had to be repeated 30 times for only the first gel filtration step, and the total number of chromatography runs required for complete purification was more than 60. An extract of 50,000 jellyfish yielded only 150–200 mg of purified aequorin in the early '70s, but as the techniques improved, the yield gradually increased, exceeding 500 mg by 1980. Since 1975, all of the steps in the purification have been done by my wife, Akemi, who is highly knowledgeable in handling aequorin.

The purified aequorin was used in various studies of luminescence in our laboratory. Thus, the chemical structure of the light-emitter was determined in 1973. Then the structure of the aequorin chromophore "coelenterazine" was elucidated and the regeneration of spent aequorin into active aequorin was accomplished, both in 1975. The molecular characterization of various aequorin isoforms was reported in 1986. The improved forms of aequorin—"semisynthetic aequorins" with widely different calcium sensitivities—were produced in 1988–1989. Purified aequorin has also been supplied to hundreds of cell biologists and physiologists who study intracellular calcium, leading to many important findings about intracellular calcium. Aequorin was cloned in 1985 by two groups simultaneously, one in Georgia and another in Japan. With the recent progress in molecular genetics, studies involving recombinant aequorin are now flourishing.

Acknowledgments

Our work on aequorin was initiated by Dr. Frank H. Johnson, and developed with support and encouragement from many individuals. I thank all the people who helped directly or indirectly with this project. The work was made possible by the excellent facilities of the Friday Harbor Laboratories, University of Washington, and of Princeton University, and was financially supported by research grants from the National Science Foundation and the National Institutes of Health.

Literature Cited

Ridgway, E. B., and C. C. Ashley. 1967. Calcium transients in single muscle fibers. *Biochem. Biophys. Res. Commun.* **29:** 229–234.