Properties of the Bioluminescent Protein Aequorin*

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ABSTRACT: A protein, aequorin, having no more than the usual, slight fluorescence of proteins in general, gives a bright, blue, light-emitting reaction with Ca^{2+}, leading irreversibly to a strongly blue fluorescent protein product; the reaction mechanism presumably involves an unusual intramolecular chemical change catalyzed by Ca^{2+}. A functional moiety in this bioluminescence reaction can be separated from the protein in the form of either of two blue fluorescent compounds designated as "AF-350" and "AF-400." The former has an absorption maximum at 350 mμ, and molecular weight of 277 by mass spectrometry; its separation from aequorin is by treatment with urea and mercaptoethanol at alkaline pH, leaving the protein moiety as "apoaequorin-SH." The latter fluorescent compound (AF-400) has an absorption maximum at 400 mμ; its separation from aequorin is by treatment with $NaHSO_3$, leaving apoaequorin-SO. Critically determined properties of aequorin include: $E^{1\%}_{1\,cm}$ at 280 mμ = 27.0; $s_{20,w} = 2.90 \times 10^{-13}$ sec; $D_{20,w} = 8.70 \times 10^{-7}$ cm^2/sec; MW by Sephadex G-75 gel filtration = 23,000; MW_{sD} = 30,000 (using partial specific volume = 0.729); MW by amino acid analysis = 32,000; and luminescence activity = 4.5×10^{15} photons per mg dry weight at 25°. The chemical composition of aequorin includes, in addition to the above-mentioned functional moiety, 18 different amino acids, an unidentified other amino compound, glucose, no phosphate, and no acetylneuraminic acid.

The minimum components required for a light-emitting reaction in the bioluminescence[1] system of the hydromedusan jellyfish *Aequorea* are a protein "aequorin" and either calcium or strontium (Shimomura *et al.*, 1962, 1963a). Unlike most other bioluminescence systems (Harvey, 1952; Johnson, 1967; Cormier and Totter, 1968), molecular oxygen is not required. Other basic differences between this and most other bioluminescence systems which have been obtained in cell-free extracts include the facts that (1), the aequorin system involves no diffusible organic factor and (2), it involves no direct participation of enzyme activity in the usual sense (Shimomura *et al.*, 1962). Thus, according to available evidence, the protein aequorin is not to be considered an enzyme, and the system itself is not to be considered as belonging among the various bioluminescent, substrate–enzyme systems which for many years have been designated in general as luciferin–luciferase systems.

With a given amount of purified aequorin, the rate of light emission depends upon the available Ca^{2+} or Sr^{2+}, subject to influence by such factors as the presence of EDTA, and of ions such as Mg^{2+} which, though inert in causing light emission to occur, evidently compete with Ca^{2+} or Sr^{2+} (Shimomura *et al.*, 1963b). The total light-emitting capacity, in contrast to the rate, is proportional to the amount of aequorin over a wide range of concentrations of both the protein and of calcium.

The sensitivity of aequorin to Ca^{2-} provides the basis for microdetermination of free Ca^{2+} in biological systems (Shimomura *et al.*, 1963b; Blinks *et al.*, 1969); 10^{-7} M, or less, of Ca^{2+} in a small volume can be readily detected, with a response time of the order of milliseconds (van Leeuwen and Blinks, 1969). Recent applications of the test for calcium by luminescence of aequorin include rapid changes in concentration of this ion on stimulation and contraction of single muscle fibers (Ridgway and Ashley, 1967; Ashley and Ridgway, 1968) and rate of Ca^{2-} uptake by mitochondria (Chance *et al.*, 1969).

The mechanism of this light-emitting reaction, whereby calcium or strontium can trigger the release of over 70 kcal of energy as visible radiation through a single transition, involving a single kind of protein, remains obscure. The reaction is not inhibited by most enzyme-inhibitory substances. It is accompanied by a change in absorption spectrum, and by a change from a scarcely fluorescent aequorin to a product which fluoresces under ultraviolet light with almost the same spectral distribution as that of light emitted in the reaction with Ca (Johnson *et al.*, 1962; Shimomura *et al.*, 1963a). As an

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[1] The term "bioluminescent protein," is used here as it was in the first report on purification of this system (Shimomura *et al.*, 1962). More recently, the general term "photoprotein" was suggested (Shimomura and Johnson, 1966) to designate the type of bioluminescence system, including this and at least two others, wherein the total light emitted under optimum conditions is proportional to the amount of a specific protein component, in distinction to the classical "luciferin–luciferase" type of system (Harvey, 1952; Johnson, 1967) wherein the total light is proportional to the amount of an organic, diffusible substrate (luciferin) which undergoes an oxidative decomposition, catalyzed by an enzyme (luciferase), and accompanied by emission of visible light. In luciferin–luciferase systems, the substrate and enzyme are usually highly specific, and in the few instances that the structure of the substrate has been established, the luciferins from different biological sources are chemically quite different. Although about a dozen luciferin–luciferase systems have been obtained in cell-free extracts, which have been purified to various extents, only three photoprotein systems have been described and purified thus far, namely, those of (1) *Aequorea* (and a fully analogous system in the closely related hydromedusan, *Halistaura*), (2) the "paddle worm" *Chaetopterus* (Shimomura and Johnson, 1966, 1968a), and (3) the deep sea euphausiid shrimp ("krill") *Meganyctiphanes* (Shimomura and Johnson, 1967, 1968b). Suggestive evidence of analogous photoprotein systems in several species of the hydroid *Obelia* as well as in the ctenophore *Mnemiopsis* has recently been reported in an abstract (Hastings and Morin, 1968).

approach toward understanding the reaction mechanism, we have undertaken a critical study of the properties of aequorin. Information in this regard should be helpful also in regard to the use of aequorin in the test for calcium. The present paper summarizes the information gained through experiments with approximately 165 mg of very highly purified aequorin obtained from some 60,000 specimens of *Aequorea* collected during the past two summer seasons at Friday Harbor, Washington.

Materials and Methods

A couple of general points regarding procedures should be emphasized. Since the luminescence activity of aequorin is irreversibly destroyed not only in the light-emitting reaction with Ca^{2+} or Sr^{2+} but very easily also through nonlight-emitting reactions with a great variety of other inorganic as well as organic substances, even at refrigerator temperatures, chemicals of highest purity were used throughout and whenever possible the temperature was kept close to 0°. Moreover, although once-distilled water was satisfactory for the $(NH_4)_2SO_4$, EDTA, and other solutions used in the extraction and initial steps of purification, deionized distilled water was used throughout the remainder of the work. Parenthetically, all EDTA solutions were made from the disodium salt, with pH adjusted to 5.5 (except where stated otherwise) by addition of $NaHCO_3$. The urea used in various experiments was Mann's Ultra-Pure.

Extraction and Purification of Aequorin. Modification of the originally described procedures (Shimomura *et al.*, 1962, 1963a) resulted in an improved yield, amounting to as much as 15-fold. The details are as follows.

The circumoral ring canal with adjacent tissues, which include the nearly microscopic photogenic organs, was cut off with a pair of scissors in as thin a ring as feasible and dropped into sea water in an ice bath. Usually, 1000 rings were squeezed through 2 linen handkerchiefs (500 rings each) into 700 ml of cold saturated $(NH_4)_2SO_4$ containing 0.05 M EDTA (pH 5.5) with stirring. The average volume per ring was 0.3 ml in the squeezate, which was about half that of the previous report; considerable mucous and other substances which interfered with stability and purification of aequorin were thus eliminated. The average luminescence activity in the squeezate was 0.9–1.2 $\times 10^{14}$ quanta/specimen of *Aequorea*.

Approximately 350 ml of Celite (Johns Manville analytical filter-aid) was added to the squeezate and the mixture was filtered on a Büchner funnel. The filter cake was vigorously shaken in 700 ml of cold 0.05 M EDTA at pH 5.5 and then filtered on a Büchner funnel into a suction flask containing 480 g of powdered $(NH_4)_2SO_4$, followed by washing with 100 ml of 0.05 M EDTA into the same flask. The resulting precipitate of crude aequorin contained *ca.* 82% of the luminescence activity of the initial squeezate. Preliminary purification, essential to stability of the active component during storage at −20 to 0° for periods up to 3 or 4 months, was accomplished by a batch method with DEAE-cellulose as follows.

The above precipitate was collected with a small amount of Celite on a Büchner funnel. The filter cake was extracted with 0.05 M EDTA (pH 5.5) first with 100 ml then with three portions of a second 100 ml. The combined extracts were diluted with 3.5 l. of cold 0.005 M EDTA (pH 6.0), then 60 g of DEAE-cellulose by dry weight, pretreated with 0.01 M EDTA at pH 6.0, was stirred in. After 10–30 min of stirring, when 90–95% of the luminescence activity was adsorbed on the DEAE-cellulose according to assay of a small volume of the supernatant, the mixture was quickly filtered and the filter cake was extracted first with 500 ml of 0.01 M EDTA–0.3 M NaCl (pH 6.0) followed by extraction with additional 400 ml in three to four portions. The combined extracts were saturated with $(NH_4)_2SO_4$ and the precipitate which formed was harvested with the aid of centrifugation, for storage in a plastic bottle in a freezer, pending further purification. The yield of luminescence activity at this stage was about 65% of that in the squeezate, with an increased stability.

The above procedure was satisfactory for a larger number of specimens, up to a maximum of about 2000 at one time, merely by increasing the scale at each step proportionally. For a considerably smaller number of specimens, *e.g.*, 200–300, the DEAE-cellulose batch method could be conveniently replaced by gel filtration on a column of Sephadex G-75 or G-100.

Further purification of aequorin was accomplished by chromatographing alternately on DEAE-cellulose and Sephadex G-100 columns three times on each. The DEAE-cellulose chromatographies were performed in 0.01 M EDTA–0.03 M sodium acetate (pH 6.0) eluting with a gradient concentration of NaCl. Sephadex G-100 chromatographies were performed in 0.01 M EDTA–0.03 M sodium acetate–0.3 M NaCl (pH 6.0). Each fraction of eluate from each column was checked for specific activity, *i.e.*, (total quanta of light/ml)/(absorbance at 280 mμ/cm), and the fractions which had the highest specific activity, aggregating 80–85% of the activity applied to the column, were pooled and added to the next following column. The eluate from the Sephadex G-100 columns could be added directly to the next DEAE-cellulose column after diluting with five volumes of 0.005 M EDTA. The eluate from the DEAE-cellulose column was saturated with $(NH_4)_2SO_4$, centrifuged, and the precipitate was dissolved in a small amount of 0.05 M EDTA before adding to the next Sephadex G-100 column. The active fractions from the last Sephadex G-100 column showed an almost constant specific activity of (1.64 ± 0.03) $\times 10^{15}$ at 25°. The final yield of activity was approximately 12% of that of the initial squeezate, in contrast to only 0.8% in the previous report (Shimomura *et al.*, 1962).

Criteria of Purity of Aequorin. In addition to the constant specific activity in the elution curve of the final chromatography mentioned above, the ultracentrifugal patterns of the purified aequorin showed a highly symmetrical, single peak.

The results of disc electrophoresis are shown in Figure 1. The electrophoresis was carried out at pH 9.5 using an apparatus and the standard gel obtained from Canal Industrial Corp., Bethesda, Md. Because of the difficulty of preventing aequorin from any inactivation, a condition which would give a complete inactivation was preferred. Under usual conditions (A), a small side peak appeared close to the main peak. Approximately 10% of the initially applied activity was found before staining at the position of the side peak but no activity was found at the position of the main peak. When a sample gel was left 12 hr (B), the side peak disappeared along with the activity. The weak tailing is probably due to denatured aequorin formed in the course of the experiment. Thus, the homogeneity of the inactivated aequorin strongly supports the homogeneity of the active aequorin.

Fluorescent Compounds Split from Aequorin. Although aequ-

orin is scarcely fluorescent prior to the light-emitting reaction with Ca^{2+}, fluorescent products evidently derived from the group which functions in the bioluminescence reaction could be separated from the protein moiety of aequorin by the following two methods.

(1) An $(NH_4)_2SO_4$ precipitate containing 10 mg of aequorin was dissolved in 2.5 ml of 0.01 M EDTA and the pH of the solution was adjusted to 8.0 by adding solid Tris. Then 0.1 ml of mercaptoethanol and 2.5 g of urea were added. After standing at room temperature for 2 hr, 2 ml of water was added and the pH was readjusted to 5.2–5.5 by acetic acid, followed by extraction of the mixture three times with 1-butanol. A blue fluorescent compound (AF-350) was completely extracted in butanol, and the protein part (apoaequorin-SH) remained in the urea–water solution. The butanol solutions were combined and mixed with 1.5 volumes of ether. This mixture was washed three times with water to remove urea, and after evaporating most of the ether and butanol under reduced pressure, AF-350 could be purified on a silica gel column with ether as the solvent. Apoaequorin-SH was exhaustively dialyzed against water, then freeze dried.

(2) An $(NH_4)_2SO_4$ precipitate containing 10 mg of aequorin was dissolved in 1.5 ml of 0.01 M EDTA (pH 6.0) and mixed with 1.5 ml of 0.02 M $NaHSO_3$ (pH 5.5). The mixture was then left at room temperature for 1.5 hr. A heavy cloudiness which developed was precipitated by centrifugation, and the supernatant was passed through a column of Sephadex G-25 (fine) prepared with water, resulting in a complete separation of a blue fluorescent compound (AF-400) and the protein part. The protein fractions from the column and the precipitate of initial centrifugation were combined (apoaequorin-SO), exhaustively dialyzed against water, and finally freeze dried.

Weight of Protein Samples. The protein samples, *i.e.*, active aequorin, apoaequorin-SH, and apoaequorin-SO, were freeze dried and stored in a vacuum desiccator over P_2O_5. Weighings were usually made with such freeze-dried samples, and corrections for water content were made to arrive at the dry weight. With active aequorin, which is too unstable when completely desalted, desalting was done by passing through a column of Sephadex G-25 (fine) prepared with 10^{-4} M EDTA and a correction for residual EDTA was made after freeze drying.

The water content was determined by drying the freeze-dried samples at 110° until constant weight (4–5 hr). Both apoaequorin-SH and apoaequorin-SO gave a weight loss of 5%, whereas aequorin gave a loss of 6%. Since aequorin contains a volatile moiety (*vide infra*), however, a value of 5% was taken for water content of all three freeze-dried proteins.

Sedimentation and Diffusion Coefficients. Both coefficients were obtained with a Spinco Model E centrifuge. Sedimentation velocity was measured at 59,780 rpm at 20 and 3° for several concentrations of aequorin, from 0.04 to 0.8%, and the results were extrapolated to zero concentration. The different concentrations and temperatures had very little influence either on the value of $s_{20,w}$ or on the sedimentation pattern. Thus it appears that no dissociation or polymerization took place under these conditions. The diffusion experiment was carried out at 8766 rpm at 20° in a double-sector synthetic boundary cell with a 0.6% solution of aequorin, and the coefficient was calculated according to Svedberg and Pedersen (1940). In both measurements, the solvent was 0.01 M EDTA–0.01 M sodium acetate–0.01 M NaCl (pH 5.8) and

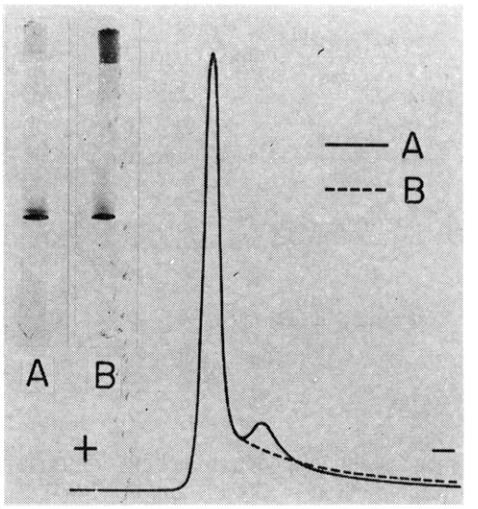

FIGURE 1: Photograph of disc electrophoresis of aequorin in polyacrylamide gel and the data from densitometer tracing. The gel columns (Canalco standard gel containing 0.01 M EDTA) were prepared at room temperature, and the electrophoresis was carried out in a cold room at 3°. (A) By ordinary procedure. (B) Sample gel was left 12 hr before the addition of stacking gel. Cathode side is the top of the gels. Staining was made by anilin black. The abscissa in the densitometer tracing was expanded 8.5 times compared with the photograph of the gel. The amount of protein was *ca.* 40 μg for each run.

the solution of protein was prepared by dialysis or with a column of Sephadex G-25. Schlieren optics were used, except for the low concentrations of aequorin in the sedimentation measurements, for which ultraviolet absorption optics were employed.

Molecular Weight Estimation by Gel Filtration. For this, a column of Sephadex G-75 (1.6 × 75 cm) at 3–4° was calibrated by chymotrypsinogen, egg albumin, and bovine serum albumin. A buffer of 0.005 M EDTA–0.015 M sodium acetate–0.2 M NaCl (pH 6.0) was used throughout.

Amino Acid Analysis. Amino acid analyses were performed with a Beckman amino acid analyzer using an accelerated method with spherical resin according to Spackman (1963, 1967). The protein samples (1 mg) were hydrolyzed with 6 N HCl (0.5 ml) in evacuated, sealed tubes at 110° for 22 and 76 hr. HCl was removed in a vacuum desiccator containing P_2O_5 and NaOH, and the residue was dissolved in citrate buffer containing α-amino-β-guanidinopropionic acid and β-2-thienylalanine as internal standard to correct for slight fluctuations in the sensitivity of the analyzer according to Walsh and Brown (1962). A hydrolysate approximately equivalent to 0.4 mg of protein was applied to each column.

Tryptophan in apoaequorin-SH was determined by the ultraviolet absorption method of Beaven and Holiday (1952). Tryptophan in aequorin, for which the ultraviolet method is not applicable because of interfering absorption, was determined colorimetrically by procedure N of Spies and Chambers (1949).

The sum of cysteine and half-cystine (total half-cystine) was obtained by amino acid analysis as *S*-carboxymethylcysteine after reduction and carboxymethylation of the proteins by the method of Crestfield *et al.* (1963), as well as by the amino acid analysis of cysteic acid after performic acid oxidation of the proteins by the method of Moore (1963).

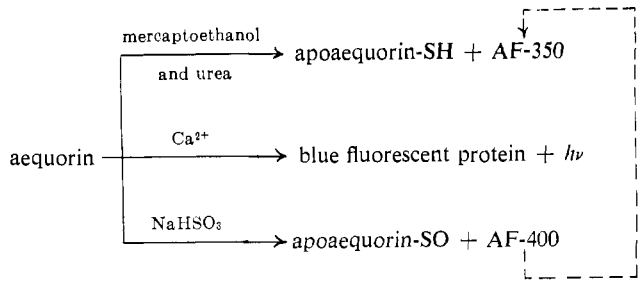

FIGURE 2: Reactions of aequorin.

The cysteine residue was determined by omitting the reduction step in the method of Crestfield *et al.* (1963). To 1 ml of 1 M Tris (pH 8.5) containing 1 mg of protein, 1 g of urea and 0.05 ml of iodoacetic acid solution (68 mg in 1 ml of 0.2 N NaOH) was added. After 15 min at room temperature, excess iodoacetate was decomposed by addition of 5 μl of β-mercaptoethanol, followed after 5 min by desalting according to the method of Dixon (1959) or by dialysis after acidification with acetic acid. The carboxymethylated protein was hydrolyzed and carboxymethylated cysteine was determined by amino acid analysis. After the amino acid analyses, the exact amount of protein was calculated from the alanine, leucine, and phenylalanine content.

Carbohydrates. Total carbohydrate was determined by the phenol–sulfuric acid procedure of Plummer and Hirs (1963) using glucose as the standard. *N*-Acetylneuraminic acid was analyzed by the method of Svennerholm (1957).

For the analyses of neutral carbohydrates, 7 mg of apoaequorin-SH was heated with 2 ml of 1 N HCl at 100° for 3 hr in an evacuated, sealed tube. HCl was removed by a column of Dowex 2-X8 (acetate form), followed by evaporation to dryness. The residue was redissolved in water, then amino acids, peptides, and proteins were removed by a column of Bio-Rad AG-50WX8. In all steps, precautions were taken for quantitative recovery of neutral carbohydrates. Half of the resulting solution was used, after concentration, for identification of carbohydrates by paper chromatography. Arabinose, galactose, glucose, levulose, mannose, rhamnose, ribose, sorbitol, and xylose were used as references, with three kinds of solvent systems: butanol–pyridine–water (6:4:3, v/v), butanol–pyridine–0.1 N HCl (5:3:2, v/v), and ethyl acetate–pyridine–water (10:4:3, v/v). The remaining half of the solution was used for determination of neutral carbohydrates by the anthron method of Scott and Melvin (1953).

Phosphate Groups. Estimations were made by the Fiske–Subbarow method modified by Bartlett (1959) and by Gerlach and Deuticke (1963), as described by Cohen *et al.* (1967).

Results and Discussion

Stability of Aequorin. As noted above and previously (Shimomura *et al.*, 1962), aequorin even with EDTA, is extremely sensitive to Ca^{2+}, the solutions usually faintly luminescing spontaneously (presumably due to contaminating Ca^{2+}) resulting in a gradual loss of capacity to luminesce. When an aequorin solution containing a low concentration of EDTA, *e.g.*, less than 10^{-4} M, was transferred from a thoroughly cleaned plastic to a thoroughly cleaned Pyrex glass container, light emission brightened at once to a new steady level, probably indicating the presence of Ca^{2+} leached from the glass. Spontaneous luminescence, with the accompanying decrease in activity, could be practically eliminated by saturating the aequorin plus EDTA solutions with $(NH_4)_2SO_4$ and keeping in a deep freezer at −20°, but at ordinary temperatures a highly sensitive light-measuring device could detect light being emitted at a rate which, if maintained uniformly, according to our calculations, would exhaust the calibrated photon content in about 300 years (G. Calleja and G. T. Reynolds, 1968, personal communication).

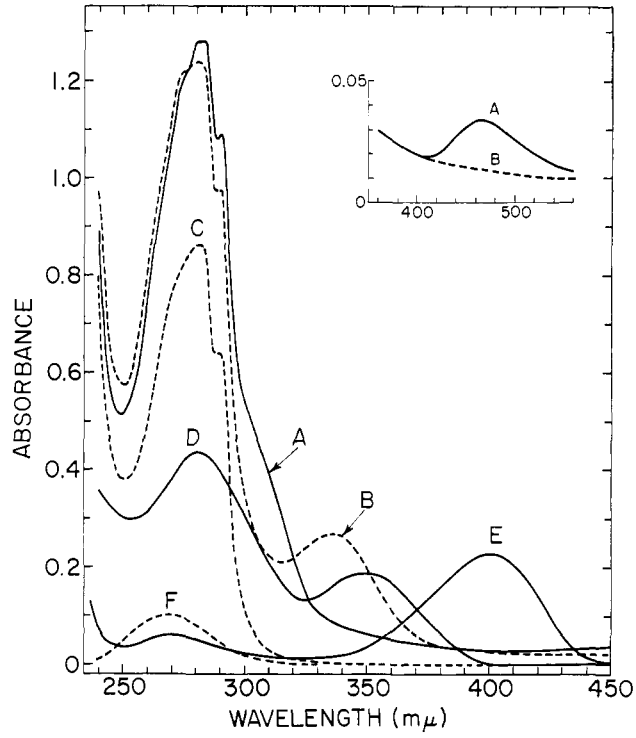

FIGURE 3: Absorption spectra of aequorin (A), blue fluorescent protein (B), apoaequorin-SH and apoaequorin-SO (C), AF-350 (D), AF-400 (E), and distillate from aequorin (F; see text). A contained 0.5 mg of aequorin/ml by the weight of freeze-dried protein (water content, 5%). For B–F, the molar concentrations were the same as A in regard to the aequorin which was used to prepare these substances. The solvent for A and B was 0.01 M EDTA, pH 6.0; for C, 0.03 M sodium phosphate, pH 7.2; for D, ethanol; for E and F, water. Light path: 1 cm.

Aequorin solution (1 ml) in 10^{-4} M EDTA, which had an absorbance of 2.56 at 280 mμ, was found to contain 1 mg of protein (corrected for EDTA present) after freeze drying in a vacuum desiccator over P_2O_5, in a room at 25°. However, this 1 mg of protein was found to have an absorbance of only 2.25 at 280 mμ when redissolved in 1 ml of buffer, indicating that a chemical change in the aequorin had taken place. Thus, when aequorin in 10^{-4} M EDTA was freeze dried as above, 50–60% loss of activity was found, and the same percentage of activity was again lost after redissolving and redrying. When aequorin was in 10^{-3} and 10^{-2} M EDTA, the loss in activity by freeze drying was 20 and 5%, respectively, and when the solution with 10^{-2} M EDTA contained a small amount of $(NH_4)_2SO_4$, almost no loss in activity was detected.

Spectral Properties of Aequorin and Derivatives (Figures 2 and 3). Purified aequorin, which was colorless in dilute solutions and lightly yellowish when concentrated, having no special fluorescence, is converted, by addition of Ca^{2+}, into a blue fluorescent protein, accompanied by light emission. The ultraviolet spectra of aequorin and the blue fluorescent protein (Figure 3) correspond to the data in the previous report (Shimomura *et al.*, 1962, 1963a), but the present specimen was of a higher purity.

The very weak peak at 465 mμ, which disappears after the luminescence reaction, is thought to be characteristic of aequorin.

When a solution of aequorin was treated with urea and β-mercaptoethanol, a blue fluorescent compound (AF-350), with absorption maxima at 280 and 350 mμ, and a protein (apoaequorin-SH) with the absorption spectrum of a simple protein were obtained as described under Methods. AF-350 could even be formed by merely shaking a solution of aequorin with 1-butanol, but with less (about half) the yield, indicating that at least one factor of the reaction is denaturation. Treating aequorin with $NaHSO_3$ also gave a fluorescent compound (AF-400) with an absorption maximum at 400 mμ and a protein (apoaequorin-SO). Treatment of AF-400 with HCl afforded a substance which showed absorption maxima at 280 and 350 mμ, similar to AF-350, though the possible identity of the two substances was not confirmed by evidence beyond absorption spectra. The chromophores of the blue fluorescent protein (product of the luminescence reaction) and that of AF-350 or AF-400 originate, we believe, from the same group in aequorin, a group which functions in the light-emitting reaction with Ca^{2+}. Efforts to split off the fluorescent group from the blue fluorescent protein, however, were not successful.

AF-350 could be distilled under reduced pressure at 150–200°, and a preliminary experiment with mass spectrometry indicated that the molecular weight of this compound was 277. The absorption spectrum of AF-350 did not change on addition of ammonia or acetic acid.

A molecular absorption coefficient of approximately 13,000 was obtained from both the 336-mμ peak of the blue fluorescent protein and the 350-mμ peak of AF-350 (Figure 3), taking 32,000 as the molecular weight of aequorin (see below). This indicates that, if the structure of the fluorescent chromophore in the blue fluorescent protein or in AF-350 is similar to DPNH, aequorin should have two such DPNH-like chromophores per molecule of protein.

When a solution of aequorin in 10^{-4} M EDTA was freeze dried and then heated at 70° for 30 min under a vacuum, all in a specially built, completely glass apparatus equipped with a trap which was immersed in a Dry Ice–acetone mixture, a substance having an absorption maximum at 270 mμ was distilled into the trap. This substance is most likely attributable to decomposition of the chromophore of aequorin, because (1) in the splitting off of apoaequorin-SH, apoaequorin-SO, AF-350, and AF-400, no other product absorbing above 230 mμ was found; (2) none of these four products gave a distillate which absorbed at or near 270 mμ; and (3) the dry protein treated as above gave a very poor yield of AF-350.

Chemical Composition of Aequorin. These results are summarized in Table I. The molecular weight of 32,000 used in the calculations was obtained as described in the next section from the content of methionine and the total half-cystine; special care was taken to ensure precise accuracy of the data. The amino acid content of aequorin, apoaequorin-SH, and apoaequorin-SO were essentially the same.

TABLE I: Composition of Aequorin.[a]

	Residues/ 10^5 g of Protein	Residues/ 32,000 g of Protein	Nearest Integral No./ 32,000 g	Integral No. × Formula Wt of Residues
Lysine	61.0	19.5	20	2,564
Histidine	18.6	6.0	6	823
Ammonia[b,c]	80.0	25.6	26	
Arginine	30.8	9.9	10	1,562
Aspartic acid	105.5	33.8	34	3,913
Threonine[c]	40.0	12.8	13	1,314
Serine[c]	39.2	12.5	13	1,132
Glutamic acid	99.2	31.8	32	4,132
Proline	32.5	10.4	10	971
Glycine	64.5	20.6	21	1,198
Alanine	55.2	17.7	18	1,279
Valine	40.2	12.9	13	1,289
Methionine	18.75[d]	6.00	6	787
Isoleucine	42.4	13.6	14	1,584
Leucine	56.6	18.2	18	2,037
Tyrosine	30.2	9.7	10	1,632
Phenylalanine	33.4	10.7	11	1,619
Tryptophan[e]	21.8	7.0	7	1,304
Half-cystine (total)	15.65[f]	5.01	5	516
Cysteine[g]	8.95	2.86	3	
Glucose[h]	7.12	2.28	2	324
Acetylneuraminic acid[g]	Negligible		0	
Phosphate[g]	0.3	0.1	0	
Total				29,980

[a] Average of more than triplicate determinations, except for the last two in the list. [b] Corrected by control. [c] Extrapolated to zero time of hydrolysis. [d] The same result obtained from methionine sulfone after performic acid oxidation. [e] By ultraviolet absorption for apoaequorin-SH. [f] By carboxymethylation. Analysis of cysteic acid after performic acid oxidation gave 15.55, at a reaction yield of 88%. [g] See the section on Methods. [h] By the phenol–sulfuric acid method for apoaequorin-SH.

Analysis of aequorin for tryptophan by the method of Spies and Chambers (1949) gave 6 residues/32,000 g of protein, which is 1 residue less than the result by the uv method for apoaequorin-SH, indicating the presence of an interfering group in aequorin.

After performic acid oxidation and acid hydrolysis of aequorin and apoaequorin-SH, an unidentified peak was found in the amino acid analysis at exactly the same position as β-2-thienylalanine (internal standard) and in an amount of probably 1 residue/32,000 g of protein as judged by the size of the

TABLE II: Properties of Aequorin.

$E^{1\%}_{1\,cm}$ at 280 mμ	2.70[a]
$s_{20,w}$ (sec)	2.90×10^{-13}[b]
$D_{20,w}$ (cm²/sec)	8.70×10^{-7}
MW by gel filtration[c]	23,000
MW_{sD}	30,000[d]
MW by amino acid analysis	32,000
Luminescence activity	4.5×10^{15} photon/mg at 25°[e]

[a] By dry weight, for active aequorin. For both apoaequorin-SH and -SO; 18.2. [b] Extrapolated value for zero concentration of protein. [c] By Sephadex G-75. [d] Partial specific volume of 0.729 was used. [e] For dry weight of protein. The actual value obtained was 4.43×10^{15}, whereas the accuracy of calibration of the photomultiplier apparatus in absolute units was approximately ±10%.

peak. This result suggests the presence of an unidentified amino compound in the apoaequorin. Although the presence of amino sugars such as glucosamine or galactosamine would seem a likely possibility, none were detected by amino acid analysis.

The fraction of neutral carbohydrates isolated from apoaequorin-SH was found by paper chromatography to contain only glucose, and the determination of this glucose fraction by the anthrone method agreed with the result of the phenol–sulfuric acid method applied directly to apoaequorin-SH. The result of the phenol–sulfuric acid method for aequorin, however, was approximately half of that for apoaequorin-SH, indicating again the presence of an interfering group in aequorin, presumably in a group corresponding to AF-350.

The total weight of residues, approximately 30,000 g, is nearly 2000 g less than the molecular weight used in the calculation. This difference may be due, in a rough estimation, to (a) 100–300 g of an unidentified amino compound mentioned above, (b) 280 (for 1 residue)–560 g (for 2 residues) of the group which comes off as AF-350, (c) 800–1400 g of the other unidentified component(s), for which we suspect lipids, and (d) 100–300 g of inert contaminants, such as SiO_2.

Molecular Weight of Aequorin (Table II). The calculation from the content of methionine and total half-cystine (Table I) gave three possible molecular weights for aequorin: 26,100 ± 500, 31,950 ± 50, and 37,800 ± 500. The middle value, 32,000, was chosen as the molecular weight obtained by amino acid analysis, because of closeness to MW_{sD} as well as the best fit to the number of cysteine residues calculated from the cysteine content.

The partial specific volume, $\bar{V}$, of 0.729, which was used in computing MW_{sD}, was calculated from only the known components listed in Table I, according to Cohn and Edsall (1943) using a value of 0.65 ml/g for glucose (Lansing and Kraemer, 1935). If the unidentified components mentioned above were added in this calculation of $\bar{V}$, the result could be different from 0.729, with much more likelihood of an increase than a decrease. If the suspected presence of lipids is real, a considerable increase would be expected for $\bar{V}$, and consequently for MW_{sD}.

Thus, considering the molecular weight obtained by amino acid analysis, the molecular weight calculated from s and D, as well as the presence of unidentified components, the true molecular weight of aequorin is most likely in the range of 31,000–32,000.

The molecular weight obtained by gel filtration with Sephadex G-75 did not agree with the above result. The molecular weight of 23,000 for aequorin and the blue fluorescent protein is even smaller than the molecular weight calculated for a spherical protein with $s_{20,w} = 2.90$ S (24,000 at $\bar{V} = 0.73$, 26,500 at $\bar{V} = 0.75$). The discrepancy became smaller after modification of protein, *e.g.*, a value of 27,000 was obtained for apoaequorin-SO and carboxymethylated apoaequorin-SH, although the true molecular weight of both proteins should be practically the same as that of aequorin, because, for the latter, carboxymethylation of other than cysteine was not detected in the amino acid analysis. Relatively low molecular weights by gel filtration have been reported independently by Hastings and Morin (1968) and by Blinks *et al.* (1969).

Although the unusual behavior of aequorin in the gel filtration experiments must be explained by its shape, size, or adsorptivity, or their combination, no definite explanation can be offered at this moment.

Quantum Yield of Aequorin. The maximum wavelength and the spectral distribution of the luminescence of aequorin is very similar to those of *Cypridina* luminescence (Johnson *et al.*, 1962; Shimomura *et al.*, 1963a). The photomultiplier apparatus used in the present work was calibrated by *Cypridina* luminescence, and the value of luminescence activity (Table II) could therefore be directly converted into quantum yield, giving the value of 0.23 photon/molecule of aequorin at 25°, a value higher than the 0.14 estimated earlier on the basis of less information, less material available, and less purity.

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Regulation of Hepatic Glycogen Synthetase of *Rana catesbeiana*. I. The Effect of Insulin Treatment*

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ABSTRACT: Tadpole liver glycogen synthetase occurs only in a glucose 6-phosphate dependent form. The enzyme activity was increased two- to threefold after insulin administration. This stimulation was the result of the conversion of one enzyme form into another which still requires glucose 6-phosphate. Insulin treatment of the animal lowered the K_m for uridine diphosphate glucose from $(2.5 \pm 0.7) \times 10^{-3}$ to $(1.2 \pm 0.2) \times 10^{-4}$ M without significant changes in V_{max}. K_m's for glucose 6-phosphate for two enzyme forms were identical at 5×10^{-3} M.

Using adenosine triphosphate, which behaves kinetically as a partially competitive inhibitor of glucose 6-phosphate, additional differences in the kinetic properties of the two enzymes were determined.

Uridine diphosphate glucose:α-1,4-glucan α-4-glucosyltransferase (EC 2.4.1.11) (glycogen synthetase) has been observed to occur in two forms: one glucose 6-phosphate (G-6-P) dependent and the other independent of glucose 6-phosphate (Rosell-Perez *et al.*, 1962, 1964; Friedman and Larner, 1963; Hizukuri and Larner, 1964; Danforth, 1965; Traut and Lipmann, 1963). The ratio of the two activities occurring in different biological systems varies markedly (Goldemberg, 1966). Insulin has been reported to effect the transformation of dependent form (D form) to independent form (I form) which is accompanied by an increase in substrate affinity (Bishop and Larner, 1967). The transformation between D and I forms, which appears to involve dephosphorylation and phosphorylation of the enzyme, seems to be associated with control of glycogen synthesis (Bishop and Larner, 1967).

Mersmann and Segal (1967), however, presented evidence that the difference between these two forms of the enzyme is in their affinity for UDP-glucose (UDPG) and G-6-P and not in strict dependence upon G-6-P. Activation of the enzyme is

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