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STEREOSPECIFICITY AND FIREFLY BIOLUMINESCENCE, A COMPARISON OF NATURAL AND SYNTHETIC LUCIFERINS

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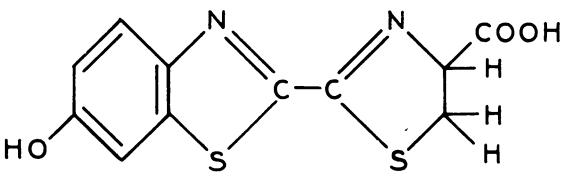
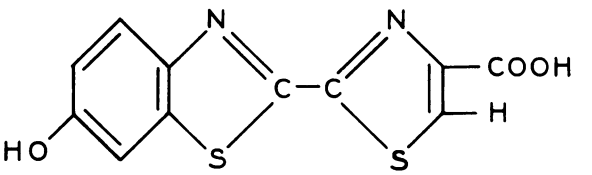
Synthetic firefly luciferin and dehydroluciferin (oxyluciferin) have been prepared for the first time and the structures are given in Figure 1. The complete chemical analysis and method of synthesis will be given in a separate paper by E. White *et al.*[1] In the last step of the chemical synthesis of luciferin, 2-cyano-6-hydroxybenzthiazole is reacted with cysteine. When D-cysteine is used, a luciferin (D-LH_2) is obtained which is activated by ATP, forming the adenylate derivative and releasing pyrophosphate. The enzyme complex formed then reacts with oxygen and bioluminescence is observed. When L-cysteine is used in the final step of synthesis, the resulting luciferin, L-LH_2, also reacts with ATP to form the adenylate derivative and to release pyrophosphate. The unusual feature of this L-enantiomorph, however, is that it is subsequently oxidized by molecular oxygen in a manner indistinguishable from D-luciferin or natural luciferin but *without light emission.* It is the purpose of this paper to present the physical and biochemical evidence establishing the identity of the synthetic with the natural products and to describe the remarkable stereospecificity in the bioluminescent reaction.

Enzyme Reactions.—The following reactions are catalyzed by firefly luciferase:

$$LH_2 + ATP + E \rightleftarrows E{\cdot}LH_2{\text{-}}AMP + PP \qquad (1)$$



FIREFLY LUCIFERIN



FIREFLY OXYLUCIFERIN
(DEHYDRO-LUCIFERIN)

FIG. 1.—Structures of firefly luciferin and dehydroluciferin.

$$E{\cdot}LH_2\text{-}AMP + O_2 \rightarrow E{\cdot}L\text{-}AMP + \text{Light} \quad (2)$$

$$L + ATP + E \rightleftarrows E{\cdot}L\text{-}AMP + PP \quad (3)$$

$$E{\cdot}L\text{-}AMP + CoA \rightleftarrows L{\cdot}CoA + E + AMP \quad (4)$$

In the initial activation step the carboxyl group of luciferin (LH_2) reacts with adenosine triphosphate (ATP) to form a luciferyl-adenylic acid-enzyme complex and inorganic pyrophosphate (PP). The progress of these reactions can be followed by observing light emission, pyrophosphate release, or fluorescence intensity changes.[2,3] For example, consider reactions 3 and 4. If a small amount of luciferase (E) is added to natural dehydroluciferin (L) with excess adenosine triphosphate (ATP) and magnesium ion (Mg^{++}) reaction 3 can be followed by observing the decrease in intensity of the 544 mμ fluorescence of free L. The binding of L onto the enzyme in the activation step with ATP produces essentially a nonfluorescent species (dehydroluciferyl-adenylic acid-enzyme complex). The addition of excess coenzyme A as shown by reaction 4 will release enzyme and eventually by virtue of 3 and 4 all of the L present will be converted to L·CoA. Since L·CoA is also a nonfluorescent molecule, we add excess cysteine which results in the following nonenzymatic reaction:

$$L{\cdot}CoA + \text{cysteine} \rightarrow L{\cdot}\text{cysteine} + CoA. \quad (5)$$

L·cysteine is a fluorescent molecule and the progress of reaction 5 can be followed as a fluorescence increase with time. This three-stage fluorescence assay technique has now been demonstrated for synthetic dehydroluciferin.

Synthetic D-luciferin reacts with ATP, luciferase, Mg^{++}, and O_2 to give bioluminescence. The bioluminescence emission spectrum of the *in vitro* natural luciferin-luciferase reaction at neutral pH has a peak emission which, for various samples, varies between 562 mμ and 565 mμ. Using the same purified enzyme preparation the shapes and the peak positions of the bioluminescence emission

spectra were identical for both natural luciferin and synthetic D-luciferin. At acid pH the bioluminescence emission spectrum of synthetic D-luciferin exhibited the same red-shift as reported previously for natural luciferin;[4] there is a disappearance of the 565 mμ yellow-green band and the appearance of the new 616 mμ red band.

An equal amount of synthetic L-luciferin under identical conditions gives essentially no light emission. Synthetic L-luciferin, while not effective for the light-emitting reaction, is a potent competitive inhibitor of luminescence for both natural luciferin and synthetic D-luciferin. The inhibition data would suggest that in reaction 1 the luciferase makes no distinction between the L- and D- forms of luciferin. To test this hypothesis equal amounts of synthetic D- and L-luciferin were reacted separately, and the amount of pyrophosphate released was determined as a function of time. The initial rate of pyrophosphate release is the same for both the D- and the L-luciferin. In addition, chromatographic and fluorometric analyses of the reaction products indicate that dehydroluciferin is formed from both D- and L-luciferin. Thus, chemically there does not appear to be any difference between D- and L-luciferin; the only difference being that bioluminescence attends the enzymatic oxidation of D-luciferin while the enzymatic oxidation of L-luciferin is a dark reaction.

We have been able to convert both synthetic L- and D-luciferin to dehydroluciferin with equal facility in alkaline solution by heating or by oxidation at room temperature with ferricyanide ion. The chromatographic separation both on paper and on celite columns of the initial synthetic D- and L-luciferins and of the dark-oxidized (dehydroluciferin) products showed the same R.F.'s and bands as the natural products.

As expected from the structure it has been possible to convert synthetic L-luciferin into D-luciferin by an alkali-catalyzed isomerization. If L-luciferin is heated to approximately 80°C in 1*N* sodium hydroxide in the absence of oxygen one obtains significant amounts of D-luciferin as judged by its ability to produce bioluminescence. Similarly a solution of synthetic D-luciferin treated as above will produce less light even though there is no apparent loss of luciferin as measured by absorbance.

Absorption and Fluorescence Spectroscopy and Optical Rotation.—The normalized absorbance spectra of natural luciferin and dehydroluciferin are shown in Figure 2. At neutral pH or below, natural luciferin has an absorption peak at 327 mμ and at alkaline pH the absorption peak shifts to 381 mμ. The corresponding peaks for natural dehydroluciferin are 348 and 393 mμ, respectively. These shifts, with a pK of 8.25 correspond to the ionization of the OH group of the molecule. The effect of the ionic forms of the molecules is evident in Figure 3 which shows the large increases in the fluorescence quantum yields of both natural luciferin and dehydroluciferin at alkaline pH; the pK of the fluorescence yield shifts is identical with that of the absorption peak shifts. Both the acid-base shifts in absorbance and the acid-base shifts in fluorescence yields have been observed for both synthetic D- and L-luciferin and for synthetic dehydroluciferin.

The fluorescence emission spectra of natural luciferin and dehydroluciferin have peaks at 535 mμ and 544 mμ, respectively. These peak positions remain independent of pH changes even though the ionization resonance lowers the absorption peak energies by 12.5 and 9.3 kcal/mole, respectively. Insofar as absorbance and

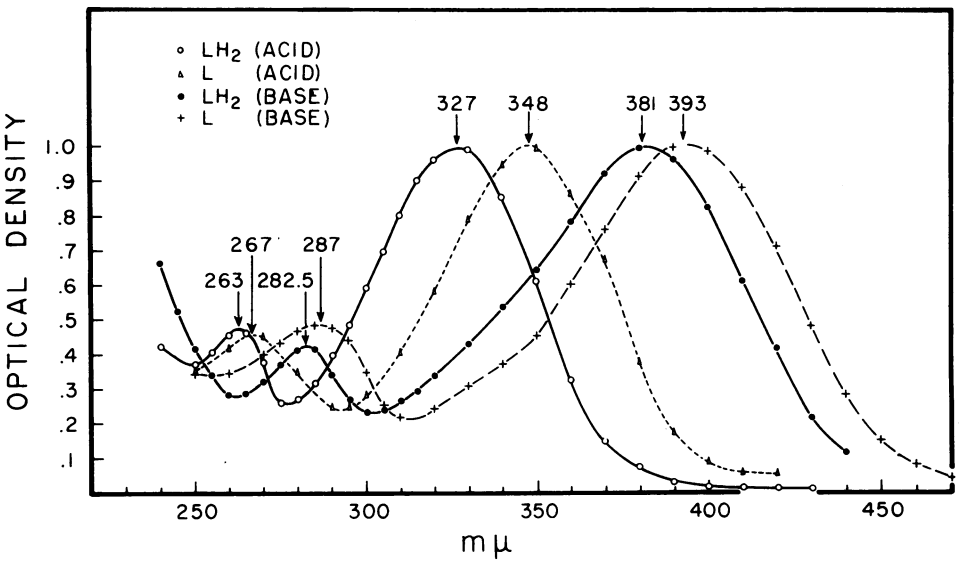


FIG. 2.—Normalized absorbance spectra of luciferin and oxyluciferin. The points of crossing of the acid and base curves are isobestic points.

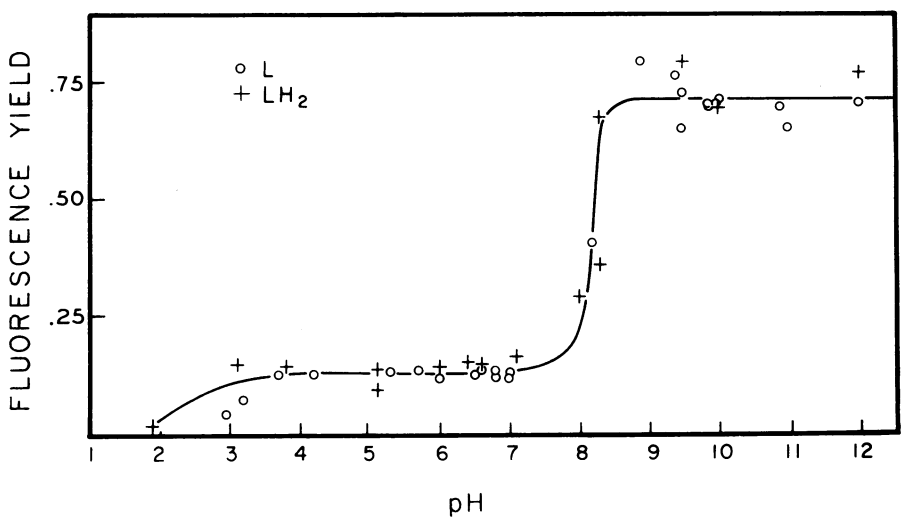


FIG. 3.—Fluorescence yields of luciferin and dehydroluciferin as functions of pH. The measurements were complicated by the absorption shifts shown in Figure 2. The quality of the emission, however, was unchanged by pH. Measurements were made relative to fluorescein in alkaline solution.

fluorescence are concerned, synthetic D-luciferin, synthetic L-luciferin, and natural luciferin are identical, as are synthetic and natural dehydroluciferin.

The synthetic D- and L-luciferins are enantiomorphs with specific rotations for the sodium D doublet in dimethyl formamide solvent of minus and plus approximately 30 degrees, respectively. The maximum rotation observed depends somewhat upon the method of synthesis since racemization can take place. Natural

luciferin is not readily available in the concentrations normally required for optical rotation measurements and therefore measurements have not been too accurate. The natural luciferin that we have measured is levo-rotatory indicating that natural product is predominantly the D-isomer.

Discussion.—It is surprising that natural firefly luciferin is D-luciferin in view of the preponderance of L-amino acids over D-amino acids in biological systems. Unfortunately, we have no evidence concerning the biosynthesis of firefly luciferin.

The quantum yield (number of light quanta emitted per luciferin molecule oxidized) of purified natural firefly luciferin was found to be 0.88 ± 0.25.[5] Since the assay of the initial amount of luciferin present was based on absorbance spectrophotometry and since any L-luciferin present would not have contributed to the light emission, this value is a lower limit, depending upon the isomeric purity of the luciferin samples used.

The stereospecificity for the bioluminescence reaction is a biological example of a general mechanism of chemiluminescence proposed earlier.[6] That is, in an exothermic chemical reaction such as oxidation by molecular oxygen, luminescence is specifically the fluorescence of the product molecule which upon formation is in a highly excited vibrational state. If the product molecule is not a fluorescent species or if it is bound in such a way as to be quenched, chemiluminescence will not be observed. Here, apparently, in the case of firefly luciferin the L-isomer complex of luciferin, after undergoing oxidation, is either nonfluorescent or very strongly quenched.

Unfortunately, we do not know the exact oxidative mechanism which leads to light emission. We are certain of products indicated in reaction 2 but there are transients which have not as yet been identified. The continuous spectrophotometric assay of the luciferin oxidation in excess enzyme (reactions 1 and 2) indicates that as the reaction proceeds the 327 mμ absorption peak of luciferin decreases and is replaced by a new absorption peak at 383 mμ. The new absorption band is presumably due to the "E·LAMP" complex indicated in reaction 2. However, a comparable set of absorbance curves for the enzyme reaction with dehydroluciferin show relatively little decrease in the 348 mμ absorption peak of dehydroluciferin and no new absorption peaks corresponding to "E·LAMP" of reaction 2. Thus, at least in our *in vitro* enzyme reactions the enzyme-intermediate complex formed is different from E·LAMP formed directly as in reaction 3. It may be significant that the enzyme complex formed in reaction 2, at neutral pH, behaves in absorption as though the product were closely related to luciferin in basic solution. The crossover point between the disappearance of the original 327 mμ peak and the appearance of the new 383 mμ peak is just the 348 mμ isobestic point for luciferin as shown in Figure 2.

While we cannot yet identify the enzyme complex responsible for the new 383 mμ absorption peak we have been able to verify that both synthetic D- and L-luciferin in the enzymatic reaction give this same 383 mμ absorption peak with a crossover point at 348 mμ. Synthetic dehydroluciferin gives the same results as natural dehydroluciferin.

The luminescence stereospecificity which we have observed seems to be a most unusual type of enzyme stereospecificity, inasmuch as the D- and L-luciferins appear to combine with the enzyme with the same affinity. Both forms have the same

Briggs-Haldane constants and dehydroluciferin is formed from both to the same degree. We are forced to conclude, therefore, that the intermediate formed in the oxidation of L-luciferin is either a different nonfluorescent species or is the same molecule as that resulting from D-luciferin oxidation but vibrationally quenched with very high efficiency. This difference might be ascribed to the mode of binding of the intermediate to the enzyme.

It is of interest to compare this observed luminous stereospecificity with the fluorescence of the α- and β- forms of reduced diphosphopyridine nucleotide (DPNH). It has been found that the 260 mμ adenine absorption results in fluorescence emission at 460 mμ of β-DPNH. However, the α-glycoside linkage of the nicotinamide riboside grouping alters the molecular configuration so that, although the adenine moiety absorbs as strongly at 260 mμ in α-DPNH, there is no resulting 460 mμ fluorescence.[7] Perhaps on the enzyme the D- and L-forms of LH_2-AMP give rise upon oxidation to a fluorescent and nonfluorescent intermediate species respectively. Alternatively, it is known that different enzymes catalyze the formation of the A and B stereoisomers of DPNH.[8] It may be that the D- or L-luciferyl adenylate can react with molecular oxygen in different ways, the former resulting in a highly resonant complex and the latter participating in a dark oxidation similar to that observed by heating in alkali in the presence of oxygen.

It would, therefore, be of considerable interest to look for possible differences in absorption or fluorescence of intermediates which might be formed in other enzyme reactions where optically active substrates are available. Quite possibly there may be bioluminescence observed upon substitution of stereoisomers into normal oxidase reactions.

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