Biochemistry

Volume 12, Number 10 May 8, 1973

Synthesis of a New Substrate Analog of Firefly Luciferin. An Active-Site Probe†

Lemuel J. Bowie,‡,* Vaclav Horak, and Marlene De Luca

ABSTRACT: The synthesis of a new substrate analog of firefly luciferin is reported. The compound, 2-(6′-hydroxy-2′-benzothiazolyl)-4-hydroxymethylthiazole, is an analog of dehydroluciferin, a competitive inhibitor of firefly luciferase. The spectral and enzymatic properties of this analog suggest that it should be an ideal active-site probe for firefly luciferase.

The reactions catalyzed by firefly luciferase are shown below. Reaction 1 is the formation of enzyme bound luciferyl adenylate. Reaction 2 is the oxidation of luciferyl adenylate by molecular oxygen. This gives rise to light emission, CO_2, and other products. Dehydroluciferin (L)[1] which is a competitive inhibitor of luciferase can also be activated to form dehydroluciferyl adenylate (reaction 3) but it cannot be oxidized and does not produce light.

$$E + LH_2 + ATP\text{-}Mg^{2+} \rightleftharpoons E\cdot LH_2\text{-}AMP + PP_i\text{-}Mg^{2+} \quad (1)$$

$$E\cdot LH_2\text{-}AMP + O_2 \longrightarrow E + AMP + CO_2 + \text{products} + h\bar{\nu} \quad (2)$$

$$E + L + ATP\text{-}Mg^{2+} \rightleftharpoons E\cdot L\text{-}AMP + PP_i\text{-}Mg^{2+} \quad (3)$$

Dehydroluciferin is a fluorescent molecule and its emission spectrum is dependent both on the solvent composition and the pH of the media (Morton *et al.*, 1969). There is a large change in the emission spectrum of L when it is bound to luciferase. This change in fluorescence appears to be due to the fact that L is bound in a very hydrophobic environment (Denburg *et al.*, 1969). However, when L reacts with ATP to form the adenylate the fluorescent intensity of the enzyme-bound product is very low, about 5% that of free L. It was desirable to have an anlog of L which would not react with ATP to form an adenylate so that the effect of ATP on the binding of the analog could be studied. We have synthesized the analog of dehydroluciferin, 2-(6′-hydroxy-2′-benzothiazolyl)hydroxymethylthiazole, in which the carboxyl function has been converted to a primary alcohol. This alcohol analog of dehydroluciferin has been given the trivial name dehydroluciferol (L-OH). The structure of dehydroluciferol, dehydroluciferin, and luciferin are shown in Figure 1.

Materials and Methods

Reagents. Dihydropyran was obtained from Eastman Organic Chemicals and was distilled prior to each use. $LiAlH_4$ was obtained as a 4.1 M solution in ether from Alfa Inorganics (Ventron). $NaAlH_2(OCH_2CH_2OCH_3)_2$ was obtained as a 70% benzene solution from Eastman Organic Chemicals. [^{32}P]PP_i was purchased from ICN Chemical and Radioisotopes Division and ATP was obtained from P-L Biochemicals Inc. All other chemicals were of reagent grade quality.

Experimental Methods. Firefly luciferase was prepared as described by Green and McElroy (1956) and recrystallized four to five times to a constant, maximal specific activity of 4–5 × 10^5 light units/mg of protein. Protein concentrations were determined by measuring the absorbance at 278 nm, where 1 mg/ml of luciferase gives an absorbance of 0.75 in a 1-cm path-length cell.

Luciferase enzymatic activity in the overall bioluminescent reaction (eq 1 and 2) was assayed by monitoring the height of the initial flash of light emitted resulting from the rapid

† From the Department of Biochemistry, Georgetown University Schools of Medicine and Dentistry, and the Department of Chemistry, Georgetown University, Washington, D. C. *Received December 27, 1972.* The research was supported in part by the U. S. Public Health Service and the National Science Foundation.

‡ Recipient of National Institutes of Health Training Grant GM-57, John Hopkins University.

* To whom to address correspondence at the Veterans Administration Hospital, San Diego, Calif. 92161.

[1] Abbreviations used are: LH_2, luciferin; L, dehydroluciferin; LH_2-AMP, luciferyl adenylate; L-AMP, dehydroluciferyl adenylate; L-OEt, dehydroluciferyl ethyl ester; T-L-OEt, tetrahydrapyranyldehydroluciferyl ethyl ester; T-L-OH, tetrahydropyranyldehydroluciferol; L-OH, dehydroluciferol.

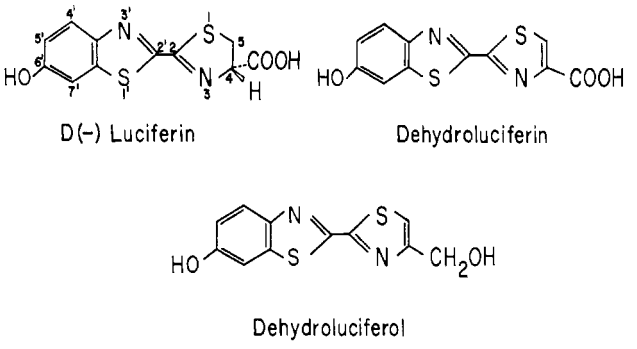

FIGURE 1: Structures of luciferin, dehydroluciferin, and dehydroluciferol.

injection of excess ATP into a solution of luciferase and excess LH_2 (McElroy and Seliger, 1961). The normal assay mixture contained 2.0 ml of 0.025 M glycylglycine buffer (pH 7.8), 0.1 ml of 0.1 M $MgSO_4$, 0.1 ml of 9×10^{-4} M LH_2, and 2–10 μg of luciferase. The reaction was then initiated by the rapid injection of 0.2 ml of 0.02 M ATP (pH 7.0–7.2). The height of the initial flash was taken as a measure of the initial velocity and expressed in arbitrary light intensity units.

The enzyme-catalyzed activation of L (eq 3) in the presence of ATP was measured by the ATP–PP_i exchange reaction. The assay is performed by measuring the incorporation of [^{32}P]PP_i into ATP in the presence of luciferase and L. The normal assay mixture contained 0.8 ml of 0.025 M glycylglycine buffer (pH 7.8), 0.1 ml of 0.025 M ATP, 0.05 ml of 0.1 M $MgSO_4$, 0.1 ml of approximately 8×10^{-5} M L or L-OH, and 0.05 ml of [^{32}P]PP_i in 0.04 M PP_i, The reaction is initiated by the addition of 2.05 μg of luciferase and allowed to proceed 15 min at 37°. It is then quenched by the addition of 7% perchloric acid containing 0.2 M PP_i. One-half milliliter of acid washed Norit A (30 mg/ml) was added to adsorb the nucleotide, and the entire mixture was washed on Millipore filters (5 μ), dried, and counted on a Nuclear-Chicago gas-flow counter.

All assays of luciferase activity were performed under conditions where the initial velocities increased linearly with enzyme concentration. Inhibition studies were performed in 0.025 M glycylglycine buffer (pH 7.8). Under these conditions the association constant for the Mg–ATP complex is 20,000. The concentrations of Mg–ATP complex (the active substrate) were, therefore, calculated according to the mass law equation (eq 4), where $[Mg_T]$ and $[ATP_T]$ are the total

$$[\text{Mg–ATP}] = \frac{\left[\left([Mg_T] + [ATP_T] + \frac{1}{K_a}\right) - \sqrt{\left([Mg_T] + [ATP_T] + \frac{1}{K_a}\right)^2 - 4[Mg_T][ATP_T]}\right]}{2} \quad (4)$$

concentration of Mg and ATP, respectively, and K_a is the association constant for the Mg–ATP complex.

Crystalline D(−)-luciferin and dehydroluciferin were synthesized according to the method of Seto *et al.* (1963) by Dr. Peter Plant and Dr. George Fields, respectively. The concentrations of L, LH_2, and ATP were determined by measuring optical densities at their respective absorption maxima at neutral pH using the following extinction coefficients: L:ϵ_{353} (20,600), LH_2:ϵ_{327} (18,000), and ATP:ϵ_{260} (15,400). The concentration of L-OH was determined using an extinction coefficient at 353 of 20,600 cm^{-1} M^{-1}, the value for dehydroluciferin at this wavelength.

Fluorescent titrations to determine L-OH binding parameters were done on a Turner Model 111 fluorometer. Exciting light was passed through a Turner 110-811 (7-60) narrow pass filter which peaks at 360 nm. Emitted light was measured after passage through a Turner 110-812 (405) narrow pass filter which peaks at 405 nm. In order to insure against inner filter effects under the experimental conditions, all emissions were corrected for self-absorption of the exciting light by the method of McClure and Edelman (1967) using

$$I_{cor} = I_{obsd} 2.303 E_\lambda C_T / (1 - 10^{-E_\lambda C_T})$$

I_{cor} and I_{obsd} are the corrected and observed fluorescence intensities respectively, E_λ is the extinction coefficient of the chromophore at the exciting wavelength, and C_T is the total chromophore concentration. The titrations were done in 0.05 M phosphate buffer (pH 7.5) at 10°.

All steady-state fluorescence excitation and emission spectra were obtained on a Perkin-Elmer MPF-2A (Hitachi) fluorescence spectrophotometer using a constant-temperature jacketed cell holder. The spectra were obtained as quanta per unit wavelength and replotted as quanta per unit wave number (Seliger and McElroy, 1966) to allow direct comparison of bandwidths and peak areas.

Ultraviolet absorption spectra were obtained on a Bausch and Lomb (Model 505) spectrophotometer and a Beckman ACTA V.

Analytical Methods. All elemental analyses were performed by Geller Microanalytical Laboratories, Saddle River, N. J.

Infrared (ir) spectra were measured on a Perkin-Elmer (Model 337) spectrophotometer as solid samples in KBr pellets and nuclear magnetic resonance (nmr) spectra were obtained on a Varian 60 MHz spectrometer, peak positions being given in τ (τ = 10 ppm) downfield from Me_4Si as the internal standard.

The purity of luciferin and dehydroluciferin was checked by ascending paper chromatography using a mixture of 95% ethanol–1 M aqueous ammonium acetate (7:3, v/v), adjusted to pH 7.5, and by thin-layer chromatography (tlc) on silica gel using commerical plates with fluorescent indicator ("Baker-flex," silica gel IB-F) using solvent system II below. Dehydroluciferin showed no detectable contaminants and luciferin showed only minor traces of two other fluorescent spots on the paper chromatograms which had no effect on its activity in the luciferase bioluminescent reaction. Progress of reactions and purity of nonpolar compounds were monitored by tlc on silica gel (same as above) visualizing with both long and short ultraviolet excitation using two solvent systems for development: system I, chloroform–ethyl acetate (5:1, v/v; system II, ethyl acetate–methanol (10:1, v/v). Melting points (uncorrected) were taken in open capillaries with a Thomas Hoover capillary melting apparatus.

Chemical ionization mass spectrometry (Munson and Field, 1966) proved very useful in confirming the structures of the final products (III and IV) whose physical properties greatly hampered confirmation by other methods. It should be noted

that the spectra produced using this technique are frequently different from those produced by conventional electron impact methods. Secondary ions of the reaction gas either donate a proton or abstract a hydride ion when they collide with the sample molecules giving rise to "quasimolecular" (QM^+) ions at *m/e* $(M + 1)^+$ or ions at *m/e* $(M - 1)^+$. Therefore, in the case of the spectra reported here, note that the relative abundances of the QM^+ ions are much higher than those of the M^+ conventional molecular ions. All chemical ionization mass spectra were done by Dr. George Milne at the National Institutes of Health.

Procedures. DEHYDROLUCIFERYL ETHYL ESTER (L-Et; I). Dehydroluciferin (500 mg, free acid, 1.80 mmol) was added to 100 ml of absolute ethanol and 75 drops of concentrated H_2SO_4 and the mixture was refluxed 3.5 hr and allowed to react overnight at room temperature after which the reaction was only 70% complete (by tlc). The solution was then refluxed an additional 8 hr and the large needle-like crystals were collected after sitting overnight (379 mg, 1.23 mmol). The filtrate was then neutralized with $NaHCO_3$, filtered again, and carefully evaporated to dryness under reduced pressure and gentle heating. The crude product was then extracted with ether until no additional blue fluorescence extracted (tlc indicates pure product). Ether was removed yielding an additional 26 mg, 0.09 mmol, of product I. An analytical sample was prepared by recrystallizing from ethanol: mp 255–256°; R_F (solvent system I), 0.33; uv spectra (CH_3OH), ϵ_{353} (21,600), ϵ_{277} (8500); ir spectra 3410, 2940 (weak), 1725, 1020 cm^{-1}; chemical ionization mass spectra *m/e* 306 (molecular ion, M^+), 14%; *m/e* 307 (QM^+), 55.57%; *m/e* 308, 12%; *m/e* 309, 7%; *m/e* 279 ($QM^+ - 28$, loss of ethyl group), 2%. *Anal.* Calcd for $C_{13}H_{10}N_2O_3S_2$: C, 50.97; H, 3.27; N, 9.15. Found: C, 50.74, H, 3.26; N, 8.93.

TETRAHYDRAPYRANYLDEHYDROLUCIFERYL ETHYL ESTER (T-L-OEt; II). Dihydropyran (50 ml; freshly distilled and stored in dark until used) was deaerated with nitrogen and placed in an ice bath. Product I (500 mg; 1.3 mmol) was added and the solution was stirred while continuously bubbling nitrogen. Dissolution of L-OEt took approximately 30 min after which *p*-toluenesulfonic acid (10 mg, oven dried, 0.058 mmol) was added. The ice bath was then removed and the reaction was allowed to proceed 5 hr at room temperature with continuous nitrogen bubbling. The flask was then stoppered, covered with aluminum foil, and stored overnight at 4°. Subsequently, stirring at room temperature for an additional 6 hr showed no further progress of the reaction (by tlc). The solution was then concentrated to 10 ml at 30–35° under vacuum followed by the addition of 50 ml of ethyl ether. The organic layer was then extracted twice with a total volume of 25 ml of 10% NaOH and washed twice with cold water (total volume 25 ml). This procedure yielded 400 mg (1.03 mmol) of about 90% pure tetrahydropyranyl derivative of L ester (product II) which was isolated as a crude product from ether. Traces of product I were removed by dissolving the crude product in dry ether, applying it to a silica gel column and eluting with ethyl acetate. Removal of the solvent yielded product II: mp 136.5–137°, R_F (solvent system I) 0.63; uv spectra (CH_3OH), ϵ_{348} (22,200), ϵ_{273} (9100); ir spectra 2940 (strong), 1725, 1020 cm^{-1}; chemical ionization mass spectra *m/e* 390 (M^+), 9%; *m/e* 391 (QM^+), 45%; *m/e* 392, 13.5%; *m/e* 393, 7%; *m/e* 306 ($M^+ - 84$, loss of tetrahydrapyranyl group), 4%; *m/e* 307 ($QM^+ - 84$), 5%.

TETRAHYDRAPYRANYLDEHYDROLUCIFEROL (T-L-OH; III). Method 1. Product II (400 mg; 1.03 mmol) was dissolved in 10 ml of dry ethyl ether. $LiAlH_4$ (0.5 ml; 2.05 mmol) in ether was added dropwise with stirring followed by an addition of 15 ml of ether. The reaction was complete in 10 min according to the tlc. After addition of another 5 ml of ether the residual reagent was destroyed by the addition of 5 ml of ethyl acetate. The reaction mixture was then extracted three times with a total volume of 25 ml of cold 30% NaOH and the combined NaOH extracts were reextracted with ethyl ether until no additional fluorescent material was extracted. The ether extracts were then combined, concentrated by evaporation, and allowed to sit overnight at 4°. Treatment of this solution with freshly distilled petroleum ether (bp 80°) by dropwise addition yielded only a yellow syrup. This product which could not be obtained pure through crystallization was reprecipitated from $CHCl_3$ with petroleum ether yielding product III of about 95% purity (yield 10–20%).

Method 2. Product II (400 mg; 1.03 mmol) was dissolved in 25 ml of benzene and the mixture was then placed in an ice bath and deaerated with nitrogen for 30 min. A total of 6.6 ml (33 mmol) of $NaAlH_2(OCH_2CH_2OCH_3)_2$ was added with stirring to give complete conversion of the starting material of the corresponding product. The addition of reducing agent was accompanied by a transient formation of a red color which in later phases of the reaction persisted for longer periods of time and finally became permanent with excess of the reagent. Excess hydride was then destroyed by the cautious addition of 10 ml of cold water. The reaction mixture was extracted four times with a total of 40 ml of 10% NaOH. Residual product III in the NaOH layer was retrieved by seven extractions with a total of 150 ml of benzene or until no additional fluorescent products were extracted. Attempts at crystallization were unsuccessful and product III was obtained by precipitation with petroleum ether in the form of a syrup (approximately 300 mg): R_F (solvent system I), 0.13; uv spectra max (CH_3OH) 353 nm, 274 nm; ir spectra 3450, 2940 (strong), 1020 cm^{-1}; chemical ionization mass spectra *m/e* 348 (M^+), 5%; *m/e* 349 (QM^+), 46%; *m/e* 350, 9%; *m/e* 351, 5.5%; *m/e* 264 ($M^+ - 84$, loss of tetrahydrapyranyl group), 3%; *m/e* 265 ($QM^+ - 84$), 2.5%.

DEHYDROLUCIFEROL (L-OH; IV). Since it was desirable to use product III for the preparation of other derivatives of the primary alcohol group by reactions requiring a protected phenolic hydroxyl, product IV was prepared, as needed, by hydrolysis of small aliquots of product III (5–25 mg). The hydrolysis was performed in 0.1–2 N HCl, MeOH–HCl, and in dioxane–HCl (1 N) at room temperature and, in all cases, the reaction was complete in less than 5 min (by tlc).

In a typical hydrolysis, 21.4 mg of product III was added to 0.5 ml of 2 N HCl. Monitoring the reaction by tlc showed the reaction to be complete in less than 2 min. One-half milliliter of H_2O was then added and the solution was then extracted four times with 2 ml of ethyl acetate. The ethyl acetate extracts were combined and dried with anhydrous $MgSO_4$. The ethyl acetate layer was carefully removed and evaporated in a stream of nitrogen in a carefully weighed tube. The residue was then dried at 77° under vacuum in a drying tube containing phosphorus pentoxide for 3 hr. The tube was again weighed and the weight of the product was determined: yield 10.1 mg, 47%; R_F (solvent system I) 0.05; uv spectra max (CH_3OH) 353 nm, 274 nm; ir spectra 3200 cm^{-1} (very broad), 1020 cm^{-1}; chemical ionization mass spectra *m/e* 264 (M^+), 9%; *m/e* (QM^+) 265, 61%; *m/e* 266, 12%; *m/e* 267, 7.3%; *m/e* 247 ($QM^+ - 18$, loss of H_2O), 2.5%.

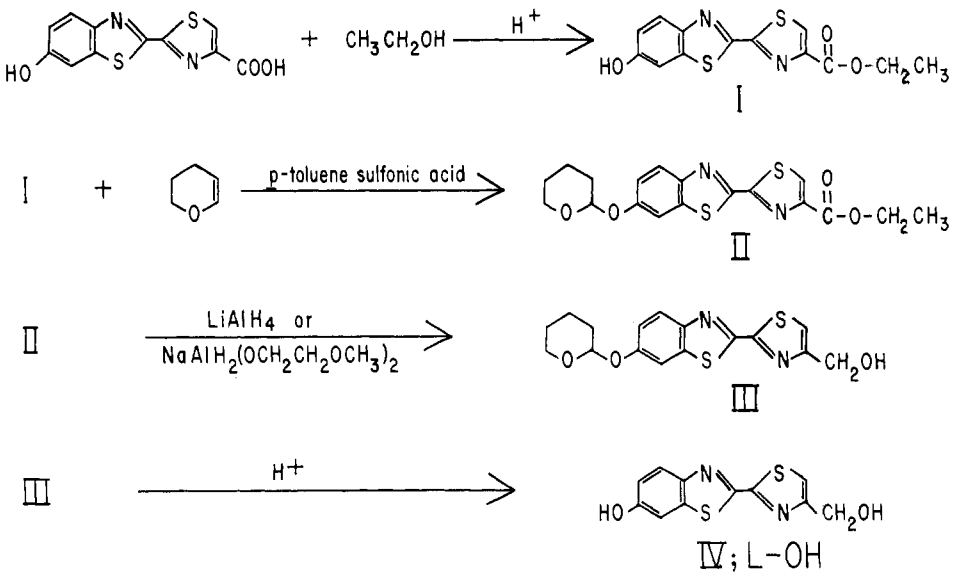

FIGURE 2: Reaction sequence for the synthesis of dehydroluciferol.

Results and Discussion

Synthesis of dehydroluciferol (L-OH) was accomplished by the sequence of reactions shown in Figure 2.

In addition to the main step of the reaction scheme, the reduction, there were included additional steps involving modification of the starting molecule. The esterification of the carboxylic group and blocking of the phenolic OH group not only make the reduction more economic but also facilitates this step by changing the polarity of the dehydroluciferin molecule which is highly insoluble in nonpolar solvents. The last step of the scheme represents removal of the tetrahydropyranyl blocking group. All steps were accomplished using well-established reaction procedures which generally give high yields of a single reaction product. The hydride reduction was accomplished with two different reagents. Also the hydrolysis of III was accomplished under various conditions (see Procedures).

Each step of the reaction scheme was followed by means of chromatographic and spectroscopic methods. These not only characterized the progress of the particular reaction, but also indicated the uniformity of the product and its structural characteristics. Reaction products from individual reaction steps were purified to the degree of purity necessary for its use in the next step with the main aim of minimizing losses of material. For the same reason, the only product which was purified to the highest degree and for which the elemental analysis was undertaken was the ester I, while the structural identity of the further reaction intermediates and also the reaction products were deduced from unambiguous results of uv, ir, nmr, and mass spectra along with R_F changes from tlc.

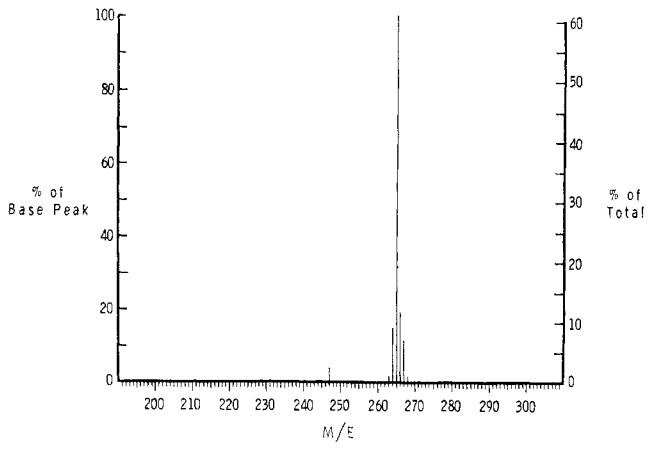

FIGURE 3: Chemical ionization mass spectrum of dehydroluciferol.

The esterification of dehydroluciferin proceeded smoothly in acidified absolute ethanol to yield I and subsequent reaction of this ester in dihydropyran (under nitrogen) gave the corresponding tetrahydropyranyl derivative (II). Reduction of II with $LiAlH_4$ was rapid; however, the yield was somewhat low. Therefore, the reduction was also tried with a new, less vigorous, more selective reductant, $NaAlH_2(OCH_2CH_2OCH_3)_2$ in benzene, which gave III in reasonable yield. Product III was easily hydrolyzed with acid under a variety of conditions to give IV. Nmr spectroscopy was very useful for the characterization of the structure of the reduced product (III). The spectrum of III in Me_2SO-d_6 showed aromatic absorptions in the region from τ 1.85 to 2.4, quite similar to what had been observed for LH_2 (White *et al.*, 1965). A broad absorption (three protons) was observed at τ 5.25 which was attributed to the overlapping absorptions of the primary alcohol methylene protons and the acetal proton of the tetrahydropyranyl acetal linkage, in agreement with reported data on known compounds with similar types of protons. A broad absorption band was observed in the region centering around τ 6.4 which was attributed to alcohol "OH" proton water "OH" proton exchange. The absorption of the eight shielded methylene protons of the tetrahydropyranyl group were observed, as expected, in the region from τ 8 65 to 8 80 as a broad multiplet. Upon integration of the absorption peaks, the expected ratios for the relative number of protons in each were observed. The hydroxyl absorption did not fit with these ratios, as would be expected for the postulated proton exchange.

Inspection of infrared spectra was particularly useful in following the structural changes resulting from the individual reactions and aiding in the identification of products and also

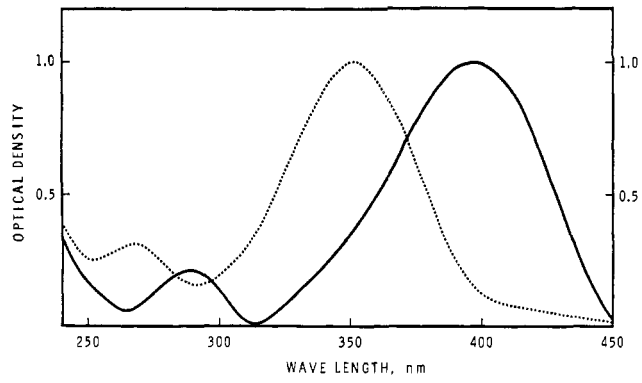

FIGURE 4: Absorption spectra (normalized) of dehydroluciferol in 0.1 M Tris buffer (pH 11.5) (——), and in 0.1 M Tris buffer (pH 7.5) (- - - - - -).

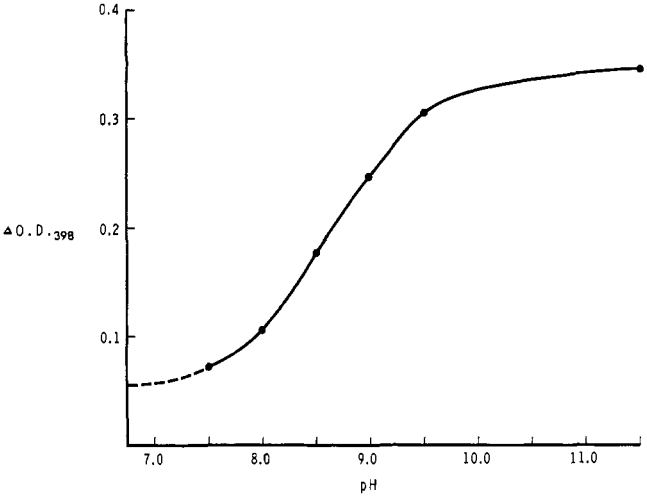

FIGURE 5: Effect of pH on the ionization of the 6′ hydroxyl of dehydroluciferol following the increase in OD_{398} as a function of pH in 0.1 M Tris buffer. The concentration of dehydroluciferol is 1.5×10^{-5} M.

deserves some comment. The infrared spectra of dehydroluciferin and products I, II, III, and IV show the dehydroluciferin backbone to be rich in detail as previously reported for luciferin (White *et al.*, 1965). For dehydroluciferin there is a broad band in the OH stretching region, having a peak of 3100 cm^{-1} and a strong absorption at 1725 cm^{-1} in the region of carboxylic carbonyl stretch. Upon esterification of the carboxyl, the carbonyl stretch band undergoes little or no change while the phenolic OH stretch band shifts to slightly higher frequency and a new band at 1020 cm^{-1} now appears due to CO stretch of the ester group. Upon formation of the tetrahydropyranyl derivative (II), the broad OH stretch band is obliterated and a new strong absorption appears at 2940 cm^{-1} due to aliphatic CH stretch (a weak 2940-cm^{-1} absorption was also observed for the ester I). The ir spectrum of III was obtained as a liquid film of the clear oil remaining after removal of solvent at 10^{-5} mm. In contrast to II, there is now a broad band in the OH stretching region which peaks at 3450 cm^{-1} due to the OH stretch of the primary alcohol group while the CH stretch band at 2940 cm^{-1} and the CO stretch at 1020 cm^{-1} remain unperturbed. In addition, the strong carbonyl absorption at 1725 cm^{-1} of II is now absent. The acid hydrolyzed product compound, IV, exhibits an even broader absorption in the OH stretching region while the aliphatic CH stretch band disappears. The CO band at 1020 cm^{-1}, however, still remains.

The chemical ionization mass spectrum of product IV is shown in Figure 3. The molecular ion occurs at *m/e* 264 while the large quasimolecular ion (QM^+), due to proton donation, occurs at *m/e* 265 as expected. The significant absorptions at $(M + 2)^+$ and $(M + 3)^+$ result from the isotopic contributions from the ring heteroatoms sulfur and nitrogen. The presence of a $(QM^+ - 18)$ peak at *m/e* 247 is a characteristic primary alcohol fragmentation pattern resulting from the loss of H_2O. All other analytical data are summarized in Materials and Methods.

Spectral Properties of Dehydroluciferol. The absorption spectra of dehydroluciferol at pH 7.5 and pH 11.5 are shown in Figure 4. The shift in the absorption maximum with pH is very similar to what has been reported for dehydroluciferin (Morton *et al.*, 1969) and is indicative of absorption by the phenol form in which the 6′-hydroxyl group of the benzothiazole ring is protonated at acid and neutral pH's. At strongly alkaline pH's absorption is primarily from the ionized phenolate. The shift in absorption maximum with pH as shown in Figure 5 indicates that this group has a pK_a of 8.6. Since the pK_a for this group of dehydroluciferin is also approximately 8.7, chemical modification at the other end of the molecule seems to have had little or no effect on the

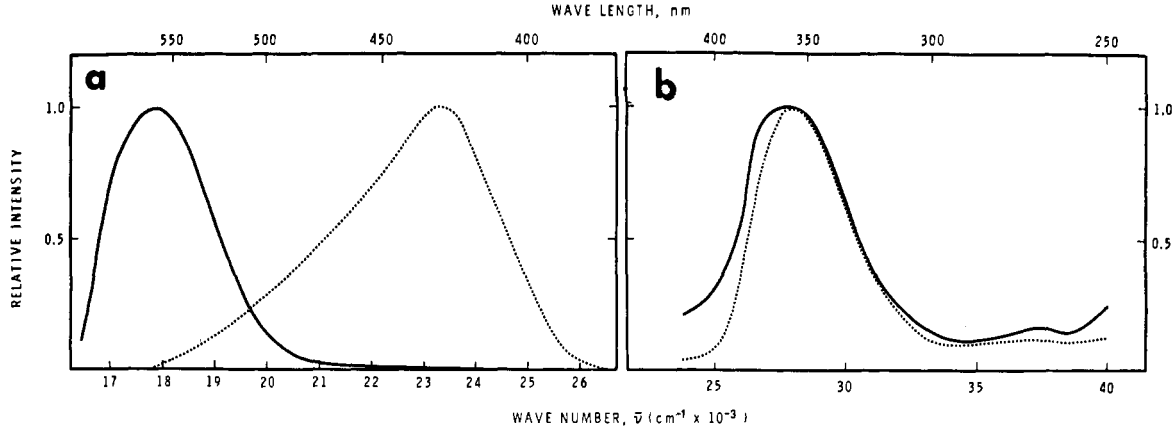

FIGURE 6: (a) Fluorescence emission spectra (normalized) of dehydroluciferol in 0.05 M phosphate buffer (pH 7.5) (——), and in absolute methanol (. . . . .). (b) Fluorescence excitation spectra (normalized) of dehydroluciferol in 0.05 M phosphate buffer (pH 7.5) (——) and in absolute ethanol (. . . . .).

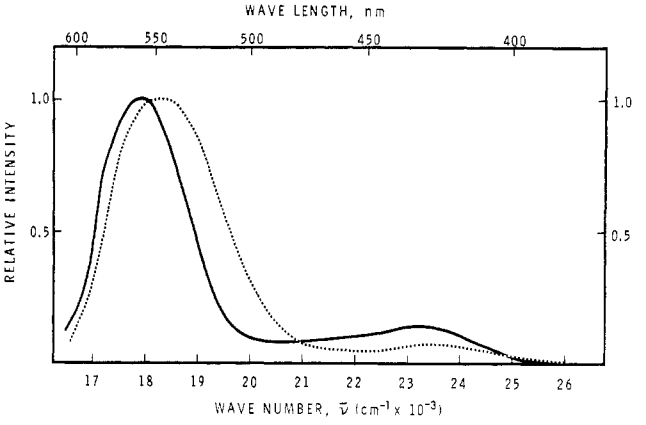

FIGURE 7: Fluorescence emission spectra (normalized at the peak of the phenolate emission) of luciferase-bound dehydroluciferin (—) and dehydroluciferol (- - - - -) in 0.05 M phosphate buffer (pH 7.5). The concentration of luciferase is an 8-fold excess over that of L or L-OH.

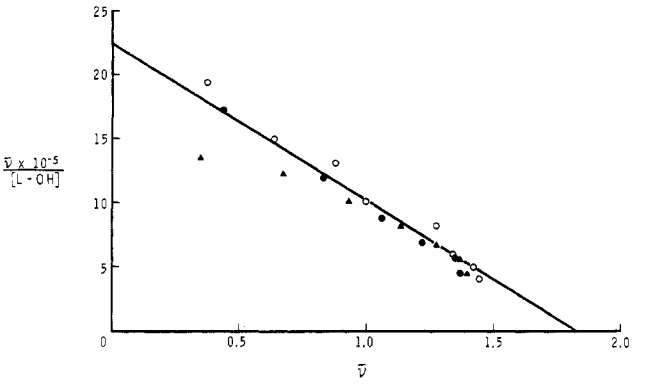

FIGURE 8: Data obtained from a representative titration of luciferase with dehydroluciferol and plotted according to the method of Scatchard. All titrations were performed in 0.05 M phosphate buffer (pH 7.5) and at 10°. For the titrations, the luciferase concentrations are: (○) 4.1×10^{-7} M, (▲) 12.3×10^{-7} M, (●) 8.2×10^{-7} M. Under these conditions, $n = 1.8$ and $K_a = 1.5 \times 10^6$.

acidity of this group, at least in the ground state. The fact that the spectral shifts are due to ionization at the 6′ position is also indicated by the observation that compound III, whose phenolic hydroxyl is tied up as the tetrahydropyranyl derivative, shows no pH-dependent shift in its absorption. Its absorption maximum is 348 nm at both neutral and alkaline pH's. Measurement of spectra for this compound in acidic media was not possible due to the lability of the acetal linkage.

Dehydroluciferol is highly fluorescent as is dehydroluciferin and exhibits similar excitation and emission spectra. Figure 6 shows the normalized excitation and emission spectra of dehydroluciferol in methanol and in 0.05 M phosphate buffer (pH 7.5). Although both spectra show excitation maxima at approximately 353 nm, indicating absorption of the phenol form, the predominant emission occurs at 425 nm (23,350 cm^{-1}) in methanol and 555 nm (17,850 cm^{-1}) in 0.05 M phosphate (pH 7.5). These results indicate that dehydroluciferol, like dehydroluciferin and luciferin, also undergoes excited-state ionization (Morton *et al.*, 1969), *i.e.*, although the excited state *phenol* form is the species which is excited in both cases, the emission occurs from the excited state *phenolate* in aqueous buffer. Therefore, in buffer (pH 7.5), ionization occurred during the lifetime of the excited state due to the proton accepting character of the aqueous environ.

Denburg *et al.* (1969) has shown that when dehydroluciferin binds to luciferase there is a large enhancement of the 440 nm (uncorrected) emission. He suggested that this was due to binding in a hydrophobic pocket at the luciferase active site. Figure 7 shows the emission spectra of dehydroluciferol and dehydroluciferin bound to luciferase. The emission spectra for both show a significant enhancement of fluorescence in the region around 425 nm (23,400 cm^{-1}); however, the enhancement is slightly greater for dehydroluciferin.[2]

[2] Throughout this and the subsequent paper, all fluorescence excitation and emission spectra are corrected for differences in photomultiplier sensitivity as a function of wavelength (unless otherwise noted). This procedure gives a truer representation of the fluorescence intensities as a function of wavelength and, as a result, the corrected spectra often differ, not only in relative intensities for emission in different regions of the spectrum, but also in the wavelength of the emission or excitation maxima. Therefore, the relative intensities of the blue (425 nm) and yellow green (555 nm) emissions reported here are significantly different from those reported by Denburg. The wavelength maxima are also shifted.

Two additional effects are apparent. The bandwidth as judged by the full bandwidth at half-maximum intensity (FWHM) is much narrower for enzyme bound dehydroluciferin (1900 cm^{-1}) than for enzyme bound dehydroluciferol (2350 cm^{-1}). However, the broader band for dehydroluciferol is not a result of binding to luciferase since it has essentially the same FWHM in buffer at pH 7.5. Dehydroluciferin also does not undergo any changes in bandwidth when it binds to luciferase. There is, however, a blue shift in the phenolate emission from 555 nm (17,850 cm^{-1}) in buffer to 540 nm (18,300 cm^{-1}) when L-OH is bound to luciferase. Such blue shifts in emission are indicative of emissions from excited-state forms in which the dipole moment increases upon excitation, emitting in a more hydrophobic environment. Dehydroluciferin undergoes no shift in the emission maximum when it binds. Since L and (as will be shown later) L-OH bind at the luciferin binding site, dehydroluciferol seems to be even more sensitive to solvent polarity than L. It is, however, possible that L-OH could be binding more deeply in the hydrophobic pocket due to the absence of repulsive interaction as with the COO^- group of L (Denburg *et al.*, 1969) or that L-OH could simply bind in a slightly different manner than L.

Using the enhancement of the 425-nm emission as a measure of bound L-OH, aliquots of dehydroluciferol were added to a solution of luciferase and the increase in 425-nm emission was measured. χ, the fraction of dehydroluciferol bound after each addition, was obtained according to Laurence (1952). The fluorescence intensity for enzyme bound dehydroluciferol was determined both in the presence of a tenfold excess of enzyme and from reciprocal plots of fluorescence intensity *vs.* protein concentration (Weber and Young, 1964), both methods yielding identical values. The average number of molecules of dehydroluciferol bound per 100,000 molecular weight of luciferase ($\bar{\nu}$) is calculated from eq 5, where ($L\text{-}OH_T$) represents the total concentration

$$\bar{\nu} = \chi \frac{(L\text{-}OH_T)}{(P_T)} \tag{5}$$

of dehydroluciferol and (P_T) represents the total concentration of luciferase. When plotted according to the method of Scatchard (1949), the data indicated two binding sites per

TABLE I: Reactivity of Dehydroluciferol in the ATP-[^{32}P]PP$_i$ Exchange Reaction.[a]

	Concn of Analog	cpm in ATP
Luciferase only		185
Luciferase + L-OH	7.62×10^{-6}	245
Luciferase + L	7.63×10^{-6}	26,260

[a] For reaction conditions, see Materials and Methods.

mol wt 100,000 with an association constant (K_a) for the analog–luciferase complex of 1.5×10^6 (Figure 8). Under similar conditions the K_a for dehydroluciferin is 1.4×10^6 thereby indicating that the analog binds to luciferase with approximately the same affinity as dehydroluciferin.

Enzymatic Activity. Although there were no unexpected changes in the excitation and emission spectra of enzyme-bound dehydroluciferol direct evidence that it was enzymatically nonreactive was necessary. Dehydroluciferol was, therefore, tested for its ability to form adenylate in the luciferase-catalyzed pyrophosphate exchange reaction. The results are shown in Table I and demonstrate that it is not active in this reaction.

The possibility of an irreversible formation of alkyl adenylate or other product was checked by subjecting aliquots of a similar reaction mixture to thin-layer chromatography on silica gel. In both highly polar solvents (7:3, 95% ethanol–1 M ammonium acetate, pH 7.5) and rather nonpolar solvents (5:3, $CHCl_3$–ethyl acetate), the only observable migrating fluorescent spot was that which comigrated with L-OH.

As expected, dehydroluciferol is inactive toward light emission since it cannot form the necessary acyl adenylate. These experiments indicate that dehydroluciferol is unaltered when it binds to luciferase, even in the presence of ATP–Mg^{2+}.

Inhibition Studies. In an attempt to further demonstrate that dehydroluciferol is binding to the luciferase active site, inhibition studies were performed to determine the kinds of inhibition obtained with respect to the two natural substrates, LH_2 and ATP–Mg. The variation of the initial velocity at different fixed values of L-OH was measured and the results were plotted according to the method of Lineweaver–Burk. Analysis of these plots (Figure 9a,b) shows that inhibition by dehydroluciferol is competitive with luciferin but noncompetitive with respect to ATP–Mg when L-OH is added simultaneously with ATP. (Preincubation of luciferase with L-OH leads to mixed inhibition with respect to luciferin; neither purely competitive nor noncompetitive.) K_i's determined from these plots give values of 6.2×10^{-7} and 8.3×10^{-7}, respectively, again in good agreement with the values for dehydroluciferin ($K_i = 1 \times 10^{-6}$ at pH 7.7).

We conclude from these experiments that the alcohol analog of dehydroluciferin does, indeed, bind at the active site. The analog is enzymatically unreactive inasmuch as it cannot form an intermediate acyl adenylate (eq 1) which precludes its participation in the light emitting step (eq 2). In addition, the analog is very sensitive to solvent effects and should prove useful for studying the luciferase active-site microenvironment.

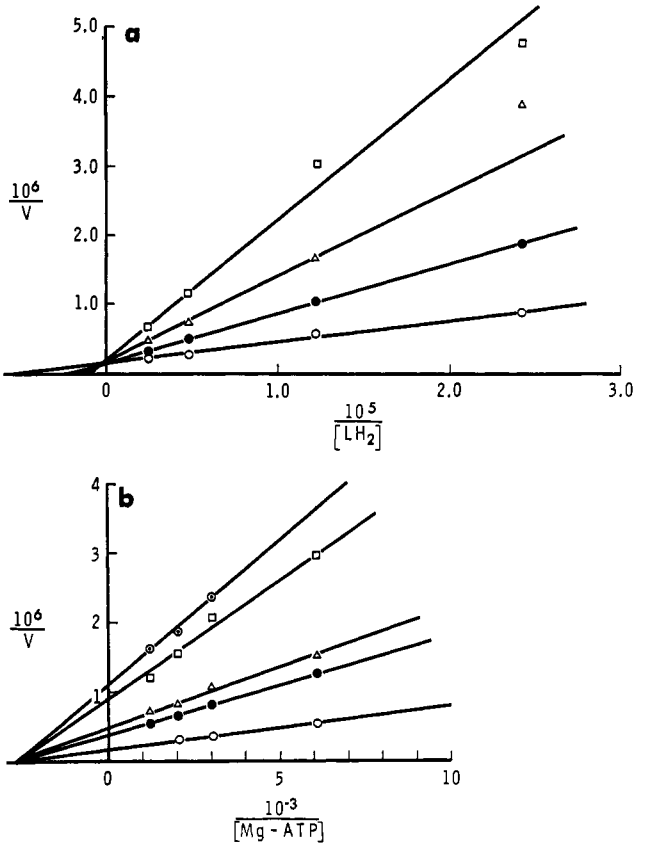

FIGURE 9: Inhibition of the luciferase bioluminescent reaction by dehydroluciferol. (a) Inhibition as a function of LH_2 concentration. The reaction mixture contained 6.7×10^{-7} M luciferase, 2×10^{-3} M ATP containing various amounts of L-OH as stated below, and 4×10^{-3} M $MgSO_4$ in 0.025 M glycylcine buffer (pH 7.8). The LH_2 concentration was varied as shown: (○) no L-OH, (●) 8.44×10^{-7} M L-OH, (△) 2.11×10^{-6} M L-OH, (□) 4.22×10^{-6} M L-OH. (b) Inhibition as a function of ATP–Mg concentration (concentration of ATP–Mg determined as described in Experimental Methods). The reaction mixture contained 6.7×10^{-7} M luciferase, various ATP–Mg concentrations (prepared by using various concentrations of ATP in the presence of 4×10^{-3} M $MgSO_4$), and 4.2×10^{-5} M LH_2 in 0.025 M glycylglycine buffer (pH 7.8): (○) no L-OH, (▲) 1.05×10^{-6} M L-OH, (△) 2.1×10^{-6} M L-OH, (□) 3.14×10^{-6} M L-OH, (⊙) 4.18×10^{-6} M L-OH. All assays were performed in triplicate.

Acknowledgments

The authors are very grateful to Dr. George Milne for kindly running the chemical ionization mass spectra. A note of appreciation also goes to Dr. George Fields for his generous gift of dehydroluciferin and to Charles Furfine for the use of the MPF-2A fluorescence spectrophotometer.

References

Denburg, J. L., Lee, R. T., and McElroy, W. D. (1969), *Arch. Biochem. Biophys. 134*, 381.

Green, A. A., and McElroy, W. D. (1956), *Biochim. Biophys. Acta 20*, 170.

Laurence, S. J. R. (1952), *Biochem. J. 51*, 181.

McClure, W. O., and Edelman, G. (1967), *Biochemistry 6*, 559.

McElroy, W. D., and Seliger, H. H. (1961), *in* Light and Life, Baltimore, Md., Johns Hopkins University Press, p 219.

Morton, R. A., Hopkins, T. A., and Seliger, H. H. (1969), *Biochemistry 8*, 1598.

Munson, M. S. B., and Field, F. H. (1966), *J. Amer. Chem. Soc. 88*, 2621.

Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.

Seliger, H. H., and McElroy, W. D. (1966), *in* Bioluminescence

in Progress, Johnson, F. H., and Hanada, H., Ed., Princeton, N. J., Princeton University Press, p 423.
Seto, S., Ogura, K., and Nishiyama, Y. (1963), *Bull. Chem. Soc. Jap. 36*, 332.
Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239*, 1415.
White, E. H., Worther, H., Field, G. F., and McElroy, W. D. (1965), *J. Org. Chem. 30*, 2344.

Excited-State Proton Transfer and the Mechanism of Action of Firefly Luciferase†

Lemuel J. Bowie,* Richard Irwin, Michael Loken, Marlene De Luca, and Ludwig Brand

ABSTRACT: The synthesis of a fluorescent substrate analog of firefly luciferin, dehydroluciferol, has been reported in the previous paper of this series. It has been shown to be enzymatically unreactive and binds at the luciferin binding sites. The spectral properties of this analog suggest that it should be a good active-site probe. In the present study our results indicate that the binding of the second substrate for the luciferase bioluminescent reaction, ATP–Mg^{2+}, causes a decrease in the rate of excited state proton transfer from the 6′ hydroxyl of bound dehydroluciferol. These results are interpreted as being due to a change in the "hydrophobicity" of the active site microenvironment. Since formation of dehydroluciferyl adenylate, an analog of the intermediate in the bioluminescent reaction, has been shown to cause a large conformational change in luciferase, our results indicate that this change manifests itself at the active site. The binding of the activator, AMP, causes changes in the fluorescence emission of bound dehydroluciferol which indicate that the microenvironment becomes even more "hydrophobic." In addition, emission occurs almost completely from the excited-state species in which the 6′ hydroxyl is ionized. Since this group is un-ionized in the ground state when natural substrate luciferin, or dehydroluciferol, binds and since bioluminescence emissions occur from an excited-state species of luciferin in which the 6′-hydroxyl group is ionized, ionization of the enzyme-bound chromophore must occur prior to light emission. Therefore, the effect of AMP may have significance in normal firefly bioluminescence since it facilitates this ionization.

Many investigations recently have demonstrated the usefulness of small fluorescent molecules as probes for certain binding sites in proteins. In the case of firefly luciferase the substrate luciferin (LH_2)[1] is a fluorescent molecule. A competitive inhibitor of the enzyme, dehydroluciferin (L), is also fluorescent and the spectral properties of these compounds are sensitive to the composition of the solvent (Morton *et al.*, 1969). Denburg *et al.* (1969) showed that the emission spectrum of L changed when it was bound to luciferase. This change was thought to be a result of dehydroluciferin binding in a very "hydrophobic" [2] site on the enzyme. Previous studies using fluorescent dyes (De Luca, 1969; Turner and Brand, 1968) also indicated the LH_2 or L binding site is very hydrophobic. Both L and LH_2 in the presence of luciferase react with ATP–Mg to form the enzyme-bound acyl adenylate. It was desirable to have a substrate analog which would not react with ATP–Mg in order to study its spectral properties when bound to luciferase in the presence and absence of ATP–Mg. We have synthesized the alcohol analog of dehydroluciferin which does not react with ATP–Mg. The structure and properties of this compound were described in the previous paper. The spectral properties of L-OH have been shown to be sensitive to both pH and solvent composition. L-OH is an inhibitor of luciferase and is competitive with respect to luciferin (Bowie *et al.*, 1972). Using this analog we have been able to study the effect of ATP–Mg and AMP on the spectral properties of the enzyme-bound L-OH. We have found changes in the active site which influence the rate of excited-state ionization of the phenolic hydroxyl group. The apparent hydrophobicity of the binding site also changes in the presence of ATP–Mg or AMP.

Materials and Methods

Firefly luciferase was isolated from lyophilized firefly lanterns as described in the previous paper. However, even after repeated recrystallization, it was not possible to completely remove small amounts of enzyme bound fluorescence. The possibility that this fluorescence might be due to small amounts of enzyme bound L-AMP was suggested by the similarity between the excitation and emission spectra of this fluorescence and the corresponding spectra resulting when enzyme-bound dehydroluciferin is incubated with ATP–Mg. In addition, the K_D for L-AMP is very low, 5×10^{-10}

† From the Department of Biochemistry, Georgetown University Schools of Medicine and Dentistry, Washington, D. C. 20007, and from the Department of Biology, Johns Hopkins University, Baltimore, Maryland. *Received December 27, 1972.* The research was supported in part by the National Institute of Health GM 11632, the National Science Foundation, and the American Cancer Society. A preliminary report of this work was presented at the 1972 meeting of the Federation of American Societies for Experimental Biology.

* To whom to address correspondence at the Veteran's Administration Hospital, San Diego, Calif. 92161.

[1] Abbreviations used are: LH_2, luciferin; L, dehydroluciferin; L-OH, dehydroluciferol; L-AMP, dehydroluciferyl adenylate; LH_2-AMP, luciferyl adenylate.

[2] The term "hydrophobicity" as used in this paper refers to the sum total of all such environmental effects on fluorescence. For a more complete discussion on hydrophobicity and solvent polarity the reader is referred to the recent review of Brand and Gohlke (1972).