Extraction, Purification and Properties of Aequorin, a Bioluminescent Protein from the Luminous Hydromedusan, *Aequorea*[1]

OSAMU SHIMOMURA,[2] FRANK H. JOHNSON AND YO SAIGA
Department of Biology, Princeton University, Princeton, New Jersey, and the Friday Harbor Laboratories, University of Washington, Friday Harbor, Washington

In experiments that have become classic in bioluminescence, Dubois (1885, 1887), first prepared from a luminous elaterid, *Pyrophorus*, and a luminous clam, *Pholas*, respectively, crude extracts containing a substrate, luciferin, and an enzyme, luciferase, which luminesced on mixing in aqueous solution containing dissolved oxygen. In the years that have followed, efforts have repeatedly been made to separate functionally similar components from numerous other, divers types of luminous organisms (Harvey, '52; '55). Although the majority of these efforts proved unsuccessful, about a dozen biologically specific, chemically different luciferin-luciferase systems have by now been obtained in varying degrees of purification (Johnson, Sie and Haneda, '61). The present investigation has resulted in the discovery of a new type of luminescent system, differing from those hitherto extracted in being comprised of a single organic component, with the properties of a protein. In aqueous solution either devoid of, or saturated with, oxygen, this protein gives a light-emitting reaction on addition of Ca^{++}. With the procedures employed, nearly 10,000 individual specimens of the hydromedusan, *Aequorea*, yielded about 5 mg of the highly purified, active substance which we have named "Aequorin" (Shimomura, Johnson and Saiga, '62).

While certain aspects of the phenomenon of luminescence in medusae have long been known, the biochemistry involved has remained very much of an enigma. As early as the first century, Pliny (cf. Harvey, '57) described the light of "Pulmo Marinus," evidently *Pelagia noctiluca*, and observed that luminous slime from the bell could be rubbed *onto* various surfaces making them glow as if on fire. Spallanzani (1794, 1798) noted that luminescence of this organism continued after death, and that a dark, almost liquefied specimen luminesced on addition of fresh water. At the turn of the nineteenth century, von Humboldt (1799–1804; cf. von Humboldt, 1853) and Macartney (1810) found that luminescence of medusae could be elicited by electrical stimuli, a phenomenon that has only recently been investigated from a modern viewpoint (Davenport and Nicol, '55; Nicol, '60). Macartney (1810) also found that no diminution in luminescence could be detected in a vacuum, as compared to aerobic conditions. The lack of a free oxygen requirement for luminescence was convincingly demonstrated by Harvey ('26; Harvey and Korr, '38), not only for medusae but also radiolarians and ctenophores. Luminescence under strictly anaerobic conditions, however, is an unusual feature among the various types of luminescent systems now known (Harvey, '52; '55).

Though unsuccessful in attempts to obtain a luciferin - luciferase reaction with extracts of *Aequorea*, Harvey ('21) found that the photogenic tissues dried over $CaCl_2$ would luminesce when moistened. Moreover, strips of the margin of the umbrella, where the photogenic organs are

Received Jan. 19, '62. Accepted Feb. 8, '62.

[1] Aided in part by National Science Foundation grant number G-12857, Office of Naval Research Contract Nonr 1353(00), Project NR 165-253, and the Eugene Higgins Fund allocated to Princeton University. Publication in whole or in part by or for any purpose of the United States Government is permitted.

[2] On leave from the Pharmacy Department, University of Nagasaki, Japan.

situated, could be squeezed through cheese cloth yielding a brei that luminesced for a maximum of several hours, after which light was momentarily restored by cytolytic agents such as fresh water, saponin, chloroform and certain others. No further light could then be elicited by any method tried. Later efforts by Johnson (cf. Johnson, Sie and Haneda, '61) to obtain luciferin and luciferase from *Aequorea*, through a variety of approaches, were again unsuccessful. Suggestive evidence, however, for the presence of a luciferin-luciferase system was found in partially reversible inhibitions of light emission, by urea and other agents, in the brei or "squeezate," as well as in preparations that had been dried in *vacuo* or in cold acetone. Furthermore, active fractions could be precipitated by $(NH_4)_2SO_4$. Diffusible factors known to function in other systems, viz, nucleotides of adenosine, flavin and pyridine (cf. Johnson (ed.), '55; McElroy and Glass (eds.), '61), did not restore or enhance the luminescence of squeezates or other preparations, though a long chain, aliphatic aldehyde, required in bacterial luminescence, caused a flash of luminescence, evidently through a non-specific cytolytic action.

MATERIALS AND METHODS

Raw material

The raw material used throughout this study consisted of specimens of *Aequorea aequorea* collected in the environs of Friday Harbor, Washington. The photogenic organs consist primarily, if not exclusively, of oval shaped, slightly yellowish masses of tissue, in pairs, one on either side of the tentacular bulbs associated with the marginal canal; on gentle mechanical stimulation, a greenish luminescence arises from these masses, and when a quiescent animal is viewed in the dark under the ultraviolet from a Mineralite lamp, the same masses exhibit a greenish fluorescence that resembles the bioluminescence resulting from stimulation (Forbes, 1848; Harvey, '21; Davenport and Nicol, '55; and present observations). The photogenic cells are apparently quite delicate and easily ruptured, accompanied by the release of granules, which luminesce on contact with water. Evidently for this reason, large numbers of specimens could not be satisfactorily collected at one time by means, for example, of a herring net, in which considerable trauma during the collecting process is unavoidable. The best results were obtained with specimens collected with a hand net, treated as gently as feasible, and processed as soon as possible. The luminescence potency of a given individual could be roughly judged in advance by the intensity of fluorescence of the photogenic organs under the Mineralite lamp.

Measurement of luminescence

The rate and total amount of light emission were measured, usually at room temperature (24 to 25°C), by means of a photomultiplier-amplifier with Sanborn automatic recorder, in terms of "Light Units" (L.U.), one L.U. being arbitrarily defined as the integrated amount of light that would give one tenth a full scale deflection on the recorder with the amplifier gain set at 1×10^{-5} coulombs. The specimen holder was shielded in such a manner that solutions could be introduced without exposing the phototube to extraneous light. Assays of the amount of light in strips of photogenic tissues or in brei prepared therefrom were usually made by adding 5 ml of distilled H_2O or saponin solution to the specimen. During purification, 5 ml of 0.05 M Ca-acetate solution, except where stated otherwise, were added to initiate the light of a small volume of specimen. For purposes of comparison, the luminescence activity of crystalline *Cypridina* luciferin with purified *Cypridina* luciferase was measured under the same conditions usually employed for assays of *Aequorea* extracts; on adding 5 ml of luciferase in 0.05 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, to a small volume of a methanolic solution of luciferin, the total light was computed to be 6.4 million L.U./mg of luciferin.

Extraction procedure

Most of the non-luminescent tissue, together with the bulk of the organism, was eliminated at the start by cutting off with a pair of scissors a 3 to 5 mm wide strip

from the margin of the umbrella and discarding the remainder. Such strips, from a desired number of organisms, were dropped into cold sea water in which they could be kept several hours or longer with gradually diminishing luminescence potency.

A squeezate was prepared from an aggregate of cold, excised strips by placing them in a cotton handkerchief with ends twisted together to form a bag, then kneading, rolling and squeezing until most of the activity came through, usually leaving about a fourth or less of the bulk of starting material in the bag. On the average, 0.6 ml of squeezate, with an activity of some 3,000 L.U., was obtained per strip.

Preliminary experiments showed that when the squeezate was quickly filtered with the aid of filtercel (Johns-Manville Celite analytical filter-aid) on a Buchner funnel, most of the luminescence activity remained in the filter cake, which was found to emit light as a result of mechanical stimulation, rapid heating or cooling, freezing, or contact with organic solvents. Attempts to obtain active extracts with various organic solvents and salt solutions failed, but about 10% of the activity in the original squeezate could be recovered by extracting the filter cake with dilute acetic acid at pH 3.5 and neutralizing the filtered extracts with sodium bicarbonate. The rate of light emission on neutralizing, however, was much slower than that of a squeezate on adding fresh water. The rate on neutralizing could be greatly increased only by adding calcium salts. In place of acetic acid, subsequent filter cakes were extracted with EDTA solution to chelate the Ca^{++}, with the result that about half of the activity in the original squeezate could be recovered on addition of calcium salts, i.e., a five-fold better yield than with acetic acid. Based on these observations, the procedure developed for preparing extracts on a large scale was as follows.

A total of 11 batches of extracts were prepared from 500 to 1,200 strips at the time, aggregating over 9,000 strips in all. The squeezate in each instance was drained directly into a continuously stirred, saturated ammonium sulfate solution in an ice bath, immediately extinguishing the luminescence. The volume of ammonium sulfate solution used at this step was equivalent to the total volume of squeezate as estimated in advance. Filtercel in an amount equivalent to 30% by volume of the squeezate plus ammonium sulfate solution was added and the resulting slush quickly filtered on a pre-chilled, large Buchner funnel with the aid of a vacuum pump. The filter cake was transferred to a 1 or 2 L flask and a cold 0.05 M solution of the disodium salt of EDTA (EDTA-2Na) was added in a volume corresponding to that of the original squeezate. The flask was shaken vigorously for 10 seconds and the solution filtered through a Buchner funnel into a pre-cooled suction flask containing enough ammonium sulfate to saturate the filtrate. The filtrate became cloudy with a whitish precipitate which was centrifuged down in 30 minutes at 12,000 rpm in a refrigerated centrifuge at 0°C. Fifty per cent of the activity of the initial squeezate was recovered in the precipitate.

The centrifuged precipitates of the 11 separate extracts were combined in groups of 3 to 4, in 3 polyethylene bottles which were kept in a large thermos with cracked ice for transportation from Friday Harbor, Washington, to Princeton, N. J. During transportation and subsequent storage in this manner for about a month before proceeding with purification, a loss of some 40% in activity took place.

Purification

Purification was accomplished by repeated chromatography on DEAE columns. In preparation for chromatography, the stored contents of one polyethylene bottle were first centrifuged and the precipitate dissolved in about 5 volumes of 0.05 M EDTA-2Na solution which was then dialyzed against 0.01 M EDTA-2Na solution overnight at 3°C in cellophane tubing. More than 30% of the luminescence activity was lost in this process, but no co-factor activity was found in the dialysate. The dialyzed solution was adsorbed on a column of 25 g of DEAE (Calbiochem.) which had been preliminarily washed with 0.01 M EDTA-2Na. The substance active in luminescence was eluted with several

0.01 M EDTA-2Na solutions containing successively higher concentrations of NaCl from 0.05 M to 0.3 M. The eluates containing most of the activity were combined and saturated with amonium sulfate. A further loss of up to 50% in activity occurred during the process of chromatographing.

A cloudy precipitate, which formed in the ammonium sulfate solution, was difficult to sediment by centrifugation but was recovered by filtration on a Buchner funnel with the aid of filtercel that had been previously treated with EDTA-2Na solution. The luminescence substance was extracted from the filter cake with a small volume of 0.05 M EDTA-2Na solution at 3°C for 5 hours, with a 10% loss in activity, then purified on a 5 g DEAE column by means of NaCl concentration gradient chromatography, using a combination of 150 ml of 0.01 M EDTA-2Na in 0.03 M NaCl and 0.01 M EDTA-2Na in 0.4 M NaCl at pH 5.5. The effluent was collected by a fraction collector in approximately 5 ml portions which were tested for luminescence activity as well as absorption at 280 mμ, with the results shown in figure 1, indicating the presence of a large amount of impurities. The fractions having the highest activity, aggregating up to 80% of the total activity, were pooled and saturated with ammonium sulfate which again gave rise to a cloudy solution.

By the above procedure, the remaining two lots of material stored in polyethylene bottles were each partially purified and the cloudy, ammonium sulfate solutions resulting thereby were combined with the first. The precipitate in the combined solutions was centrifuged down at 10,000, rpm in 20 minutes at 0°C and dissolved in a small volume of 0.05 M EDTA-2Na solution, which was then dialyzed against 4 liters of 0.01 M EDTA-2Na for 4 hours. A white precipitate, which formed during dialysis, was removed by centrifugation, leaving a clear, almost colorless solution containing the substance with light-emitting activity. The latter was further purified by two successive NaCl concentration gradient chromatographings, using a 2 gm DEAE column each time, with a combination of 110 ml of 0.01 M EDTA-2Na in 0.03 M NaCl and 0.01 M EDTA-2Na in 0.5 M NaCl at pH 5.8. The effluent was collected in 2.5 ml fractions. Excess NaCl in the pooled most active fractions of the first run was removed by dialysis before repeating the procedure. Data on the effluent from the second run, illustrated in figure 2, indicate a high degree of purity of

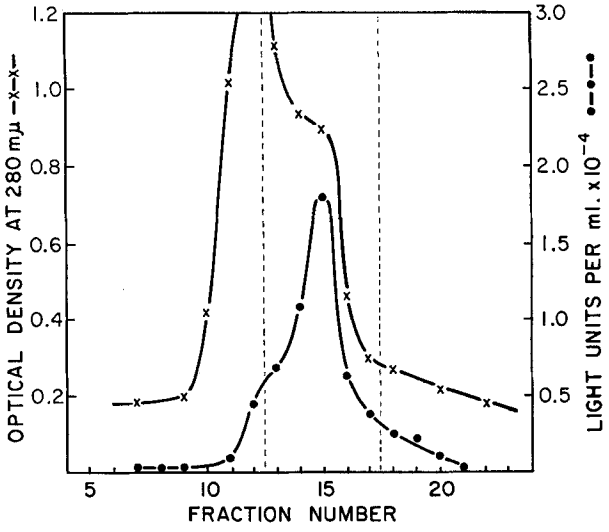

Fig. 1 Optical densities and luminescence activities of the fractions from the first gradient concentration chromatography of one of the three lots of stored EDTA extracts. At the peak of luminescence activity, the specific activity (L.U./ml divided by O.D) is 20,000. The portion between the broken lines was saved for further purification.

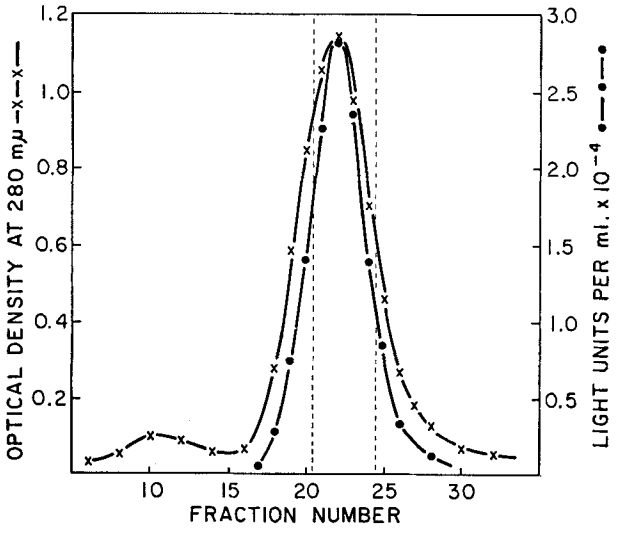

Fig. 2 Results of the final (third) gradient concentration chromatography, plotted in the manner of figure 1. The 4 fractions numbering 21 to 24, i.e. the material indicated between the broken lines, of highest purity were combined, giving a total volume of 10 ml with a specific activity of 23,200. The small peak in O.D. at the tenth fraction probably represents denatured aequorin, inasmuch as it recurred in each of repeated chromatographings.

the luminescence substance. Fractions 21, 22, 23, and 24, which were considered the most highly purified, were pooled and used for the major experiments on the characteristics of aequorin, the substance involved. The total light of these combined fractions amounted to 225,000 L.U., representing 0.8% of the total activity of the initial squeezates. The dry weight was estimated to be 5.3 mg after dialyzing a small portion, evaporating at room temperature and drying for 2 hours at 105°C. A better yield would undoubtedly have resulted except for the loss of material necessitated by exploratory and other experiments, as well as loss of activity during transportation and storage prior to purification. The remaining fractions, less highly purified, were used chiefly in experiments on the action of quenching and other agents.

PROPERTIES OF AEQUORIN

General

Aequorin has the general nature of a protein, as judged by positive Biuret and Ninhydrin reactions, precipitation by ammonium sulfate, and rapid loss of luminescence activity at elevated temperatures. It is unstable at pH lower than 4, even at 0°C. It is relatively stable, however, in 0.01 M EDTA-2Na. A solution containing 5 mg in 0.5 ml of solvent is clear and almost colorless, though saturation with ammonium sulfate yields a slightly yellowish precipitate. Separate components with light-emitting activity could not be obtained by dialysis, DEAE chromatography, ammonium sulfate precipitation, or paper electrophoresis, so it is reasonable to conclude that aequorin is a single substance. Because of adsorption of aequorin on paper, a precise isoelectric point was difficult to determine by paper electrophoresis, but it was evidently below pH 5.5. Although the amount available was insufficient for analysis of ultracentrifugal behavior, one run at 59,780 rpm in a Spinco E indicated that the molecular weight must be relatively low, and no detectable amount of any high molecular weight substance was present. Solutions exhibit no special fluorescence in the ultraviolet of a Mineralite lamp, but acquire a bluish, moderately bright fluorescence after the luminescent reaction that results on adding Ca^{++}. The quality of the light in the luminescent reaction itself is also bluish, differing in this

respect from the greenish luminescence of the whole organism or fresh squeezates.[3] The light-emitting potency was calculated to be 42,500 L.U./mg, which is equivalent to the total light of 6.6 μg of *Cypridina* luciferin whose molecular weight is 469 (Hirata, Shimomura and Eguchi, '59).

Ultraviolet absorption spectrum

Except for a slight bulge at 310 mμ, the ultraviolet absorption spectrum (fig. 3) is similar to that of simple proteins, with a peak at 280 mμ. After the luminescent reaction the bulge at 310 mμ disappears and a new absorption maximum shows up at 333 mμ.

Requirement of Ca^{++} for luminescence, and effects of other cations

The calcium requirement has been referred to above. Thirteen other cations in the form of salts of chloride, sulfate or acetate were tested for a possible activating effect in the luminescent recation by adding 5 ml of 0.01 M salt solution to 0.05 ml of aequorin in 0.01 M EDTA-2Na solution at pH 6.0. Among them, no activation was found with ions of magnesium, barium, potassium, ammonium, zinc, cobalt, manganese, ferric or ferrous iron, copper, or lead. A slight activation that occurred with cadmium was probably due to the presence of impurities, inasmuch as the purest available substance had the least effect. Some activating effect of strontium could either be real or due to impurities; the evidence is not conclusive.

In regard to calcium, the influence of concentration is illustrated by the data shown in figure 4,(A). When the concen-

[3] A protein giving solutions that look slightly greenish in sunlight though only yellowish under tungsten lights, 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 sustance could be detected. Studies of the emission spectra of both this protein and aequorin are in progress.

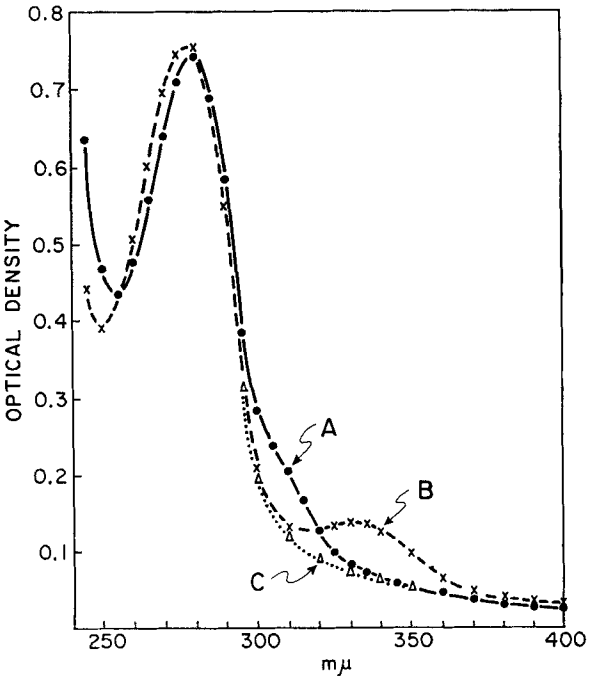

Fig. 3 Ultraviolet absorption spectra of aequorin (A), the product of the luminescent reaction (B), and the product of the reaction between aequorin and $NaHSO_3$ (C). The concentration of aequorin used for curve A was 0.41 mg/ml by dry weight; for curve B, 20 μl of 8% $Ca(CH_3COO)_2H_2O$ solution were added to a 3 ml portion of aequorin solution to complete the luminescent reaction; for Curve C, 10 μl of 10% $NaHSO_3$ solution were added to a 1 ml portion of aequorin solution.

tration of EDTA exceeds that of Ca^{++}, no luminescent reaction occurs. When the molarity of Ca^{++} is the same as that of EDTA, the volocity of the luminescent reaction is about half maximum, whereas only a slight excess of Ca^{++} results in nearly maximum velocity. The reaction accords with first order kinetics in each case (fig. 4, B).

Lack of an oxygen requirement

The quantitative effects of absence of oxygen, as compared to equilibration with air and pure oxygen, were investigated with the aid of the specially constructed vessel assembly diagrammed in figure 5. One ml containing 3 μg of aequorin in EDTA-2Na solution was added to the sample tube, and 10 ml of ca-acetate solution to the other. The gas inlet was connected by lead tubing, sealed on with DeKhotinski cement, to a source of hydrogen puri-

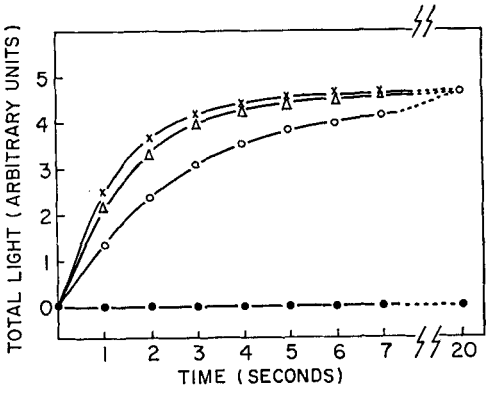

Fig. 4 (A) Influence of Ca^{++} concentration on the luminescent reaction of aequorin. To 0.1 ml aliquots of a 0.05 M EDTA-2Na solution of aequorin, were added 5 ml of calcium acetate solutions at concentrations of 0.1 M (crosses), 0.01 M (triangles), 0.001 M (hollow circles), and 0.0001 M (solid circles), respectively. The final concentration of EDTA was thus 0.001 M.

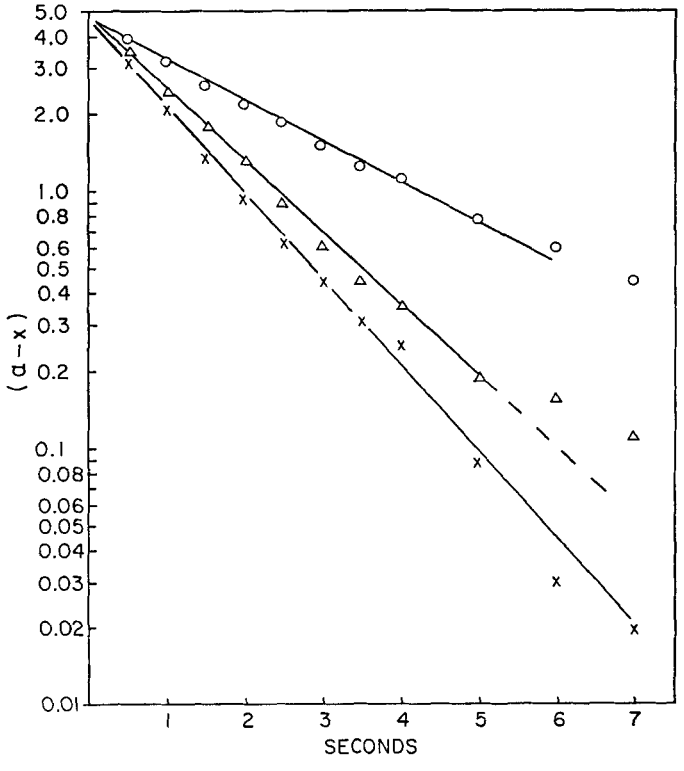

Fig. 4 (B) The data of figure 4 (A) plotted in the manner of a first order reaction, "a" representing the final total of light produced, and "x" the amount produced up to the times indicated on the abscissa.

fied over hot platinized asbestos. The vessels were placed in an ice bath and were evacuated followed by filling with hydrogen, 5 times in succession. Pure hydrogen was then passed through the system for 30 minutes, with occasional shaking of the vessels. The assembly was quickly removed from the ice bath, the sample tube placed in the holder of the photomultiplier, and the luminescence reaction set off with calcium acetate by rotating the other tube through 180° on the ground glass connection. Corresponding experiments except that evacuation of the system was omitted, were carried out with the solutions equilibrated with a stream of air and pure oxygen, respectively. The results are graphed in figure 6 which shows that the velocity of the reactions, as well as the total light finally emitted, are within about 10% of being the same, or well within the limits of experimental error under the conditions involved.

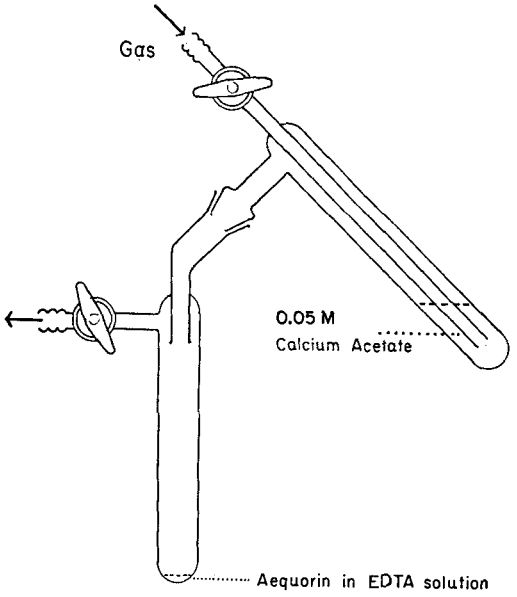

Fig. 5 Vessel assembly for measuring the luminescent reaction of aequorin solutions equilibrated with pure hydrogen, air, and oxygen, respectively.

Tests for catalytic properties and the influence of products of the reaction

Conceivably, aequorin might have catalytic properties characteristic of enzymes and act in the manner of a luciferase in the luminescent reaction. Evidence in this regard was sought through the following experiment, which at the same time pro-

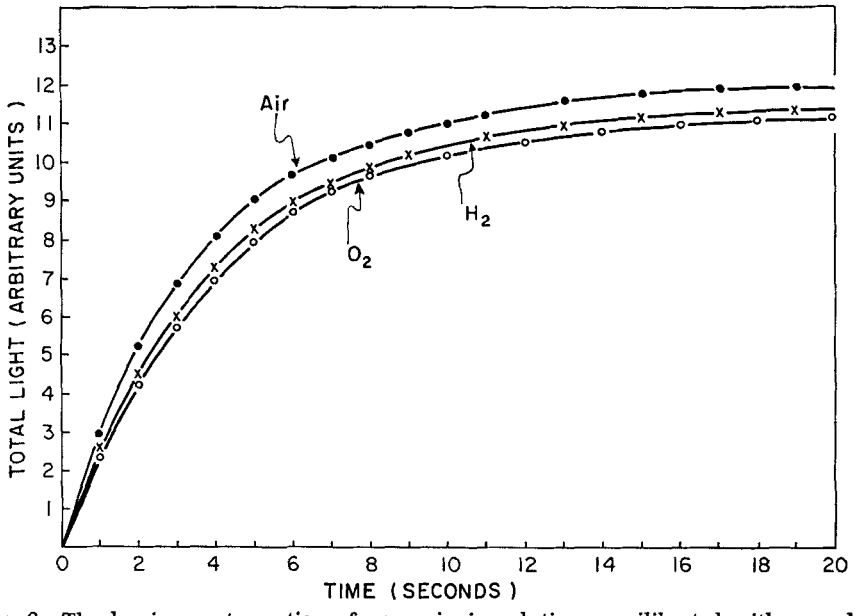

Fig. 6 The luminescent reaction of aequorin in solutions equilibrated with pure hydrogen, air and oxygen, respectively, as described in the text.

vided information concerning the influence of products of the reaction.

Twenty μg of aequorin were added to 5 ml of 10^{-4} M Ca-acetate containing 0.01 M Na-acetate and the mixture was used as a test solution after luminescence had ceased. The luminescent response of 2 μg of aequorin, on adding 5 ml of acetate solution of the same composition, was recorded and compared with the luminescent response of 2 μg of aequorin on adding 5 ml of the test solution. The results

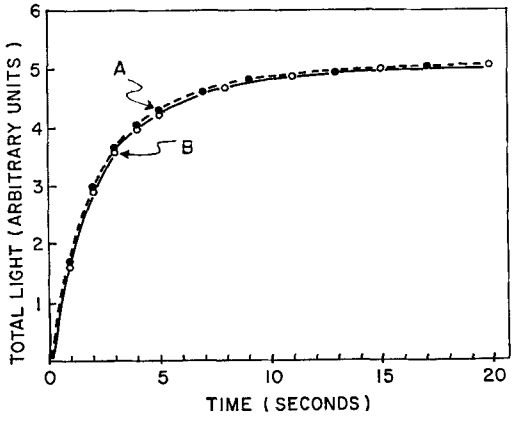

Fig. 7 The luminescent reaction of aequorin on adding solutions containing salts of calcium (A) and salts of calcium plus the reaction products of the luminescent reaction (B), as described in the text.

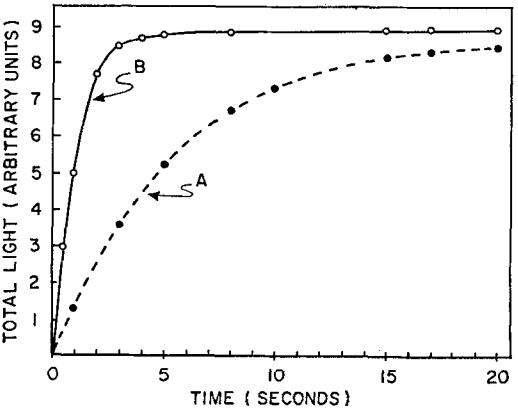

Fig. 8 Luminescence of the *Cypridina* system on adding to 0.02 μg of luciferin 10 ml of a 0.1 M sodium phosphate buffer solution, pH 7.0, containing 2 μg of luciferase (curve A), and the same buffer solution containing the reaction products of 0.2 μg of luciferin with 20 μg of luciferase (curve B).

are illustrated in figure 7, which shows a close agreement in both the velocity and final total of light emitted. Repeated experiments with various concentrations of aequorin showed a similarly close, or even closer agreement, but in each instance the velocity of light emission with the test solution was very slightly less than that of the control, possibly indicating a very slight inhibitory effect of reaction products. For comparison, an experiment was carried out in the same manner as the above, using components of the *Cypridina* system, with quite different results, as illustrated in figure 8. The activity of the luciferase in the test solution of a spent reaction speeds the process of light emission, as expected. Thus it is clear that the test solution of aequorin does not have properties of an enzyme for the aequorin reaction. It must be recalled, however, that the initially active aequorin becomes altered, as witnessed by the changes in absorption spectrum mentioned above, as a result of the luminescent reaction, and the possibility cannot be excluded that such alteration is accompanied by the loss of a catalytic property; by way of an analogy, the luciferases of certain kinds of fireflies are extremely unstable, at least in crude extracts (McElroy and Harvey, '51). While the evidence is insufficient to conclude unequivocally that aequorin does not act in the manner of an enzyme during the course of its luminescent reaction, if it did it would constitute an almost unique situation in which an enzyme undergoes destruction at a rate proportional to the reaction it catalyzes, and this not only appears extremely unlikely, but could scarcely result in the first order rate characterizing the light-emitting reaction of aequorin.

Influence of pH

For determining the influence of hydrogen ion concentration, portions of a 0.01 M solution of Ca-acetate were adjusted to various pH values by addition of acetic acid or sodium hydroxide, and added in 5 ml volumes to small aliquots of an aequorin solution in EDTA-2Na; the pH of the mixture was taken immediately after the resulting luminescent reaction. The data of figure 9, curve A, show that

the total light is independent of pH over a wide range, from 5.1 to 8.3. The relative velocity constants (fig. 9, curve B) are influenced differently, in that they increase with alkalinity from a near plateau between about pH 6.5 and 7.5, whereas they decrease much in the manner of an enzyme's activity as the pH is lowered from 6.5. The shape of the curve is indicative of more than one reaction being involved in the influence of pH on the observed velocity constant.

Influence of temperature

The influence of temperature on total light was determined by adding 0.05 ml of aequorin solution to an assay tube previously equilibrated in a water bath at the desired temperature, transferring quickly to the holder of the photomultiplier, and immediately adding 5 ml of a Ca-acetate solution that had been equilibrated in the same water bath. The results illustrated in figure 10, curve A, show that total light decreases with rise in temperature, much

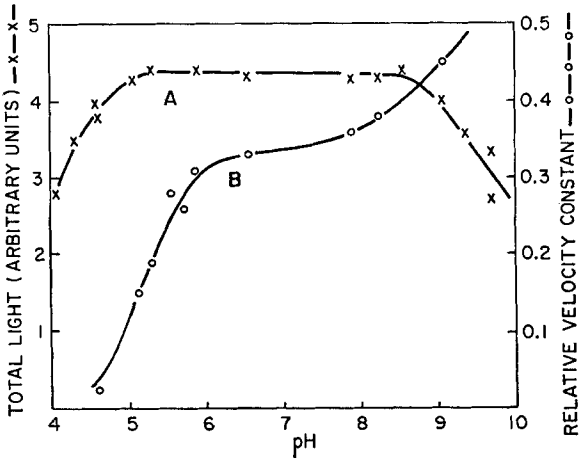

Fig. 9 Influence of pH on the total light produced (A), and rate of emission (B), as described in the text.

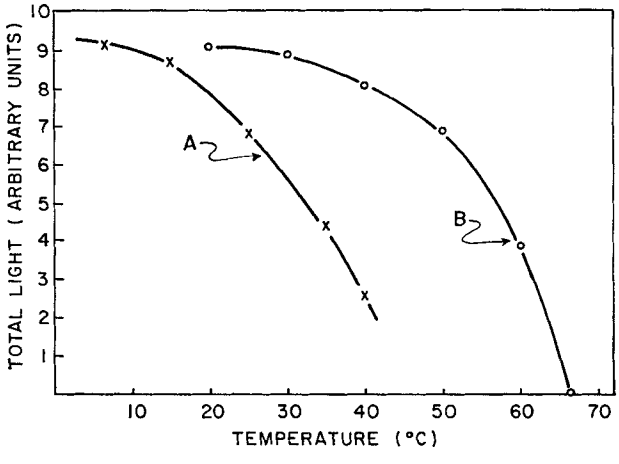

Fig. 10 Influence of temperature on the total light produced (curve A), and stability of aequorin in EDTA-2Na solution after one minute exposures to the temperatures indicated in Curve B, as described in the text.

in the manner of the *Cypridina* reaction (Chase and Lorenz, '45; cf. also, Johnson, Eyring and Polissar, '54, p. 154).

Data on the stability of aequorin as a function of temperature are represented in figure 10, curve B. The method in this instance consisted of first equilibrating the assay tube in a water bath at the desired temperature, then adding 0.1 ml of aequorin solution to the tube, leaving it in the water bath for one minute, and cooling quickly to 20°C in tap water. Total light was measured at this temperature by adding Ca-acetate solution. By this procedure, the temperature for half inactivation was 58°C. Similar experiments with the aequorin in saturated ammonium sulfate revealed a considerably increased stability, the temperature for half inactivation being about 25°C higher, or 83°C as compared to 58°C.

Inhibition and activation by various substances

Table 1 lists the results of experiments with various substances which at room temperature either reduced the total light or had no effect, and table 2 substances which, for the most part, caused appreciable increases in total light. The method consisted of adding 2 to 3 μg of aequorin to 1 ml of a solution of test substance in 0.01 M EDTA-2Na solution whose pH had been adjusted to between 6.0 and 6.5 by

TABLE 1

Influence on total light produced by adding 5 ml of 0.05 M calcium acetate solution to 2 to 3 μg of aequorin that had stood 5 minutes in 1 ml of various substances at the concentrations listed. The final concentration of each substance during the luminescent reaction was 1/6th of that given in the table

Number	Substance	Initial molar concentration	Per cent reduction in total light
1	$NaHSO_3$	0.0005	69
2	Sodium hydrosulfite	0.0005	60
3	$Na_2S_2O_3$	0.05	38
4	$NaAsO_2$	0.02	0
5	KCNS	0.05	0
6	KCN	0.05	0
7	KI	0.05	53
8	I_2-2KI	0.001	46
9	Br_2	0.00001	45
10	H_2O_2	0.05	0
11	KNO_2	0.05	0
12	$K_3Fe(CN)_6$	0.025	44
13	$K_4Fe(CN)_6$	0.025	0
14	$HgCl_2$	0.00001	65
15	*p*-Chloromercuribenzoic acid[1]	0.00001	45
16	Glutathione (GSSG)	0.01	0
17	Glutathione (GSH)	0.01	0
18	Diphosphopyridine nucleotide (DPN)	0.01	12
19	Diphosphopyridine nucleotide (DPNH)	0.01	0
20	N^1-Methyl nicotinamide chloride	0.001	39
21	Hydroquinone	0.05	54
22	Benzoquinone	0.000001	53
23	Benzaldehyde	0.03	34
24	*p*-Dimethylamino benzaldehyde	0.0004	40
25	Cinnamaldehyde	0.01	47
26	Pyridoxal hydrochloride	0.01	50
27	Dinitrofluorobenzene-$NaHCO_3$	0.002	68
28	Histidine hydrochloride	0.05	16
29	Cyanoacetic acid	0.05	24
30	Pyridine	0.05	0
31	Malonic acid[2]	0.05	0
32	Malonic acid, pyridine[2]	0.05 each	21
33	Barbituric acid	0.05	0
34	Barbituric acid, pyridine	0.05 each	7

[1] Not reversed by GSH.
[2] Rate of light emission reduced.

TABLE 2

Influence of alcohols on the total light produced by aequorin, according to the same procedure described in table 1

Number	Alcohol	Initial molar concentration	Per cent increase or decrease (−), in total light
1	*n*-Butyl alcohol	0.05	8
2	*n*-Amyl alcohol	0.05	7
3	*iso*-Amyl alcohol	0.05	3
4	*n*-Hexyl alcohol	0.01	10
5	*n*-Heptyl alcohol	0.01	30
6	*n*-Octyl alcohol	saturated	18
7	Cyclohexanol	0.05	5
8	Benzyl alcohol	0.05	−10

sodium hydroxide or acetic acid. The mixture was allowed to stand for 5 minutes, then 5 ml of Ca-acetate solution were added and the luminescence recorded.

Except for the inhibitory action of EDTA, which has already been discussed, available data are not sufficient to distinguish clearly in some instances between different possible mechanisms of action such as inhibition of the active groups, denaturation of the protein, quenching by reaction of excited molecules with components in the solvent, etc. (cf. Johnson, Eyring and Polissar, '54). Specific points of interest, however, with respect to the data in tables 1 and 2 appear worthy of comment, as follows:

In general, it is noteworthy that among the considerable number of substances listed in these tables, only one, viz., malonic acid, reduced the rate of the luminescent reaction. Only two other examples, EDTA (fig. 4) and a series of aliphatic aldehydes (fig. 11) reduce the rate, by a reversible inhibition. These results are in contrast to the susceptibility of extracted enzymes, involved in luciferin-luciferase systems of other luminous organisms, to inhibition by a large number of chemical agents (Harvey, '52; McElroy and Hast-

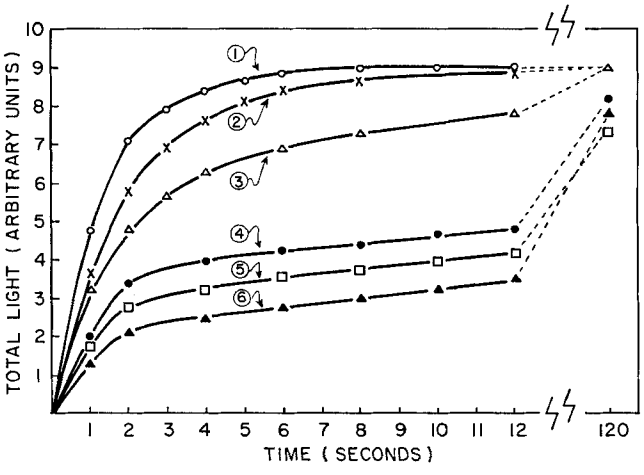

Fig. 11 Influence of aliphatic aldehydes on the luminescence of aequorin. Ten μl of EDTA-2Na solution containing 2 to 3 μg of aequorin were incubated 5 minutes at room temperature with 1 ml of the respective aldehyde solutions before initiating the luminescent reaction by adding 5 ml of 0.05 calcium acetate solution. The final aldehyde concentration, during luminescence, was thus 1/6th the following initial concentrations: none (control, with water; curve (1); 0.05 M propionaldehyde (2); 0.05 M isobutylaldehyde (3); 0.002 M caprylaldehyde (4); saturated capraldehyde (5); and saturated caproaldehyde (6).

ings, '55; Strehler, '55; Hastings and McElroy, '55; Airth, '61; Hastings and Bode, '61; Shimomura, Johnson, and Saiga, '61).

The effect of sodium hydrosulfite is weaker than that of sodium bisulfite. It is reasonably likely that the effects of the former are due to the bisulfite ion formed by decomposition of hydrosulfite in water.

Strong oxidative reagents such as halogens reduce activity as expected, whereas mild oxidative agents such as ferricyanide have little effect, in contrast to the action of the latter on the *Cypridina* system (Anderson, '36, '37). Much of the effect of ferricyanide on the aequorin system may be attributed to the absorption of emitted light by the colored solution. No influence of 0.05 M H_2O_2 was observed, and pure oxygen at 1 atm has no influence, as mentioned earlier (fig. 6).

The inhibitory action of $HgCl_2$ and *p*-chloromercuribenzoic acid, respectively, is possibly indicative of functionally active sulfhydryl groups. Similarly, the effect of dinitrofluorobenzene is possibly indicative of amino groups. The influence of various aromatic aldehydes, such as benzaldehyde, *p*-dimethylamino - benzaldehyde, cinnamaldehyde and pyridoxal, and aliphatic aldehydes, may be considered indicative of the presence, among other possibilities, of amino or active methylene groups. The mechanism of the potent action of benzoquinone is probably similar to that of aromatic aldehydes. The action of aliphatic aldehydes in particular calls for further comment, as follows.

The kinetics of light emission in the presence of aliphatic, but not aromatic, aldehydes show a change in specific reaction rate during the course of the reaction, and only a minor reduction in the final total of light produced (fig. 11). The major action of these substances evidently does not involve quenching in the usual sense of the word. A likely interpretation is that aliphatic aldehydes form a reversible combination with an amino or active methylene group of aequorin. Thus, the initial, rapid light emission which occurs on addition of Ca^{++}, is attributable largely to the fraction of aequorin uncombined with aldehyde. As the reaction proceeds, this fraction decreases and at the same time further, uncombined aequorin is released by dissociation of aldehyde in accordance with the equilibrium constant of the reversible combination. The situation is somewhat analogous to the luminescent reaction of partially purified *Cypridina* extracts containing at the start various amounts of reversibly oxidized luciferin which presumably undergo gradual reduction, accompanied by a light-emitting oxidation of the reduced form, during the course of the reaction (Anderson, '36).

Figure 11 shows that, as often obseved with a homologous series, the quantitative inhibition increases with length of the aliphatic chain. A somewhat similar relationship exists for activating effects of alcohols (table 2).

The influence of the last 6 substances in table 1 suggest the presence in aequorin of a carbonyl group active in luminescence, pyridine possibly catalyzing the reaction between the substance and this group.

The inhibitory effects of some substances increase at lower pH, e.g., bisulfite and I_2-2KI, especially the latter, which caused a 50% inhibition with a 2.5×10^{-6} M solution at pH 4.5, in contrast to a 46% inhibition with a 1×10^{-3} M solution at pH 6.4.

The inhibition by KI gradually increases with time of standing with aequorin prior to the luminescent reaction; this action is perhaps a general denaturation of protein. Similarly, the effects of water-insoluble organic solvents are possibly due to protein denaturation. Thus when aequorin in 1 ml of 0.01 M EDTA-2Na solution, pH 6.0, was shaken for 5 seconds with 1 ml of benzene, ether, methylene dichloride or ethyl acetate, the loss of total light amounted to 60, 60, 100, and 100 per cent, respectively.

The increase in total light in the presence of alcohols (table 2) is not readily understandable, particularly since macroscopic particles of photogenic material, such as occur in squeezates and emit light under the influence of various cytolytic agents including alcohols, are not present in homogenous solutions of aequorin as judged, for example, by the method of preparation. On the other hand, small

concentrations of ethyl alcohol increase the intensity of bacterial luminescence both in intact cells and in extracts (Johnson et al., '45; Strehler and Johnson, '54), though only at relatively low temperatures. At certain concentrations of propyl alcohol, the luminescence of bacterial cells or extracts undergoes a marked increase at low temperatures and marked decrease at higher temperatures (Johnson and Plough, '59). No unique interpretation can be offered in explanation of these and related phenomena at the present time.

Some other reagents tested, such as 8-hydroxyquinoline-5-sulfonic acid, *p*-aminobenzoic acid, and adenosine triphosphate (ATP) each in an initial concentration of 0.01 M, or hydrazine hydrochloride, hydroxylamine hydrochloride, sodium formate, potassium oxalate, ascorbic acid, resorcinol, methanol and ethanol, each in a concentration of 0.05 M, had no observed effect at the temperature and concentrations of the respective substances involved.

DISCUSSION

Nature of the luminescence system

The evidence indicates that the system active in purified extracts is comprised of a single organic component which in aqueous solution undergoes a luminescent reaction in the presence of Ca^{++}. In contrast to all other systems extracted from luminescent organisms thus far, no free oxygen or added peroxide is required, no peroxide is produced during the reaction,[4] and no separate substrate and enzyme, i.e., luciferin and luciferase in the usual sense of the terms, are involved (Harvey and Tsuji, '54; Harvey, '55; Johnson, Sie and Haneda, '61); indeed, the single organic substance, aequorin, does not even act as an enzyme in the light-emitting reaction. The over-all reaction in water can be written simply as follows:

Aequorin + Ca^{++} $\rightarrow$ products + light + Ca^{++}. Thus, at least in terms of minimal requirements for luminescence, the aequorin system is the simplest yet discovered. Moreover, it is reasonable to believe that comparably simple systems exist among certain other types of luminescent organisms from which attempts to extract a luciferin-luciferase system have been unsuccessful.

In living *Aequorea*, the photogenic substance is confined to densely packed, nonsecretory cells, which in turn are packed with granules of some 0.5 μ in diameter or smaller, ranging down to the limits of optical resolution, and luminescence appears to be normally intracellular (Davenport and Nicol, '55). According to the available evidence, intracellular luminescence can be elicited by gentle mechanical stimulation or directly by electrical stimulation without the participation of nerve activity except, at most, very locally. Unless such means of stimulation cause momentary, and perhaps reversible, increases in permeability to Ca^{++}, which is a reasonable possibility, the nature of the excitatory process is difficult to account for. On the other hand, luminescence resulting from traumatic stimulation, or the action of cytolytic agents, is easily understandable in terms of the release of aequorin into aqueous solution containing Ca^{++}. In fact, the luminous slime released by handling these organisms emits a distinctly bluish light, in contrast to the greenish light emanating from the small masses of intact photogenic cell masses, or from granules and particles of tissues in fresh squeezates. Although other explanations are possible, it is reasonable to suppose that the greenish quality results from a light-filtering effect and fluorescence of the green protein[5] which is highly concentrated together with aequorin in the photogenic cells. If this supposition is correct, perhaps the green protein has some biological significance, through its influence on the quality of the light of bioluminescence.

The functional group and molecular weight of aequorin

The changes in absorption spectrum of aequorin accompanying the luminescent reaction, i.e., the disappearance of the bulge at 310 mμ and the appearance of a new peak which attains a maximum at 333 mμ on completion of the reaction, very likely represent structural changes of

[4] Negative results were obtained in tests for peroxide by the KI-starch method and others.

[5] See footnote 3.

groups that function in light emission. As shown in figure 3, the bulge at 310 mμ quickly disappears, along with luminescence activity, on addition of very small amounts of sodium bisulfite or sodium hydrosulfite. Further reduction with a little more hydrosulfite resulted in a slight increase in absorption at about 340 mμ. On the other hand, the peak at 333 mμ in the product of the luminescent reaction is not affected by hydrosulfite, though it is slightly decreased by propionaldehyde. It will be recalled that the product of the luminescent reaction fluoresces blue under ultraviolet light, and that this fluorescence is not removed by dialysis. Moreover, the fluorescent substance is decomposed by acidity below about pH 4.

From the foregoing data, it is a likely assumption that an electron transfer system with a pyridinium skeleton, probably N^1-substituted nicotinamide, familiarly encountered in pyridine nucleotide co-enzymes is present. The peak at 333 mμ would thus be attributed to absorption by a reduced pyridinium derivative combined with protein, analogous to the diphosphopyridine nucleotide (DPN) complex of alcohol, aldehyde and other dehydrogenases (cf. Velick, '61). The assumption of the presence of N^1-substituted nicotinamide in aequorin is supported by the inhibitory action of DPN and N^1-methyl nicotinamide chloride. This action is understandable in terms of electron transfer from one of at least two possible functional groups of aequorin to the externally added electron acceptor which, in the above instances, is presumably related chemically to the other functional group.

Assuming one functionally active pyridinium group in aequorin, and a value of 6 for the millimolar extinction coefficient of its peak at 333 mμ as a reasonable value for reduced DPN in a protein complex from the data of Theorell and Bonnichsen ('51) and later investigators, the molecular weight of aequorin can be computed from the data shown in figure 3. On this basis, a molecular weight of 35,000 was calculated, which, it is interesting to note, is close to the equivalent molecular weights of several dehydrogenase-DPN complexes (Velick, '61).

Using the above value for molecular weight, and data on total light emitted by known amounts of aequorin, the luminescence efficiency per molecule of aequorin was calculated to be one half that of *Cypridina* luciferin under the conditions involved.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the helpful cooperation of Dr. Robert Fernald, Director, and Dr. Arthur Whiteley, who facilitated the progress of the work at the Friday Harbor Laboratories in various ways.

SUMMARY

When the brei, obtained by squeezing through cloth marginal strips of *Aequorea*, is filtered with filtercel, photogenic material in the filter cake can be extracted in solutions of the di-sodium salt of EDTA (EDTA-2Na) and will luminesce on addition of Ca^{++}.

Purification by ammonium sulfate precipitation and column chromatography on DEAE yields a substance, aequorin, with properties of a protein having an estimated molecular weight of 35,000 which emits a flash of light in aqueous solution on addition of Ca^{++} in an amount at least molar equivalent to that of EDTA present. A large number of other cations could not replace Ca^{++} though questionable activation of luminescence occurred with Sr^{++}.

The rate of light emission follows first order kinetics and is maximal with a slight molar excess of Ca^{++}. Quantitatively the same rate and total light produced occur in solutions equilibrated with pure hydrogen, air, or pure oxygen. The products of the reaction have essentially no influence. Components active in light emission are not separated from EDTA solutions of aequorin by dialysis, ammonium sulfate precipitation, DEAE chromatography, or paper electrophoresis.

The total light produced decreases with rise in temperature from 0° to 40°C. Stability decreases with rise in temperature, half of the activity disappearing during one minute exposure of aequorin in EDTA-2Na solution to 58°. When saturated with ammonium sulfate, half the activity is lost in one minute at 83°C. At room temperature, the total light produced is independ-

ent of pH between 5.1 and 8.3, but decreases at more acid or more alkaline pH. The rate of light emission has a near plateau between about pH 6.5 and 7.5, decreasing at more acid pH but increasing at more alkaline pH.

Among a large number of substances tested for their influence on the rate and total of light emission, only three types of substances, *viz.*, malonate, EDTA-2Na, and a series of aliphatic aldehydes, were found reversibly to inhibit the rate but not the total. A considerable number of substances had quenching effects, in reducing the total light without affecting the rate, e.g., aromatic aldehydes, inorganic reducing agents or strong oxidizing agents, *p*-chloromercuribenzoic acid, $HgCl_2$, N^1-methyl nicotinamide chloride, hydroquinone, benzoquinone, histidine hydrochloride, dinitrofluorobenzene-$NaHCO_3$, and cyanoacetic acid. Added H_2O_2 had no influence, and no H_2O_2 could be detected in the luminescent reaction mixture. Substances which had no effect, under the conditions involved, include such inorganic agents as arsenite, cyanide, ferrocyanide, nitrite and thiocyanate, and such organic agents as ATP, *p*-aminobenzoic acid, ascorbic acid, barbiturate, DPNH, GSH, GSSG, ethanol, hydrazine hydrochloride, hydroxylamine hydrochloride, 8-hydroxyquinoline-5-sulfonic acid, malonate, methanol, pyridine, potassium oxalate, resorcin, and sodium formate, respectively. The total light emitted was increased by a series of aliphatic alcohols, by an amount tending to increase with chain length, whereas an aromatic alcohol, i.e. benzyl alcohol, caused a slight decrease in total light.

The ultraviolet absorption spectrum of aequorin in EDTA solution has a maximum at 280 mμ and, except for a slight bulge at 310 mμ is similar to that of simple proteins. In the luminescent reaction initiated by Ca^{++}, the bulge disappears and a new peak appears at 333 mμ, which is considered as possibly indicative of the presence of a reduced pyridinium derivative combined with the protein. The nature of functional groups and some aspects of their role in the light-emitting reaction is discussed.

In terms of minimal components required for a light-emitting reaction in aqueous solution, namely aequorin plus Ca^{++}, this luminescent system extracted from *Aequorea* is the simplest yet discovered. Its efficiency is computed to be one-half that of the *Cypridina* system, in terms of the amount of light emitted per molecule of aequorin and *Cypridina* luciferin, respectively, under the conditions involved.

LITERATURE CITED

Airth, R. L. 1961 Characteristics of cell-free fungal bioluminescence. In McElroy, W. D., and B. Glass (eds.), Light and Life, pp. 262–273. Johns Hopkins Univ. Press., Baltimore, Md.

Anderson, R. S. 1936 Chemical Studies of bioluminescence. III. The reversible reaction of *Cypridina* luciferin with oxidizing agents and its relation to the luminescent reaction. J. Cell. Comp. Physiol., *8:* 261–276.

——— 1937 Chemical Studies of bioluminescence. IV. Salt effects on the total light emitted by a chemiluminescent reaction. J. Amer. Chem. Soc., *59:* 2115–2117.

Chase, A. M., and P. B. Lorenz 1945 Kinetics of the luminescent and non-luminescent reactions of *Cypridina* luciferin at different temperatures. J. Cell. Comp. Physiol., *25:* 53–63.

Davenport, D., and J. A. C. Nicol 1955 Luminescence in hydromedusae. Proc. Roy. Soc., B, *144:* 399–411.

Dubois, R. 1885 Fonction photogénique des pyrophores. Compt. ren. soc. biol., *37:* 559–562.

——— 1887 Note sur la fonction photogénique chez les Pholades. Ibid., *39:* 564–566.

Forbes, E. 1848 A monograph of the naked-eyed Medusae with figures of all the species. Ray. Society, London.

Harvey, E. N. 1921 Studies on bioluminescence. XIII. Luminescence in the coelenterates. Biol. Bull., *51:* 89–97.

——— 1952 Bioluminescence. Academic Press, New York.

——— 1955 Survey of luminous organisms: problems and prospects. In, F. H. Johnson (ed.), The luminescence of biological systems, pp. 1–24. Amer. Assoc. Adv. Sci., Washington, D. C.

——— 1957 A history of luminescence. Amer. Philosoph. Soc., Philadelphia.

Harvey, E. N., and I. M. Knorr 1938 Luminescence in absence of oxygen in the ctenophore, *Mnemiopsis*. J. Cell. Comp. Physiol., *12:* 319–323.

Harvey, E. N., and F. I. Tsuji 1954 Luminescence of *Cypridina* luciferin without luciferase, together with an appraisal of the term, luciferin. Ibid., *44:* 63–76.

Hastings, J. W., and V. C. Bode 1961 Ionic effects upon bioluminescence in *Gonyaulax* extracts. In, McElroy, W. D., and B. Glass (eds.), Light and Life, pp. 294–306.

Hastings, J. W., and W. D. McElroy 1955 In, Johnson, F. H. (ed.), The luminescence of bio-

logical systems, pp. 257–264. Amer. Assoc. Adv. Sci., Washington, D. C.

Hirata, Y., O. Shimomura and S. Eguchi 1959 The structure of *Cypridina* luciferin. Tetrahedron Letters, No. 5, pp. 4–9.

von Humbolt, A. 1853 Personal narrative of travels to the equinoctial regions of America during the years 1799–1804. Three vols, Trans. by T. Ross, London.

Johnson, F. H. (ed.) 1955 The luminescence of biological systems. Amer. Assoc. Adv. Sci., Washington, D. C.

Johnson, F. H., H. Eyring and M. J. Polissar 1954 The kinetic basis of molecular biology. John Wiley and Sons, Inc., New York.

Johnson, F. H., H. Eyring, R. Steblay, H. Chaplin, C. Huber, and G. Gherardi 1945 The nature and control of reactions in bioluminescence. With special reference to the mechanism of reversible and irreversible inhibitions by hydrogen and hydroxyl ions, temperature, pressure, alcohol, urethane, and sulfanilamide in bacteria. J. Gen. Physiol., *28:* 463–537.

Johnson, F. H., and B. Plough 1959 The action of DFP and related drugs on bacterial luminescence. J. Cell. Comp. Physiol., *53:* 279–306.

Johnson, F. H., H-C. Sie and Y. Haneda 1961 The luciferin-luciferase reaction. In, McElroy, W. D., and B. Glass (eds.), Light and Life, pp. 206–218. Johns Hopkins Univ. Press, Baltimore.

Macartney, J. 1810 Observations upon luminous animals. Phil. Trans., *100:* 258–293.

McElroy, W. D., and B. Glass (eds.) 1961 Light and Life. Johns Hopkins Univ. Press, Baltimore.

McElroy, W. D., and E. N. Harvey 1951 Differences among species in the response of firefly extracts to adenosine triphosphate. J. Cell. Comp. Physiol., *37:* 83–90.

McElroy, W. D., and J. W. Hastings 1955 Biochemistry of firefly luminescence. In, F. H. Johnson (ed.), The luminescence of biological systems, pp. 161–198. Amer. Assoc. Adv. Sci., Washington, D. C.

Nicol, J. A. C. 1960 The regulation of light emission in animals. Biol. Rev. Camb. Phil. Soc., *35:* 1–42.

Shimomura, O., F. H. Johnson and Y. Saiga 1962 Purification and properties of aequorin, a bio-(chemi-) luminescent protein from the jellyfish, *Aequorea aequorea*. Fed. Proc., April, 1962, *21:* 401.

Shimomura, O., F. H. Johnson and Y. Saiga 1961 Purification and properties of *Cypridina* luciferase. J. Cell. Comp. Physiol., *58:* 113–124.

Spallanzani, L. 1794 Memoria sopra le Meduse fosforiche. Mem. d. Mat. e Fis. d. Soc. Ital. d. Sci. Modena e Verona, *7:* 271–290. ,

——— 1798 Travels in the two Sicilies and some parts of the Apennines. Trans. from original Italian (1793). Chap. 27, on phosphorescent medusae. London.

Strehler, B. L. 1955 Factors and biochemistry of bacterial luminescence. In, Johnson, F. H. (ed.), The luminescence of biological systems, pp. 209–240. Amer. Assoc. Adv. Sci., Washington, D. C.

Strehler, B. L., and F. H. Johnson 1954 The temperature-pressure-inhibitor relations of bacterial luminescence *in vitro*. Proc. Nat. Acad. Sci.. (Wash.), *40:* 606–617.

Theorell, T., and R. Bonnichsen 1951 Studies on liver alcohol dehydrogenase. I. Equilibria and initial reaction velocities. Acta Chem. Scand., *5:* 1105–1126.

Velick, S. F. 1961 Spectra and structure in enzyme complexes of pyridine and flavin nucleotides. In, McElroy, W. D., and B. Glass (eds.), Light and Life, pp. 108–143. Johns Hopkins. Univ. Press, Baltimore.