Light-Induced Bioluminescence. Isolation and Characterization of a Specific Protein Involved in the Absorption and Delayed Emission of Light*

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ABSTRACT: A protein has been isolated and purified from extracts of luminous bacteria which emits a long-lived (10 sec) delayed light following irradiation with an intense flash of ultraviolet light. The light emission appears the same in several ways to the normal chemically initiated bioluminescence catalyzed by bacterial luciferase. The light-inducible protein has been shown to be a modified luciferase molecule, having a noncovalently bound fluorescent chromophore (λ_{max} 490 nm). Its removal yields active native luciferase.

The free chromophore has spectral and chromatographic properties which resemble those of a flavin; it will recombine with native luciferase to form active light-inducible protein with a concomitant loss of luciferase activity. The original spectral properties are not restored, however. It is hypothesized that the luciferase-bound chromophore is an intermediate or product of the bioluminescent reaction which serves as the emitter in the normal reaction.

Bioluminescent emission catalyzed by bacterial luciferase is normally obtained by adding reduced flavin mononucleotide ($FMNH_2$) to the luciferase preparation in the presence of oxygen and a long-chain aliphatic aldehyde (Hastings and Gibson, 1963; Hastings *et al.*, 1966; Hastings, 1968). However, it has been recently shown that bioluminescence can also be induced by flash irradiation of luciferase in the absence of added flavin. If the luciferase preparation is exposed to a short, intense flash of ultraviolet light, one obtains a bioluminescence which is virtually identical (both spectrally and otherwise) with the emission resulting from the addition of $FMNH_2$ (Gibson *et al.*, 1965). Although the light-induced emission (λ_{max} 490 nm) requires both oxygen and aldehyde, neither need be present at the time of irradiation (Gibson *et al.*, 1965; Hastings and Gibson, 1967).

An unusual and diagnostic property of bacterial bioluminescence is the fact that emission continues to occur for many seconds, long after all the substrate ($FMNH_2$) has been oxidized. The characteristic lifetime of the emission is of the order of 10 sec at 25° with dodecanal ($k = 0.05$ sec^{-1}). This long lifetime is attributed to the existence of an enzyme intermediate which is formed through the interaction of luciferase and $FMNH_2$, but whose exponential decay and concomitant photon production continue over an extended period of time. The fact that the lifetime of light-induced bioluminescence is the same as the flavin-initiated emission indicates that the same intermediate is involved in both cases.

The reaction scheme hypothesized to account for these results (Hastings and Gibson, 1967) was based on the assumption that the protein responsible for light-induced bioluminescence is luciferase. However, the experiments reported here show that the light-inducible protein is distinct from bacterial luciferase and that the two activities can be physically separated. The evidence indicates that light-inducible bioluminescence is a luciferase which has been altered in charge due to the presence of a noncovalently bound small molecule, which molecule may be implicated in the bioluminescent emission; luciferase on the other hand may be prepared in the active form devoid of such species. The results further indicate that although luciferase itself lacks light-induced bioluminescence activity, it may be converted to a light-inducible protein by the addition of the small molecule. This results in a concomitant decrease in its bioluminescence activity with $FMNH_2$.

Materials and Methods

The luciferase and the light-inducible protein used in most experiments were isolated from a luminous bacterium temporarily designated as *Photobacterium fischeri*, strain MAV (Mitchell and Hastings, 1969; Hastings *et al.*, 1969). At pH 7.0 light-inducible protein from the MAV strain is positively charged with respect to the luciferase whereas the light-inducible protein from *P. fischeri* (ATCC 7744) has a charge more nearly equal to that of the luciferase. Thus a separation of the two activities is more easily achieved with MAV. The isolation and purification procedures were modified from those previously described (Hastings *et al.*, 1965a, 1969).

Routine measurements of light-inducible protein activity were made with a small flash apparatus similar to that described earlier (Hastings *et al.*, 1965b). A Vycor syringe containing the sample was mounted in the center of a helical flash tube; after irradiation the sample was rapidly injected into a vial positioned above the photocathode of a photomultiplier tube, calibrated with the standard of Hastings and Weber (1963). The output signal was amplified and recorded graphically; measurements of luminescence intensity began about 100 msec after irradiation. The receiving vial contained

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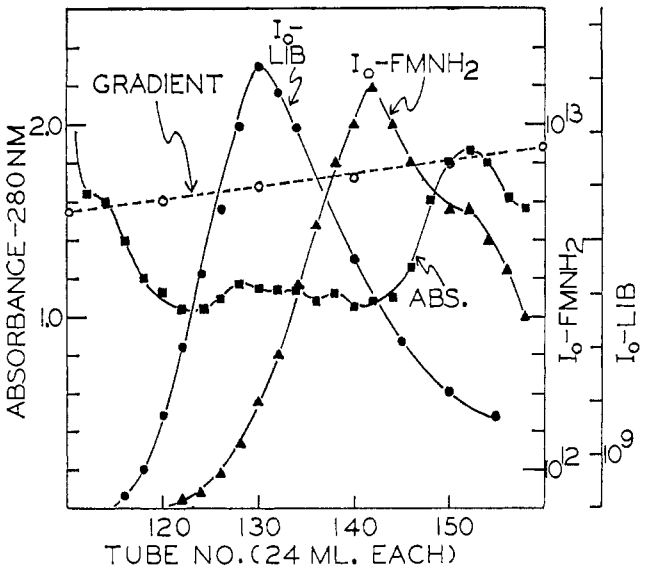

FIGURE 1: The elution pattern of the two bioluminescence activities upon DEAE chromatography. An extract of 250 g of cell paste, purified by $(NH_4)_2SO_4$ fractionation (see text), was applied to the column (4 × 60 cm) in a volume of 114 ml containing 9.4×10^3 units of absorbance at 280 nm. The column was developed with a shallow linear gradient formed from 4 l. each of 0.05 and 0.4 M sodium potassium phosphate buffer at pH 6.25 with 10^{-4} M dithiothreitol. The scale at the left is the absorbance at 280 nm of the undiluted fractions; the same scale multiplied by 0.1 gives the gradient molarity. The light-inducible and flavin-initiated activities (I_0 − LIB and I_0 − $FMNH_2$) are presented in this and all subsequent figures in terms of the initial intensity in quanta sec^{-1} ml^{-1} of each fraction.

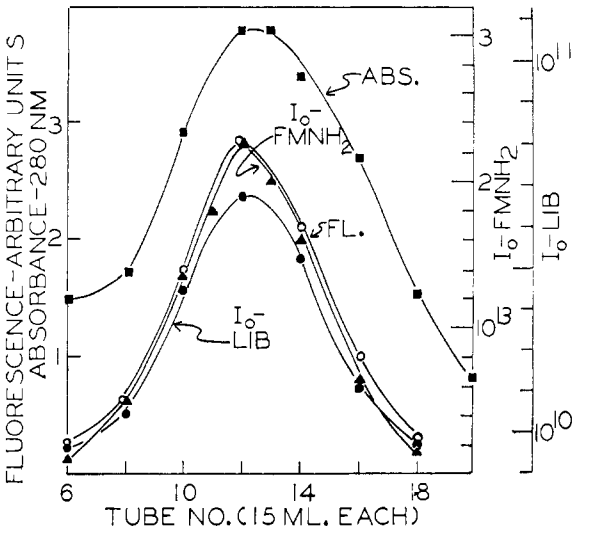

FIGURE 2: Elution pattern of the two bioluminescence activities upon gel filtration on a Sephadex G-100 column (3 × 80 cm) equilibrated with 0.05 M phosphate buffer (pH 6.25) with 10^{-4} M dithiothreitol. The material applied (see text) was selected from a DEAE column as in Figure 1 on the basis of having a relatively high light-induced bioluminescence activity; a total of 10^3 $A_{280\ nm}$ was applied. A fluorescent material was also eluted; its quantity was measured and plotted in arbitrary units.

1 ml of 0.05 M phosphate buffer (pH 7) and 0.1 ml of a dodecanal suspension prepared by ultrasonic treatment of 0.01 ml of dodecanal in 10 ml of H_2O. The irradiation syringe contained an amount of protein in 1.4 ml of the same buffer such that the absorbance at 280 nm did not exceed 0.15/cm.

Measurements of $FMNH_2$-initiated bioluminescence were made as previously described (Hastings *et al.*, 1965a). Reaction mixtures contained 0.02 ml of 1.25 M phosphate buffer (pH 7), 0.01 ml of the dodecanal suspension, 0.2 ml of 1% bovine serum albumin, luciferase as specified, and water to a volume of 1.3 ml. The reaction was then initiated by injecting from a syringe 1.0 ml of $FMNH_2$ (5×10^{-5} M), reduced by bubbling hydrogen in the presence of platinized asbestos. Both the $FMNH_2$ and light-induced bioluminescence (LIB) assays were done at 23 ± 2°, and the activities are expressed in terms of the initial maximum intensity of the bioluminescence (I_0-$FMNH_2$ and I_0-LIB), which occurs about 1 sec after the initiation of the reaction.

Sephadex chromatography in 5 M Gd·HCl,[1] and the renaturation of protein fractions so treated, were done according to the methods of Friedland and Hastings (1967a,b). Before use, 5 M Gd·HCl (Aldrich Chemical Co.) was slurried with activated charcoal followed by Millipore filtration until the absorbance at 280 nm was less than 0.05. Urea was purified by deionization of 10 M solutions using mixed-bed ion-exchange resin (Bio-Rad AG 501-X8D) followed by filtration through Millipore. Florisil (Fisher Scientific Co.) was stirred in 30% pyridine in water followed by repeated washing with water. Dithiothreitol was purchased from Calbiochem and used without further purification. All other reagents were of analytical quality where available.

Fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer modified by us to provide a ratio recording of the exciting light intensity *vs.* fluorescence intensity and fitted with gratings ruled at 1200 lines/in. The fluorescence spectra are presented uncorrected for monochromator and phototube efficiency as a function of wavelength. These corrections are small and if applied would shift the maxima to the red by less than 5 nm.

Fluorescence polarization measurements were made with an analyzer which is available as an accessory from the American Instrument Co. Polarization measurements are presented as $P = (I_{||} - I_{\perp})/I_{||} + I_{\perp})$, where $I_{||}$ represents the fluorescence intensity with the excitation and emission polarizer axes oriented parallel to one another and $I_{\perp}$, the intensity with the polarizers oriented perpendicularly at maximum extinction. FMN (1.5×10^{-5} M) in 0.05 M phosphate buffer (pH 7) at 25° was used to establish a base line for the measurements.

The action spectrum for light-induced bioluminescence was determined using monochromatic radiation from the spectrophotofluorometer incorporating the xenon light source and grating monochromator. The relative photon output of the light falling on the sample was determined at each wavelength by the use of a fluorescent solution as a proportional quantum counter (Weber and Teale, 1958; Birks and Kuchela, 1961). It was possible to utilize the relatively low-intensity source because the intermediate giving rise to bioluminescence can be accumulated by continued

[1] Abbreviations used are: Gd·HCl, guanidine hydrochloride; PMB, *p*-mercuribenzoate.

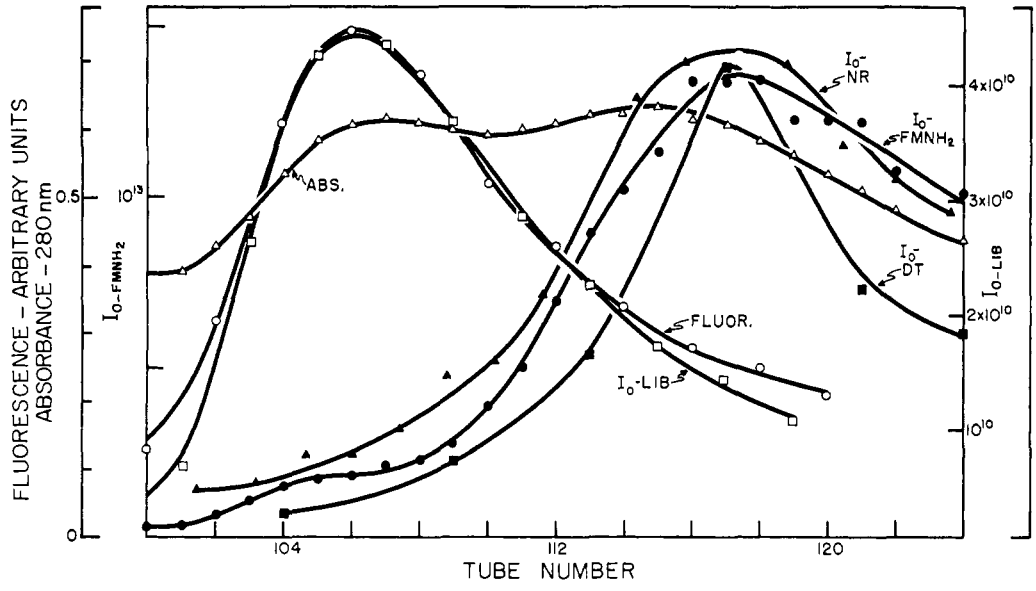

FIGURE 3: The elution pattern of light-induced bioluminescence and $FMNH_2$ activities upon further DEAE chromatography. The active fractions from several Sephadex G-100 columns (similar to Figure 2) were pooled and applied directly to a 3 × 70 cm DEAE-cellulose column in a volume of 436 ml containing a total of 1×10^3 $A_{280\,nm}$. The column was developed with a linear gradient formed from 2 l. each of 0.05 and 0.4 M sodium potassium phosphate buffer at pH 6.25 with 10^{-4} M dithiothreitol. The initial maximum light intensity obtained using dithionite (DT; prepared by dissolving 1 mg of sodium hydrosulfite in 50 ml of water saturated with hydrogen) and catalytically reduced neutral red (NR; 5×10^{-5} M) as alternate reductants is shown for individual tubes. The intensity of the green fluorescence parallels the light-induced bioluminescence activity.

irradiation of the protein if it is suspended as a precipitate in a high salt concentration. A sample (0.5 ml) of light-inducible protein in 5 M phosphate buffer (pH 7) was irradiated for 2 min at the desired wavelength and transferred to a vial (situated in a photomultiplier housing) containing 1.0 ml of the 5 M phosphate. Light emission was obtained by adding 3 ml of a saturated aqueous solution of dodecyl aldehyde. The initial maximum intensity was taken as the amount of intermediate formed during irradiation, and plotted on the basis of equal photon flux at each wavelength.

Results

Luminous bacteria were grown in a high salt medium and lysed osmotically in distilled water (Hastings *et al.*, 1965a). To this crude lysate ammonium sulfate was added to 40% of saturation and the cell wall debris and precipitated proteins were removed by centrifugation. The addition of $(NH_4)_2SO_4$ to 75% of saturation precipitated the active material, which was removed by centrifugation and dissolved in 0.05 M phosphate buffer (pH 7.0). After treatment with DNase and RNase (Hastings *et al.*, 1969) the active material was precipitated a second time with $(NH_4)_2SO_4$, and dialyzed overnight against 0.05 M phosphate buffer (pH 6.25) with 10^{-4} M dithiothreitol added. This material was then applied to a DEAE column and eluted with a shallow linear gradient of phosphate buffer (Figure 1). A separation of the two bioluminescent activities occurs under these conditions, and the material was divided at this stage to enrich separately for luciferase and light-inducible material. The two fractions were precipitated with ammonium sulfate and dissolved in a minimal quantity of buffer.

Additional purification of light-inducible protein was achieved by gel filtration using Sephadex G-100. Although this procedure does not resolve the two bioluminescent activities, it shows that the proteins are closely similar in molecular weight (about 80,000), and separates the active materials from contaminating proteins having lower and higher molecular weights. A protein-bound fluorescent species (emission λ_{max} 490 nm) whose quantity closely parallels the bioluminescence activities is evident at this stage (Figure 2).

Further purification was attempted by repeated chromatography on DEAE. From each successive column the fractions having light-induced bioluminescence activity were selected and rechromatographed. The results (Table I) make it evident that additional purification was not achieved subsequent to

TABLE I: Specific Activities of Light-Induced Protein and Luciferase during Purification of Inducible Protein.

Description of Stage of Purifcn	quanta sec^{-1} per $A_{280\,nm}$ I_0-LIB[a]	I_0-$FMNH_2$	Ratio
After first $(NH_4)_2SO_4$ precipitation	3.0×10^8	3.0×10^{11}	1:1000
First DEAE	6.0×10^9	2.8×10^{12}	3.8:1000
	4.9×10^9	1.3×10^{12}	
After Sephadex	1.9×10^{10}	5.3×10^{12}	3.6:1000
After second DEAE	7.0×10^{10}	3.0×10^{12}	23:1000
After third DEAE	5.8×10^{10}	3.0×10^{12}	20:1000
After fourth DEAE	4.9×10^{10}	3.0×10^{12}	16:1000
After second DEAE	5.0×10^{10}	2.1×10^{12}	24:1000

[a] LIB = light-induced bioluminescence.

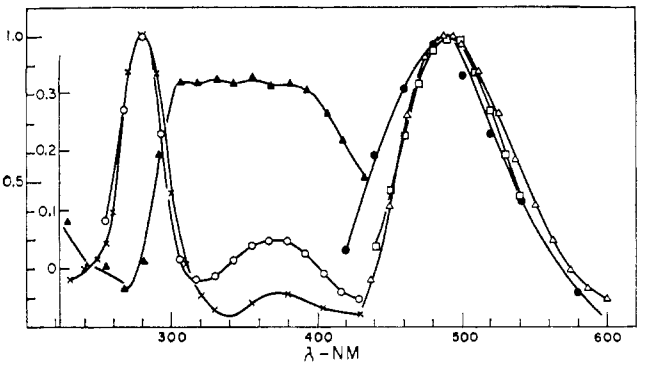

FIGURE 4: The spectral properties of light-inducible protein. The fluorescence excitation (○) and emission (△) spectra for light-inducible protein were determined in 0.05 M phosphate buffer (pH 7) at 25°. The action spectrum for light-induced bioluminescence (×) was performed as described in Materials and Methods. The light-induced bioluminescence emission spectrum (●) was determined using a Bausch & Lomb high-intensity monochromator fitted to record the ratio of the light intensity at the entrance slit to the intensity at each particle wavelength (Mitchell and Hastings, 1969) and is presented uncorrected for monochromator efficiency and phototube sensitivity *vs.* wavelength. The emission spectrum for flavin-induced bioluminescence (□) was taken from an earlier publication (Mitchell and Hastings, 1969) and is also uncorrected. All curves are normalized to a value of 1 (outer scale) their respective wavelengths of maximum intensity. The fluorescence polarization (inner scale on ordinate) of light-inducible protein is plotted (▲) as a function of exciting wavelength.

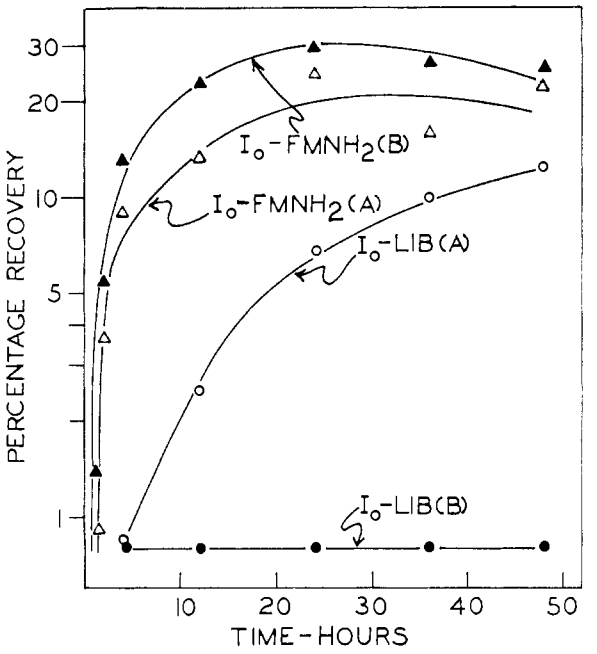

FIGURE 5: The kinetics of recovery of light-induced bioluminescence and luciferase activities following denaturation in Gd·HCl. A 1.5-ml sample containing 100 mg of light-inducible protein and luciferase was denatured by dialysis against 5 M Gd·HCl. This sample (1.0 ml) was applied at room temperature to a Sephadex G-50 column (1 × 25 cm) in 5 M Gd·HCl and the column washed with 5 M Gd·HCl to elute the protein. The remaining portion (0.5 ml) was diluted with 5 M Gd·HCl to an absorbance at 280 nm equal to the pooled protein from the Sephadex column. A 1.0-ml portion of each preparation was then diluted 1 to 100 to lower the guanidine concentration (Friedland and Hastings, 1967a), and the reappearance of luciferase and light-induced bioluminescence activities followed with time. The curves labeled A represent the recovery of activities for the untreated protein; those labeled B correspond to the Sephadex-treated material. Recovery is expressed as the percentage of the activity which the protein possessed before denaturation. The renaturation was carried out at 4°. No activity could be detected for the light-induced bioluminescence (B) sample. It is plotted simply to indicate that determinations were made and that the activity, if any, was below the detection limit of the apparatus.

the second DEAE column, where a ratio of specific activities of about 25–1000 was obtained. The activities quoted in Table I for the $FMNH_2$-initiated bioluminescence are those for the fractions having maximum light-inducible bioluminescence. Maximum specific activities for the $FMNH_2$-initiated bioluminescence (*e.g.*, Figure 3, where it is 2.3×10^{13} quanta sec^{-1} mg^{-1} for tube 118) indicate that this luciferase has a purity of about 75%.

On the assumption that the best fractions of light-inducible protein have a purity of 50%, pure material would have a specific activity of about 1.5×10^{11} quanta sec^{-1} mg^{-1}. The ratio of light-induced bioluminescence to flavin-initiated activities in crude cell lysate is 1:1000; one can thus calculate that there may be as much as 0.25 mg of light-inducible protein for every mg of luciferase. Luciferase constitutes about 4% of the total soluble protein of the cell (Hastings *et al.*, 1965a). The light-inducible protein would thus be about 1%. Given such a large amount of light-inducible protein, it is difficult to ignore its presence.

The protein-bound fluorescence noted in Figure 2 migrates on DEAE with the light-inducible protein (Figure 3), and is present in quantities almost exactly in proportion to the light-induced bioluminescence activity. The fluorescence emission is excited at 280 and 375 nm and emits maximally at 490 nm (Figure 4). Since this fluorescent emission corresponds closely to the light-induced bioluminescence emission spectrum it is possible that the chromophore responsible for the fluorescence may be involved in the bioluminescence. The 490-nm chromophore is also implicated in the process of light-induced luminescence, because the action spectrum for light-induced bioluminescence corresponds closely to the fluorescence excitation spectrum of the chromophore (Figure 4).

If the chromophore is firmly attached to protein then one would expect the fluorescence to show some degree of polarization. A plot of polarization *vs.* wavelength of excitation (Figure 4) shows that the fluorescence is indeed highly polarized when excited at the 375-nm transition but that the 280-nm transition is essentially depolarized, indicating that there is energy transfer to the chromophore from another molecule which absorbs at 280 nm. An emission polarization of 30% for the 375-nm transition is consistent with values found for other chromophores attached to proteins of a molecular weight in the range of 80,000 (Weber, 1953).

Some earlier observations are of interest in connection with the possible role of this chromophore in bioluminescence. It has been known for some time that luciferase will give some light with reductants other than $FMNH_2$, not only flavins such as reduced FAD or riboflavin, but also others such as neutral red, or even dithionite (Mitchell and Hastings, 1969). Although it seemed clear that this activity should be attributed to the mediation of some contaminating protein-bound pigment (Mitchell and Hastings, 1969), these experi-

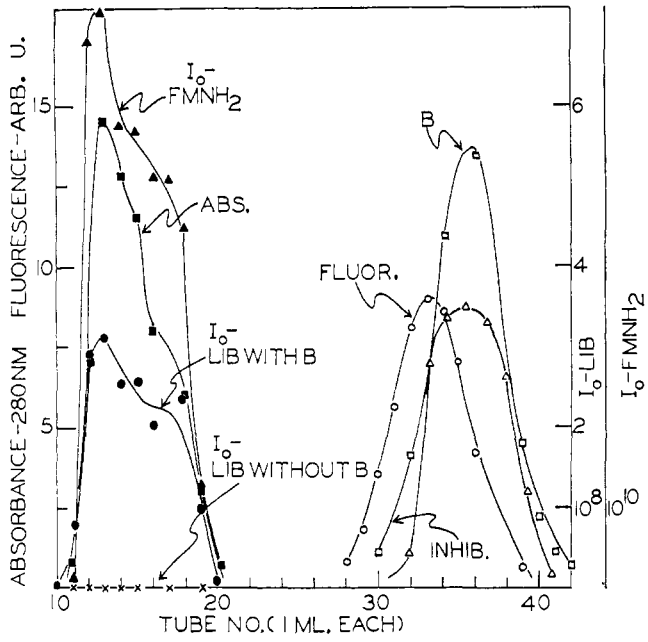

FIGURE 6: Sephadex G-50 chromatography of light-inducible protein in the presence of 5 M Gd·HCl. After elution, the luciferase and light-inducible protein of individual tubes were renatured by dilution; the activity of the latter was determined by adding a 0.05-ml sample of the B material to aliquots of the tubes and assaying for light-induced bioluminescence activity. The inhibitory capacity (on flavin-initiated luciferase) of the material in the tubes was determined by assaying samples of luciferase (*e.g.*, tube 12) for flavin-induced activity with and without a 0.01-ml aliquot from each of the tubes indicated. Fluorescence is presented as the relative intensity of emission at 525 nm, excited at 380 nm.

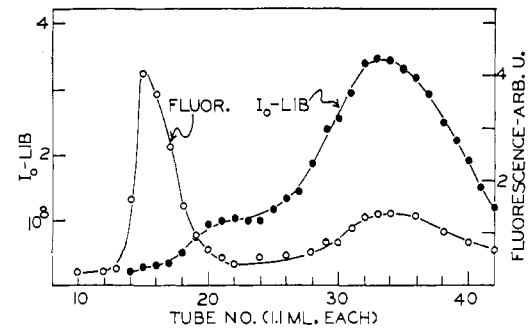

FIGURE 7: Florisil chromatography of the flavin-like material and B activity. Guanidine-denatured light-inducible protein and luciferase (150 mg) prepared by dialysis as in Figure 5 were applied to a 1 × 12 cm column of Florisil equilibrated with 5 M Gd·HCl. Both the fluorescent material and the B activity were absorbed to the column while the protein was washed through. Gd·HCl was then removed with a wash using 100 ml of water, and the column developed with a linear water–pyridine gradient (100 ml of water–100 ml of 30% pyridine in water). Fluorescence and B activity (I_0 − LIB) were assayed in the same manner as in Figure 6.

ments indicate that it cannot be identical with the 490-nm pigment. The experiment of Figure 3 shows, first, that while the 490-nm pigment migrates with the light-induced bioluminescence activity, the luciferase activity obtained with alternate reductants parallels instead the flavin-inducible bioluminescence. This result indicates that the activity of luciferase with nonflavin reductants cannot be attributed to the major contaminating 490-nm pigment. This result also makes it unlikely that the mechanism of light-induced bioluminescence involves a photochemical reduction of the fluorescent pigment.

Although there appear to be two distinct protein species associated with the two kinds of bioluminescence, the purification procedures described have not resulted in a complete separation of the two activities using extracts from normal bacteria. It has been possible, however, to obtain luciferase preparations lacking or nearly lacking light-inducible bioluminescence. One method involves the removal of a low molecular weight species, which we have arbitrarily designated as B. The recombination of the "stripped" protein with B restores activity.

Reversible denaturation and subunit dissociation of luciferase occur readily in 8 M urea or 5 M Gd·HCl (Friedland and Hastings, 1967a,b). A protein preparation containing both luciferase and light-inducible protein was treated with 5 M Gd·HCl and renatured by dilution. The activities of both light- and $FMNH_2$-initiated bioluminescence were restored slowly (Figure 5). But in the same experiment no light-induced bioluminescence activity could be regained with a sample of the protein which prior to renaturation was chromatographed on Sephadex G-50 (in 5 M Gd·HCl) to remove the low molecular weight component. We have thus concluded that light-induced bioluminescence activity requires the presence of a noncovalently bound small molecule which can be removed when the protein is unfolded in 5 M Gd·HCl.

The existence of this small molecule and its reactivity with renatured protein were demonstrated by Sephadex–Gd·HCl chromatography of a sample of protein containing both luciferase and light-inducible protein (Figure 6). Protein whose luciferase activity was recoverable by renaturation eluted in the void volume. This material lacked light-induced bioluminescence activity, but samples from later tubes added to it either before or after its renaturation resulted in the recovery of light-induced bioluminescence activity. This species corresponds to the low molecular weight material which was referred to above and designated as B. In addition to the B activity, fluorescence analysis of the tubes revealed that a flavin-like material (λ_{max} (emission) 525 nm; λ_{max} (excite) 280, 380, and 450 nm) was also released from the denatured protein. This was further purified by chromatography on Florisil, which resolved the flavin-like fluorescence into two components, one of which cochromatographed with B activity (Figure 7). The fluorescence excitation and emission spectra of both were the same, within error. Neither has been chemically identified, but FMN has no B activity. In addition, riboflavin, lumiflavin, lumichrome, and 6,7-dimethyl-9-formylmethylisoalloxazine were assayed for B activity and found to be inactive.

It is possible that B is identical with the 490-nm chromophore associated with native light-inducible protein. However, when light-inducible protein was denatured in Gd·HCl, the 490-nm fluorescence was completely quenched and did not reappear upon renaturation and recovery of light-induced bioluminescence. Likewise, the addition of B to "stripped" protein did not result in the reappearance of the 490-nm fluorescence. Resolution of this problem must await further work.

The identity of the protein component associated with

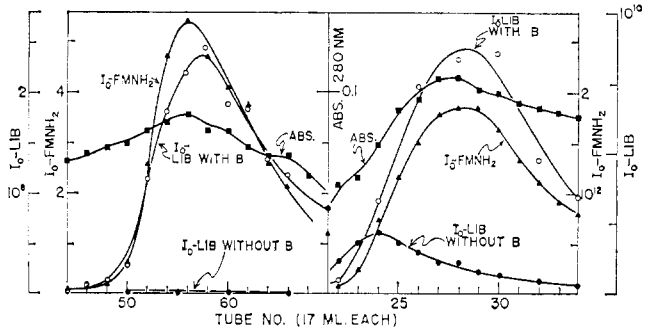

FIGURE 8: DEAE-cellulose chromatography. (A) Of light-inducible protein after removal of B and renaturation. Light-inducible protein was treated as in the experiment of Figure 6 and then applied to a 2.5 × 25 cm column of DEAE-cellulose. The column was developed with a shallow linear gradient of increasing phosphate buffer concentration as in Figure 1. Luciferase activity (I_0-FMNH$_2$) was measured in the normal manner, bioluminescence (I_0-LIB) activity was measured with and without added B. Luciferase activity was inhibited by the addition of B (see Figures 6 and 9). (B) Of a luciferase preparation possessing low light-induced bioluminescence. The luciferase-rich fractions from a preparative DEAE column (see text) were pooled, desalted by chromatography on Sephadex G-100, and applied to a 2.5 × 25 cm DEAE-cellulose column. The activity was eluted with a gradient identical with that used for part A. A clear separation of the residual bioluminescence (I_0-LIB) and the luciferase (I_0-FMNH$_2$) activities occurred. A sample of each fraction was then incubated with B and reassayed for LIB. The species of the protein which would accept B and become light inducible co-chromatographed with luciferase.

light-induced bioluminescence has been difficult to study because of the presence of relatively large quantities of authentic luciferase. However, there is considerable evidence to support the hypothesis that the combination of luciferase with B forms light-inducible protein, which thereby reversibly modifies both the charge and activity of the luciferase.

"Stripped" protein was prepared using Sephadex–Gd·HCl chromatography as in Figure 6 and was renatured to restore luciferase activity. This material was then applied to a DEAE column and eluted with a shallow linear phosphate gradient as in Figure 1. The species of protein which would accept B

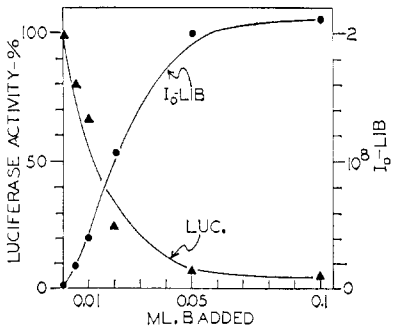

FIGURE 9: The reaction of B with protein to form light-induced bioluminescence activity results in a concomitant loss of the flavin-initiated luciferase activity. B was prepared by Florisil chromatography as in Figure 7. The pyridine was removed by extraction with chloroform, B remaining in the aqueous layer. Luciferase lacking light-induced bioluminescence activity was titrated with B and the material assayed for the two activities after each successive addition.

TABLE II: Effect of PMB upon Luciferase and Light-Induced Bioluminescence Activities (in Arbitrary Units).

Material and Treatment	I_0-LIB[a]	I_0-FMNH
1. Purified light-inducible protein only	0.33	26
2. Purified light-inducible protein + 5 × 10^{-5} M PMB	0.27	0.54
3. Luciferase (1–5a) only	0.04	
4. Luciferase + "B"	1.3	
5. Luciferase + 10^{-4} M PMB, then add B	0.05	
6. Luciferase + B, then add PMB	0.6	

[a] See footnote to Table I.

and become light-inducible cochromatographed with luciferase (Figure 8a). This result could be demonstrated equally well with native luciferase. The late fractions of a DEAE preparative column (*e.g.*, tubes 150–160 in Figure 1) contain relatively large amounts of luciferase but little light-inducible protein. These fractions were combined, and after chromatography on Sephadex G-100, were subjected to a second DEAE step. Separation of the residual light-inducible protein and luciferase occurred normally (Figure 8b). A large stimulation of light-induced bioluminescence was obtained by the addition of B and the stimulation closely paralleled the luciferase activity.

When B was added to stripped protein prior to the DEAE chromatography, the resultant light-inducible protein, like "native" light-inducible protein, migrated ahead of the luciferase. Thus luciferase is more negatively charged than light-inducible protein.

When B was added to a luciferase preparation, there occurred a loss of luciferase activity in parallel with the increase in light-induced bioluminescence activity (Figure 9). The effectiveness of B as an inhibitor in this regard also paralleled and cochromatographed with B activity (Figure 6).

Luciferase was found to be more susceptible to inactivation than was the light-inducible protein. MAV luciferase contains 15 free SH groups, most or all of which must be in the reduced state in order for the enzyme to be active. Reagents such as PMB, which combine with free SH groups, are potent inhibitors of luciferase. However, the light-induced bioluminescence activity is far less sensitive to PMB (Table II, lines 1 and 2). Moreover, when B-free luciferase was treated with PMB, it could not then be converted into light-inducible protein by the addition of B (Table II, lines 3–6). When B was added before PMB, the light-induced bioluminescence activity obtained was stable to PMB. These results implicate the sulfhydryl groups in the binding of B to luciferase.

It was found that a differential destruction of luciferase activity could be achieved in other ways. Incubation of luciferase at 41° resulted in a loss of 90% of the activity within 50 min, whereas only 20% of the light-induced bioluminescence activity was lost during that time. Precipitate formed by reaction with luciferase-specific antibody is

TABLE III: Purification of Luciferase and Light-Inducible Protein from the Aldehyde Mutant.

Stage of Purification	Sp. Act.-LIB[a]	Sp. Act.-$FMNH_2$	Ratio
A. No aldehyde added during growth of cells			
1. First $(NH_4)_2SO_4$		3.0×10^{11}	
2. Sephadex G-25	$<5.0 \times 10^5$	5.6×10^{11}	<0.001:1000
3. DEAE-cellulose	1.2×10^8	4.0×10^{12}	0.03:1000
B. Aldehyde added during growth			
1. First $(NH_4)_2SO_4$		3.5×10^{11}	
2. Sephadex G-25	3.0×10^8	5.7×10^{11}	0.53:1000
3. DEAE-cellulose	4.3×10^9	1.1×10^{12}	3.8:1000
C. Luciferase from line A-3 to which B was added followed by chromatography on DEAE-cellulose			
1. DEAE-cellulose	3.0×10^9	1.8×10^{12}	1.7:1000

[a] See footnote to Table I.

inactive with reduced flavin but was found to be active in the light-induced bioluminescence assay. Thus, protein in the soluble state does not appear to be required for the reactions involved in light-induced bioluminescence.

On the basis of the results obtained it was hypothesized that the light-inducible protein is a product-inhibited luciferase molecule. This theory predicts that a dark mutant whose lesion involves something other than the luciferase would contain normal quantities of luciferase but little or no light-inducible protein. Mutants fitting this requirement have been described and were available (Nealson, 1969), and the predicted result was indeed observed. Mutant 1–5a is a dark mutant which has nearly normal luciferase levels, but it remains unexpressed *in vivo:* only when long-chain fatty aldehyde is added to a cell suspension will the cells emit a luminescence. Moreover, the intensity of the light emission upon the addition of aldehyde is proportional to the luciferase content of the cells. Luciferase from these mutant cells was deficient in light-inducible protein.

This mutant permitted a further test of the hypothesis, for by simply adding aldehyde to the growing culture, the cells could be stimulated to emit bioluminescence throughout the period of growth. Extracts of such cells were then found to possess light-inducible protein (Table III).

To assure ourselves of the reality of the observation and to obtain quantitative data, the extracts from mutant cells grown in the presence and in the absence of added aldehyde were subjected a normal purification procedure. In Figure 10 the elution profiles of the DEAE chromatography step are presented, showing the virtual absence of light-induced bioluminescence activity in the enzyme preparation from the mutant grown without aldehyde (see also Table III). In the one caused to luminesce during growth by adding aldehyde, it is evident that the light-inducible protein formed had charge properties similar to that from the wild type cells, and is thus believed to be identical with the normal light-inducible protein.

Although it cannot be said for all aldehyde mutants, the luciferase of this mutant (1–5a) appeared identical with the wild-type enzyme. Its migration upon electrophoresis on acrylamide appeared the same. It gave a normal and quantitatively similar precipitation with antibody prepared with purified wild-type luciferase. Its ability to react with B and give light-induced bioluminescence activity was the same (Figure 10a). Furthermore, the light-inducible protein formed by the addition of B (isolated from wild-type cells) to this luciferase migrated on DEAE-cellulose in a manner similar to the migration of the light-inducible protein from wild-type cells (Table IIIc and Figure 11).

The hypothesis that the light-inducible protein is a product-inhibited luciferase molecule also predicts that it should be possible to form the light-inducible protein in the *in vitro* reaction. Several attempts to demonstrate this using pure luciferase, dodecanal, and $FMNH_2$ have been unsuccessful. This might be due to differences in the yield of product-inhibited enzyme *in vivo* and *in vitro*, as a result of the different conditions. Alternatively, the yield might be low in both cases, but the material accumulates only *in vivo* as a consequence of continued turnover. Still another uncertainty relates to the fact that neither the flavin nor the aldehyde which is actually utilized in the *in vivo* reaction has been positively identified. Thus, the reaction may not be exactly duplicated in our *in vitro* experiments.

Discussion

Although our first experiment showed that the protein which is responsible for the light-induced bioluminescence is not identical with luciferase, a close relationship between the two proteins has now been demonstrated. On the basis of these results it is hypothesized that light-inducible protein is a luciferase molecule possessing a small molecule (designated "B") strongly but noncovalently bound to it, thereby changing its physical (charge) and activity properties. Luciferase itself emits no bioluminescence upon irradiation but is highly active with reduced FMN as a substrate; light-inducible protein has less (or no) activity with reduced FMN but emits a characteristic bioluminescence following flash irradiation with ultraviolet light.

One is immediately concerned with understanding the nature of the reactions which lead to the formation of this curiously modified luciferase. The fact that it is apparently present in such large amounts (see Results; about 1% of the soluble protein of the cell) indicates that it does not arise

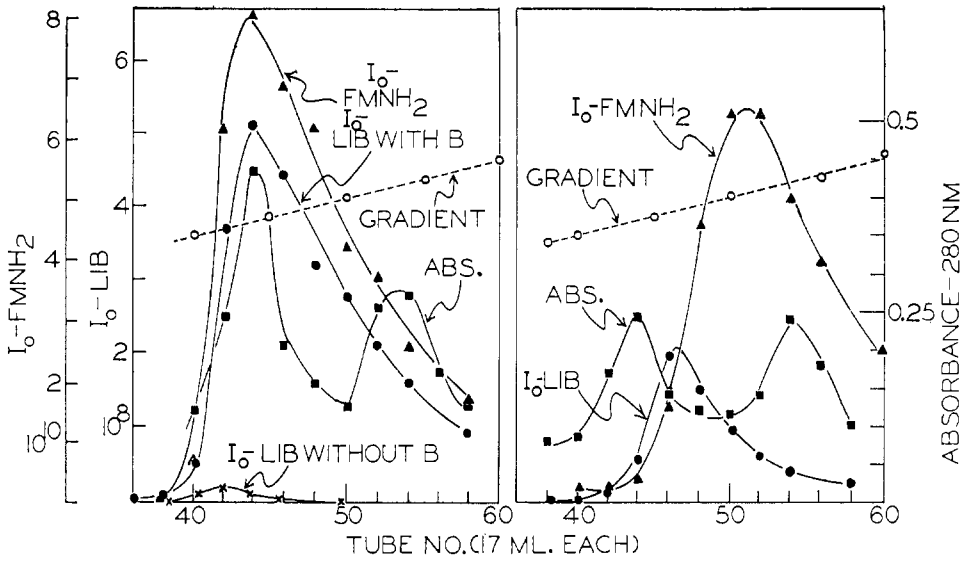

FIGURE 10: DEAE chromatography of partially purified extracts from aldehyde mutant cells (1-5a) which were grown in the absence (A) and in the presence (B) of added aldehyde. The elution profile for the extract of cells grown without added aldehyde shows that a negligible amount of light-inducible protein was formed and that protein (luciferase) could be converted into light-inducible protein by the addition of B. The cells to which aldehyde was added during growth produced a light-induced bioluminescence activity which migrated like the light-inducible protein from wild-type cells.

via some trivial side reaction. Dark luciferase-positive mutants, which grow and synthesize luciferase but emit very little light unless aldehyde is added, lack the light-inducible protein. This strongly suggests that the formation of light-inducible protein occurs as a consequence of the luminescent reaction. This idea was supported by the demonstration that light-inducible protein does occur in extracts from cells of the same mutant which were caused to emit luminescence during growth by adding aldehyde.

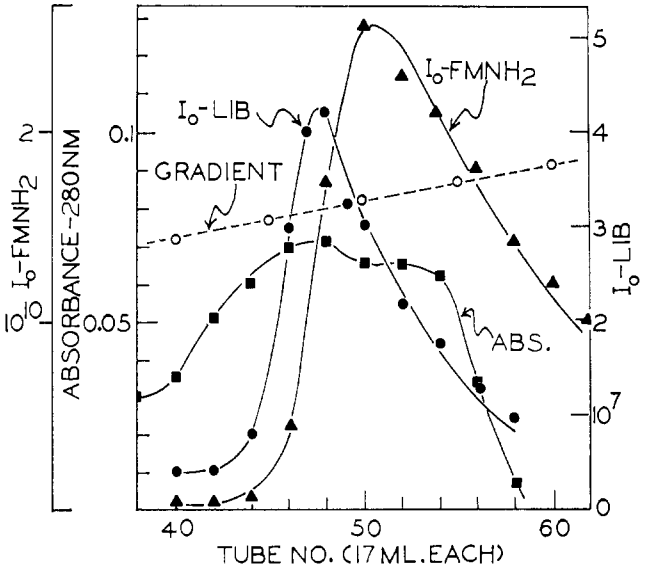

FIGURE 11: Chromatography of light-inducible protein formed by the reaction of B with luciferase. The luciferase was obtained from the dark (aldehyde) mutant, 1-5a, purified (Table IIIA), and treated with enough B to convert a substantial fraction of the luciferase into light-inducible protein. Upon subsequent chromatography (Table IIIC) both the luciferase and light-inducible protein migrated as do luciferase and light-inducible protein from wild-type cells.

Information concerning the exact structure of B would clearly be of value in further resolving this matter. Its flavin-like properties (spectral and chromatography on Florisil) lead us to suspect that B might be formed from the *in vivo* flavin, perhaps modified in the course of the bioluminescent reaction. Although FMN turnover has been demonstrated in the *in vitro* reaction (Cormier and Totter, 1957; Hastings, 1968) it is not known for sure that FMN itself is the *in vivo* bacterial flavin. Irrespective of that, the yield of B as a reaction product could be quite low so that flavin turnover could occur while at the same time an accumulation of a considerable quantity of the B-inhibited luciferase could occur.

Although the relationship between B and the 490-nm chromophore is not completely certain, we postulate that the chromophore is B and that its spectral properties change considerably upon removal from the luciferase. The binding (but not the spectral changes) is apparently reversible. Free B will bind to free luciferase to give light-inducible protein whose activity and chromatographic properties are indistinguishable from native light-inducible protein. However, we have not yet observed a restoration of the characteristic 490-nm fluorescent emission. The 530-nm flavin-like fluorescence of B persists after its recombination with luciferase; no quenching or color change occurs. Whether or not the color of the light-induced bioluminescence obtained from reconstituted light-inducible protein is the same as that of native light-inducible protein is of interest in this connection but it has not yet been determined.

The excited state in the bacterial bioluminescent reaction (λ_{max} 490 nm) has never been identified. Thus, the isolation of luciferase with a bound chromophore whose fluorescence closely corresponds spectrally to the bioluminescence is very interesting. The evidence that this material is a specific

product of the *in vivo* bioluminescent reaction, and that the chromophore has flavin-like properties, suggests that this is the emitter and that it is formed in the course of the bioluminescent reaction. We thus postulate that a chemiluminescent reaction mechanism explicitly involving the flavin molecule is involved. This is consistent with the observation that different flavins can have a marked effect on both the color and the lifetime of the bioluminescence, so that the flavin must remain complexed with luciferase through the complete process of light emission (Mitchell and Hastings, 1969). Finally, it will be recalled that pure active luciferase requires no noncovalently bound chromophore for its activity.

From these observations we conclude that authentic luciferase is indeed void of any bound chromophore necessary for flavin-initiated bioluminescence. It is well known, for example, that it exhibits no binding whatsoever with flavins, as judged by fluorescence polarization (Hastings *et al.*, 1965). Yet as shown in Figure 3, some light is obtained with alternate reductants such as neutral red or dithionite, and this activity is proportional to the luciferase and not to the modified luciferase possessing the bound (490-nm) chromophore. We have no idea what the emitter in the dithionite-stimulated reaction might be unless there are alternate low quantum yield pathways in which other species (*e.g.*, an aromatic amino acid) serve as the excited state. It seems scarcely possible to attribute the emission to an impurity. The intensity is very low and although the spectrum has not been accurately determined, it was found to emit maximally at about 475 nm (Mitchell and Hastings, 1969).

The experiments with alternate reductants also are of interest in connection with the photochemical mechanism involved in the light-induced bioluminescence. Photoreduction of B to form BH_2 was and in some ways still does appear to be an appropriate and logical mechanism. The reduced chromophore would presumably react *via* the pathway normally involved in the reduced FMN reaction, the major difference being that the oxidized B is initially bound to the active site of the luciferase. However, since the light-inducible protein is not stimulated to bioluminescence by either dithionite or neutral red, the mechanism involving simple photoreduction seems questionable. It is conceivable that B is bound in such a way as to make it inaccessible to reduction by means other than light.

The light-induced bioluminescent system is of interest and importance not only because it gives us insight concerning the mechanism of the normal chemically induced bioluminescence. It also provides a possible model system for the study of delayed light emission, such as that which occurs in green plants.

References

Birks, J. B., and Kuchela, K. N. (1961), *Proc. Phys. Soc. 77*, 1083.

Cormier, M. J., and Totter, J. R. (1957), *Biochim. Biophys. Acta 25*, 229.

Friedland, J. M., and Hastings, J. W. (1967a), *Biochemistry 6*, 2893.

Friedland, J. M., and Hastings, J. W. (1967b), *Proc. Natl. Acad. Sci. U. S. 58*, 2336.

Gibson, Q. H., Hastings, J. W., and Greenwood, C. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 187.

Hastings, J. W. (1968), *Annu. Rev. Biochem. 37*, 597.

Hastings, J. W., and Gibson, Q. H. (1963), *J. Biol. Chem. 238*, 2537.

Hastings, J. W., and Gibson, Q. H. (1967), *J. Biol. Chem. 242*, 720.

Hastings, J. W., Gibson, Q. H., Friedland, J., and Spudich, J. (1966), *in* Bioluminescence in Progress, Johnson, F. H., and Haneda, Y., Ed., Princeton, N. J., Princeton Univ. Press, p 151.

Hastings, J. W., Gibson, Q. H., and Greenwood, C. (1965b), *Photochem. Photobiol. 4*, 1227.

Hastings, J. W., Riley, W. H., and Massa, J. (1965a), *J. Biol. Chem. 240*, 1473.

Hastings, J. W., and Weber, G. (1963), *J. Opt. Soc. Am. 53*, 1410.

Hastings, J. W., Weber, K., Friedland, J., Eberhard, A., Mitchell, G. W., and Gunsalus, A. (1969), *Biochemistry 8*, 4681.

Mitchell, G. W., and Hastings, J. W. (1969), *J. Biol. Chem. 244*, 2572.

Nealson, K. H., (1969), Ph.D. Thesis, University of Chicago, Chicago, Ill.

Weber, G. (1953), *Advan. Protein Chem. 8*, 415.

Weber, G., and Teale, F. W. J. (1958), *Trans. Faraday Soc. 54*, 460.