STUDIES ON BIOLUMINESCENCE

II. The Partial Purification of Cypridina Luciferin

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Kanda has used several methods for purifying *Cypridina* luciferin (1–3), from the last of which he has reported a crystalline product. His method (2) when repeated with 10 gm. lots concentrated the luciferin from 15 to 30 times. His last method (3) was less successful because most of the luciferin was extracted from the final benzene solution or oxidized, by the recommended washing with "large amounts" of water. By making use of this extractability of luciferin from benzene solutions or residues by 0.5 M hydrochloric acid instead of water, particularly in the presence of small amounts of ethyl alcohol, and the 10 or 20 to 1 distribution ratio of luciferin between N-butyl alcohol and the acid, preparations 200 or 300 times as active as the initial dry *Cypridina* were obtained. Results were similar when the luciferin was precipitated from the dilute acid solutions, alcohol free, with an excess of flavianic acid. However, losses from oxidation and incomplete separations were large. Also, although Kanda considered the benzene solubility as good support for his suggestion that luciferin is a phospholipid, it would go into benzene only under limited conditions and much of the behavior indicated that other materials present played an important part both in the transfer into and out of benzene.[1]

[1] For instance when a benzene solution was shaken with an equal volume of HCl, no alcohol being present, less than half of the luciferin transferred to the HCl during a few minutes of shaking. However, when once in the HCl no appreciable quantities have ever been found to transfer back from it into pure benzene. On the other hand although luciferin is extracted much more slowly from the original powder by alcohol than by water, when once extracted the greater solubility in any of the lower alcohols compared with benzene, ether, or aqueous solutions is the most striking solubility characteristic up to any degree of purity so far obtained.

The idea of purifying oxyluciferin (4–6) failed when the several attempts to reduce it with H_2 and platinized asbestos or sodium hydrosulfite ($Na_2S_2O_4$), produced a very small percentage of reduction. In HCl solutions, using $Na_2S_2O_4$, considerable apparent reversal was finally obtained but only within a narrow range of reagent concentrations and when the luciferin was in the oxidizing medium, ceric sulfate, for about a minute or less.

A more successful approach was opened by preparing apparent derivatives of luciferin, more stable in the presence of oxygen than luciferin itself. Under some conditions either acetic anhydride or benzoyl chloride produced such, largely inactive, forms of luciferin. Most of the acetylated luciferin was reactivated by cold 0.5 M HCl. Up to 86 per cent of the benzoylated luciferin was reactivated by hot 0.5 M HCl in the absence of oxygen but in a procedure certainly permitting some losses.[2] The properties of this latter compound have made it very useful in the purification work. For the rest, conditions and solvents have been chosen that have been found to allow a minimum velocity of oxidation of the free luciferin.[3]

Procedure

The method of estimating the quantity of luciferin present was that previously described (7) except that runs to be compared were made in the presence of a constant amount of sodium chloride. This was found desirable because of the

[2] Although it is realized that absolute proof of compound formation has not been obtained, the evidence presented by the reversible inactivation of the light-producing substrate by two compounds under conditions where an active hydrogen would be removed and returned, the striking apparent change in solubility, and the greatly increased resistance to oxidation by atmospheric oxygen, is sufficiently strong to justify thinking of it in those terms until something inconsistent with such a view appears.

[3] The method of extraction, the degree of purity, and in some cases the concentration of luciferin were found to influence enormously its rate of oxidation in a given solvent. In addition smaller variations of unknown origin were apt to occur. The rate of oxidation previously given (7) for certain hydrochloric acid solutions is, therefore, not general. Some experiments indicated that at least a part of this complicated behavior was due to other materials present in the various preparations. That the rate of loss of luciferin was actually sensitive to some compounds was shown by the great increase in rate obtained after the addition of small amounts of hydroquinone and especially cuprous chloride to certain solutions.

surprisingly large specific effect of very small concentrations of sodium chloride on the total light emitted, in spite of the 0.067 M phosphate always present in the reaction mixture. The final concentration, including any sodium chloride formed from the neutralization of hydrochloric acid, was usually about 0.011 M. The concentration of luciferase added was 0.8 per cent throughout. The volumes of luciferin used were such as to make the total light emitted by samples from different solutions of the same order of magnitude.

The whole, dry organisms were powdered in a ball mill and extracted in a soxhlet with ether and then with benzene. The luciferin remained in the solid and was stable in this condition in the absence of water.

Since the quantity of material is limited, originally only 10 gm. lots were used from this point on. Later this was increased to 50 gm. with the same procedure except as indicated.

Two different methods of extraction of luciferin from this preextracted material have been used successfully on the small lots. In the first method the *Cypridina* powder was placed in a Buchner funnel and the luciferin extracted by slowly pouring through it 300–400 ml. of a boiling 0.1 M NaCl solution containing 10 per cent ethyl alcohol, keeping the solid covered with liquid at all times. The filtrate containing the luciferin, filtered into a chilled mixture of 2.5 M HCl and butyl alcohol. This is a modification of Harvey's original method. The ethyl alcohol increased the amount extracted and the rest of the procedure quickly transferred the luciferin from the extremely unstable hot aqueous solution to the much more stable cold acid or butyl alcohol solution. The second method was to follow Kanda and extract with methyl alcohol. This was slower but gave somewhat better yields and was less liable to accidents. It was used for all of the later work.

The extractions were made with from 5 to 10 ml. of methyl alcohol per gram of *Cypridina* powder and continued under hydrogen for about 24 hours. The solution was filtered and the residue washed with methyl alcohol. This step must be rapid as considerable losses, particularly with the 50 gm. lots, occurred here. 25 ml. of N-butyl alcohol were added to the filtrate and the methyl alcohol removed from the deaerated solution *in vacuo* at room temperature. The residual liquid was decanted from the precipitate which formed and the precipitate washed with several 15 ml. portions of butyl alcohol. The suspension was centrifuged down each time. Since it was difficult to wash this precipitate free of luciferin it was sometimes dissolved in 0.5 M HCl and the luciferin extracted from that solution with butyl alcohol. This was easier and faster but considerably more of a red pigment was carried into the butyl alcohol. The mixed butyl alcohol extractions, totaling 50 or 60 ml., were chilled and then benzoylated with 2 ml. of benzoyl chloride. The relatively unfavorable conditions for benzoylation were probably an advantage from the purification standpoint. After 15 minutes in the ice bath the solution was tested for free luciferin. If it was present to more than 1 or 2 per cent, more benzoyl chloride was added. If not, the solution was washed with three successive equal volumes of water during about one-half hour to allow time for the excess benzoyl chloride to hydrolyze. The butyl alcohol fraction

was then dissolved in ten volumes of water. A highly colored material, apparently dissolved in the butyl benzoate formed in the above reaction, remained as a separate phase. The inactive luciferin in this suspension was then extracted with 80 ml. of ether[4] followed by three portions of 40 ml. each. When a stable emulsion formed, as was frequently true during the first extraction, it was broken by centrifuging. The several ether fractions were mixed and the ether removed *in vacuo* without deaeration. The residual solution of inactive luciferin in butyl alcohol was then mixed with 250 ml. of 0.55 M HCl and carefully deaerated. This suspension was heated with a water bath kept between 95–100° for 1 hour and then cooled in an ice bath. The hydrogen was left bubbling through during these operations. When thoroughly cooled, the mixture was washed with ether as above except that centrifuging was not ordinarily necessary. Most of the pigment, which was concentrated in a small film on top of the aqueous solution, went into the ether. Most of the luciferin remained in the aqueous phase although up to 4 per cent appeared in the first ether washing. No significant amounts appeared in the later washings. These washings also removed the butyl benzoate and the benzoic acid, formed from the excess benzoyl chloride, quite completely from the aqueous phase. The luciferin was extracted from the HCl solution by 40 ml. of N-butyl alcohol followed by four portions of 20 ml. each. The benzoylation and hydrolysis were then repeated in essentially the same way. Sometimes more benzoyl chloride was required the second time because of a greater volume of butyl alcohol and the presence of some HCl.

Where obvious accidents had not occurred about an 80 per cent yield was obtained for each complete cycle of benzoylation, hydrolysis, and return to butyl alcohol. Apart from that mentioned above, no discarded fractions contained more than 1 or 2 per cent of the luciferin present. Much of the loss was probably from oxidation although some may have occurred during the heating with HCl.

The final butyl alcohol solutions were yellow, as were many of the discarded fractions, and had an activity in arbitrary light units[5] of from 40,000 to 60,000 per gm. of dry weight. After one benzoylation cycle the activity was from 13,000 to 30,000 units. The dry *Cypridina* used for these preparations had in the same units an activity from 21 to 33. This was based on the most active initial methyl alcohol extracts obtained from small samples of a given bottle of material.

[4] Great care must be taken to avoid oxidizing agents in the ether which may easily attain a sufficient concentration to destroy much of the luciferin. Some lots may be kept safely for months after opening, while others apparently must be purified the same day as used.

[5] This unit was a reading of 1 volt on the potentiometer in the particular experimental arrangement previously described (7).

SUMMARY

Some solubility, oxidation, reduction, and compound-forming characteristics of extracts of *Cypridina* luciferin have been presented.

A method of purification has been described which increased the amount of luciferin per unit of dry weight, as measured by the total light emitted, to about two thousand times that in the dry starting material. The best yields were from 50 to 65 per cent.

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