Properties of Mnemiopsin and Berovin, Calcium-Activated Photoproteins from the Ctenophores *Mnemiopsis* sp. and *Beroë ovata*†

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ABSTRACT: By methods previously described, the calcium-activated bioluminescent photoproteins were extracted with EDTA from the ctenophores *Mnemiopsis* sp. and *Beroë ovata* and then purified extensively. Two forms of the *Mnemiopsis* photoprotein, mnemiopsin-1 and mnemiopsin-2, and the single *Beroë* photoprotein, berovin, have been characterized and compared with respect to their physical and spectral properties. Ctenophore photoproteins react *in vitro* with calcium ions in molar excess of EDTA to produce a rapid flash with first-order decay kinetics. The rates and total light yields of these reactions are differently dependent on pH, temperature, and ionic strength. Mnemiopsin activation is specific for calcium ions. However, under certain assay conditions and at low metal ion concentrations a large number of cations appear to activate mnemiopsin. But at higher concentrations, most of these cations are inhibitors. Among these, only Mg^{2+} and Ba^{2+} are completely reversible competitive inhibitors of bioluminescence. Total photon yields are enhanced by preincubation with certain hydrophilic alcohols but reduced with long-chain aliphatic alcohols. Molecular weights were estimated by gel filtration on Sephadex and Bio-Gel and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The most reliable molecular weight estimates were 24,000, 27,500, and 25,000 awu for m-1, m-2, and berovin, respectively. *In vitro* emission spectra for the three photoproteins are identical, having a common peak at 485 nm. The *in vivo* emission spectra have peaks at 488 nm (*Mnemiopsis*) and 494 nm (*Beroë*). Pure mnemiopsin-2 has a broad absorption band near 435 nm which disappears upon calcium addition with the concomitant appearance of a new absorption band at 335 nm. *Mnemiopsis* photoprotein is inactivated by exposure to light over its entire absorption spectral range. Photoinactivation is accompanied by a loss in the 435-nm absorption band. Physical and spectral similarities among these photoproteins and the bioluminescent systems of the jellyfish *Aequorea* and the sea pansy *Renilla* suggest that similar biochemical mechanisms may exist among all of the bioluminescent coelenterates.

Photoproteins isolated from the hydrozoan jellyfish, *Aequorea* and *Halistaura* (Shimomura *et al.*, 1962, 1963a,b), the colonial hydroid, *Obelia* (Morin and Hastings, 1971a,b), and the ctenophores, *Mnemiopsis* and *Beroë* (Morin and Hastings, 1971a,b; Ward and Seliger, 1973a,b, 1974a) are low molecular weight proteins (~30,000 awu) which react with calcium ions to produce light (λ_{max} 460–485 nm). A characteristic of these reactions is the lack of exogenous requirements for molecular oxygen and diffusable organic substrates. Photoproteins (and certain of the luciferases) are further distinguished from enzymes by their failure to turn over *in vitro*. The divalent cation which triggers bioluminescence is assumed to induce a change in conformation or charge distribution in the protein resulting in the oxidation of a tightly bound chromophore (prosthetic group). Based on analogy to known bioluminescent reaction mechanisms of the firefly and sea pansy (McElroy and Seliger, 1963; DeLuca and Dempsey, 1970; DeLuca *et al.*, 1971), it is further assumed that the photoprotein chromophore exists as a stable hydroperoxide derivative (Seliger and Morton, 1968). The nature of the binding of this chromophore to the protein and the mechanism of the bioluminescent reaction of photoproteins have not yet been established. However, recent reports by Shimomura and Johnson (1972) and Hori and Cormier (1973a,b) show the chromophore of aequorin (the *Aequorea* photoprotein) to be very similar to luciferin from the anthozoan coelenterate, *Renilla*. It is further proposed (Hori *et al.*, 1973) that the electronic excited state of an oxyluciferin monoanion similar to that of *Renilla* is responsible for the light emission from coelenterate photoproteins.

Recently we have succeeded in purifying to virtual homogeneity, similar photoproteins from two species of ctenophores, *Mnemiopsis* sp. and *Beroë ovata* (Ward and Seliger, 1974a). These photoproteins are termed mnemiopsin and berovin after the generic name of the organisms from which they were isolated. Mnemiopsin has been resolved into two distinct "isoproteins," mnemiopsin-1 and mnemiopsin-2 (abbreviated m-1 and m-2), by alkaline DEAE-cellulose chromatography. At pH 5.5 these are further subdivided by high-resolution DEAE-cellulose chromatography into m-1α and -β and m-2γ, -δ, and -ϵ. The present work describes the biochemical, physical, and spectral characteristics of these purified ctenophore photoproteins.

Materials and Methods

Photoprotein Extraction and Purification. Photoproteins from *Mnemiopsis* sp. and *Beroë ovata* were extracted and purified in EDTA-containing buffers by the methods previously described (Ward and Seliger, 1974a). The most highly purified preparations, which are essentially homogeneous by

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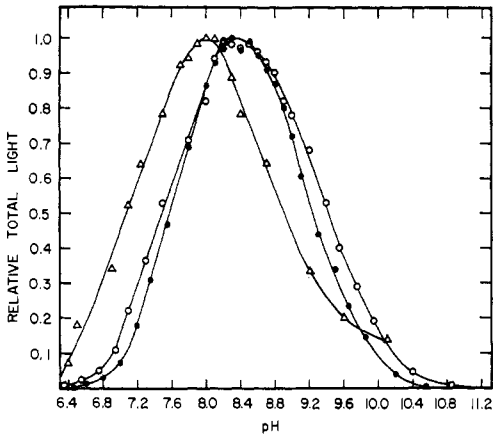

FIGURE 1: pH optima for total light assays of m-1 (open circles), m-2 (closed circles), and berovin (triangles) photoproteins. Photoprotein (0.2 ml in 5×10^{-3} M Tris–EDTA at pH 8.0) was injected into 2.0 ml of assay buffer containing 0.1 M $CaCl_2$ and 0.1 M Tris–maleate (for the pH range 6.4–8.0) or 0.1 M Tris–glycine (pH 8.0–11.5). Each point was determined by a minimum of two assays (near the peaks in the pH curves 3–5, assays were performed at intervals of 0.1 pH unit).

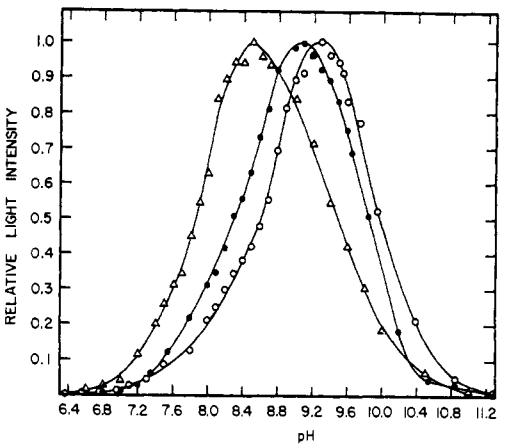

FIGURE 2: pH optima for light intensity assays of m-1 (open circles), m-2 (closed circles), and berovin (triangles) photoproteins. Procedures are those described in the legend to Figure 1.

polyacrylamide gel electrophoresis, have specific photon yields of 4×10^{14} photons mg^{-1}.

In Vitro Assay Methods. Standard calcium assays for photoprotein activity were performed as previously described (Ward and Seliger, 1974a). For determinations of the temperature optima of *in vitro* reactions, the assay tube was housed in a water-jacketed assay box. The tube was bathed with a rapid flow of water pumped from a constant-temperature bath. Light from the reaction was received by a 1P21 phototube mounted outside a double glass window on the side of the assay box.

Interference Filter Spectral Analysis. An apparatus which permits simultaneous measurement of light by two phototubes, each with its own interference filter (Seliger and McElroy, 1964; Fuller *et al.*, 1972) was used to compare the relative shapes of emission spectra.

Bioluminescence Emission Spectra. The emission spectra for *Mnemiopsis* and *Beroë* were measured *in vivo* and *in vitro* using a 1-m f/3 grating spectrometer designed by W. Fastie (Morton *et al.*, 1969). For *in vivo* measurements, strips of bioluminescent tissue, dissected from the meridional canals of dark-adapted animals and frozen in 10×75 mm test tubes, were allowed to thaw in front of the spectrometer entrance slit. This produced a diffuse glow with a half-life of several minutes, suitable for continuous spectral recording. A steady glow of *in vitro* light was obtained by injecting magnesium ion into a photoprotein solution containing a slight excess of EDTA over calcium ion. The slow release of EDTA-bound calcium was sufficient to maintain a reasonably steady glow. Spectral data were corrected for phototube and spectrometer sensitivity and for changes in the source intensity.

Fluorescence Spectra. Fluorescence was measured with an Aminco-Keirs spectrophotofluorometer equipped with an X–Y recorder.

Absorption Spectra. The absorption spectra of purified photoproteins were determined on a Cary Model 14 spectrophotometer using the 0.0- to 0.1-absorbance slide-wire.

Photoinactivation Measurements. Photoinactivation of mnemiopsin was measured with an apparatus to be described elsewhere (Ward and Seliger, 1974b).

Preparation of "Calcium-Free" Water and Glassware. Water for metal activation and inhibition studies was purified first by deionization and then was distilled twice in an all-glass Corning still. It was finally passed through a bed of Chelex-100 (Bio-Rad Laboratories) in an acid-washed polyethylene column. Polyethylene containers were soaked 3 days in 1 N HCl at 70° and 3 days in deionized water at 70° and finally rinsed five times with "Ca^{2+}-free" water. The same procedure was followed for preparing glassware except that 6 N HCl was used.

Chemical Reagents. Proteins for molecular weight standards were purchased from the following suppliers: bovine serum albumin, catalase, ovalbumin, aldolase, carbonic anhydrase, myoglobin, lysozyme, ribonuclease A, cytochrome *c*, and soybean trypsin inhibitor (Sigma); liver alcohol dehydrogenase, creatine kinase, horseradish peroxidase, α-chymotrypsinogen, and carboxypeptidase (Worthington), and β-lactoglobulin (Schwarz/Mann). Salts for cation studies were Baker Analyzed Reagent chemicals with the exception of $BeSO_4 \cdot 4H_2O$ and $SnCl_2 \cdot 2H_2O$ which were purchased from Fisher Chemical Co. Alcohols were kindly provided by Dr. William Sofer of our department. Other reagents used in these studies have been listed previously (Ward and Seliger, 1974a).

Experiments and Results Section

Optima for in Vitro Assays. Optimum conditions for *in vitro* assays were determined with respect to pH, temperature, and ionic strength. Highly purified mnemiopsin and partially purified berovin with the following specific photon yields were used in these experiments: m-1 (7.1×10^{13} photons mg^{-1}), m-2 (1.7×10^{14} photons mg^{-1}), and berovin (1.3×10^{11} photons mg^{-1}). The pH optima were determined by injecting 0.2 ml of purified photoprotein (in dilute buffer) into 2.0 ml of assay buffer previously adjusted to the desired pH. The pH profiles for all three photoproteins (Figures 1 and 2) are unusually sharp, each with a width at half-maximum of less than 2 pH units. This is in marked contrast with aequorin pH profiles which have half-widths greater than 6 pH units (Shimomura *et al.*, 1962). As with aequorin, the intensity maximum occurs at higher pH than the total light optimum. The pH optima for total light emission are: m-1 (pH 8.4), m-2 (pH 8.3), berovin (pH 8.0). The intensity optima are: m-1 (pH 9.3), m-2 (pH 9.1), berovin (pH 8.5).

Temperature optima were determined by injecting 0.2 ml of purified photoprotein into 2.0 ml of standard assay buffer held at a given temperature. The temperature of the assay tube was regulated by water circulation through a jacketed photometer (see Materials and Methods). Corrections were made

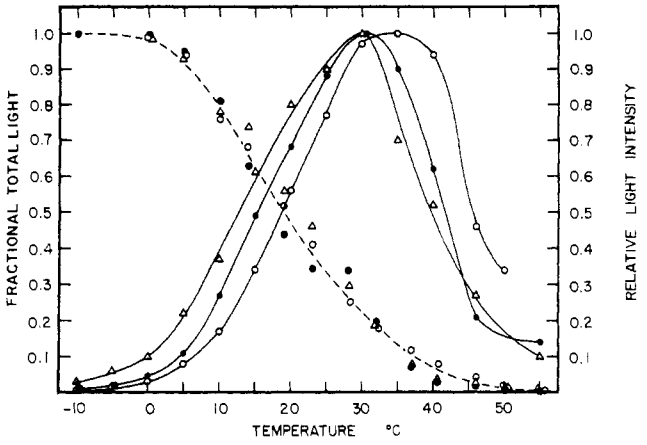

FIGURE 3: Temperature effect on total light assays (broken line) and on light intensity assays (solid lines) of m-1 (open circles), m-2 (closed circles), and berovin (triangles) photoproteins. Temperature was maintained (±1°) in a constant temperature photometer. Each point is the average of three assays.

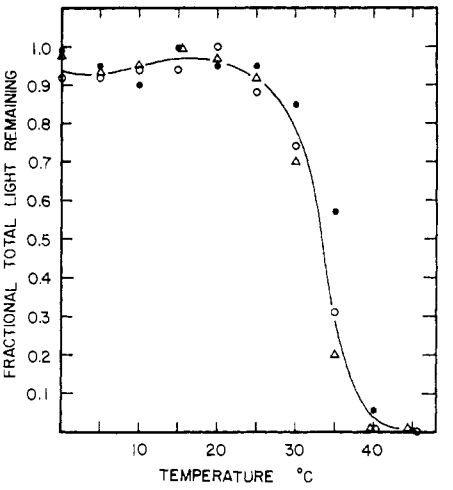

FIGURE 5: Thermal stability curve for ctenophore photoproteins m-1 (open circles), m-2 (closed circles), and berovin (triangles). Incubation was for 5 min in a constant-temperature bath followed by rapid cooling in ice water. Assays were performed in triplicate.

for the small temperature reduction caused by injection of the cold photoprotein sample into a warmer assay tube. These results (Figure 3) agree qualitatively with those of Shimomura *et al.* (1962), who reported a temperature optimum for total light approaching 0°. The ctenophore photoprotein is more sensitive to temperature than the aequorin photoprotein. The quantum yield is reduced to 50% of maximum at 20°C for the ctenophores as compared with 34° for aequorin. The total light optimum for all three photoproteins is 0°. (m-2 gives the same total light at −10°.) A rather sharp intensity optimum at 30° was found for m-2 and berovin, while the maximum intensity for m-1 is nearer 35° and the curve is somewhat broader.

The relations between ionic strength and total light and light intensity were determined for m-2 photoprotein over four orders of magnitude in ionic strength and four orders of magnitude in calcium concentration, at a constant pH of 8.5. Tris concentration was adjusted to produce the desired ionic strength. At a given calcium concentration, total light and intensity vary as the log of the ionic strength (Figure 4). The graphs for 10^{-5} and 10^{-4} M Ca^{2+}, however, show a drop off at higher ionic strength.

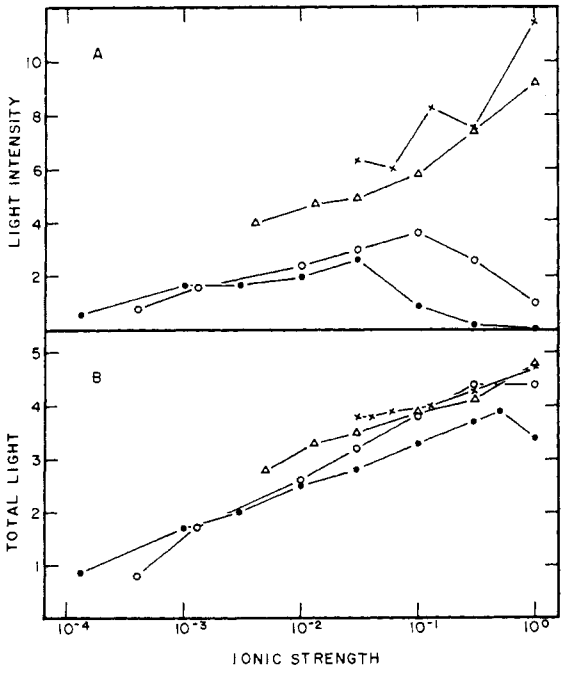

FIGURE 4: Ionic strength dependence for light intensity (A) and total light (B) assays at calcium concentrations of 10^{-5} M (solid circles), 10^{-4} M (open circles), 10^{-3} M (triangles), and 10^{-2} M (X's). Duplicate assays were performed in pH 8.5 buffer. Tris concentration was varied to achieve the ionic strength desired.

Thermal Stability. This was determined by incubating a 50-μl sample of photoprotein in 2.0 ml of 0.01 M Tris–EDTA buffer (pH 8.0), previously adjusted to the desired temperature. After 5 min in a constant-temperature bath, the tube was rapidly cooled in ice water and later assayed for irreversible thermal inactivation (Figure 5). Highly purified mnemiopsin and partially purified berovin (see preceding section) were used for this study. The results show that activity was stable from 0 to 25° but fell to less than 5% at 40°. A significant increase in thermal stability was observed at higher ionic strength, however. In 0.3 M NaCl, 70% of the original activity was retained after 30-min incubation at 35°. Magnesium ion, a competitive inhibitor of calcium activation, failed to confer greater thermal stability than sodium ion at the same ionic strength.

Kinetics of Photoprotein Reactions. The *in vivo* and *in vitro* flashes of *Mnemiopsis* and *Beroë* were recorded on a high-speed Sanborn recorder (10 msec for full-scale deflection). *In vivo* flashing was stimulated by tapping the side of the assay box containing a dissected ctenophore meridional canal in a 10 × 75 mm test tube. *In vitro* flashes were initiated by rapid manual injection of photoprotein into assay buffer containing a large excess of calcium (0.1 M). Figure 6 shows the *Mnemiopsis* flash *in vivo* at 22 ± 2°. The rise time is 50 msec and the pseudo-first-order rate constant for decay from maximum intensity was calculated to be 25 sec^{-1}. This is in agreement with a previously reported value of 24 sec^{-1} (Morin and Hastings, 1971a). The decay curve for the *in vivo* flash (Figure 6) is first order over at least two decades. Identical rise time and first-order decay constant were found for flashes of the live meridional canal tissue from *Beroë ovata*. (A mechanical probe was required to stimulate the *Beroë* tissue.)

In vitro kinetics of the three purified photoproteins are not identical, however, as shown in Figure 7. At constant temperature (22 ± 2°), constant pH (8.2), and constant ionic strength

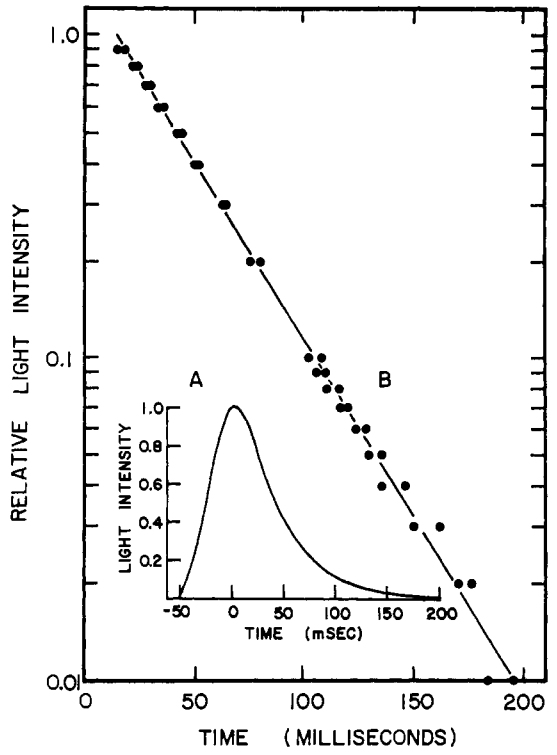

FIGURE 6: (A) An *in vivo* flash recording from *Mnemiopsis* meridional canal tissue stimulated mechanically. (B) Data from two such flashes is plotted as a first-order luminescent decay curve.

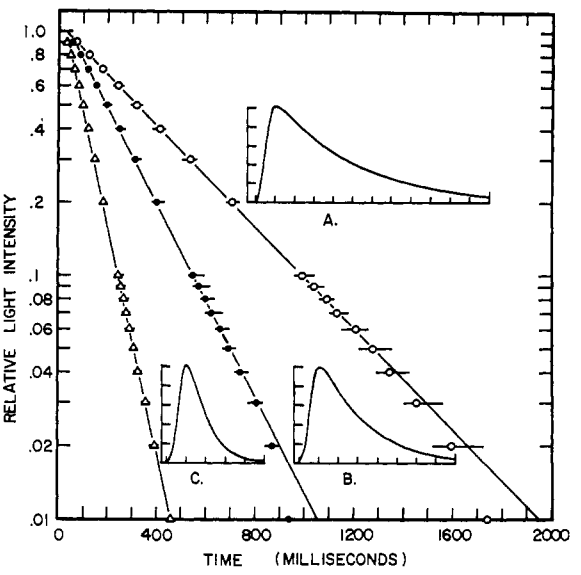

FIGURE 7: *In vitro* flash recordings of mnemiopsin-1 (A), mnemiopsin-2 (B), and berovin (C) at pH 8.2 and 22 ± 2°. Divisions on the abscissas of these graphs are at 0.1-sec intervals. The corresponding first-order decay curves are based on data from five flash recordings of m-1 (open circles), five recordings of m-2 (solid circles), and three recordings of berovin flashes (triangles). Horizontal bars indicate the range of the measurements.

(10^{-1} M $CaCl_2$, 2×10^{-1} M Tris), the three photoproteins have very different decay rates. The first-order decay constants were found to be 2.4, 4.5, and 11 sec^{-1} for m-1, m-2, and berovin, respectively, and these were independent of photoprotein concentration. Large differences among these decay constants are apparent over wide ranges in temperature and pH, as illustrated in Table I. In all cases (except at pH 10) the magnitude of these decay constants followed the order: berovin > mnemiopsin-2 > mnemiopsin-1. The data from Figure 3 and from Table I (A) are represented in Figure 8 in the form of Arrhenius plots. For flash height data (8A), the plot is linear from −10 to 15° after which the slope begins to level off. A departure from linearity occurs near 15° (a temperature at which ctenophore photoproteins are quite stable). The plot of first-order decay rates (8B) however is linear to 30°. The nonlinearity above 30° is suggestive of thermal instability. Energy of activation (E_a) was calculated from the data of Figure 8A using the equation

$$\ln \frac{k_2}{k_1} = \frac{E_a}{R}\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

where R is 1.98 cal deg^{-1} mol^{-1} and T is the absolute temperature. Unusually high activation energies of 18.4, 25.4, and 25.3 kcal per mol were found for berovin, mnemiopsin-2, and mnemiopsin-1, respectively. A value of 14.2 kcal/mol for mnemiopsin E_a (based on first-order decay rates) has been reported by Girsch and Hastings (1973).

TABLE I: First-Order Decay Constants for Ctenophore Photoproteins at Various Temperatures and pH's (Values Given in sec^{-1}).

Photoprotein	Temperature (°C)[a]					
	−10	0	10	20	30	40
	Part A					
Mnemiopsin-1	0.018	0.073	0.58	3.2	24	62
Mnemiopsin-2	0.014	0.10	0.90	5.7	31	76
Berovin	0.15	0.82	3.9	12	39	79
	pH[b]					
	7.0	8.0	9.0	10.0		
	Part B					
Mnemiopsin-1	0.91	2.4	18	63		
Mnemiopsin-2	1.6	4.2	20	73		
Berovin	3.1	10	24	61		

[a] pH adjusted to 8.5 at 25° with Tris buffer. No corrections were made for temperature dependence of pH. [b] Temperature was maintained at 25 ± 1°. The assay buffer contained 0.1 M $CaCl_2$, 0.1 M Tris, 0.05 M citric acid, and 0.05 M glycine.

Activation and Inhibition of Mnemiopsin Bioluminescence by Cations. Coelenterate photoproteins have been reported as highly specific for the divalent cations calcium and strontium (Shimomura *et al.*, 1962, 1963a,b, 1970; Morin and Hastings, 1971a). Shimomura *et al.* (1962) tested thirteen cations in addition to calcium for possible activation of bioluminescence from aequorin. At a metal ion concentration of 0.01 M (100-fold excess over EDTA), only calcium, strontium, and cadmium were effective. Strontium produced 1% of the calcium-activated light intensity while the slight effect of cadmium was attributed to contamination by calcium. Morin and Hastings (1971a) verified these observations with photoproteins from *Obelia* and *Mnemiopsis* without, however, reporting the concentrations of metals or EDTA used. They also reported that magnesium inhibits the reaction while sodium, potassium, zinc, copper, and iron cations neither inhibit nor stimulate these photoproteins. Magnesium inhibition of the aequorin reaction had previously been reported by van Leeuwen and Blinks (1969). In a preliminary communication (Ward and Seliger, 1973a), we reported a total of nine divalent cations

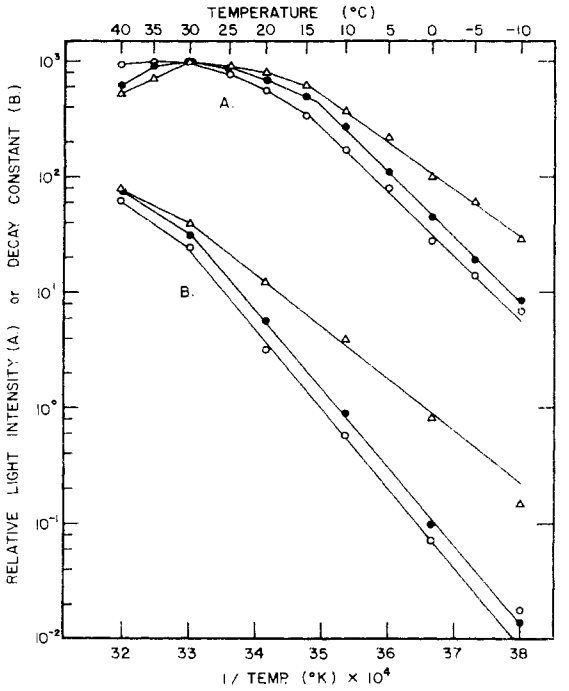

FIGURE 8: (A) Arrhenius plot of light intensity data (from Figure 3) for mnemiopsin-1 (open circles), mnemiopsin-2 (solid circles), and berovin (triangles). (B) A similar plot of first-order decay constants (listed in Table IA) for the three photoproteins.

which activate mnemiopsin-2, noting that with most cations the effect occurs only at low metal ion concentration (5×10^{-5} M). Similarly, Izutsu *et al.* (1972) have demonstrated a large number of divalent and trivalent cations apparently capable of triggering luminescence from "EDTA-free" solutions of aequorin.

In view of these findings we have extended our study of metal activators of mnemiopsin to lower concentrations of both metal ion and EDTA. With the specially prepared distilled water and acid-washed glassware used throughout these experiments (see Materials and Methods), mnemiopsin was stable for only a matter of seconds in 10^{-7} M EDTA, apparently reacting with ubiquitous calcium contaminants. At 10^{-6} M EDTA, however, spontaneous light emission was undetectable and we selected this as the lowest practical concentration. Total light assays for possible activating metals were performed at final concentrations of 10^{-6} M EDTA, 2×10^{-6} M metal salt, and 10^{-3} M Tris buffer at pH 8.5. The results of this survey showed that of 22 cations tested, including calcium, 17 produced significant levels of light with mnemiopsin-2. Six of these, lanthanum, mercury, strontium, cobalt, copper(II), and zinc were at least 50% as effective in total light production as calcium under the conditions described. As previously reported (Ward and Seliger, 1973a), the same effects are not observed at higher cation concentrations. Only 5 of the original 17 cations (calcium, cadmium, copper, mercury, and strontium) activate mnemiopsin-2 at 10^{-4} M. At still higher concentrations (10^{-3} or 10^{-2} M), the list of activators is reduced to three—calcium, strontium, and cadmium—in the order of relative effectiveness. These latter results are in exact agreement with those of Shimomura *et al.* (1962) on the aequorin system. None of the cations tested have reaction rates comparable to the rate at which calcium triggers mnemiopsin bioluminescence.

A possible reason for decreasing effectiveness of metal ions with increasing concentration became more apparent with the

TABLE II: Inhibition by Various Cations of the Calcium Activation of Mnemiopsin.

	Cation	Initial Fraction of the Total Light[a]	Fraction of Total Light after Ca^{2+} Addn[b]	Overall % Inhibn
1	Ag^{+}	0.85	0.00	15
2	Al^{3+}	0.15	0.55[c]	30
3	Ba^{2+}	0.05[c]	0.95	0
4	Be^{2+}	0.10	0.25[c]	65
5	Ca^{2+}	1.00	0.00	0
6	Cd^{2+}	0.05	0.35[c]	60
7	Co^{2+}	0.00	0.40[c]	60
8	Cu^{2+}	0.30	0.00	70
9	Fe^{2+}	0.00	0.05[c]	95
10	Fe^{3+}	0.05	0.05	90
11	Hg^{2+}	0.00	0.20	80
12	K^{+}	1.00	0.00	0
13	La^{3+}	0.05	0.00	95
14	Li^{+}	1.00	0.00	0
15	Mg^{2+}	1.00	0.00	0
16	Mn^{2+}	0.15[c]	0.55	30
17	Na^{+}	1.00	0.00	0
18	Ni^{2+}	0.00[c]	0.60	40
19	Pb^{2+}	0.00	0.00	100
20	Sn^{2+}	0.50	0.00	50
21	Sr^{2+}	0.70	0.00	30
22	Zn^{2+}	0.00	0.20[c]	80
23	Blank	1.00	0.00	0

[a] Total light was integrated over a one minute period following the injection of photoprotein into a solution containing 10^{-2} M Tris (pH 8.5), 10^{-6} M EDTA, 10^{-5} M Ca^{2+}, and 10^{-3} M metal ion. Results are expressed as a fraction of calcium-activated bioluminescence. [b] Additional light production, subsequent to the addition of 10^{-2} M Ca^{2+}, was integrated for 2 min and then expressed as a fraction of calcium-activated bioluminescence. [c] Indicates that light was still being produced at a detectable rate at the completion of the light collecting period.

results of an ion competition experiment. A mnemiopsin-2 sample was injected into a solution containing 10^{-2} M Tris (pH 8.5), 10^{-6} M EDTA, 10^{-5} M Ca^{2+}, and 10^{-3} M metal salt, and total light was recorded for 1 min. Calcium ion concentration was then increased to 10^{-2} M by a second injection, total light being recorded for an additional 2 min. It is seen from the results of this experiment (shown in Table II) that certain cations which are activators at low concentration (2×10^{-6} M) are strong inhibitors of bioluminescence at high concentration (10^{-3} M). This is especially true for cobalt, mercury, lanthanum, and zinc, which prevent activation of mnemiopsin by calcium and whose effects are only partially reversed by the addition of excess calcium. Two alkaline earth metals, Mg^{2+} and Ba^{2+}, are completely reversible competitive inhibitors of calcium activation. Figure 9 demonstrates magnesium inhibition kinetics of m-2 photoprotein and the complete reversibility of inhibition with excess calcium. At a 200-fold excess of magnesium to calcium, the half-time for total light emission is increased from 250 to 3100 msec and the initial intensity of the flash is reduced by 90%. The barium

TABLE III: Effect of Various Alcohols on the Total Light Production of M-2 Photoprotein.[a]

Alcohol	% Enhancement or Inhibn (−) 0.01 M	0.05 M	Alcohol	% Enhancement or Inhibn (−) 0.01 M	0.05 M
Primary (linear chain)			Amino alcohols		
1. Methyl	4	0	29. 2-Aminoethanol	−6	−3
2. Ethyl	4	−1	30. 1-Amino-2-propanol	7	5
3. *n*-Propyl	8	9	31. 3-Amino-1-propanol	−4	−1
4. *n*-Butyl	9	6	32. Histidinol		5
5. *n*-Pentyl	3	6	Diols		
6. *n*-Hexyl	−2	−36 (±3)[c]	33. Ethylene glycol	12	16
7. *n*-Heptyl[b]	−32		34. 1,3-Butanediol	11	12
8. *n*-Octyl[b]	−26		35. 2,3-Butanediol	7	9
9. *n*-Nonyl[b]	−22		36. 1,5-Pentanediol	1	14
10. *n*-Decyl[b]	−17		37. Glycerol	−2	0
11. Cetyl (*n*-hexadecyl)[b]	−17		Chloro alcohols		
Primary (branched chain)			38. 1,3-Dichloro-2-propanol	10	28 (±3)[c]
12. Isobutyl	11	12	Cyclic alcohols		
13. Isoamyl	5	3	39. Cyclopentanol	5	12
Primary (aromatic)			40. Cyclohexanol	−1	1
14. Benzyl	9	6	Oxy-Alcohols		
Secondary			41. 2-Methoxyethanol	1	0
15. Isopropyl	1	8	42. 2-Ethoxyethanol	−1	3
16. *sec*-Butyl	0	−2	Amino acids		
17. *sec*-Hexyl	−3	−3	43. Serine		13
18. 3-Methyl-2-butyl	21	37 (±5)[c]	44. Homoserine		19
19. 3-Pentanol	6	13	45. Threonine		4
20. 2-Octanol[b]	−3		46. Hydroxyproline		14
Tertiary			Hydroxy acids		
21. *tert*-Butyl	15	8	47. Lactic acid		14
22. *tert*-Pentyl	10	25	48. Malic acid		−1
Unsaturated			49. β-Hydroxybutyric acid		16
23. 1-Buten-3-ol	7	25	Polymers		
24. Crotyl (2-buten-3-ol)	20	35 (±2)[c]	50. Poly(vinyl alcohol)[b]	−5	
25. 1-Penten-3-ol	3	16	51. Poly(ethylene glycol)	−2	6
26. 3-Penten-2-ol	7	8	52. Dextran T-500	1	4
27. 1-Hexen-3-ol	9	6	53. Ficoll	−1	−2
28. 1-Pentyn-3-ol	9	3			

[a] Alcohols were incubated with photoprotein at 0° for 5–8 min. Alcohol concentrations were reduced by a factor of 10 during the calcium assay. All numbers are the average of 5 assays. [b] Saturated solution at <0.01 M. [c] Numbers in parentheses are representative standard deviations.

ion effect is similar, except that a much smaller ratio of barium to calcium is required for the same results.

Effects of Alcohols on Bioluminescent Quantum Yields. It was reported by Shimomura *et al.* (1962) that long-chain aliphatic alcohols enhance (by as much as 30%) the total light yield of aequorin bioluminescence. Repeating their experiment with mnemiopsin and berovin, we have found the above alcohols to be inhibitory by approximately the same percentage. In surveying more than 50 alcohols and hydroxy compounds we have found five which enhance total light in mnemiopsin m-2 by 25% or more (Ward and Seliger, 1973a). All are branched alcohols, one is a tertiary alcohol, two are unsaturated, and one is halogenated. The five are: 2-methyl-2-butanol (*tert*-pentyl), 3-methyl-2-butanol, 1-buten-3-ol, 2-buten-3-ol (crotyl), and 1,3-dichloro-2-propanol. The strongest inhibitors among the 50 alcohols surveyed are the unbranched primary alcohols, 6–10 carbon atoms in length. Saturated solutions of these alcohols, and also cetyl alcohol (*n*-hexadecanol), gave more than 15% reduction in total light.

Alcohols were surveyed by dispensing 50 μl of dilute mnemiopsin-2 into 2.0 ml of 0.01 M Tris–EDTA (pH 8.0) containing the test alcohol at 0.01 or 0.05 M concentration. After a 5- to 8-min incubation at 0°, the sample was assayed for total light by the standard procedure. Five assays of the experimental and five assays of a control were performed for each alcohol. The coefficient of variation was less than 15% for most of these determinations (Table III).

Three alcohols with the greatest enhancement, 3-methyl-2-butanol, crotyl alcohol, and 1,3-dichloro-2-propanol, and one with the greatest inhibition, 1-hexanol, were studied more closely with respect to concentration, incubation time, and reversibility. The maximum enhancement occurs at alcohol concentrations between 0.04 and 0.06 M, but at higher concentrations all three alcohols are inhibitory. The alcohol enhancement requires an incubation time of 2–5 min for maximum effect, whereas inhibition occurs very rapidly, reaching 90% of the maximum effect in less than 20 sec. Reversibility of the alcohol effect was determined by incubating a mnemiopsin-2 solution in 0.05 M alcohol for 5 min and then diluting the

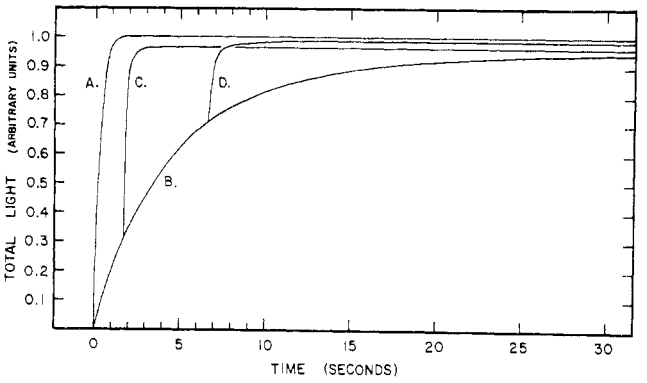

FIGURE 9: Magnesium inhibition kinetics of m-2 photoprotein bioluminescence. (A) Total light curve without added magnesium (0.1 M $CaCl_2$–0.2 M Tris at pH 8.0). (B) Inhibition kinetics with buffer containing 0.001 M $CaCl_2$, 0.2 M $MgCl_2$, and 0.1 M Tris at pH 8.0. Reactions C and D were initiated with the same high magnesium buffer, however at 1.7 and 6.6 sec, respectively, the calcium concentration was increased tenfold (sufficient to overcome the inhibition completely). The reactions were run at 20 ± 1°.

solution with buffer 5:1 (expt A) or 50:1 (expt B). Within experimental error, the stimulatory and inhibitory effects of alcohols are completely reversible (Table IV). The alcohol interaction has no effect on the bioluminescence emission spectrum as determined by a sensitive interference filter spectral analysis (see Materials and Methods for a description of the apparatus). The observed effects of alcohols on total light assays cannot be explained, therefore, on the basis of shifts in the bioluminescent emission spectrum relative to the phototube spectral sensitivity curve. The enhancement of total light with alcohol and the increase in total light by assaying at reduced temperature are not additive effects. No alcohol enhancement of m-2 photoprotein with crotyl alcohol or 1,3-dichloro-2-propanol was observed when assays were performed at 0°. The total light yields for mnemiopsin-1 and

TABLE IV: Reversibility of Alcohol Enhancement and Inhibition of m-2 Photoprotein Total Light Yield.

	Alcohol Concn (M)[a]		% Enhancement or Inhibn (−)	
	Initial	Final	1,3-Dichloro-2-propanol	1-Hexanol
Experiment A				
Experimental	0.05	0.01	17	−10
Control 1	0.05	0.05	26	−19
Control 2	0.01	0.01	15	−11
Control 3	0.00	0.00	0	0
Experiment B				
Experimental	0.05	0.001	2	
Control 1	0.05	0.05	16	
Control 2	0.001	0.001	0	
Control 3	0.00	0.00	0	

[a] Photoprotein was incubated in alcohol 5 min at the concentration listed in the column headed "Initial." The sample was diluted 5:1 (expt A) or 50:1 (expt B) to the concentrations of alcohol listed in the column marked "Final." After 5 min at this concentration, standard assays were performed.

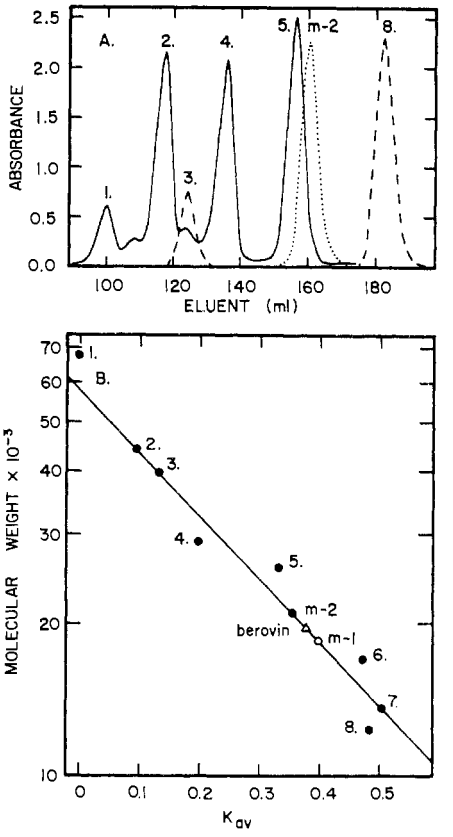

FIGURE 10: (A) Elution pattern of mnemiopsin-2 and six simultaneously chromatographed protein standards from a Sephadex G-75 (superfine) column. The column (118 × 1.7 cm) was equilibrated with a pH 8.0 buffer solution containing 10^{-3} M EDTA, 10^{-2} M Tris, and 5×10^{-2} M NaCl. A 2.0-ml sample was eluted with this buffer at a flow rate of 1.8 ml cm^{-2} hr^{-1}. Sixty-five 1.5-ml fractions were collected after the void volume. Protein was measured by absorbance at 280 (solid line) or 405 nm (dashed lines). Photoprotein activity was measured by total light assay with standard calcium buffer (dotted line). (B) Semi-log plot of molecular weight *vs.* K_{av} for m-2, berovin, and m-1 photoproteins and eight protein standards. These standards are bovine serum albumin (1), ovalbumin (2), horseradish peroxidase (3), carbonic anhydrase (4), α-chymotrypsinogen (5), myoglobin (6), ribonuclease A (7), and cytochrome *c* (8). This graph is a composite of four separate runs with elution profiles similar to part A.

berovin were also enhanced by incubation with the three alcohols most effective with mnemiopsin-2.

Estimations of Molecular Weight by Gel Filtration. Molecular weight estimations for ctenophore photoproteins were made by gel filtration chromatography on Sephadex G-75 (superfine grade) and Bio-Gel P-100 (−400 mesh). The columns were standardized with eight or more globular proteins of known molecular weight. High-resolving power of the columns and the use of protein standards with strong Soret absorption bands (horseradish peroxidase, myoglobin, cytochrome *c*) made it possible to include nearly all of the standards in each run. On G-75 Sephadex columns, the ctenophore photoproteins elute soon after the chymotrypsinogen peak in the order: m-2, berovin, and m-1 (Figure 10). The same relative positions with respect to chymotrypsinogen were found in more than a half-dozen runs on this and other G-75 columns. A plot of log molecular weight *vs.* K_{av}[1] for eight protein standards is also shown in Figure 10. The ctenophore photoprotein K_{av}'s correspond to apparent molecular weights

[1] $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e = elution volume, V_0 = void volume, and V_t = total volume.

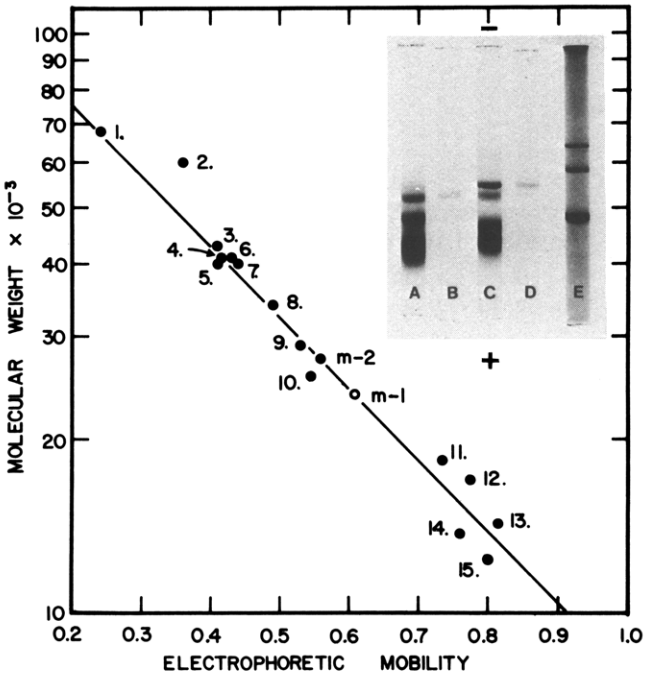

FIGURE 11: Molecular weight determination of mnemiopsin-1 and mnemiopsin-2 by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein standards are bovine serum albumin (1), catalase (2), ovalbumin (3), soybean trypsin inhibitor (4), creatine kinase (5), liver alcohol dehydrogenase (6), aldolase (7), carboxypeptidase (8), carbonic anhydrase (9), α-chymotrypsinogen (10), β-lactoglobulin (11), myoglobin (12), lysozyme (13), ribonuclease A (14), and cytochrome *c* (15). The insert is a photograph of five gels from which some of the data for this graph was taken. Samples which were applied to the gels were as follows: (A) 70 μg of partially purified m-1; (B) $\sim$10 μg of pure m-1; (C) 40 μg of partially purified m-2; (D) $\sim$10 μg of pure m-2; (E) 6 μg of liver alcohol dehydrogenase, 10 μg of α-chymotrypsinogen, and 15 μg of myoglobin.

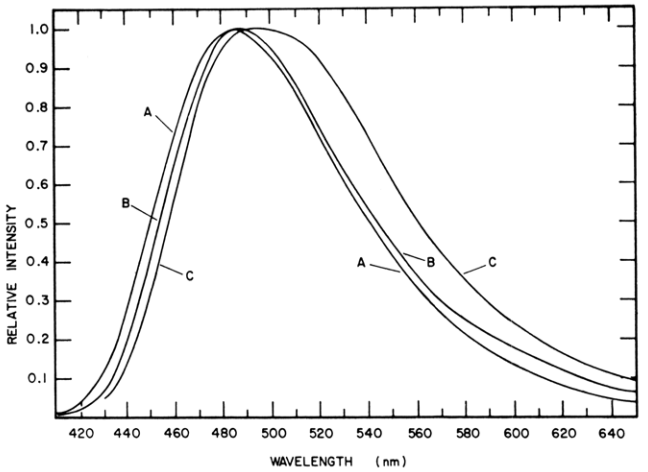

FIGURE 12: *In vivo* and *in vitro* bioluminescence emission spectra of *Mnemiopsis* sp. and *Beroë ovata*. Spectra represented are: (A) *in vitro* emission for m-1, m-2, and berovin photoproteins, (B) *in vivo* emission of *Mnemiopsis* (freeze-thaw technique), and (C) *in vivo* emission of *Beroë* (freeze-thaw technique). Spectra marked A and B are both composites of 10 complete spectral determinations. Spectrum C is the average of two spectral determinations.

of 21,000 (m-2), 19,500 (berovin), and 18,500 (m-1). Similar values have been reported for the gel filtration molecular weight of aequorin (Shimomura and Johnson, 1969; Blinks *et al.*, 1969; Hastings *et al.*, 1969) and *Obelia* photoprotein (Morin and Hastings, 1971a). The apparent scatter of points in Figure 10B is highly reproducible, indicating that deviations from the semi-log plot are the result of nonspherical shape of the proteins or chemical adsorption to the dextran polymer.

The results of gel filtration with Bio-Gel P-100 were similar to those with Sephadex. Each protein standard fell on or near a straight line semi-log plot with the exception of chymotrypsinogen, a standard which chromatographs reproducibly as a protein with the molecular weight of 16,000 on our P-100 column. By this technique mnemiopsin-2 has an apparent molecular weight of 22,000. This is in contrast to the results of Shimomura and Johnson (1969) who found a much higher molecular weight for aequorin on Bio-Gel P-100 (31,000) than on Sephadex G-100 (21,000).

Molecular Weight Determination by Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. Samples of m-1 and m-2 photoprotein, purified several hundredfold by a procedure similar to the one we have described (Ward and Seliger, 1974a), were electrophoresed in the presence of sodium dodecyl sulfate using a modified Weber and Osborn (1969) technique as previously described (Ward and Seliger, 1974a). Also included were samples of electrophoretically pure mnemiopsin-1 and mnemiopsin-2 with specific light yields of 3×10^{14} photons mg^{-1}. Fifteen pure proteins of known molecular weight were run under the same electrophoretic conditions to serve as standards for determining the photoprotein molecular weights. Figure 11 is a plot of log molecular weight *vs.* electrophoretic mobility for the two mnemiopsin "isoproteins" and each of the fifteen standards. The insert is a photograph of five gels from which some of the data for this graph was taken. Relative mobilities of photoproteins and protein standards were highly reproducible in the four or five separate runs. By this method m-1 and m-2 have apparent molecular weights of 24,000 and 27,500, respectively. These values are about 30% higher than those determined by Sephadex gel filtration. Again there is an interesting contrast between the functionally identical photoproteins from *Mnemiopsis* and *Aequorea*, as molecular weight determinations for aequorin agree by these two methods while the mnemiopsin determinations do not.

Bioluminescence Emission Spectra. Figure 12 shows corrected emission spectra for the *in vivo* and *in vitro* bioluminescence from *Mnemiopsis* sp. and *Beroë ovata*. The *in vivo* maximum for *Mnemiopsis* is at 488 nm and the full width at half-maximum intensity (FWHM) is 90 nm. The *Beroë in vivo* spectrum is broader (FWHM = 102 nm) and shifted to the red, with a peak at approximately 494 nm. Although there is significant species difference between the *in vivo* spectra, the *in vitro* spectra for mnemiopsin-1, mnemiopsin-2, and berovin are all identical. This was also verified with a sensitive interference filter spectral analyzer (see Materials and Methods). The common *in vitro* spectrum (Figure 12A) has a peak at 485 nm (FWHM = 90 nm). A slight but statistically significant red shift to 487 nm occurs when strontium is used to activate m-2. No spectral shift occurs in the presence of 3-methyl-2-butanol, an alcohol which enhances total light yield for m-2 photoprotein. The *Mnemiopsis* emission spectra shown in Figure 12A,B are composites of ten complete *in vitro* spectra and ten complete *in vivo* spectra. The *in vivo* red shift of *Mnemiopsis* (λ_{max} 488 nm) is apparently not of experimental origin, but it may be the result of absorption by pigments which are concentrated in the meridional canal tissue (but absent from purified photoprotein samples). This may also account for the substantial red shift *in vivo* for *Beroë* bioluminescence.

Fluorescence Spectra. Purified mnemiopsin at a concentration of 0.4 mg/ml in pH 8.0 Tris–EDTA buffer shows no

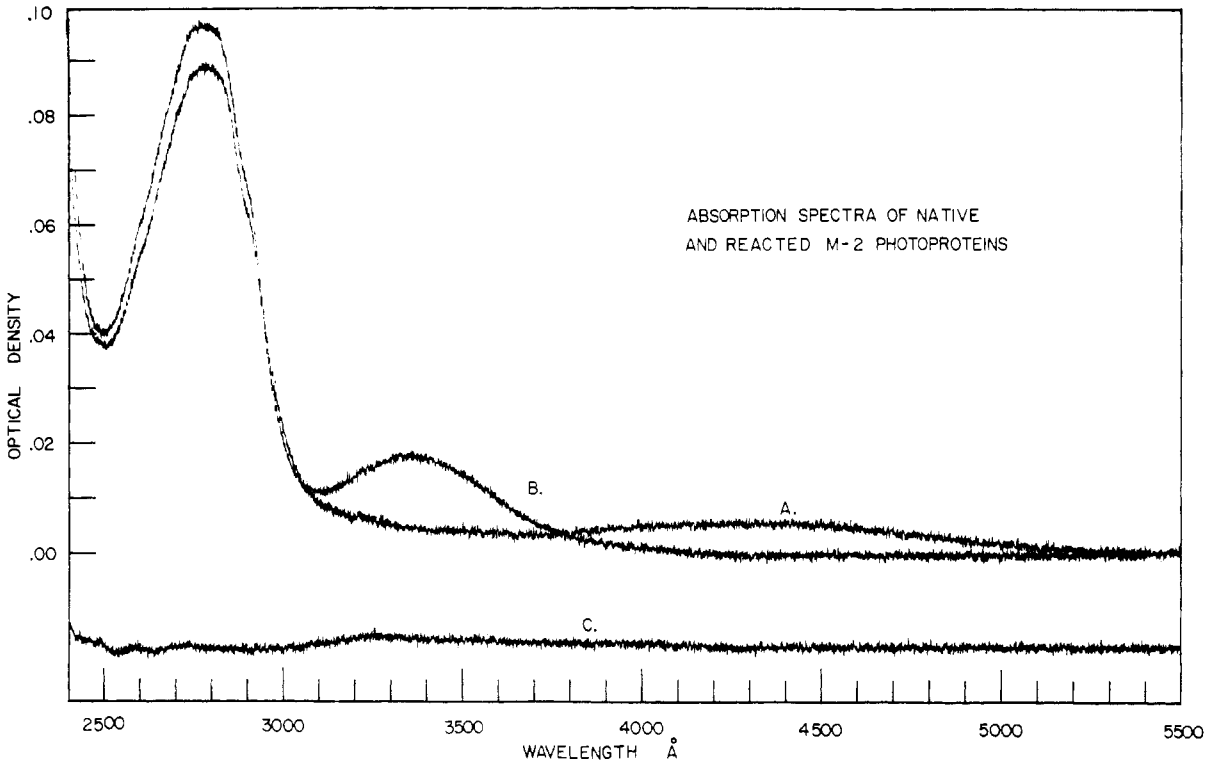

FIGURE 13: Absorption spectra of pure m-2δ photoprotein and its calcium reaction product. Spectrum A is native photoprotein; spectrum B is the calcium reaction product of pure m-2δ; line C is the base line measurement. Spectra were recorded on a Cary Model 14 spectrophotometer using the 0–0.1-absorbance slide-wire. The sample contained 0.081 mg/ml of photoprotein in 10^{-3} M EDTA, 5×10^{-2} M NaCl, and 10^{-2} M Tris (pH 8.0).

detectable fluorescence except the typical tryptophan fluorescence of proteins. No fluorescence other than that of tryptophan was found for the bioluminescent reaction products in the presence of calcium or for light-inactivated mnemiopsin.

Absorption Spectrum of Pure Mnemiopsin-2. The absorption spectrum was determined from a sample of m-2δ photoprotein, chromatographically pure on Sephadex G-75 and essentially homogeneous by sodium dodecyl sulfate gel electrophoresis (Ward and Seliger, 1974a). The native photoprotein has an absorption maximum at 277 nm and a shoulder at 290 nm, typical of proteins containing tryptophan. A broad absorption band, with a maximum at approximately 435 nm (millimolar extinction coefficient = 1.8), is the only other chromophore detected in the photoprotein (Figure 13). Upon the addition of calcium and subsequent light emission, this chromophore disappears and a new absorption band at 335 nm appears (millimolar extinction coefficient = 5.3). At the same time, the ultraviolet absorption maximum shifts to 278 nm with 8% decrease in the extinction.

Quantum Yields for Bioluminescence. Quantum yields for purified ctenophore photoproteins were determined with reference to the radioactive light standard previously described (Ward and Seliger, 1974a; Biggley *et al.*, 1969), assuming molecular weights of 27,500 (m-2), 24,000 (m-1), and 25,000 (berovin). Assayed under the optimum conditions, the quantum yields were found to be 0.04, 0.04, and 0.05 for the most highly purified samples of m-1, m-2, and berovin, respectively. These are minimum values assuming the photoprotein samples are fully "charged" with the luciferin-like chromophore.

Photoinactivation of Ctenophore Photoproteins. Ctenophore photoproteins are rapidly inactivated by light (Morin and Hastings, 1971a). An action spectrum for photoinactivation of mnemiopsin-2 extending from 230 to 570 nm includes two major peaks at 270 and 435 nm (Ward and Seliger, 1973a,b). Complete inactivation by light from a cool white fluorescent bulb bleaches the 435-nm absorption band of mnemiopsin without producing any other spectral changes. Details of these photoinactivation studies will be presented elsewhere (Ward and Seliger, 1974b).

Discussion

We have reported that mnemiopsin-2 photoprotein can be triggered to luminesce by 16 of the 21 cations tested (in addition to calcium), but only at low metal ion concentrations. At higher concentrations all 21 of these metals, except Li^+, Na^+, and K^+, are inhibitors of calcium-activated bioluminescence. Under the assay conditions we described, the concentration of free calcium, if released from EDTA chelation, could be sufficient to trigger mnemiopsin bioluminescence. It therefore would appear that the 16 effective cations activate mnemiopsin indirectly by replacing the calcium bound to EDTA. At higher concentrations, these metals inhibit calcium activation of mnemiopsin either by competing with calcium for its active site on mnemiopsin (as appears the case with magnesium and barium) or by noncompetitive binding to other functional groups on the protein. We conclude from these results that mnemiopsin activation is specific for calcium and that apparent activation by other cations including strontium is an artifact of the assay method.

Izutsu *et al.* (1972) performed similar experiments on the photoprotein aequorin in solutions which they stated to be free of EDTA. If however, these solutions contained on the order of 10^{-6} or 10^{-6} M residual EDTA (as might be expected from their gel filtration desalting procedure), then the many ions they report as aequorin activators may simply be releasing bound calcium from EDTA, as we believe occurs with our

assay system. They reported that nine cations have maximum reaction rates equal to or greater than that for Ca^{2+}, an observation we have not been able to substantiate with our studies on mnemiopsin. These authors also state that Shimomura *et al.* (1962, 1963a) failed to observe an activation of aequorin by lead and cadmium because EDTA in their assay solutions suppressed the effects of these ions. In stating this argument, Izutsu *et al.* have failed to consider the fact that Shimomura's ion survey was performed with a 100-fold excess of metal ion concentration to EDTA concentration.

Our initial survey of alcohols (Ward and Seliger, 1973a) was extended to more than 50 compounds in this report to determine if a pattern exists to the chemical structure of alcohols which interact with mnemiopsin. It was observed that the most effective inhibitors are linear aliphatic alcohols 6–10 carbon atoms in length. However, the alcohols which enhance mnemiopsin light yields fit into no specific structural category. The most effective alcohols are relatively hydrophilic and their carbon chains are generally branched. This low degree of structural specificity and the high concentration requirement, long incubation period, and complete reversibility of the alcohol enhancement indicate that alcohols modify the environment of the protein or the bound chromophore rather than bind specifically to an "alcohol site." The effective alcohols may be those whose three-dimensional structure and hydrogen bonding characteristics permit access to and loose interaction with an active site of the protein. Such interactions could stabilize the electronic excited state sufficiently to produce a slight increase in quantum yield of bioluminescence. At higher concentration or over longer incubation times deleterious interactions may occur.

It has recently been demonstrated (Cormier *et al.*, 1973) that a number of bioluminescent coelenterates possess components required for the *Renilla* bioluminescent reaction. Depending on the organism these include one or more of the following: luciferyl sulfate, luciferase, and luciferin sulfokinase. On the basis of several criteria, these components are indistinguishable from the authentic *Renilla* components. More recently it has been shown (Hori and Cormier, 1973a,b; Hori *et al.*, 1973) that the aequorin functional group AF-350, chemically identified by Shimomura and Johnson (1972), is identical with *Renilla* etioluciferin. Hori *et al.* (1973) have also pointed out the absorption spectral similarities among coelenterate bioluminescent systems. The bioluminescent systems of *Aequorea*, *Renilla*, and *Mnemiopsis* have long-wavelength absorption bands at 465, 433, and 435 nm, respectively, which are lost during the bioluminescent reaction. Subsequent to the light reactions, products are formed which absorb in the near-ultraviolet region at 335 nm (aequorin product), 333 nm (*Renilla* oxyluciferin), and 335 nm (mnemiopsin product). Furthermore, our action spectrum for mnemiopsin photoinactivation (Ward and Seliger, 1974b) closely resembles the absorption spectrum of *Renilla* luciferin (Hori and Cormier, 1973a).

These data suggest that all of the bioluminescent coelenterate systems, including those of *Aequorea* and *Mnemiopsis*, contain substrate molecules similar to *Renilla* luciferin. Furthermore, the products of these reactions appear to be similar or identical to *Renilla* oxyluciferin.

Acknowledgments

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A Study of the Parameters Which Determine the Conformation of Linear Polypeptides in Solution by Synthesis of Models and Determination of Thin Film Dialysis Rates†

Michael J. Harris and Lyman C. Craig*

ABSTRACT: A series of hexapeptides with maximum conformational flexibility have been synthesized and studied by the technique of thin film dialysis. The results indicate that the conformation may not be completely random in a given solvent environment and temperature although a degree of randomness has not been excluded. The major contribution to the conformation, within the restrictions expected from conformational mapping, has been shown to be the solvent effect. Electrostatic effects between charged groups were shown to be of little importance when 0.1 M sodium chloride is the solvent. The most extended conformation resulted when the terminal amino acid residues were highly polar.

Recently because of the rapid advances made in high-resolution nuclear magnetic resonance and attempts to calculate minimal energies by computer, considerable attention is being directed toward the conformation in solution of all types of polypeptides (Deber *et al.*, 1969). Such studies are progressing from the simpler problem of the interpretation of results with the rigid cyclic representatives to the more difficult problem of reliable interpretation with the linear ones.

Linear polypeptides without stabilizing covalent cross-linkages have in the past generally been referred to as "random coils." Nonetheless extensive studies (Craig and Harfenist, 1963; Craig, 1967; Craig *et al.*, 1971) by thin film dialysis have shown that in favorable solvent environments such peptides give straight line escape plots indicative of a single diffusional size or at least a narrow distribution of sizes or conformations too similar to be detected by a deviation from linearity. However, the rate of dialysis was found to be highly dependent on the solvent environment and could easily be altered by a small change in pH, ionic strength, or addition of a low concentration of alcohol, urea, guanidinium chloride, or formamide. On the other hand, the rates of dialysis of rigid covalently bound polypeptides such as bacitracin or gramicidin SA were not influenced this way or influenced to a much less extent. It was concluded that in the case of the linear polypeptides much less conformational stability was the reason for the difference.

Simple dipeptides were of special interest in the general study because the conformations of the side chains rather than that of the peptide backbone would be expected to have the greatest influence on the dialysis rate if the way the side chains are folded is determined by the particular solvent environment. The data obtained (Burachik *et al.*, 1970) gave strong support to this theory but nonetheless there were a number of ambiguities in the interpretation. It seemed apparent that the hydrophobic interactions of the side chains were important and that electrostatic interaction played an important role where the side chains contained charged groups of like sign. A later study with lysylglutamic acid or glutamyllysine did not support the theory of a primary effect of electrostatic interaction of the side chains.

In order to ascertain if the same parameters which influence the interactions of the side chains in dipeptides and their conformations would also be those influencing the conformations of longer peptides it seemed of interest to synthesize a series of hexapeptides, X-Gly_4-Y, in which the same amino acids used in the dipeptide study were separated by four glycine residues and to study their rates of dialysis by thin film dialysis in different solvent environments. Glycine peptides theoretically and by dialysis (Burachik *et al.*, 1970) are known to have the least restrictions on their "allowed" (Ramachandran and Sasisekharan, 1968) conformations. This paper reports such a study. It also reports data with two further series of hexapeptides where the two terminal peptide residues are connected by alanine and proline residues. The latter would be expected to have the most rigid conformation and to be the most extended.

The X-Ala_4-Y peptides would be expected to be less compact than the X-Gly_4-Y series but more compact than the X-Pro_4-Y series. Polyproline peptides have not been studied previously by thin film dialysis. With these peptides there exists the opportunity of either cis or trans forms but with a series of adjacent proline residues solvent conditions will usually favor the all-cis form with a right-handed compact helix or all trans forms in a left-handed extended helix (Strassmair *et al.*, 1969). The extended peptide with the trans arrangement of proline residues would be expected to be the most stable form in water.

Experimental Section

The peptides in this study were prepared by the Merrifield solid phase method (Stewart and Young, 1969) from protected

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