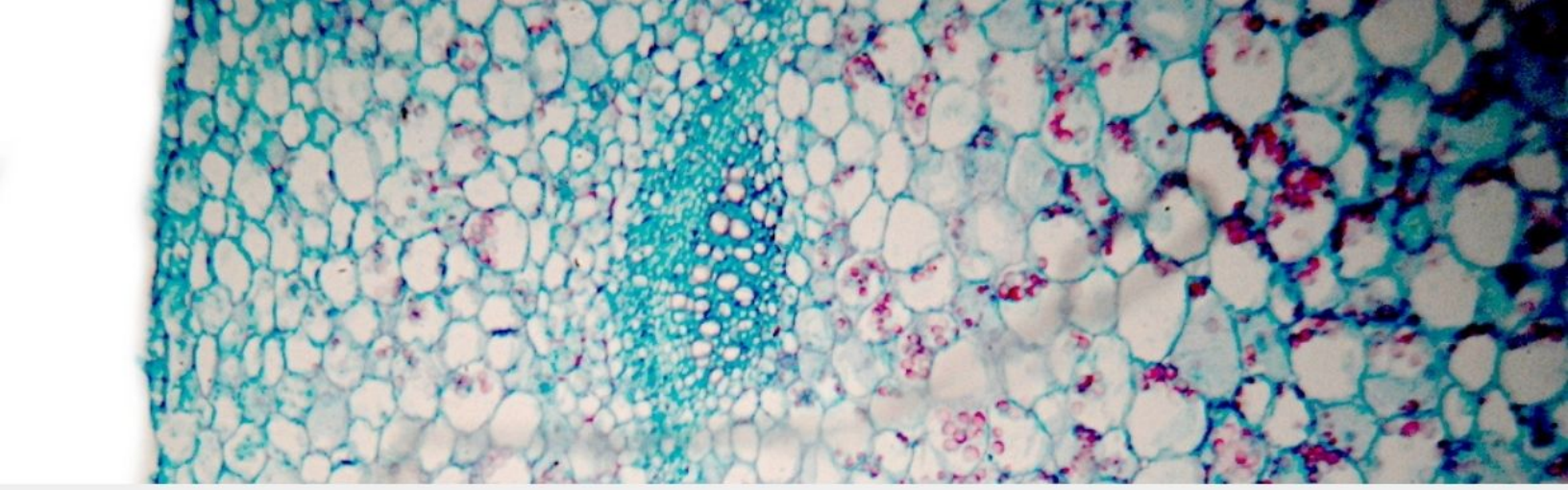

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Early History, Discovery, and Expression of *Aequorea* Green Fluorescent Protein, With a Note on an Unfinished Experiment

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KEY WORDS bioluminescence; photoprotein; aequorin; green and blue fluorescent proteins; energy transfer; chromophore structure; green light emission; protein marker

ABSTRACT The bioluminescent hydromedusan jellyfish, *Aequorea victoria*, emits a greenish light (λ_{max} = 508 nm) when stimulated electrically or mechanically. The light comes from photocytes located along the margin of its umbrella. The greenish light depends on two intracellular proteins working in consort: aequorin (21.4 kDa) and a green fluorescent protein (27 kDa). An excited state green fluorescent protein molecule results, which, on returning to the ground state, emits a greenish light. Similarly, a green light emission may be induced in the green fluorescent protein by exposing it to ultraviolet or blue light. Because the green light can be readily detected under a fluorescence microscope, the green fluorescent protein, tagged to a protein of interest, has been used widely as a marker to locate proteins in cells and to monitoring gene expression. This article reviews the work that took place leading to the discovery, cloning, and expression of the green fluorescent protein, with a note on an unfinished experiment. *Microsc. Res. Tech. 73:785–796, 2010.*

INTRODUCTION

With the 2008 Nobel Award in Chemistry to Osamu Shimomura, Martin Chalfie, and Roger Y. Tsien, readers may be interested in learning the early history, the discovery, cloning, and expression of the green fluorescent protein (GFP) from the jellyfish, *Aequorea victoria*. This jellyfish is commonly found during the late summer months in the waters surrounding the University of Washington's Friday Harbor Laboratories on San Juan Island, Friday Harbor, Washington. It can be easily collected using a small hand-held dip-net. The live *Aequorea* emits a greenish light from photocytes located along the margin of its umbrella. The light is produced by the interaction of two closely associated proteins present in the photocyte: aequorin and GFP (Johnson and Shimomura, 1978; Kojima et al., 1997).

Aequorin is a calcium-binding photoprotein with three calcium-binding sites. It consists of a complex of apoaequorin (apoprotein), coelenterazine (a small organic molecule), and molecular oxygen. The molecular oxygen is bound to the C-2 carbon of coelenterazine and forms a peroxy bridge to an amino acid residue in apoaequorin. It is not easily removed even under high vacuum. The binding of calcium ions to the calcium-binding sites triggers the aequorin luminescence reaction leading to the emission of blue light. During the reaction, a change in the conformation of aequorin takes place, accompanied by the rapid oxidation of coelenterazine by the bound molecular oxygen via a dioxetanone mechanism. The products of the reaction are CO_2, a blue fluorescent protein (BFP), and 60 kcal/mol required for the blue light emission (λ_{max} = 470 nm). With GFP in close proximity, resonance (radiationless) energy transfer takes place from the excited state of BFP (donor) to GFP (acceptor) according to Förster's theory (1959, 1960). A hypothetical scheme for the *Aequorea* bioluminescence systems is shown in Figure 1. Aequorin itself has been widely used as a biological indicator of calcium in living cells (Blinks et al., 1976, 1978, 1982), including its first use by Ashley and Ridgway (1968) to study calcium transient in single muscle fibers.

Following the end of World War II, I was able to resume my education, receiving a PhD degree in biochemistry from Cornell University in 1950. As a student, I learned my biochemistry from James B. Sumner (recipient of the 1946 Nobel Prize in Chemistry for isolating and crystallizing urease and showing that enzymes are proteins). In 1952, I was appointed a postdoctoral research assistant (1952–1955) in the laboratory of E. Newton Harvey, Department of Biology, Guyot Hall, Princeton University. Howard S. Mason (co-discoverer of oxygenase with Osamu Hayaishi) was leaving Harvey's lab and I filled his position. Harvey was the leading authority on bioluminescence, and I felt fortunate to be working under his guidance. Harvey was the Henry Fairfield Osborn Professor of Biology at Princeton where he devoted more than 40 years of his life to teaching and research, writing four books and some 125 articles on bioluminescence. He made the Department of Biology a world-renowned

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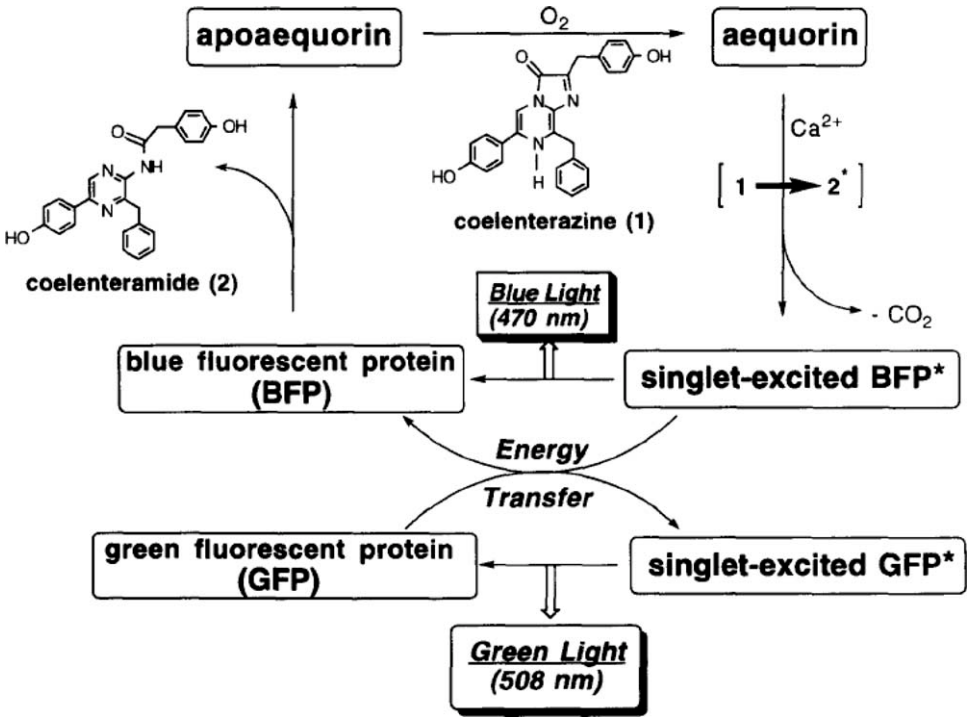


Fig. 1. Bioluminescence system of the Jellyfish, *A. victoria*. From Kojima et al., 1997.

center for bioluminescence research and is remembered by former students and colleagues as a true gentleman and dedicated researcher. My assignment in his lab in 1952 was to isolate, purify, and study the properties of luciferin (substrate) and luciferase (enzyme) responsible for the luminescence of the tiny Japanese marine ostracod crustacean, *Cypridina hilgendorfii* (now called *Vargula hilgendorfii).* Two students who had recently finished their PhD degrees under Harvey were William D. McElroy, who worked on *Cypridina* and firefly bioluminescence, and J. Woodland Hastings, who worked on *Cypridina* and bacterial bioluminescence. The *Cypridina* used for our research were obtained from Dr. Yata Haneda, Director of the Yokosuka City Museum, Yokosuka, Japan, who had hired fishermen to collect and air-dry them in large amounts. Haneda then shipped the dried organisms to Harvey in a bottle with a small amount of anhydrous calcium chloride in a cotton bag as desiccant. When stored under such conditions, the luciferin and luciferase in the organisms remained highly active almost indefinitely. The *Cypridina* specimens were powdered with mortar and pestle and the luciferin and luciferase extracted and partially purified before being used in experiments. Thus, the *Cypridina* organisms provided by Haneda became an important resource for the training of students and for research on bioluminescence. Haneda was a physician who gave up medicine soon after graduating from medical school and remained active in research on the natural history of bioluminescent organisms for the remainder of his life.

I also came to know two other members of the Department of Biology who were also working with *Cypridina*, namely, Frank H. Johnson and Aurin M. Chase, both professors in the Department. Johnson had been a student of Harvey and his collaborators were Henry Eyring (Department of Chemistry, noted for his Theory of Absolute Reaction Rates) and George T. Reynolds (Department of Physics, known for imaging luminescent organisms). Johnson also worked on problems in bacterial luminescence, notably on the effect of high hydrostatic pressure on bioluminescence. Johnson had built a special apparatus for this work, and it interested me. Later, when I had my own laboratory, I had a duplicate apparatus constructed to measure volume change of activation in the *Cypridina* luciferase-antibody reaction (Tsuji et al., 1966).

During this time, Harvey published two seminal books: Bioluminescence (Harvey, 1952) and A History of Luminescence (Harvey, 1957). The first reviewed the literature on the phylogeny and biology of luminous organisms, and the second on the history of bioluminescence "from the earliest times until 1900." Publication of the 1952 book was important because it brought together in one volume information that was widely scattered in the literature, and which, even today, still serves as a valuable reference source. Another consequence of its publication was that a committee, consisting of L. R. Blinks, E. N. Harvey, F. H. Johnson (Chairman), W. D. McElroy, and C. E. Zobell, met and decided to hold a Conference on Luminescence at Asilomar, Pacific Grove, California, from March 28–April 2, 1954. The conference was held under the auspices of the National Academy of Sciences-National Research Council and the National Science Foundation. There were 34 participants and the proceedings of the conference were subsequently published (Johnson, 1955). Those attending are shown outside the Scripps Conference Hall (Fig. 2).

Fig. 2. Group photograph of members attending Conference on Luminescence of Biological Systems in 1954 held at Asilomar, Pacific Grove, CA. Photograph taken by Yata Haneda using camera set for auto-exposure and Kodachrome film. Individuals are identified by numbers: (1) Frank H. Johnson (Princeton University), (2) J. Woodland Hastings (Northwestern University and presently Harvard University), (3) L. M. V. Duysens (University of Utrecht, Holland), (4) C. J. P. Spruit (Landbouwhogeschool, Wageningen, Holland), (5) Frederick I. Tsuji (Princeton University), (6) John Buck (National Institutes of Health, Bethesda, MD), (7) Claude E. ZoBell (Scripps Institution of Oceanography), (8) J. A. C. Nicol (Marine Biological Laboratory, Plymouth, England), (9) Yata Haneda (Yokosuka City Museum, Yokosuka, Japan), (10) Aurin M. Chase (Princeton University), (11) William V. Consolazio (National Science Foundation, Washington, DC), (12) Arthur Giese (Stanford University), (13) Howard S. Mason (University Oregon Medical School), (14) Walter J. Kauzmann (Princeton University), (15) E. Newton Harvey (Princeton University), (16) E. J. Ferguson-Wood (Marine Biological Laboratory, New South Wales, Australia), (17) E. R. Baylor (University of Michigan), (18) Rubert S. Anderson (Army Chemical Center, MD), (19) C. Stacy French (Carnegie Institution of Washington and Stanford University), (20) Paul C. Wilhelmsen (University of Utah), (21) Lawrence Blinks (Stanford University and Hopkins Marine Station, Pacific Grove, CA), (22) C. B. van Niel (Stanford University), (23) Havre Carlson (Office of Naval Research, Washington, DC), (24) William Arnold (Oak Ridge National Laboratory), (25) Demorest Davenport (University of California, Santa Barbara), (26) Ralph S. Becker (Florida State University, Tallahassee), (27) John H. Ryther (Woods Hole Oceanographic Institution), (28) William D. McElroy (Johns Hopkins University), (29) Bernard L. Strehler (University of Chicago), and (30) H. Burr Steinbach (National Science Foundation, Washington, DC). Others not shown in photograph but who participated in the conference were Francis T. Haxo (Scripps Institution of Oceanography) and Beatrice M. Sweeney (Scripps Institution of Oceanography). In addition, Prof. Michael Kasha of Florida State University, Tallahassee, FL and Prof. Henry Eyring of Princeton University and University of Utah, who were invited but did not attend. They presented joint articles, Kasha with Ralph S. Becker (26) and Eyring with Paul C. Wilhelmsen (20) and Rufus Lumry of the University of Minnesota, respectively.

The conference opened with an introductory lecture by Harvey (1955) on luminescent organisms, ranging from the Bacteria to Pisces. His table showed the distribution of luminescent organisms in each of the major groups. Luminescence characteristics were categorized for each species. All data on the luminescence of Hydromedusae were from an earlier article (Harvey, 1921) and from Bioluminescence (Harvey, 1952).

In the 1921 article, Harvey recounts his trip to the Puget Sound Marine Station, Friday Harbor, Washington, where he studied four species of hydromedusans: *Aequorea forskalea* (now called *A. victoria), Mitrocoma* (*Halistaura*) *cellularia*, *Phialidium gregarium,* and *Stomatoca atra*. Regarding *Aequorea*, Harvey writes: "*Aequorea* . . . produced light of a bright bluish–green color. . .from spots along the edge of the umbrella at the base of the tentacles. No other regions of *Aequorea*. . . phosphoresce . . .." (pp. 280–281). "Examined with the microscope in the daytime, the margin of the umbrella of *Aequorea* discloses oval masses of yellow tissue corresponding in position with the luminous areas at night." (p. 281). "Examined at night under the microscope, the luminous spots present a beautiful appearance. Under conditions which cause a cytolysis of the cell, such as addition of fresh water or saponin, one can clearly see that the light comes from granules which

are rather large and have a definite boundary, – light discs [sic]. They are not mere points of light. They vary in size and will luminesce for some time, then flash out very brightly and the light intensity slowly fade." (p. 281). "The luminous material of *Aequorea*...can be dried over $CaCl_2$ and will give a bright light when again moistened." "A strip of the margin of the umbrella of *Aequorea* ... is easily cut off with scissors, giving a mass of tissue containing as little non-luminous material as it is possible to obtain. If this is squeezed through four layers of cheesecloth, there is obtained a luminescent extract which glows for some hours." "When the luminescence disappears on standing, the addition of fresh water, gentle heating or cytolytic agents...again calls forth the luminescence." (p. 282). Other results obtained by Harvey using these extracts were presented in his introductory lecture at the conference (Harvey, 1955). Harvey's conclusions were: (1) *Aequorea* luminescence does not require molecular oxygen, (2) the reaction is not of the luciferin-luciferase type (as in *Cypridina*), (3) there is no reciprocal light-emitting cross-reaction between the luciferins (hot-water extracts) and luciferases (cold-water extracts) of *Cypridina* and *Aequorea*, and (4) the light of *Aequorea* when examined with a spectroscope "...discloses a band of light extending from about $\lambda = 0.46\ \mu$ to $\lambda = 0.60\ \mu$." (p. 281). (Harvey, 1921).

Preceding the Asilomar conference, there were few investigators studying bioluminescence, and they were mostly confined to the United States, Europe, and Japan. In the United States, some of the topics being studied and discussed were the role of molecular oxygen in bacterial and *Cypridina* bioluminescence (Hastings, 1952a,b), the role of ATP in firefly luminescence (McElroy, 1947), the chemical properties of luciferin and luciferase in *Cypridina* (McElroy and Chase, 1951; Tsuji et al., 1955), and the kinetics of the *Cypridina* and bacterial reactions (Harvey, 1952; Johnson et al., 1954). All these studies showed that the light emitted is due to a luciferin-luciferase reaction in which "luciferin" (a small organic molecule serving as substrate) is oxidized by molecular oxygen, catalyzed by the enzyme "luciferase" (Harvey and Tsuji, 1954). Some reactions (bacteria and firefly) required a co-factor.

The hydromedusan, *Aequorea*, was an exception in not possessing a lucierin-luciferase reaction (Harvey, 1921) and not requiring molecular oxygen for luminescence (Harvey, 1952). Much earlier Macartney (1810) had also come to the same conclusion working with the luminous medusa, *Medusa hemispherica* (now known as *Clytia hemisphaerica* or *Phialidium hemisphaericum*, courtesy of Dr. Claudia Mills, Friday Harbor Laboratories). He described the following experiment: "Experiment 9—Some of the scintillating and hemispherical species of medusa, contained in a small glass jar, were introduced into the receiver of an air pump, and the air being exhausted, they shone as usual when shaken; if any difference could be perceived, the light was more easily excited, and continued longer in vacuum." (p. 285). Figure 5 in the article (p. 292) shows a drawing of the jellyfish with the legend: "The luminous medusa, discovered by me, which I conceive to be the medusa hemispherica: it is shewn of the largest size I met with." (p. 291). These facts were known to Johnson, Chase, and me from discussions with Harvey.

Harvey died unexpectedly in 1959, but because of his influence the faculty and students around him were inspired to continue the study of bioluminescence and many subsequently went on to make a career in bioluminescence research. However, the post-World War II history of bioluminescence is outside the purview of this review and is generally not included herein.

After the Asilomar conference, Johnson and his students (Johnson, 1959; Sie et al., 1958) began work on the luminescent system of *Chaetopterus variopedatus*, a polychaete worm found both at the Marine Biological Laboratory, Woods Hole, MA, and the Hawaii Marine Station of the University of Hawaii at Kaneohe Bay, HI. The worm, 2–8 inches long, lives in a tube made of a parchment-like material and discharges a luminescent slime on mechanical stimulation, which can be collected and studied.

However, probably influenced by the earlier work of Harvey (1921) and the discussions that took place at the Asilomar Conference regarding the unique aspects of the *Aequorea* bioluminescence system, Johnson changed his focus of research to *Aequorea*. It is worth noting that soon after the Asilomar Conference ended, an article on *Aequorea* luminescence was published by Davenport and Nicol (1955), reporting the results of their research carried out at the Friday Harbor Laboratories, University of Washington. In the summary of the article, which confirmed the earlier work of Harvey (1921, 1952), the following statement appears: "In *Aequorea* the luminous points correspond to yellow–green fluorescent masses in the marginal canal."—the first evidence appearing in the literature that a green fluorescent molecule is present in the luminous organ of *Aequorea*. Both Davenport and Nicol were present and participated in discussions at the Asilomar Conference.

At the time the Asilomar Conference was held, work on the purification and characterization of *Cypridina* luciferin was underway in our laboratory (Tsuji, 1955; Tsuji et al., 1955), in the organic chemistry laboratories of Toshio Goto and Yoshimasa Hirata of Nagoya University, and in Noboru Sugiyama's laboratory at the Tokyo University of Education, Japan. Osamu Shimomura, after obtaining his PhD degree in organic chemistry in 1960 from Nagoya University for his work on crystallizing *Cypridina* luciferin (Shimomura et al., 1957), came to work in Johnson's laboratory as a Research Associate, while Ms. Yo Saiga arrived from Sugiyama's laboratory as a Laboratory Assistant. The three then started studies of the *Aequorea* bioluminescence system. Johnson obtained specimens of *Aequorea* from the Friday Harbor Laboratories shipped to Princeton and from subsequent visits to Friday Harbor during the summer collecting season.

The work by Johnson, Shimomura, and Saiga on *Aequorea* resulted in two articles: Shimomura et al. (1962) and Johnson et al. (1962). In the Shimomura et al. (1962) article, a "note 3" appears on p. 228 which reads: "A protein giving solutions that look slightly greenish in sunlight though only yellowish under tungsten light, and exhibiting a very bright, greenish fluorescence in the ultraviolet of a Mineralite, has also been isolated from squeezates. No indications of a luminescent reaction of this substance could be detected. Studies of the emission spectra of both this protein and

aequorin are in progress." The article, however, does not give details of any chemical or physical evidence linking the observed fluorescence to a "protein," nor is the method of isolation of "protein" from "squeezate" [extract obtained by squeezing luminous tissue through a cotton handkerchief—similar to the method used by Harvey (1921) who used cheesecloth instead] described. In this work by Shimomura et al. (1962) and in the earlier work of Davenport and Nicol (1955), the greenish fluorescence was obtained by irradiating the photogenic tissue or extract with ultraviolet light from a Mineralite lamp.

In the Johnson et al. (1962) article, the authors also allude to a "green protein" as producing a green fluorescence, but no experimental evidence is presented establishing a chemical or physical relationship between a "green protein" and fluorescence of the "squeezate." The article states: "The results are illustrated in Figure 10, which includes data also on the luminescence of "squeezates" and the fluorescence of a "green protein," which was extracted and purified from photogenic tissue where it occurs concentrated together with aequorin." (p. 100). Further on, the article states "...that the luminescence of intact photogenic tissues of living organisms is distinctly greenish, in contrast to the blue luminescence of aequorin...." and that "... it is reasonable to believe that the greenish quality of the light emitted from living photocytes is due to the absorption by the green protein, of blue luminescence, followed by fluorescence at longer wavelengths. Evidence supporting this supposition resides in the excitation and emission peaks of fluorescence of the green protein...." and "Three excitation peaks were found, at 270, 390, and 460 mμ, respectively. Two fluorescence peaks, at 350 and 510 mμ, respectively, result from excitation at 270 mμ, whereas one fluorescence peak at 515 mμ results from excitation at either 390 or 460 mμ." (pp. 100–101). The fluorescence peak at 350 mμ may be attributed to a protein, but this point is not mentioned since it would link the fluorescence to the green protein. The summary also reads: "The emission spectrum of fluorescence of a "green protein," extracted and purified from photogenic tissues of this organism, was also studied (pp. 102–103), but no mention is made of the method of purification which would support the existence of the "green protein."

In 1966, another conference on bioluminescence was held at Hakone, Japan, organized by Johnson and Haneda (1966) and sponsored by the Japan Society for the Promotion of Science and the US National Science Foundation under the US–Japan Cooperative Science Program. However, no article on green fluorescent protein was presented.

After a hiatus of almost 10 years, Morin and Hastings (1971a,b) published two articles providing experimental evidence and the first description of the steps involved in the green light emission of the coelenterates *Aequorea* (hydrozoan), *Obelia* (hydrozoan), and *Renilla* (anthozoan). The first article dealt with extraction and partial purification of photoproteins from *Obelia geniculata*, *O. longissima*, and *Mnemiopsis leidyi*. All three photoproteins produced a flash of light with the addition of $CaCl_2$ and did not require molecular oxygen for light emission. The second article reported on the photoproteins of *Aequorea*, *Obelia,* and *Renilla*, which showed strong similarities in emitting blue light (λ_{max} = 460–490 nm) in vitro with calcium and green light (λ_{max} = 509 nm) in vivo from photocytes of the living organism. The position of the green fluorescence in the photocyte was localized by fluorescence microscopy. Since there was an overlap in the in vitro bioluminescence emission peak with the fluorescence excitation peak (λ_{max} = 460 nm), the authors concluded that the green light was due to energy transfer from the product-excited state of the aequorin reaction to GFP. Thus, in *Aequorea* the donor molecule would be the blue fluorescent protein in the excited state (BFP*) and the acceptor molecule would be GFP, which on becoming excited (GFP*) emits green light on returning to the ground state (Fig. 1). Experimental evidence to support the scheme was obtained by isolating a photoprotein and a GFP from extracts of photogenic tissue by differential centrifugation. The term "green fluorescent protein" appears for the first time in the literature in the article by Morin and Hastings (1971b). The workers found GFP to be present in granules, akin to those observed by Harvey (1921), associated with photoprotein. Lysing the photocyte with water caused the associated photoprotein-GFP complex to be released, after which the complex dissociated very rapidly, usually within 10 s or less. They hypothesized that depolarization of the photocyte in some manner facilitates the entry or release of calcium in the granule. Thus, the scheme proposed by Morin and Hastings involves the following steps: (1) oxidation of a photoprotein (specifically coelenterazine bound to apoprotein) yielding a product excited state (BFP*), (2) energy transfer by a Förster mechanism from BFP* to GFP yielding GFP*, and (3) emission of green light by GFP* on returning to the ground state.

After the articles by Morin and Hastings (1971a,b) appeared in print, Johnson's lab published an article dealing with energy transfer in the *Aequorea* bioluminescence system (Morise et al., 1974). GFP was purified, crystallized, and partially characterized. Adding calcium to a solution of aequorin containing low concentrations of GFP produced blue light (λ_{max} = 472 nm), characteristic of the blue light seen in the aequorin reaction, whereas adding calcium to aequorin and GFP co-adsorbed on DEAE-cellulose or DEAE-Sephadex, produced green light (λ_{max} = 509 nm). They interpreted this to mean that energy transfer of the Förster type occurs when the molecules are close together, rather than the absorption of blue light by the GFP as proposed earlier by Johnson et al. (1962). Subsequently, a detailed article was published by another lab (Prendergast and Mann, 1978) on the physical-chemical properties of *Aequorea* GFP, including molecular weight, sedimentation coefficient, extinction coefficient, diffusion coefficient, and amino acid composition.

Although the work on *Aequorea* GFP was in progress, Milton J. Cormier and associates at the University of Georgia began publishing articles on the bioluminescent reaction of *Renilla reniformis*, or sea pansy, another coelenterate (Cormier et al., 1974). They found that the blue light (λ_{max} = 488 nm) emitted by *Renilla* was due to the oxidation of a species-specific luciferin (coelenterate-type luciferin) by molecular oxygen, catalyzed by luciferase (Hori et al., 1973). While purifying

the luciferase, they also noticed a green chromophore "attached" to the luciferase (Karkhanis and Cormier, 1971). They wrote as follows: "It appears that only a small fraction of luciferase has this visible chromophore attached to it. This material may represent a bioluminescent oxidation product attached to the enzyme. In this regard it is of interest to note that the chromophore is highly fluorescent. Its fluorescence excitation coincides with its visible absorption and its fluorescence emission maximum lies at 510 nm when excited at either 470 or 500 nm. This emission is the same as that observed in the in vivo bioluminescence emission." (pp. 321–322). Subsequently, they isolated a product having a blue fluorescence emission similar to that observed in the in vitro reaction in which the bioluminescent oxidation of *Renilla* luciferin is catalyzed by luciferase (Hori et al., 1973). In addition, they found a protein-bound chromophore associated with luciferase having a green fluorescence emission identical to that of the in vivo emission. On the basis of spectroscopic studies of the two chromophores (or two emitters) they suggested that the blue light emitter and green light emitter should be a good donor–acceptor pair for excitation energy transfer (Wampler et al., 1971). What they were seeing during luciferase purification was GFP being co-purified as a minor component (1%). Finally, using highly purified components (luciferase, luciferin, and GFP), they were able to reconstitute the in vivo reaction in vitro and show radiationless energy transfer from the excited state of oxyluciferin (bound to luciferase) to the GFP (Ward and Cormier, 1978, 1979).

Toward the end of 1970, while research on GFP was continuing, Johnson suffered a debilitating stroke, which had unforeseen consequences. Not only did it affect Johnson personally but also his collaboration with Shimomura. We were all stunned and saddened by the tragic news. In a handwritten letter addressed to the author in 1979, Johnson (Fig. 3) wrote about his physical and mental condition. Johnson never fully recovered and passed away 11 years later in September 1990 at age 82. Johnson was an indefatigable worker. He was imaginative and creative, with an innate ability to recognize important problems and solve them. His research career in bioluminescence spanned almost 20 years, during which time he collaborated with Shimomura on numerous articles, which serves as a testament to their accomplishments. Outside the laboratory, Johnson had a strong interest in art, being a painter himself, and in literature. Brief information about some of his colleagues is given in a footnote.[1]

After Johnson's death, Shimomura moved from Princeton to the Marine Biological Laboratory at Woods Hole, MA, with an adjunct appointment as a professor at Boston University. I have known both Shimomura (Figs. 4a and 4b) and Johnson as friends and colleagues since the time they worked together, but have not co-authored an article with either one of them.

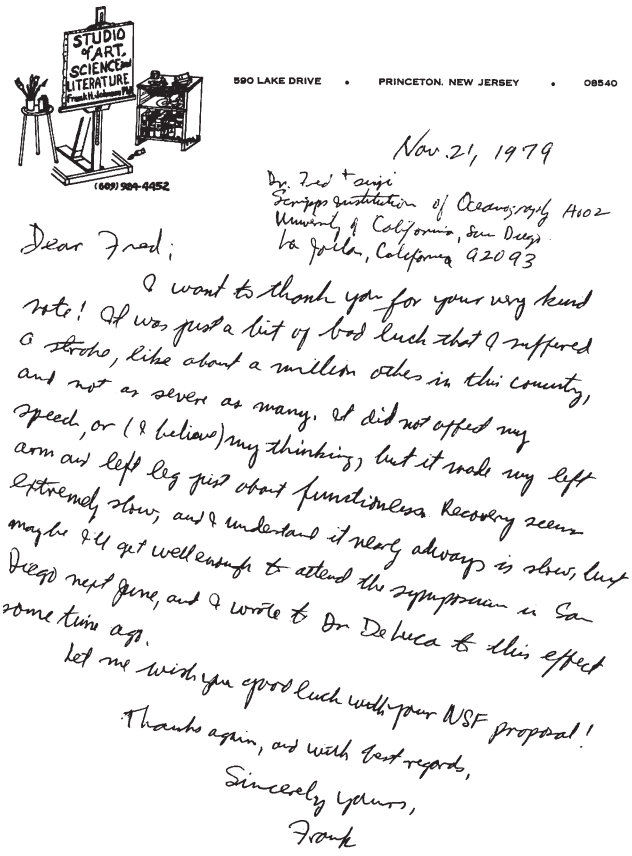
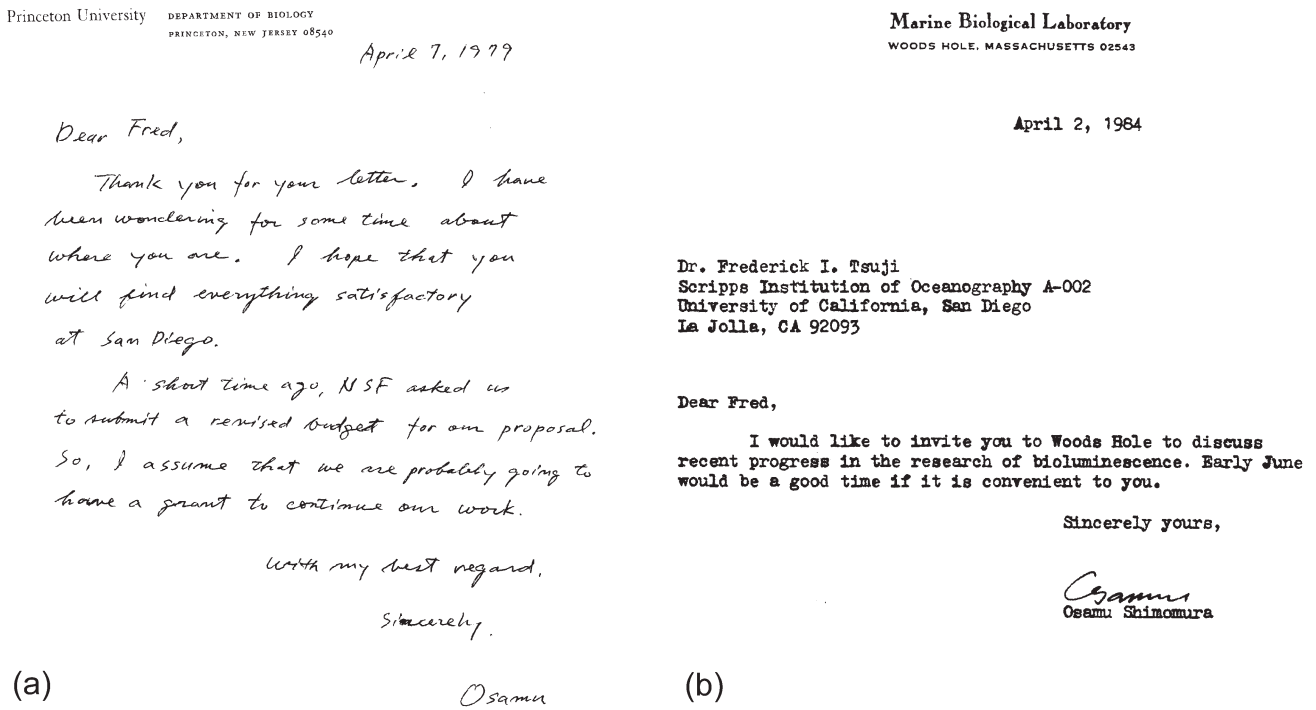


Fig. 3. Copy of letter sent by Frank H. Johnson to the author.

From approximately 1979–1992, before the cloning of the *Aequorea* cDNA for GFP by Prasher et al. (1992), many articles were published on various aspects of *Aequorea* GFP, including the structure and identity of the chromophore of GFP (Cody et al., 1993; Levine and Ward, 1982; McCapra et al., 1988; Shimomura, 1979; Ward et al., 1980); properties of GFPs (Ward, 1981); proton NMR of chromophore of GFP (Nageswara Rao et al., 1980); denaturation and renaturation of GFP (Bokman and Ward, 1981; Ward and Bokman, 1982); spectral properties of GFP (McCapra et al., 1988; Ward et al., 1982); X-ray diffraction of GFP crystals (Perozzo et al., 1988); evolution of the GFP molecule (Ward and McCapra, 1993).

In 1982, in a joint research project, carried out under the US–Japan Cooperative Science Program, I (with a grant from the National Science Foundation, NSF) and my Japanese colleagues Yasuyuki Takagi and Yoshiyuki Sakaki of Kyushu University (with a grant from the Japan Society for the Promotion of Science, JSPS) started our work on the biochemistry and molecular biology of the bioluminescence systems of *A. victoria* and the firefly. Satoshi Inouye was a graduate student at this time in the laboratories of Takagi and Sakaki, and obtained his PhD degree from Kyushu University

[1]Individuals with whom Johnson had a special relationship in carrying out research in bioluminescence were: Aurin M. Chase, Professor of Biology, Princeton University, kinetics of the *Cypridina* bioluminescence reaction; first to show thermal denaturation and renaturation of a protein using partially purified *Cypridina* luciferase (Chase, 1946; Johnson and Chase, 1942), died 1999; George T. Reynolds, Professor of Physics and Director of High Energy Physics, Princeton University, noted for research in high energy particle physics, cosmic rays and imaging of bioluminescent systems (Johnson et al., 1962; Reynolds, 1972, 1978), died 2005; Henry Eyring, Professor of Chemistry, Princeton University, theoretical physical chemist; first to propose the transition-state theory, one of the greatest achievements in 20th century chemistry (Johnson et al., 1945, 1954), died 1981; Yata Haneda, physician, Founder and Director, Yokosuka City Museum, Yokosuka, Japan; Editor, Science Report of the Yokosuka City Museum; author of numerous articles on the natural history of luminous organisms (Haneda, 1955; Haneda and Johnson, 1958, 1962; Johnson et al., 1960), died 1995.



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PRINCETON, NEW JERSEY 08540

April 7, 1979

Dear Fred,

Thank you for your letter. I have been wondering for some time about where you are. I hope that you will find everything satisfactory at San Diego.

A short time ago, NSF asked us to submit a revised budget for our proposal. So, I assume that we are probably going to have a grant to continue our work.

With my best regard,

Sincerely,

Osamu

(a)

Marine Biological Laboratory
WOODS HOLE, MASSACHUSETTS 02543

April 2, 1984

Dr. Frederick I. Tsuji
Scripps Institution of Oceanography A-002
University of California, San Diego
La Jolla, CA 92093

Dear Fred,

I would like to invite you to Woods Hole to discuss recent progress in the research of bioluminescence. Early June would be a good time if it is convenient to you.

Sincerely yours,

Osamu Shimomura

(b)

Fig. 4. (**a**) Copy of letter sent from Princeton by Osamu Shimomura to the author. (**b**) Copy of letter sent from Woods Hole Marine Biological Laboratory by Osamu Shimomura to the author.

in 1986. Since then, I have collaborated with Inouye on many research projects on bioluminescence.

A. victoria specimens were collected at the Friday Harbor Laboratories and our first article was published on the cloning and amino acid sequence analysis of cDNA for apoaequorin (Inouye et al., 1985). At the same time, an article on the cloning and expression of the cDNA for apoaequorin was also published by Cormier's group at the University of Georgia (Prasher et al., 1985). Subsequently, there were three other articles by the two groups on the expression of apoaequorin cDNA (Inouye et al., 1986; Noguchi et al., 1986; Prasher et al., 1986). The articles from Cormier's group made us realize that another group was also working on the molecular biology of the *Aequorea* bioluminescence system. This knowledge constrained us from working on the *Aequorea* and firefly bioluminescence systems simultaneously, so we decided to focus our efforts on the *Aequorea* system.

Aequorin possesses three Ca^{2+}-binding sites and has a high amino acid sequence identity to that of the Ca^{2+}-binding protein, calmodulin, which has four sites (Inouye et al., 1985). After our first article was published, we carried out a series of studies on aequorin, involving site-directed mutagenesis and the effects of amino acid substitution on the luminescence reaction (Hirano et al., 1994; Kurose et al., 1989; Nomura et al., 1991; Ohmiya and Tsuji, 1993; Ohmiya et al., 1992, 1993; Tsuji et al., 1986). In addition, to compare amino acid sequences in photoproteins of the aequorin family (Tsuji et al., 1995), we cloned the cDNAs for clytin from *Clytia gregarium* (Inouye and Tsuji, 1993) and mitrocomin from *Mitrocoma cellularia* (Fagan et al., 1993), both collected at Friday Harbor. We also showed aequorin to be a useful reporter in monitoring gene expression in mammalian cells (Tanahashi et al., 1990).

As our work on aequorin was coming to an end and we had shifted our research efforts to GFP, an article appeared reporting the primary structure of the *Aequorea* GFP, deduced from the nucleotide sequence (Prasher et al., 1992). The authors did not mention any fluorescence in the protein. The article by Prasher et al. (1992) was not unexpected because our and Cormier's groups were the only groups working on the *Aequorea* bioluminescence at that time.

Our cloning and expression article on *Aequorea* GFP was published (Inouye and Tsuji, 1994a) independently and simultaneously as an article on cloning and expression of *Aequorea* GFP by Chalfie's group (Chalfie et al., 1994), which served as a cover article in the issue of Science. The two articles were in press at the same time and appeared in print within a month of each other. In both articles, it was reported that the *Aequorea* GFP could serve as a marker for studying gene expression. Studies by the two groups were conceptually the same and the results were comparable. The Inouye and Tsuji (1994a) GFP expression vector, when expressed in *Escherichia coli*, produced a fused protein, which on purification by metal ion affinity chromatography gave a fluorescence excitation and emission spectra identical to those of native GFP, whereas the expression vector used by Chalfie et al. (1994) produced a fluorescent product in *E. coli* and *Caenorhabditis elegans*, and the partially purified GFP gave fluorescence and emission spectra identical to those of native GFP. One difference in the two studies was that Chalfie obtained his cDNA for GFP from

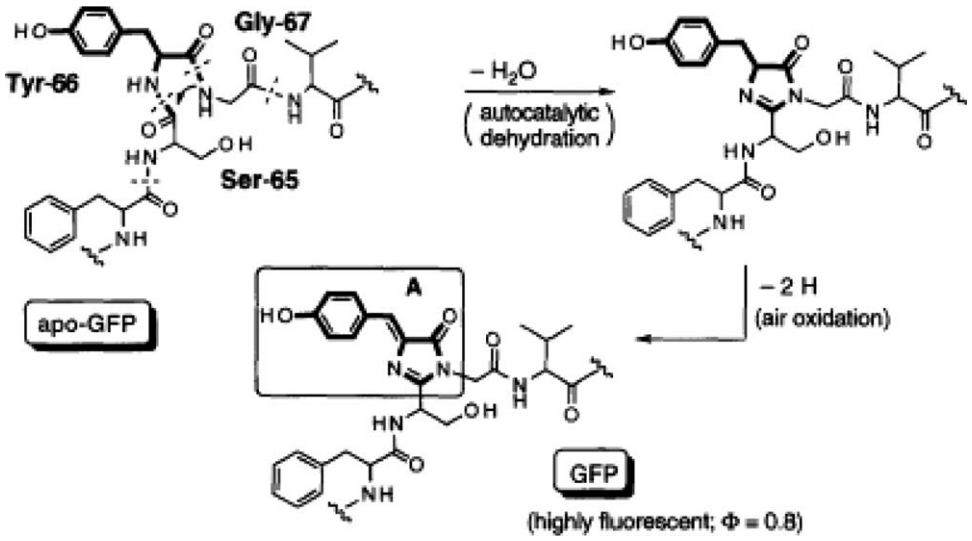

Fig. 5. Biogenesis of GFP chromophore. From Kojima et al., 1998.

Prasher, whereas our cDNA was obtained by cloning the cDNA directly from specimens of *Aequorea* we collected at Friday Harbor Laboratories. Further, our studies on GFP were done independently and without any contact with other laboratories.

We also showed that when $CaCl_2$ is added to a reconstituted mixture of purified recombinant aequorin and recombinant GFP, a greenish luminescence was clearly visible to the unaided eye (Inouye and Tsuji, 1994a), confirming the scheme of Morin and Hastings (1971) with respect to resonance energy transfer in *Aequorea* green light emission. We also reported (Inouye and Tsuji, 1994b) that: "During the purification of His-GFP, a marked increase in fluorescence intensity was observed when the harvested cells and cell-precipitate were allowed to stand overnight at room temperature. Subsequently, the intensity remained unchanged for over a month. This suggested that an oxidative step may be involved in the initial increase in fluorescence." (p. 213). We also reported that "On adding sodium dithionite, $Na_2S_2O_4$, a strong reducing reagent, to a solution of rGFP, the green fluorescence of the solution disappeared within a few minutes and, on standing for several hours, the fluorescence reappeared first as a thin band at the surface, which then spread slowly downward into the solution. On standing overnight the solution recovered its fluorescence completely. This result indicated that the GFP was involved in a redox reaction with molecular oxygen, with the oxidized form being fluorescent and the reduced form being nonfluorescent." (p. 213).

After the three articles (Chalfie et al., 1994; Inouye and Tsuji, 1994a,b) were published, four other GFP articles appeared in print in 1994: Wang and Hazelrigg (1994) in June; Heim et al. (1994) in December; Lo et al. (1994) in December; and Sengupta et al. (1994) in December. In Tsien's article (Heim et al., 1994) and in the three others, the cDNA for GFP was obtained from Prasher.

Then, after 1994, the number of GFP articles increased markedly: 16 in 1995, 16 in 1996, 11 in 1997, and 5 in 1998. Some important articles were published between 1995 and 1998, including the following: on a mutant GFP (S65T) with improved fluorescence brightness and faster rate of chromophore formation (Heim et al., 1995); on GFP for identifying and localizing intracellular proteins and organelles (Delagrave et al., 1995; Rizzuto et al., 1995; Webb et al., 1995); on GFP as a reporter for detecting viral infection (Baulcombe et al., 1995; Wu et al., 1995); on thermal sensitivity of GFP (Lim et al., 1995; Siemering et al., 1996); on altered fluorescence excitation spectra (Ehrig et al., 1995); on an improved GFP mutant for whole cell imaging (Crameri et al., 1996); on determination of the crystal structure of GFP (Ormö et al., 1996; Yang et al., 1996); on fast proton transfer in chromophore (Chattoraj et al., 1996; Kummer et al., 1998; Lossau et al., 1996; Terry et al., 1995); on kinetics of chromophore formation (Reid and Flynn, 1997); on crystal structure and spectral properties of GFP (Brejc et al., 1997; Palm et al., 1997; Wachter et al., 1997); and on dual color imaging using GFP variants (Yang et al., 1996, 1998).

During the same interval, the articles we published included the following: on the tracking of GFP promyelocytic leukemia (PML) protein in COS-1 cells and microscopy of nuclear transfer of GFP-human glucocorticoid receptor (hGR) fusion protein in COS-l cells in response to dexamethasone (Ogawa et al., 1995); on the chemical structure of the GFP chromophore, the phenolate anion as the light emitter, and *cis-trans* isomerization of the exo-methylene bond in light emission (Niwa et al., 1996); on the three-dimensional structure of GFP (Wu et al., 1996); on the mechanism of the redox reaction in GFP (Kojima et al., 1997); on the biogenesis of the GFP chromophore (Fig. 5) and fluorescence properties of model chromophores of GFP (Kojima et al., 1998) and on the chemical synthesis of the 238 amino-acid-residue-precursor molecule of GFP, subsequent folding, autocatalytic cyclization of the tripeptide –Ser^{65}-Tyr^{66}-Gly^{67} and development of fluorescence (Nishiuchi et al., 1998) (Fig. 6). The determination of the three-dimensional structure of GFP was

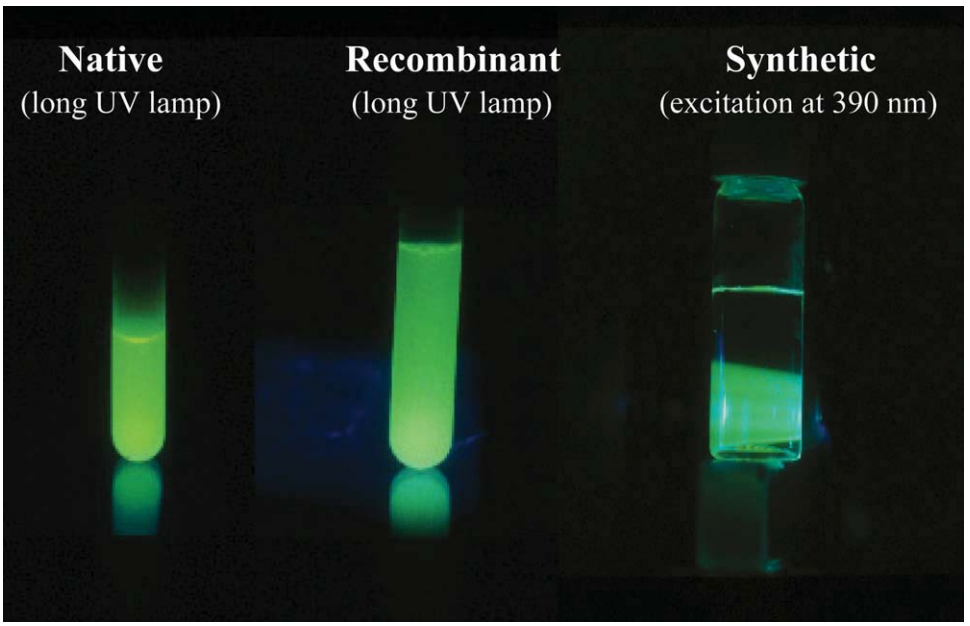

Fig. 6. Photograph of fluorescense of native, recombinant, and synthetic *Aequorea* GFP exposed to ultraviolet light. Photographs of native and recombinant GFPs were taken using 35 mm Kodachrome film; date of when film was processed is embossed on the slide as "MAR 94." Photograph of synthetic GFP is from Sakakibara (1999).

done using crystals prepared from our rGFP in B-C Wang's laboratory, University of Georgia.

NOTE ON AN UNFINISHED EXPERIMENT: STEREOCHEMISTRY OF *AEQUOREA* GREEN FLUORESCENT PROTEIN

The objective of this experiment was to synthesize and compare the physical-chemical properties of the L- and D-enantiomers of *Aequorea* GFP. Toward this end, we first succeeded in synthesizing the L-enantiomer of the precursor molecule of GFP containing all L-amino acids. This work took two and a half years and the protein folded in solution to give L- or native GFP (Nishiuchi et al., 1998). The synthesis of the D-enantiomer of the precursor molecule of GFP was not completed because of time constraints. Theoretically, the two GFPs would be optical isomers and have similar properties, rotating plane-polarized light to the same degree, but in opposite directions. We hope that GFP could serve as a useful paradigm for future studies in this area, possibly in investigating the conservation of parity in the GFP molecule.

DISCUSSION AND CONCLUSIONS

It is apparent from reviewing the published literature that the discovery of *Aequorea* GFP had its beginnings in the early observations and experiments of Harvey (1921) at Friday Harbor, Washington. On the basis of the results of Macartney (1810), his own observations on radiolarians, ctenophores, and *Pelagia* (Harvey, 1926) and on the findings of R. S. Anderson (in: Harvey, 1952), Harvey concluded that *Aequorea* did not require molecular oxygen for luminescence. Harvey (1926), therefore, hypothesized that molecular oxygen was bound in some form in *Aequorea* and that it became available during the luminescence reaction as a necessary component. Harvey (1921) was also the first to observe the green light-emitting granules released by cytolysis of photocytes. He also prepared luminescent extracts for observations under the microscope by squeezing luminous tissue through cheesecloth. Thus, many of the participants at the Asilomar Conference became aware for the first time of the nonrequirement for molecular oxygen and color of bioluminescence in the Hydromedusae (his Table I) (Harvey, 1955). Thus, *Aequorea* drew a great deal of attention because until then the concept that dominated bioluminescence studies was that of the "luciferin-luciferase" reaction, which requires molecular oxygen. It is to Johnson and Shimomura's foresight that they recognized this distinction and started a long series of studies, lasting for many years, on the *Aequorea* bioluminescence system. They went on to show that, in *Aequorea* bioluminescence, molecular oxygen is bound to coelenterazine and apoaequorin of aequorin which emits blue light on binding calcium ions (Fig. 1).

Upon initiating their studies of the *Aequorea* luminescence system, Johnson and Shimomura published two seminal articles: Shimomura et al. (1962) and Johnson et al. (1962). The Shimomura et al. (1962) article was devoted to the purification and properties of aequorin and, in a brief footnote, it alluded to the isolation of a slightly greenish "protein" with bright greenish fluorescence without providing further evidence (note also the 1955 article of Davenport and Nicol regarding the first observation of green fluorescence in the luminous organ of *Aequorea*). The Johnson et al. (1962) article was mainly devoted to the determination of the quantum efficiency of *Cypridina* luminescence,

with a note on *Aequorea* luminescence. This article, based on excitation and emission peaks of fluorescence of GFP, provided presumptive evidence, albeit fragmentary, of the existence of a GFP, "extracted and purified" from squeezates or brei of photogenic tissues of *Aequorea*. However, details regarding the extraction and purification procedures used were not described in the article. The observations in the two articles are in agreement with the early observations of Harvey (1921).

The work of Morin and Hastings (1971) deserves special mention because they were the first to conduct experiments and describe the steps involved in the *Aequorea* luminescence reaction and to point out the role played by resonance energy transfer in the emission of green light. Their article coming after a long period of inactivity in GFP research must be considered a milestone that is inseparable and central to the discovery of GFP.

The work of Cormier, Prasher, and Ward, on *Aequorea* and *Renilla* bioluminescence may be considered highly important and significant, especially with respect to the cloning and expression of the GFP gene.

Thus, the long-sought search for an answer to the question of why *Aequorea* does not require molecular oxygen for luminescence has led to the discovery of both aequorin, a calcium-binding photoprotein, which has molecular oxygen bound to it and emits blue light in the presence calcium ions, and GFP which is found in the green-light-emitting granules first described by Harvey (1921) and now used as a biological marker.

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