Crystalline Cypridina Luciferin

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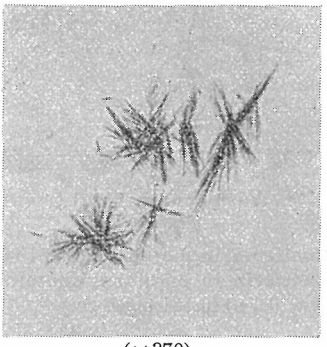
(Received June 3, 1957)

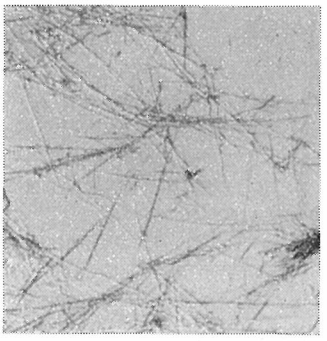
The luminescence of *Cypridina hilgendorfii* has been investigated so far by many workers[1], including Anderson, Kanda and Harvey, and it has been found that firefly luminescence requires ATP and magnesium ion as well as luciferin, luciferase and oxygen for its functioning, whereas *Cypridina* requires only the last three. *Cypridina* luciferin is therefore the simplest system and is most suited for investigating the phenomenon of biological luminescence. For the study of the chemical constitution of *Cypridina* luciferin, it is necessary to obtain pure luciferin. Among several studies concerning the purification of luciferin, the most valuable one is Anderson's benzoylation method[2] reported about twenty years ago. Recently various purification methods have been attempted by Mason[3] and by Tsuji[4]; however, luciferin has as yet not been obtained crystalline. Nevertheless many views relating to the constitution of luciferin have been submitted, including proteose, phospholipid, polyhydroxy benzene, hydroquinones, flavoprotein and chromopeptide[5]. Recently, Mason[7] and Tsuji[5] found that the acid hydrolysis of luciferin afforded many kinds of amino acids.

We have carried out the purification of luciferin and have succeeded in obtaining it crystalline. Investigations on the constitution of luciferin using the crystalline luciferin has also been carried out and preliminary results are reported herewith.

Isolation and crystallization of luciferin. — Pulverized dry *Cypridina hilgendorfii* was de-fatted with benzene and extracted with methanol in hydrogen atmosphere. The extract was purified according to a modified Anderson's benzoylation method[2] and by means of partition chromatography on cellulose powder to obtain "purified" luciferin. Acidification of the purified luciferin solution with hydrochloric acid afforded crystalline luciferin as orange-red needles (Fig. 1).



(×370)
Crystalline Luciferin



(×280)
Oxyluciferin A
Fig. 1

Crystalline luciferin melted at 182—195° (darkened at 175°) it is easily soluble in methanol and ethanol, soluble with difficulty in water and acetone, and insoluble in ether and benzene. The luminescent activity induced by luciferase is 37,000 times as intense as that of the starting material, dry *Cypridina hilgendorfii*. Ninhydrin- and *p*-dimethylaminobenzaldehyde-test were negative. When heated with lime, it decomposed with a skatole-like odor, and the molybdenum blue reaction of its residue was negative. Accordingly, phosphoric acid is probably

1) E. N. Harvey: "Bioluminescence", Academic Press Inc. Publishers, New York, 299 (1952).
2) R. S. Anderson; *J. Gen. Physiol.*, **19**, 301 (1935).
3) H. S. Mason and E. F. Davis; *J. Biol. Chem.*, **197**, 41 (1952).
4) F. 1. Tsuji; *Arch. Biochem. and Biophys.*, **59**, 452 (1955).
5) F. I. Tsuji, A. M. Chase, E. N. Harvey; F. H. Johnson: "The Luminescence of Biological Systems", American Association for the Advancement of Science, p. 127—156 (1955).

absent in luciferin. Ultra-violet and visible spectra of crystalline luciferin were nearly identical with those measured by Chase[6] and Tsuji[5] but the absorption at 435 mμ of the present sample was much stronger (Fig. 2).

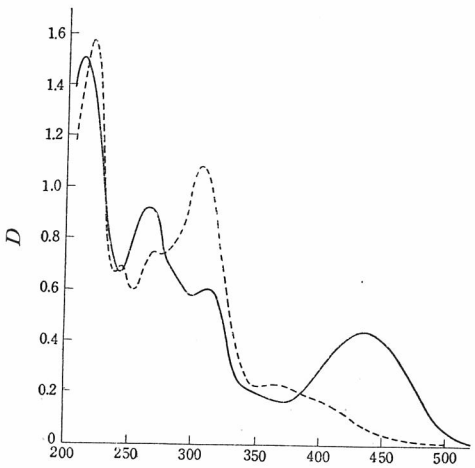


Fig. 2

—— Luciferin in methanol (0.161 mg/5 cc.)
---- Luciferin in methanol containing HCl (ca. 0.1 N) (0.161 mg/5 cc.)

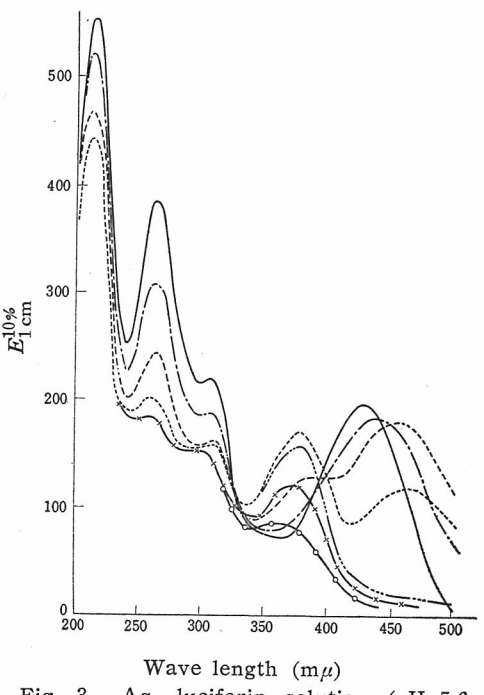


Fig. 3. Aq. luciferin solution (pH 5.6 phosphate buffer).

——— freshly dissolved
— — — after 7 hrs.
------ after 1 day
-------- after 3 days
——— after 7 days
—×— after 12 days
—○— after 52 days

Oxidation of luciferin.—When an aqueous solution of luciferin was allowed to stand in the air, its color changed gradually from yellow to red, and finally became almost colorless. The change of its UV absorption spectra is shown in Fig. 3. After being allowed to stand for 2 days, an ethanolic solution of luciferin afforded three spots as revealed by means of paper chromatography, i. e., one spot was a red colored substance and the other two were colorless blue-fluorescent substances (they were named oxyluciferin A and B) (solvent: ethyl acetate : ethanol : water, 5 : 2 : 3, Rf 0.97, 0.9 and 0.7, respectively). These substances were separated by the method of cellulose powder partition chromatography. The aqueous solutions of these substances were allowed to stand for a few days. Though the solutions of A and B were almost unchanged, the solution of the red substance was decolorized and afforded oxyluciferin A and B. These experiments and the UV absorption spectra suggest that luciferin is oxidized by at least three steps in the air; i. e., luciferin is first oxidized to the red substance ($\lambda_{max.}$ ca. 465 mμ), subsequently to oxyluciferin A and B ($\lambda_{max.}$ ca. 380 mμ in Fig. 2), and finally decomposed to unknown substances which show no fluorescence and no definite ultraviolet absorption except a maximum at 210 mμ.

The red substance has no luminescence activity, and is decolorized by addition of sodium borohydride. Its color is changed to a bluish violet by addition of concentrated sodium hydroxide solution. The red substance can be obtained easily by oxidation of luciferin with potassium ferricyanide (Fig. 4), though other oxidants oxidize luciferin to colorless substances directly.

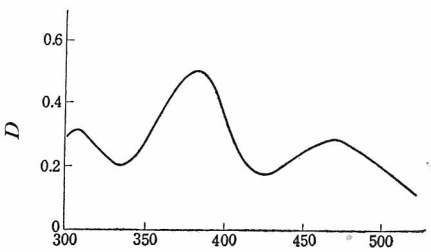


Fig. 4.

6) A. M. Chase and E. H. Brigham, *J. Biol. Chem.*, **190**, 529 (1951).
7) H. S. Mason, *J. Am. Chem. Soc.*, **74**, 4727 (1952).

Oxyluciferin A and B can be obtained in a good yield by addition of ammonia to the luciferin solution. Oxyluciferin A is obtained as yellow needles and melts at 140—148° (darkens at 135°) (Fig. 1).

Oxyluciferin B could not be obtained crystalline and became dark red upon addition of concentrated hydrochloric acid. Oxyluciferin A and B may be very similar substances, because their UV absorption spectra (Fig. 5) and their hydrolysis products (mentioned below) are very similar.

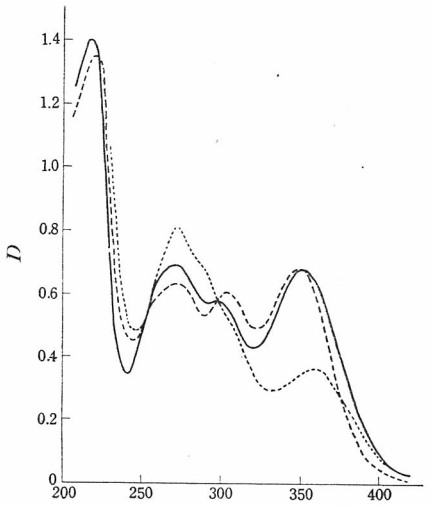


Fig. 5

—— A in phosphate buffer (pH 5.6) 0.084 mg/3 cc.
---- A in MeOH 0.123 mg./3 cc.
------ B in MeOH concn. unknown.

The oxidative decomposition of an aqueous solution of luciferin by air is also influenced by the pH of the solution. Generally, in an acidic medium the oxidation rate is slow and the red substance is stable for a long time, but in an alkaline medium the rate is very great and a yellowish green fluorescent substance is produced as well as A and B. When luciferin was decomposed by luciferase, luminescence occurred and the reaction mixture consisted mainly of oxyluciferin A and a small amount of B.

Hydrogenation of luciferin.—Hydrogenation of luciferin in methanol with Adams' platinum oxide catalyst afforded a colorless substance, the ultraviolet absorption spectrum of which was very similar to that of tryptophan or tryptamine (Fig. 6). The difference in the log D values at 220 and 280 mμ is 0.755, and this value is almost equal to that of tryptophan (0.79) or tryptamine (0.70). It is, therefore, apparent that these two absorption maxima originate in an indole type chromophore and are not caused by overlapping absorptions. Moreover the absorption intensity of hydroluciferin at 220 mμ is stronger than that of luciferin before hydrogenation. These observations suggest that the indole

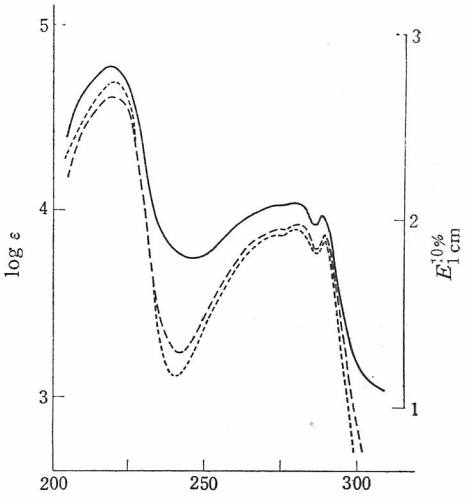


Fig. 6.

—— Hydroluciferin (right scale)
---- Tryptamine } (left scale)
------ Tryptophan }
in methanol

structure in hydroluciferin does not exist in luciferin, but is produced during hydrogenation. Hydrogenation of oxyluciferin A did not afford a substance which has an indole type absorption. The portion which is responsible for the indole type absorption after hydrogenation may be destroyed during oxidation of luciferin to oxyluciferin A. This observation is consistent with the fact that luciferin, and oxyluciferin A and B do not give positive reactions with p-dimethylaminobenzaldehyde (indole test), whereas hydroluciferin affords a positive reaction.

Hydrolysis of luciferin.—Acid hydrolysis of luciferin afforded glycine, isoleucine and a fluorescent substance. Hydrolysis of luciferin with barium hydroxide afforded glycine, isoleucine, a strong ninhydrin positive substance, a fairly large amount of anthranilic acid, and a small amount of indole or indole acetic acid, but no tryptophan. Since tryptophan is readily subject to oxidation, it was considered that tryptophan was decomposed during hydrolysis. Accordingly, the alkali hydrolysis was carried out in the presence

of small amounts of cystein as an antioxidant. Though tryptophan was not obtained either a new ninhydrin positive spot was obtained and identified to be tryptamine; in this case the amount of anthranilic acid was decreased.

Discussion

Acid hydrolysis of crystalline luciferin afforded only two amino acids, glycine and isoleucine; this reult is very different from that of Mason[7] and Tsuji[5], who obtained the following amino acids from the acid hydrolysate of "purified" luciferin: (Tsuji) arginine, leucine, isoleucine, glutamic acid, alanine, valine, sarcosine, lysine, taurine and methionine, and either aspartic acid, threonine, or serine; (Mason) taurine, aspartic acid, threonine, serine, sarcosine, glutamic acid, proline glysine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine. Acid hydrolysis of the "purified" luciferin obtained by the present authors also afforded at least eight amino acids and a fluorescent substance. Since crystalline luciferin and "purified" luciferin have the same R_f values on paper chromatography in hydrogen atmosphere, it is assumed that the extra amino acids in the hydrolysate of "purified" luciferin are impurities, which may be contained in luciferin as polypeptides. Since glycine is often produced from other substances by decomposition, care should be taken in order to identify it as one of the constituents, but by means of semi-quantitative paper chromatography it was shown that the amount of glycine was almost the sams as that of isoleucine. Thus glycine is regarded to be a component of luciferin.

The comparison of th UV absorption spectra and of the alkali hydrolysates of various indole derivatives (2-methyl indole, 3-methyl indole, 2,3-dimethyl indole, tetrahydrocarbazole, tryptophan, tryptamine, indole acetic acid, indole, etc.) suggest that anthranilic acid and indole derivatives resulting from hydrolysis of luciferin with barium hydroxide were produced from a β-substituted indole type moiety in luciferin.

The facts that hydrogenation of luciferin afforded indole type absorption and that hydrogenation of oxyluciferin A did not, suggest that the indole-type structure may be connected with the luminescent activity of luciferin. Comparison of ε values at 280 mμ of hydroluciferin and tryptophan or tryptamine leads to the result that the mole equivalent of luciferin for one indole group is ca. 500—800.

Experimental

Collected *Cypridina* was dried by being exposed to sunlight and then stored in a vacuum desiccator. Five hundred grams of completely dried *Cypridina* was pulverised and packed in the extraction apparatus (Fig. 7). It was defatted by

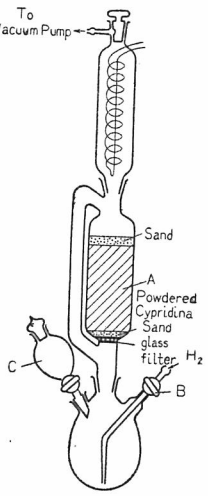


Fig. 7.

refluxing with 1.2 l. of well-dried benzene under reduced pressure (ca. 80 mm.Hg.) for 50 hr. The benzene extract was discarded, wet *Cypridina* was dried in vacuo (about 12 hr.), and then the apparatus was filled with purified hydrogen. The purified hydrogen was obtained by passing hydrogen through a soda-lime tube, red-heated metallic copper packed in a silica tube, and a washing bottle containing concentrated sulfuric acid. Absolute methanol (1.2 l.) was introduced into flask A through funnel C, and the extraction was started under reduced pressure (ca. 80 mm.Hg of hydrogen). After 50 hr. methanol was evaporated in vacuo to give a reddish brown foamy paste. The extraction apparatus was filled again with hydrogen, and 0.5 N hydrochloric acid (850 ml.) was added to the residual paste through funnel C. After being heated at 100°C on a water bath for 10 min. in hydrogen atmosphere, the yellow solution was cooled to about 0° and extracted with ether, at first with 200 ml. and subsequently thrice with 100 ml. The ether extract was discarded. Then the acid solution was extracted several times with *n*-butanol (total ca. 350 ml.), and the butanol extract was purified by a modified Anderson's benzoylation method as follows.

The butanol extract was benzoylated with benzoyl chloride (3 ml. of benzoyl chloride per 100 ml. of butanol soln.) at 0°C. After ten min. the reaction mixture was washed thrice with equal volumes of distilled water within about forty

min. and added to 8 to 10 volumes of water. The aqueous solution was extracted thrice with ether (care was taken to avoid formation of a stable emulsion; total volume ca. 400 ml.). After evaporation of the combined ether extracts to 70 ml. in vacuo, the apparatus was filled with hydrogen and 0.5 N hydrochloric acid (700 ml.) was added to the residual solution. Then benzoyl luciferin was hydrolysed by being heated on a water bath at 100° for half an hour in hydrogen atmosphere. After cooling, the acid solution was extracted with ether to remove impurities, and then with butanol (several times, total volume ca. 200 ml.) to extract luciferin.

The modified Anderson's benzoylation method (above mentioned) was repeated again on the butanol extract. The resulting butanol solution of prified luciferin was neutralized with a saturated sodium bicarbonate solution, and evaporated in vacuo to a red residue.

In the meantime, there was prepared an apparatus for partition chromatography, which was made from a cellulose powder column (2.5×20 cm.), a hydrogen inlet tube, a funnel for addition of solvent to the upper end of the column, and three flasks at the lower end to fractionate eluates. The apparatus was first completely filled with hydrogen, and the cellulose powder column was washed with a developing solvent (ethyl acetate-ethanol-water, 5 : 2 : 3). The red residue was dissolved in a small amount of methanol (2 to 3 ml.), adsorbed on the column and then developed with the developing solvent. The developing band were as follows: 1. blue-fluorescent band, 2. faintly yellow band with bluish green fluorescence, 3. yellow band with yellow fluorescence, 4. blue-fluorescent band, 5. yellow band. The eluate of band 3 contained luciferin and was evaporated to drynes in vacuo. The residue was extracted with a small amount of methanol and evaporated again, when "purified" luciferin was obtained as an orange-colored powder.

The "purified" luciferin was dissolved in dilute methanol and centrifuged from any insoluble materials. The supernatant was acidified with hydrochloric acid and allowed to stand overnight in a desiccator in which hydrogen was substituted and somewhat depressured, upon which orange-red crystals of luciferin precipitated. Yield ca. 3 mg.

Quantitative estimation of luminescent activity of luciferin. — Determination of the luminescent activity was carried out by a modified Anderson's apparatus, which will be reported in a separate paper.

Preparation of luciferase solution for luminescent test of luciferin.—The dry residue of methanol extract of *Cypridina* (20 g.) was added to 200 ml. of water, the mixture was allowed to stand overnight and filterd. The filtrate was dialysed using cellophan for 24 hr, the dialysate was once frozen, remelted and filtered. The filtrate was diluted with water to 200 ml.

Oxidation of luciferin with ferricyanide.—To the methanol solution of luciferin was added saturated potassium ferricyanide solution, and the reaction mixture was centrifuged. The supernatant was evaporated to dryness in vacuo and the residue extracted with a small quantity of methanol to measure the ultraviolet absorption spectrum (Fig. 4).

Preparation of Oxyluciferin A and B.—One drop aqueous ammonia was added to the luciferin solution and allowed to stand overnight. The reaction mixture was evaporated to dryness and chromatographed on a cellulose powder column (solvent system; ethylacetate-ethanol-water, 5 : 2:3). Oxyluciferin A and B were eluted in separate fractions. Crystalline oxyluciferin A was obtained by evaporating fraction A, dissolving the residue in water and acidifying with hydrochloric acid; the crystals were re-crystallized from a mixture of methanol and diluted hydrochloric acid. Oxyluciferin B was not obtained crystalline. The ultraviolet absorption spectra are shown in Fig. 5. Oxyluciferin A and B are easily soluble in methanol and ethanol, but insoluble in benzene and ether.

Hydrogenation of luciferin.—After measurement of the UV absorption spectrum, the methanol solution of crystalline luciferin (ca. 50 γ in 2 ml.) was hydrogenated with ca. 0.1 mg. of Adams' platinum oxide catalyst. When the solution became almost colorless (ca. 24 hr.), the catalyst was removed by filtration and the UV absorption spectrum of the filtrate measured (Fig. 6). The amount of absorbed hydrogen could not be measured because of its minute quantity.

General procedure for hydrolysis. — *Acid hydrolysis.* — The subtsance to be hydrolysed (100—200 γ) as heated with 0.5 ml. of 4 N hydrochloric acid at 100—105° for 12 hr. in a sealed glass tube, and the hydrolysate evaporated to dryness in vacuo.

Alkali hydolysis.—The substances (100—200 γ) was heated with 0.5 ml. of 10% barium hydroxide at 100—105° for 12 hr., the hydrolysate was diluted with equal volumes of water and slightly acidified with diluted sulfuric acid. After being heated on a water bath for a few minutes, the precipitated barium sulfate was contrifuged, and the supernatant was evaporated in vacuo. The hydrolysates were analysed by means of paper chromatography.

Paper chromatography of the hydrolysates.—Toyo filter paper No. 51 was used for this purpose (ascending method). Solvent butanol-acetic acid-water (4 : 1 : 1) or 2,6-lutidine-water (3 : 2).

The authors express their gratitude to Dr. Y. Haneda, the Curator of Yokosuka Museum, and Professor N. Nakamura, Tokyo University, for collection of *Cypridina*, and to Professor T. Sato, Nagoya University, for taking the photographs of the crystals.

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