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Extraction and Purification of Calcium-Activated Photoproteins from the Ctenophores *Mnemiopsis* sp. and *Beroë ovata*†

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ABSTRACT: Calcium-activated bioluminescent "photoproteins" from the ctenophores *Mnemiopsis* sp. and *Beroë ovata* have been isolated by extraction with EDTA and purified 3800-fold and 7800-fold, respectively. The steps consisted of ammonium sulfate and protoamine sulfate treatments, followed by several steps of ion-exchange, gel filtration, and solubility chromatography. The purified photoproteins are virtually homogeneous by the criterion of sodium dodecyl sulfate polyacrylamide gel electrophoresis. Approximately 30,000 adult specimens of *Mnemiopsis*, totalling more than 600 kg wet wt, yielded 2 mg of purified photoprotein. *Mnemiopsis* photoprotein has been resolved into two functionally identical forms ("isoproteins"), termed mnemiopsin-1 and mnemiopsin-2, by chromatography on DEAE-cellulose. Berovin, the *Beroë* photoprotein, chromatographs as a single symmetrical peak under the same conditions.

Bioluminescent proteins, termed "photoproteins" (Shimomura and Johnson, 1966), were originally isolated from the hydromedusae, *Aequorea* (Shimomura *et al.*, 1962, 1963a; Shimomura and Johnson, 1969, 1970) and *Halistaura* (Shimomura *et al.*, 1963b). Similar photoproteins have been isolated from the colonial hydroid, *Obelia* (Morin and Hastings, 1971a,b), and the ctenophore, *Mnemiopsis* (Morin and Hastings, 1971a,b; Ward and Seliger, 1973a,b). These photoproteins differ from the classical luciferin–luciferase system in having no exogenous requirement for diffusible organic substrates or molecular oxygen. Bioluminescence is an intramolecular reaction triggered by the addition of Ca^{2+} or Sr^{2+} (Kohama *et al.*, 1971). There is no evidence to date that these photoproteins turn over and no system has been found *in vitro* that can restore bioluminescent activity to a "spent" reaction mixture.

The photoprotein aequorin, extracted from *Aequorea* with EDTA, has been purified to homogeneity by alternate chromatography on G-100 Sephadex and DEAE-cellulose (Shimomura and Johnson, 1969). The present paper describes a

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similar extraction and purification scheme used to obtain essentially homogeneous photoproteins from the ctenophores, *Mnemiopsis* sp.[1] and *Beroë ovata*. The names mnemiopsin[2] and berovin are proposed for these photoproteins in keeping with previous nomenclature (Shimomura *et al.*, 1962). Mnemiopsin exists in multiple forms ("isoproteins") separable by high-resolution ion-exchange chromatography or by polyacrylamide gel electrophoresis. Berovin, the *Beroë* photoprotein, is not resolved into multiple forms under the same conditions. Ctenophore photoproteins, although functionally identical with the hydrozoan photoproteins, differ from aequorin and halistaurin in many of their physical properties (Ward and Seliger, 1974a). Mnemiopsin and berovin are of special interest because of their high sensitivity to and apparently irreversible photoinactivation by a broad spectrum of light from 230 to 570 nm (Ward and Seliger, 1973a,b, 1974a,b).

Materials and Methods

Collection, Handling, and Maintenance of Live Ctenophores. Two genera of ctenophore, *Mnemiopsis* sp. and *Beroë ovata*, common to the Chesapeake Bay and its tributaries (Bishop, 1972), were collected near Solomons, Md., or in the Patuxent River in the vicinity of Broome Island, Md. Ctenophores were collected in a 1-m diameter by 4-m long 16-mesh conical plankton net (nylon fabric purchased from Stern and Stern Textiles, Inc., New York, N. Y. 10018) dragged at under 2 knots. Captured organisms were transferred to 24-l. plastic pails, containing bay water at about 15°, and transported to our laboratory in Baltimore for processing. Animals collected and handled in this manner were intact and healthy as judged by their physical appearance, luminescence capacity, and ability to live for weeks in specially designed aquaria (Ward, 1974). In ten collecting trips during August and September of 1972, approximately 30,000 adult *Mnemiopsis* totalling more than 600 l. and 1,000 adult *Beroë* totalling 41 l. were collected.

A stock of *Mnemiopsis* was maintained in the laboratory during the summer and fall months of 1970, 1971, and 1972 for small-scale experiments and for the testing of extraction procedures. Twice a day the ctenophores were fed brine shrimp larvae, hatched, and harvested semiautomatically in specially designed hatchery tanks (Ward, 1974).

Absolute Light Measurements. Light measurements of *in vivo* and *in vitro* ctenophore bioluminescence were made using a radioactive light standard originally calibrated with respect to the dinoflagellate emission spectrum (Biggley *et al.*, 1969; Seliger *et al.*, 1969). The 6-nm difference between peaks of the dinoflagellate and ctenophore emission spectra is sufficiently small that the spectra were considered equivalent. Light intensity and total photon emission measurements were made with equipment as described by Biggley *et al.* (1969).

Equipment for in Vivo Light Measurements. Bioluminescence from live ctenophores is partially and reversibly inhibited by sunlight and artificial light (Harvey, 1952). To measure total *in vivo* luminescence, therefore, it was first necessary to adapt the specimens to total darkness or red light (to which they are insensitive) for 15–20 min. Under dim red light a single dark-adapted ctenophore was gently placed into a cylindrical glass reaction vessel, 6 cm in diameter by 9 cm high, with enough sea water to bring the final volume to 100 ml. The vessel was placed in a light-tight chamber, 18 cm on center from an RCA 1P21 phototube. After an additional 5-min dark adaptation in the chamber, the ctenophore was stirred with the blunt edge of an homogenizer blade for 60 sec at a speed of 100 rpm while the light emission was measured. Then the direction of the stirring motor was reversed and the animal was homogenized with the sharp edge at 1725 rpm for an additional 60 sec, during which time a further light emission was observed. A significant amount of light capacity was further obtained by reinjecting a 10-ml sample of the homogenate into a second reaction vessel now containing 90 ml of 10^{-2} M KCl. The KCl solution was stirred at full speed during the course of this injection. The sum of these three measurements was used as the value for total bioluminescence of the live animal.

Particulate Bioluminescence Measurements. The source of bioluminescence in living specimens of *Mnemiopsis* is a band of luminescent cells (photocytes) lining the walls of the eight meridional canals. These have been identified by microscopic observation (Okada, 1926), by image intensification (Reynolds, 1970; Freeman and Reynolds, 1973), and by fluorescence microscopy of an unidentified product of the bioluminescent reaction (Ward, 1968). The photocytes and perhaps subcellular particles are dislodged from the surrounding tissue by forced passage of the animal through several layers of cheesecloth. The "squeezates" so produced are sensitive to hypotonic solutions and luminesce brightly when treated with distilled water or dilute salt solutions (Harvey, 1921). The rate of hypotonic lysis is accelerated by the presence of 10^{-2} M KCl, producing a flash of 1- or 2-sec duration. Our assay for light production of the particulate system consisted of injecting 0.2 ml of "squeezate" into a 10 × 75 mm test tube containing 2.0 ml of 10^{-2} M KCl. The assay tube is housed in a light-tight box in fixed position relative to a 1P21 phototube.

In Vitro Bioluminescence Measurements. The soluble photoproteins from *Mnemiopsis* and *Beroë*, extracted in and stabilized by buffers containing EDTA, are triggered to luminesce *in vitro* by the addition of calcium ions in molar excess of EDTA. The photoprotein bioluminescent reaction is very sensitive to changes in pH, temperature, and ionic strength (Ward and Seliger, 1974a) and so these parameters were kept constant. Our standard assay consisted of a rapid syringe injection of 0.2 ml of photoprotein solution into 2.0 ml of standard calcium buffer contained in a 10 × 75 mm test tube at 22 ± 2°. The standard calcium buffer (0.1 M $CaCl_2$–0.2 M Tris) was adjusted to a pH of 8.5 for mnemiopsin assays and 8.0 for berovin assays, close to the respective pH optima for total light production.

As photoproteins do not turn over, total light measurement in absolute photons is a more direct measure of photoprotein concentration than the initial rate of light production. For this reason all light assays are expressed as total photons per assay or as photons per unit volume of solution. It is therefore appropriate to use, as a relative measure of purity, the term specific photon yield which is total photons emitted per milligram protein rather than the commonly used term, specific activity, which implies enzyme turnover.

Biochemical Assays. Crude protein solutions were assayed by the spectrophotometric method of Warburg and Christian

[1] Two Chesapeake Bay species, *Mnemiopsis gardeni* and *Mnemiopsis leidyi*, were originally reported by Mayer (1912). Ambiguities in his taxonomy have been observed by Bishop (1972) and Ziegenfuss and Cronin (1958). In addition, we have noted several taxonomic features of the Chesapeake form which are more characteristic of the warm water species *Mnemiopsis mccradyi* (Mayer, 1912). In the absence of satisfactory systematics for the genus, we will refer only to the generic name, *Mnemiopsis* sp.

[2] This name has also been used in a recent abstract (Girsch and Hastings, 1973).

(Layne, 1957) using the ratio of absorbance at 280 and 260 nm to eliminate nucleic acid interference. Dilute fractions from DEAE-cellulose columns, however, were assayed for protein by absorbance at 280 alone, a necessary precaution to avoid large errors in the 280/260 assay caused by variable concentrations in EDTA (Ward and Fastiggi, 1972). A microbiuret determination, modified from Gornall *et al.* (1949), of pure mnemiopsin-2 photoprotein was used to standardize routine A_{280} protein assays. An extinction coefficient of 1.1 at 280 nm was determined for a 0.1% solution of mnemiopsin-2 at pH 8.0 by this method. Nucleic acid concentration was measured by the same spectrophotometric method, except when EDTA interference was a factor.

Total carbohydrates were assayed by the phenol–sulfuric acid method of Dubois *et al.* (1956). Galactose, the most abundant monosaccharide in a hydrolyzed ammonium sulfate fraction of mnemiopsin (Dr. Y. C. Lee, personal communication), was used for the standard curve. Optical density changes were measured at 480 nm.

Polyacrylamide Gel Electrophoresis. Acrylamide monomer solution was prepared with 12% T (T = total monomer concentration, w/v) and 2% C (C = cross-linker, *i.e.*, bisacrylamide, w/v) and polymerized at 4° with ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine. Electrophoresis was performed in a water-jacketed tank at 3 ± 2° with a continuous buffer system of 0.05 M Tris–0.01 M EDTA (pH 8.0) or 0.025 M citrate–0.01 M EDTA (pH 5.5). After 30-min preelectrophoresis, samples in 50% sucrose were layered on the gel surfaces. This and subsequent steps were performed in dim red light. A current of 1 mA/gel was maintained for 45 min and then increased to 4 mA for the remaining 3–4 hr. Gels were stained 3–5 hr in Amido Black (1% in 7% acetic acid) or sliced into 2-mm long disks with a razor blade gel slicer. Active photoprotein could be quantitatively eluted by soaking the slices 48 hr in 10^{-3} M EDTA at pH 8.0. Stained gels were scanned with a Joyce double-beam microdensitometer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The technique of Weber and Osborn (1969) was followed except that phosphate buffer was replaced by 0.05 M Tris–0.005 M EDTA at pH 8.0.

Chemical Reagents. Chemicals used in this study were: Tris (Trizma Base) and protamine sulfate (grade I) from Sigma Chemical Co., ammonium sulfate (Ultra Pure) from Schwarz/Mann, Sephadex G-75 (superfine), G-25 (medium), and blue dextran from Pharmacia Fine Chemicals, Inc., Bio-Gel P-100 (−400 mesh) from Bio-Rad Laboratories, and Whatman microcrystalline DEAE-cellulose (DE-32) from Reeve Angel. Na_2EDTA, calcium chloride, and most of the other inorganic reagents were Baker Analyzed Reagent grade chemicals. All reagents for polyacrylamide gel electrophoresis were purchased from Eastman Kodak Co. with the following exceptions: ammonium persulfate and sodium dodecyl sulfate (Matheson, Coleman and Bell) and Coomassie Brilliant Blue (Schwarz/Mann).

Experiments and Results Section

Measurement of Total Light from Live Mnemiopsis. Large, freshly collected ctenophores, averaging 22 ml in volume, were used in the following experiments to determine the total light yield from *Mnemiopsis* and cheesecloth squeezates of *Mnemiopsis*. Dark-adapted specimens were individually stimulated to luminescence in the apparatus described in Materials and Methods. About 75% of the total light yield was generated by 2-min mechanical stimulation with the stirring–homogenizing blade. At this time, luminescence appeared to be exhausted. However an additional burst of light (25% of the total) could be produced by hypotonic lysis of the homogenate in dilute KCl (see Materials and Methods). From 15 randomly chosen specimens a total stimulable bioluminescent yield for *Mnemiopsis* was determined to be 2.9 ± 1.4 × 10^{13} photons per specimen (1.3 ± 0.6 × 10^{12} photons/ml).

Squeezates, prepared by squeezing four dark-adapted ctenophores through four layers of cheesecloth, were also tested for total luminescent capacity. A 10-ml sample of squeezate was injected into the same reaction vessel containing 90 ml of 10^{-2} M KCl. One sample from each of 10 squeezate preparations was assayed. The average value for the squeezate, 30% of the total stimulable bioluminescence, was 8.6 ± 1.5 × 10^{12} photons per specimen (4.0 ± 0.7 × 10^{11} photons/ml).

Mnemiopsis Extraction (Step 1). Prior to extraction, the ctenophores were dark adapted for 30 min in total darkness or dim red light to overcome partial photoinhibition of the *in vivo* system (Harvey, 1952). The animals were then squeezed by hand through four layers of cheesecloth. The resulting squeezate was a syrupy suspension of cells and tissue fragments containing 30% of the stimulable luminescence of whole animals. Such squeezates are stable for several hours at 0°, however, the bioluminescent capacity remains subject to partially reversible photoinactivation (Harvey, 1925). Soluble ctenophore photoprotein is also readily inactivated by exposure to light (Mortin and Hastings, 1971a; Ward and Seliger, 1973a,b; Ward and Seliger, 1974b). The light inactivation of mnemiopsin appears to be an irreversible process, although a report of a secret reducing agent has recently been presented (Girsch and Hastings, 1973). It was therefore necessary to protect the system from exposure to light at all stages of purification. Processing of the squeezate and all subsequent steps in the extraction and purification scheme were performed at 0–4° in dim red light. Bioluminescent particles in the squeezate were collected by 20-min centrifugation at 4000*g* in the Sorvall GSA rotor. The viscous supernatant, containing 95% of the volume and less than 5% of the activity, was discarded. The loosely packed pellet was removed and could be stored on ice for 2 hr. In lots of 250 ml, the pellets were homogenized with 50 ml of 0.30 M Tris–0.24 M EDTA extracting buffer at pH 9.0 for 1 min in a Waring blender set at low speed. Homogenization completely solubilized the calcium-activated photoprotein with 15–20% recovery of squeezate activity. The combined homogenate from each day's collection was centrifuged at 23,000*g* in the GSA rotor for 1 hr to remove a large mass of cellular debris. More than 95% of the mnemiopsin activity remained in the supernatant with a substantial increase in stability. The 20-fold concentrated crude extract, totalling 27 l., was stored on ice from 2 to 6 days prior to fractionation with ammonium sulfate.

Ammonium Sulfate Fractionation of Mnemiopsin (Step 2). The 20-fold concentrated crude extract, combined from two or three collections, was fractionated with ammonium sulfate at 0°. Solid ammonium sulfate was added with continuous stirring over a 60-min period. After 30-min additional stirring, precipitate was collected by centrifugation in a Sorval GS-3 rotor at 11,000*g* for 30 min. Three ammonium sulfate precipitations (0–50%, 50–60%, and 60–85%) were required for maximum purification. (Combination of the first and second steps resulted in irrecoverable coprecipitation of activity.) The first precipitate (0–50%), containing 75% of the total

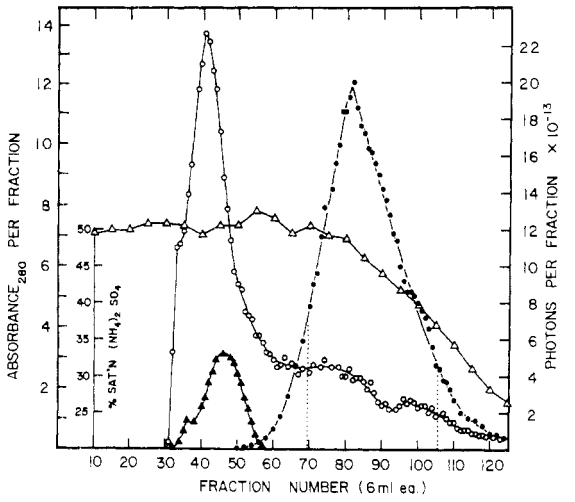

FIGURE 1: Ammonium sulfate solubility chromatography of mnemiopsin. Photoprotein activity is indicated by solid circles, total protein as A_{280} by open circles, and per cent ammonium sulfate saturation (measured by conductivity) by open triangles. The blue dextran (BD) void volume, indicated by solid triangles, was measured by absorption at 600 nm. The mnemiopsin peak elutes in the 48% saturation region of the column, well resolved from void volume proteins.

protein, collected near the mouth of the centrifuge bottle as a thick rubbery "pancake" which was easily removed by hand. The 50–60% cut removed a small volume of precipitate, rich in 260-nm-absorbing material. The 60–85% precipitate sedimented readily as a lavender granular pellet containing more than 70% of the original activity and less than 20% of the original protein. This was redissolved in 0.02 M Tris–EDTA buffer at pH 8.0 and dialyzed 48 hr against four changes of 0.01 M Tris–EDTA at pH 8.0. Treated in this way, the extract could be stored indefinitely at −20° without loss in activity.

Protamine Sulfate Precipitation (*Step 3*). After thawing, the pooled ammonium sulfate fraction was treated with 6.60 g of protamine sulfate (2 mg/mg of nucleic acid) with stirring at 0° to precipitate nucleic acids and acidic polysaccharides. The white precipitate was removed by centrifugation for 30 min at 13,000*g* in the GSA rotor, leaving a clear red solution with greatly reduced viscosity.

DEAE-cellulose I (*Step 4*). The protamine-treated extract was applied to a 50-ml column of DEAE-cellulose (1.7 × 22 cm) to further reduce the viscosity of the sample. The column was washed with 4 bed volumes of 0.05 M NaCl (in 0.01 M Tris–EDTA buffer at pH 8.0) and then eluted with a 400-ml linear salt gradient (8 bed volumes) from 0.05 to 0.40 M NaCl. The side fractions were pooled and rechromatographed on the same column. The column removed 96% of the nucleic acid and 89% of the polysaccharide and, in addition, yielded slightly better than 3-fold protein purification. The active sample, an intense burgandy-colored solution, was precipitated with ammonium sulfate at 90% of saturation. The precipitate was collected by centrifugation for 30 min at 30,000*g* and dissolved in 25 ml of 0.01 M Tris–EDTA buffer at pH 8.0, yielding a solution of low viscosity suitable for gel filtration.

Bio-Gel P-30 Gel Filtration (*Step 5*). The 25-ml sample was chromatographed on a 1280-ml column (3.7 × 119 cm) of Bio-Gel P-30. The sample was eluted with 0.01 M Tris–EDTA buffer at pH 8.0 containing 0.05 M NaCl (at a flow rate of 2.25 ml/cm^2 per hr), and fractions of 4.5 ml were collected. Active fractions were pooled and applied to a small DEAE-cellulose column (1.7 × 8 cm) to concentrate the material. The photoprotein was concentrated from 70 to 7 ml by a single step elution with 0.5 M NaCl in 0.01 M Tris–EDTA buffer at pH 8.0. In preparation for the next column purification, enough solid ammonium sulfate was added to the 7-ml sample to make it 35% saturated.

Ammonium Sulfate Solubility Chromatography (*Step 6*). The sample was further purified by solubility chromatography in ammonium sulfate (Porath, 1962; Hoffman and McGivern, 1969). Sephadex G-25 (medium), equilibrated with 0.02 M Tris–EDTA buffer at pH 8.0, containing ammonium sulfate to 50% of saturation, was packed into a 1050-ml column (3.7 × 98 cm). An 11-step decreasing gradient (20 ml/step) of ammonium sulfate from 48.75 to 36.25% was applied to the column, followed by the now 8-ml sample of photoprotein in 35% saturated ammonium sulfate. The decreasing gradient was continued with eight more 20-ml steps from 35 to 26.25% saturation, and the column was eluted with 25% saturated ammonium sulfate in 0.02 M Tris–EDTA at pH 8.0 (Figure 1). At a flow rate of 2 ml/cm^2 per hr, 125 6-ml fractions were collected. Mnemiopsin was precipitated from the pooled active fractions (indicated by vertical dotted lines in Figure 1) by addition of solid ammonium sulfate. The precipitate was collected by centrifugation at 15,000*g* for 30 min, dissolved in 10 ml of 0.01 M Tris–EDTA at pH 8.0, and dialyzed about 6 hr against the same buffer. The dialyzed sample was diluted to 40 ml in preparation for the next column. In a separate run, the void volume was determined by chromatographing blue dextran in 25% saturated ammonium sulfate.

DEAE-cellulose II (*Step 7*). We have previously reported that partially purified mnemiopsin extracts can be resolved into two components ("isoproteins" designated m-1 and m-2) by DEAE-cellulose chromatography (Ward and Seliger, 1973a). This separation was achieved by shallow salt gradient elution from a column of Whatman DE-32 previously equilibrated with 0.01 M Tris–EDTA buffer at pH 8.0 (Figure 2A). (We have observed similar separations on columns of QAE-Sephadex and Sigma DEAE-cellulose, both at pH 8.0, and also on DE-32 at pH 9.0.)

In an attempt to increase the purification of mnemiopsin on DEAE-cellulose, we subjected the 40-ml sample from solubility chromatography to high-resolution chromatography on DEAE-cellulose under acidic conditions. (We had previously found that the DEAE-cellulose elution positions of mnemiopsin-1 and mnemiopsin-2 are independent of pH from 5.5 to 9.0, whereas many of the contaminants in crude extracts chromatograph very differently in acid and base.) The sample was applied to a 20-ml column of DEAE-cellulose (0.8 × 40 cm) and then eluted with a 750-ml linear gradient (38 bed volumes) from 0.05 to 0.15 M NaCl in 0.01 M citrate–EDTA buffer at pH 5.5 (Figure 3). A flow rate of 18 ml/cm^2 per hr was maintained while 150 5-ml fractions were collected. Five bands of mnemiopsin activity were eluted from the column, a doublet of m-1 activities (α and β) and a triplet of m-2 activities (γ, δ, and ϵ). When rechromatographed on DEAE-cellulose in alkaline buffer (pH 8.5), the α and β forms of m-1 elute as one peak in a low ionic strength region of the gradient and the γ, δ, and ϵ forms of m-2 elute as one peak in a higher region. A peculiar feature of the pH 5.5 elution pattern is that while the resolution of activity into five discrete bands is excellent, the resolution of total protein is poor. The active fractions from the five bands, as indicated by vertical dotted lines in Figure 3, were pooled and dialyzed 4–5 hr against 0.01 M Tris–EDTA at pH 8.5.

DEAE-cellulose III (*Step 8*). The five samples, separated at pH 5.5, were individually rechromatographed at pH 8.5 on 1.8-ml DEAE-cellulose columns (0.6 × 6 cm) prepared in

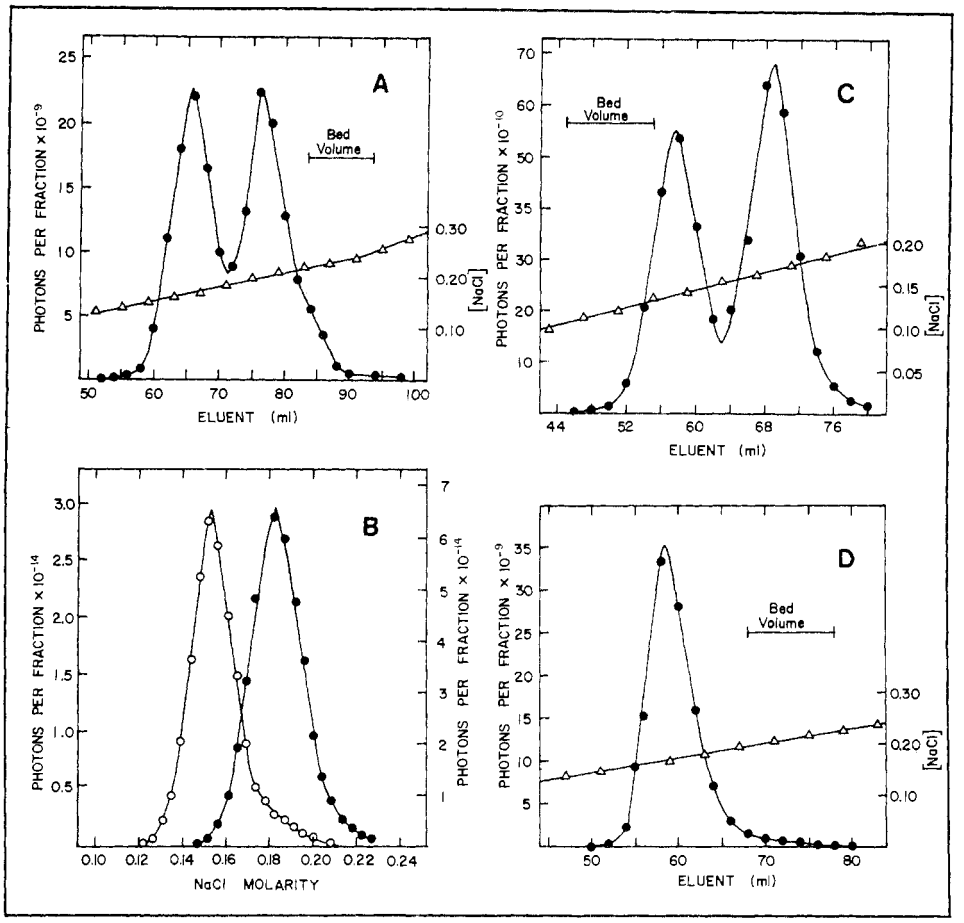

FIGURE 2: DEAE-cellulose chromatography of ctenophore photoproteins. Photoprotein activity, measured by the standard calcium assay, is indicated by circles. The salt gradient (triangles) was determined by conductivity measurements. (A) Elution profile of ammonium sulfate treated mnemiopsin at pH 8.0. The column (1 × 15 cm) was eluted with a 100-ml linear gradient of NaCl (in 0.01 M Tris–EDTA at pH 8.0) from 0.05 to 0.30 M. (B) Rechromatography of purified mnemiopsin-1 (open circles) and mnemiopsin-2 (solid circles) on identical (1 × 15 cm) DEAE-cellulose columns at pH 8.0. The columns were eluted with linear 100-ml gradients of NaCl (in 0.01 M Tris–EDTA at pH 8.0) from 0.05 to 0.25 M. Photoprotein activity is plotted as a function of NaCl concentration. Both "isoproteins" of mnemiopsin rechromatograph as single symmetrical peaks. (C) Elution profile of photoprotein extracted from a single specimen of *Mnemiopsis*. The extract was treated with protamine sulfate to reduce the viscosity and immediately chromatographed on a 1 × 14 cm column of DEAE-cellulose at pH 8.0. Activity was eluted with a 100-ml linear gradient of NaCl (in 0.01 M Tris–EDTA at pH 8.0) from 0.05 to 0.30 M. (D) Elution profile of ammonium sulfate treated berovin at pH 6.0. The column (0.8 × 20 cm) was eluted with a 100-ml linear gradient of NaCl (in 0.01 M citrate–EDTA at pH 6.0) from 0.05 to 0.30 M.

pasteur pipets. Activity was eluted with a 60-ml linear gradient (33 bed volumes) of NaCl from 0.05 to 0.20 M. Sixty 1-ml fractions were collected at a flow rate of 15 ml/cm² per hr. The salt concentrations at which the "isoproteins" eluted were as follows: m-1α (0.102), m-1β (0.106), m-2γ (0.131), m-2δ (0.144), and m-2ϵ (0.141).

Active fractions from each run were pooled, and after 1:1 dilution with 0.001 M EDTA at pH 8.0, each sample was applied to a 0.4-ml column of DEAE-cellulose (0.6 × 1.5 cm) for the purpose of concentration. Activity was eluted in a single step with 0.05 M NaCl in 0.01 M Tris–EDTA at pH 8.0, effecting a 15- to 20-fold concentration to a final volume of 0.5 ml.

Sephadex G-75, Superfine (Step 9). The five concentrated samples (0.5 ml) were applied separately to a 270-ml Sephadex G-75 (superfine) column (1.7 × 118 cm). The column was eluted with a pH 8.0 buffer containing 0.01 M Tris, 0.001 M EDTA, and 0.05 M NaCl (Figure 4). The first 130 ml were collected in a graduated cylinder and then 35–40 fractions of 1.5 ml were collected. Under a pressure head of 45 cm of H_2O, the column operated at slightly less than 2 ml/cm² per hr.

Composite Purification of Mnemiopsin. The mnemiopsin "isoproteins" were purified 3800-fold, from 27 l. of crude extract containing more than 100,000 mg of protein to 42 ml containing 1.8 mg of protein. The nine-step purification process yielded slightly less than 7% of the crude extract activity (Table I). The five "isoproteins" were virtually homogeneous as judged by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate by a modified Weber and Osborn (1969) technique. Each "isoprotein" consists of a single polypeptide chain (Figure 5) with molecular weight ranging from 24,000 to 27,500 (Ward and Seliger, 1974a).

Beroë Extraction (Step 1). The smaller total volume and lower viscosity of the *Beroë* extract made a simplified extraction procedure possible. The dark-adapted animals, in lots of 500 ml, were homogenized directly in 100 ml of extracting buffer. After 1-min homogenization in a Waring blender, the crude berovin extract was frozen in 1-l. plastic bottles and could then be stored in the deep freeze for many months. The preliminary centrifugation was eliminated because we had a much smaller volume of *Beroë* than *Mnemiopsis* and because the *Beroë* squeezate is more sensitive to mechanical shear.

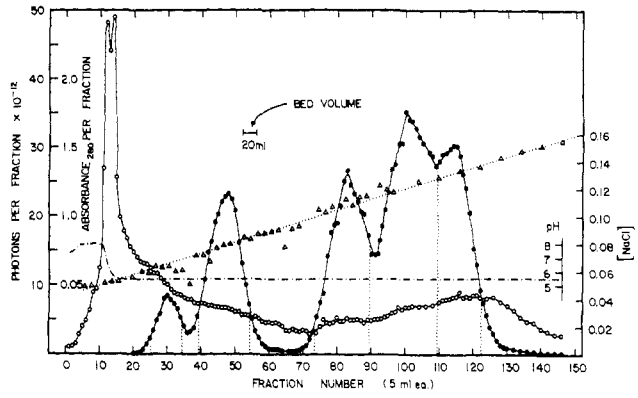

FIGURE 3: High-resolution DEAE-cellulose chromatography of mnemiopsin at pH 5.5 (DEAE-cellulose II). The column was monitored with respect to photoprotein activity (solid circles), total protein as A_{280} (open circles), NaCl concentration (dotted line), and pH (dot-dash line). Five activity bands are eluted from the 20-ml column over a 25-ml bed volume range. Two protein contaminants elute with a steep pH drop early in the gradient but the remaining protein contaminants are poorly resolved. (For further details, refer to the text.)

Little cellular debris remains after homogenization of the live animals and so the second centrifugation step was also judged unnecessary. Because the physical and chemical properties of berovin resemble mnemiopsin, a similar purification scheme was used. Significant modifications of the procedure are listed below.

Ammonium Sulfate Fractionation of Berovin (Step 2). The intermediate precipitation step (50–60%) was eliminated and the 50–85% precipitate was retained. Dialysis time was shortened to 18 hr to minimize a gradual activity loss, previously observed with *Beroë* extracts.

DEAE-cellulose Batch Adsorption (Step 3). Fractionation by ammonium sulfate removed most of the viscous nucleic acids and polysaccharides from the *Beroë* extract, so protamine sulfate treatment was unnecessary. The 1.0-l. sample was adsorbed instead onto 50 ml of packed swollen DEAE-cellulose. After 60-min equilibration, the slurry was centrifuged at 13,000*g* for 20 min. The supernatant, about 0.08 M in $(NH_4)_2SO_4$ from the previous step, contained 95% of the berovin activity but less than half of the nucleic acids and polysaccharides.

The remaining five steps of the berovin purification scheme were similar to those for mnemiopsin with two exceptions. Solubility chromatography was eliminated and, because berovin is not as stable in acidic buffers, the second DEAE-cellulose was run at pH 8.0 rather than pH 5.5. The berovin purification steps 4–8 consisted of: DEAE-cellulose I (step 4), Bio-Gel P-30 gel filtration (step 5), DEAE-

TABLE I: Summary of Purification Results for *Mnemiopsis* Photoproteins.[a]

Step	Vol (ml)	PY	Total Milligrams			280:260	SPY	Recov (%)	Purification		
			Prot	NA	CH_2O				Prot	NA	CH_2O
1	27,000	82	104,000	12,600	12,000	0.693	0.79	100	1.0	1.0	1.0
2	820	61	17,400	3,190	1,470	0.635	3.5	74	4.4	2.9	6.0
3	1,960	51	10,500	446	250	0.935	4.9	62	6.2	18	30
4	115	43	2,660	16	28	1.48	16	52	20	400	220
5	70	33	427	2.3	2.5	1.51	77	40	98	2200	1900
6	210	24	89				270	29	340		

Step	Isoproteins	Vol (ml)	PY	Prot (mg)	280:260	SPY	Recov (%) Per Step	Recov (%) Cumul	Protein Purifn Per Step	Protein Purifn Cumul
7	m-1α	40	0.47	4.0		117				
	m-1β	70	2.0	4.5		450				
	m-2γ	73	2.4	4.1		590				
	m-2δ	84	4.7	5.7		820				
	m-2ϵ	60	3.2	5.5		580				
	Totals	340	13	24		540	54	16	2.0	700
8	m-1α	9	0.39	0.58		670	83		5.7	850
	m-1β	10	1.7	1.10		1500	85		3.3	1900
	m-2γ	7	1.8	0.96		1900	75		3.2	2400
	m-2δ	7	4.0	1.81		2200	85		2.7	2800
	m-2ϵ	5	1.9	0.96		2000	60		3.4	2500
	Totals	38	9.8	5.4		1800	75	12	3.3	2300
9	m-1α	9	0.22	0.14	1.59	1570	55		2.3	2000
	m-1β	7.5	0.90	0.28	1.56	3200	53		2.1	4000
	m-2γ	9	0.57	0.16		3600	32[b]		1.9	4600
	m-2δ	7.5	2.0	0.68	1.63	3000	50		1.4	3800
	m-2ϵ	9	1.7	0.56	1.57	3000	90		1.5	3800
	Totals	42	5.4	1.8	1.59	3000	55	6.6	1.7	3800

[a] Numbers for purification steps are the same as those in the Experiments and Results Section. The following abbreviations are used: PY = total photon yield ($\times 10^{-14}$), Prot = total protein, NA = total nucleic acid, CH_2O = total polysaccharides as D-galactose, 280:260 = ratio of absorbances at 280–260 nm, SPY = specific photon yield as photons $\times 10^{-11}$/mg of protein. [b] About one-half of the sample was lost in application to the column.

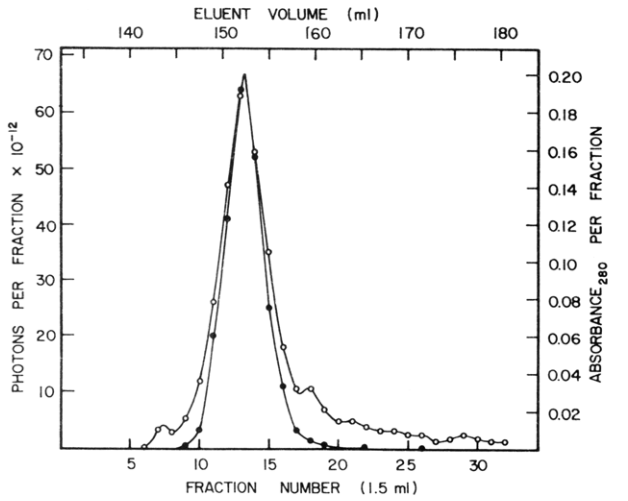

FIGURE 4: High-resolution gel filtration of m-2δ on Sephadex G-75 (superfine). The column was monitored with respect to photoprotein activity (solid circles) and total protein as A_{280} (open circles). The column was calibrated with eight protein standards (see Ward and Seliger, 1974a, Figure 11). (For further details, refer to the text.)

cellulose II (step 6), DEAE-cellulose III (step 7), and G-75 Sephadex (superfine) gel filtration (step 8; Figure 6).

Composite Purification of Berovin. The crude extract of berovin was purified 7800-fold with 7% recovery of bioluminescent activity. Nearly 100,000 mg of protein were reduced to less than 1 mg in the eight-step purification scheme (Table II). Three lower molecular weight contaminants appear on the sodium dodecyl sulfate gel despite the high degree of purification (Figure 5).

Discussion

The extraction of ctenophore photoproteins was complicated by the large volume, low specific photon yield, and high viscosity of the starting material. Later the presence of many contaminants with similar molecular weights and anion-exchange affinities made complete purification difficult. Alternative methods were tried to increase the yield and the efficiency of mnemiopsin extraction. Direct homogenization of dark-adapted *Mnemiopsis* in EDTA-containing buffer gave the same total light yield, but 20 times the volume. Addition of a saturating concentration of ammonium sulfate to the extracting buffer gave no better yield. Presoaking the animals in various solutions before homogenization or modifying the pH, ionic strength, temperature, EDTA (or EGTA) concentration, and homogenization conditions were also ineffective. Neither the organism size, physical condition, nor photoperiod had a significant effect on the yield of mnemiopsin. The principal loss of activity during extraction is nearly instantaneous. The quantum yield of a squeezate drops by a factor of 5 in 1 min as it becomes solubilized. The resulting crude extract then has a half-life for bioluminescence activity of several days at 0°. The extraction loss appears to be a reduction in bioluminescent efficiency concomitant with the disruption of cellular and subcellular organization. This is consistent with the observation that the fluorescent reaction product *in vivo* becomes nonfluorescent upon disruption of the cells (Ward, 1968). A substantial effort has been made to increase the efficiency of extraction, a laborious process requiring nearly 1000 man hr for the 600-l. collection. A variety of filtration techniques and continuous flow centrifugation procedures were tried unsuccessfully with squeezates. Direct

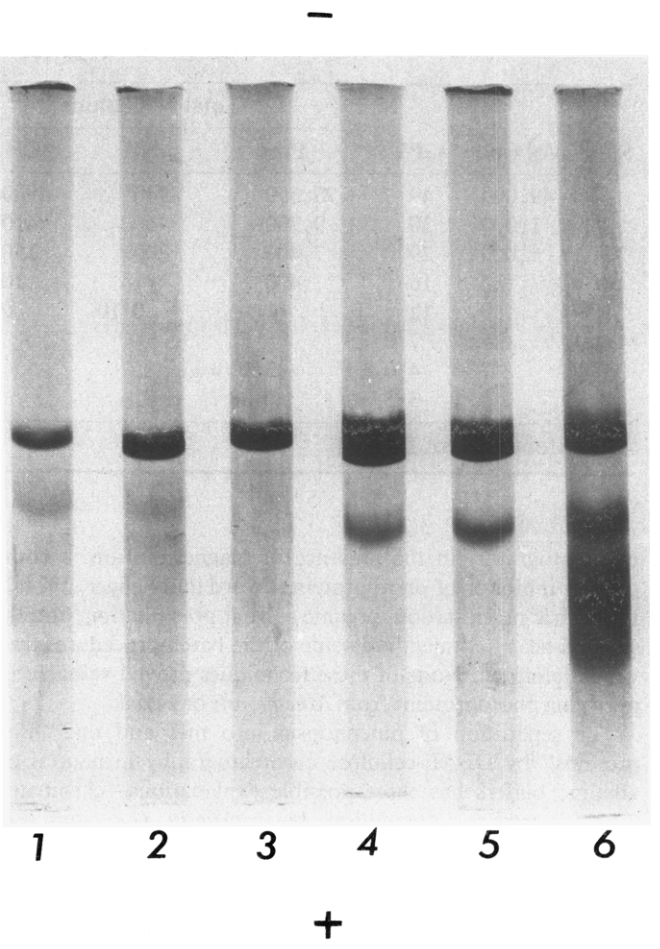

FIGURE 5: Photograph of sodium dodecyl sulfate polyacrylamide gels. Left to right the gels are: m-1α, m-1β, m-2γ, m-2δ, m-2ϵ, and berovin. The gels were intentionally overloaded and overstained to emphasize minor contaminants. (See the text for details.)

precipitation of whole ctenophores with saturated ammonium sulfate solution yielded an unworkably viscous solution. Purification techniques other than those reported above have been tried. These include chromatography on TEAE-cellulose, QAE-Sephadex, phosphocellulose, carboxymethylcellulose, and calcium phosphate gels, as well as DEAE-cellulose

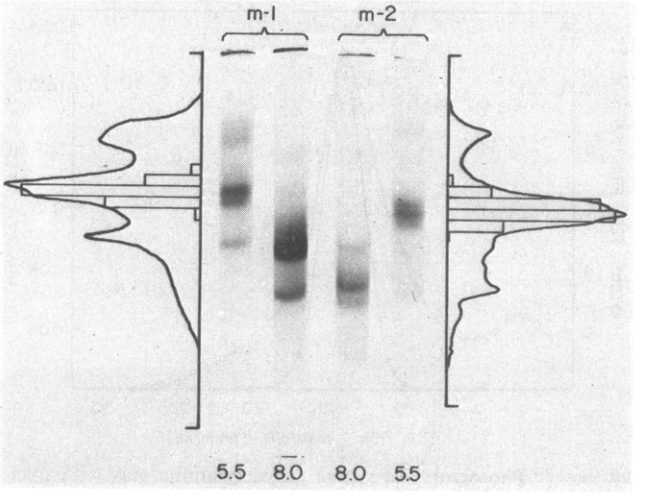

FIGURE 6: High-resolution gel filtration of berovin on Sephadex G-75 (superfine). Photoprotein activity is represented by solid circles and total protein as A_{280} by open circles. Column conditions are identical with those described in the legend to Figure 4.

TABLE II: Summary of Purification Results for *Beroë* Photoprotein.[a]

Step	Vol (ml)	PY	Total Milligrams			280:260	SPY	Recov (%)	Purification		
			Prot	NA	CH_2O				Prot	NA	CH_2O
1	49,000	49	95,550	8940	4960	0.74	0.51	100	1.0	1.0	1.0
2	1,000	30	9,700	460	410	0.89	3.1	62	6.1	12	7.5
3	1,000	29	7,400	200	150	1.06	3.9	59	7.6	27	20
4	82	16	400		10		39	32	76		160
5	59	12	41	0.10	0.63	1.63	300	25	580	22,000	2000
6	25	7.0	5.5				1260	14	2500		
7	7	4.0	3.1				1300	8.3	2500		
8	8	3.5	0.88			1.51	4000	7.1	7800		

[a] See footnote *a* of Table I.

chromatography in the presence of magnesium ion, a competitive inhibitor of photoproteins (Ward and Seliger, 1974a). Isoelectric precipitation, organic solvent precipitation, autolysis, and heat treatment are some of the batch procedures that were attempted. None of these techniques proved valuable in purifying photoproteins from *Mnemiopsis* or *Beroë*.

The separation of mnemiopsin into m-1 and m-2 "isoproteins" by DEAE-cellulose chromatography in neutral or alkaline buffers has three possible explanations—chromatographic artifact, population heterogeneity (*i.e.*, multiple species or subspecies of *Mnemiopsis* in the same collecting area), and authentic microheterogeneity (presence in a single individual of multiple forms of a protein which have identical functions).

DEAE-cellulose column artifact appears an untenable explanation for the origin of the two forms of mnemiopsin because, once separated, m-1 and m-2 do not split into additional peaks by repeated chromatography under the same conditions (Figure 2B). Also, the very closely related photoprotein from *Beroë* chromatographs as a single symmetrical peak on similar DEAE-cellulose columns at several pH values (Figure 2D). The second hypothesis, population heterogeneity, was tested by chromatographing, on separate DEAE-cellulose columns at pH 8.0, the photoprotein isolated from individual specimens of *Mnemiopsis* on three separate occasions. (Crude extract was first treated with protamine sulfate or ammonium sulfate to reduce the viscosity, a prerequisite for column chromatography.) In all three cases, two activity peaks, m-1 and m-2, were clearly resolved (Figure 2C). These experiments support the third alternative, that individual specimens of *Mnemiopsis* (of an unresolved species) contain two distinct and separable "isoproteins" of the photoprotein mnemiopsin.

Although the origin of m-1 and m-2 is well established, the origin of multiple forms of m-1 (α and β) and m-2 (γ, δ, and ϵ) remains somewhat open to question. Separation of mnemiopsin into five forms on DEAE-cellulose has been observed in acidic buffers only. This could possibly be chromatographic artifact. However further evidence for the existence of five discrete "isoproteins" comes from analysis of highly purified mnemiopsin by polyacrylamide gel electrophoresis, under conditions which fully preserve the activity (see Materials and Methods). Figure 7 shows stained gels of mnemiopsin-1 and mnemiopsin-2 which were run at either pH 8.0 or 5.5. These samples, purified several hundredfold by similar procedures, had not been treated with acidic buffer at any stage prior to this electrophoresis. The m-1 gels have two stained bands which correspond with a histogram of bioluminescent activity (shown superimposed on a microddensitometer trace) The m-2 gels have three stained bands which also correspond with an activity histogram.

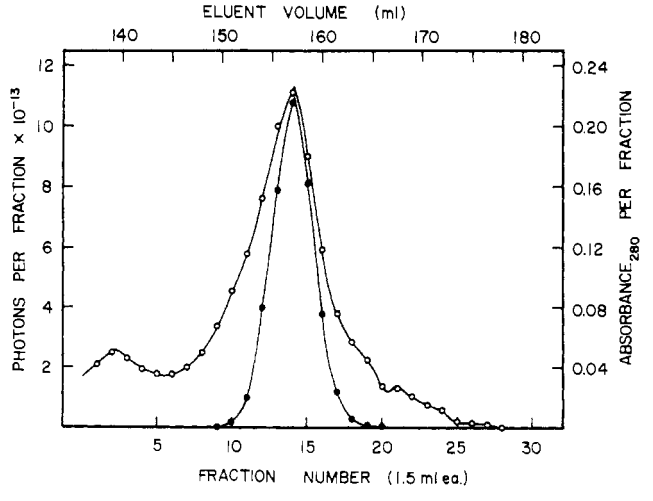

FIGURE 7: Photograph of native polyacrylamide gels with microdensitometer traces and photoprotein activity histograms corresponding to the pH 5.5 gels. Mnemiopsin-1 and mnemiopsin-2 photoproteins are labeled m-1 and m-2. The pH of the electrophoresis buffer is indicated by the number 8.0 or 5.5. (See the text for further details.)

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