STUDIES ON THE OXIDATION OF LUCIFERIN WITHOUT LUCIFERASE AND THE MECHANISM OF BIOOLUMINESCEENCE.

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In five groups of luminous animals it can be shown that luminescence is connected with the oxidation of a substance, luciferin, in aqueous solution, in presence of an enzyme, luciferase, and dissolved oxygen. In the mollusc, Pholas dactylus, Dubois (1) has been able to oxidize Pholas luciferin with luminescence by KMnO₄, H₂O₂, BaO₂, PbO₂, and hypochlorites. I have been unable to oxidize with luminescence the aqueous solutions of luciferin from an ostracod crustacean, Cypridina hilgendorfii, by the above oxidizing agents and others to be described below. Oxidation of Cypridina luciferin takes place but no light appears. The oxidation product (oxyluciferin) formed can be reduced to luciferin again, whether luciferase is present or not, by nascent hydrogen, sodium hydrosulfite, and other reducing agents (2, 3).

I have already (1918) given evidence to indicate that the reaction is of the following type:

\[ 2 \text{luciferin (LH}_2\text{)} + O_2 = 2 \text{oxyluciferin (L) + 2H}_2\text{O} \]

similar to the autooxidation of a leuco-dye, rather than the auto-oxidation of a substance like benzaldehyde, by peroxide formation. We may, as further evidence against the view that luciferin oxidizes with peroxide formation, state that diphenylamine, a negative catalyst for the oxidation of benzaldehyde, has no effect on the luminescence of an oxidizing mixture of luciferin and luciferase. As oxyluciferin is readily reduced by nascent hydrogen at cathodes (4) one might suppose that luminescence of luciferin could be observed at anodes, where nascent oxygen is formed. The following combinations of electrolytes and electrodes all gave negative results with potentials varied from 0 to 6 volts: (1)
Oxidation of Luciferin

Luciferin in Na₂SO₄ solution, anode and cathode of Mn, Al, Ag, Ni, Sn, Cu, Fe, Pb, Zn, Cd, Pt; (2) luciferin in sea water, same electrodes as above; (3) luciferin + CaCO₃ powder (to neutralize acid) in Na₂SO₄ solution, Pt cathode and anodes of platinized Pt, palladinized Pt, and palladinized Au; (4) luciferin in NaBr, NaCl, and NaI solution, Pt electrodes; (5) luciferin in NaHCO₃ solution, Pt electrodes; (6) luciferin in 0.5 M phosphate buffer, pH = 7.3, Pt electrodes.

Oxidation by colloidal solutions of metals and metal hydroxides all gave negative results also. These included Pt, Rh, Ir, Ru, V, Au, Ag, Ni, Fe, Co, and Cu, mostly prepared by the Bredig arc or by reduction methods. Luciferin with colloidal Pt or Pd through which pure oxygen is passed gives no light, nor do platinized or palladinized surfaces saturated with oxygen and placed in luciferin solution, although the same surfaces saturated with hydrogen readily reduce oxyluciferin. Platinized or palladinized surfaces in air or oxygen do oxidize luciferin readily but no luminescence appears.

There is also no luminescence when we add directly to luciferin small amounts of various oxidizing agents such as KMnO₄, K₂Cr₂O₇, CrO₃, H₂O₂, Na₂O₂, BaO₂, MnO₂, PbO₂, V₂O₅, titanium oxide (white), ozone, benzoyl peroxide, disuccinyl peroxide, Ag₂O, HgO, FeCl₃, ferricyanides, chlorates, perchlorates, persulfates, perborates, Mn or Cu salts, active catalytic (for 2 CO + O₂ = 2CO₂) Fe₂O₃, Cl₂, Br₂, or I₂, hypochlorites, hypobromites, or hypiodites. Again I must emphasize the fact that, although many of these substances oxidize luciferin, no luminescence appears. The oxidation-reduction potential of the luciferin-oxyluciferin system has been discussed by me in a recent paper (3).

Insulin is also without action on luciferin or luciferase, or the luminescence resulting from a mixture of the two.

Finally, luciferin cannot be oxidized with luminescence by oxidases, peroxidases, or various plant and animal extracts. Indeed, the luciferase of other luminous species will not oxidize Cypridina luciferin with luminescence unless the luminous animal is very closely related (5).

Some biological substances, like peptone, will luminesce with strong alkali and bromine water but crude luciferin solution gives no more luminescence under these circumstances than does au
extract of *Cypridina* containing only oxyluciferin. It is not luciferin but some other organic material in the luciferin solution which luminesces. This sort of luminescence can have no significance for the subject under discussion.

Only when luciferase is added directly to luciferin solution does luminescence occur, but even then there is no interchange of charges between luciferin and luciferase such as occurs in the oxidation of iodides by bromine water. This can be shown by a simple procedure similar to that of Ostwald’s "action at a distance" experiment. Luciferin in Na$_2$SO$_4$ solution is placed in one vessel connected with a second vessel by a salt bridge and Pt electrodes. In the second vessel is placed luciferase in Na$_2$SO$_4$ solution. No luminescence at the electrode in luciferase or in luciferin ever appears. Light emission must be an indirect result of the oxidation of luciferin, otherwise some luminescence should appear in this experiment.

All these negative results lead to the conclusion that luciferin is not the source of the luminescence but merely an accessory, and that luciferase is the body emitting light, a view I expressed (6), after study of the color of the luminescence resulting from a mixture of luciferin and luciferase prepared from different animals. Luciferin from a firefly whose light is reddish mixed with luciferase from a firefly whose light is yellow results in a yellow luminescence and *vice versa*. The color of the light from such mixtures is therefore determined by the animal supplying the luciferase (7).

It would seem that luciferase must be excited to luminesce by energy supplied by the oxidation of luciferin, in line with the view of Kautsky and Zocher (8) on luminescence in silicon compounds.

According to this view, luminescence is due to excited or energy-rich molecules returning to the normal state, just as we know luminescence of atoms is due to the change from the excited (energy-rich) to the normal state. Siloxene is a silicon compound whose oxidation by KMnO$_4$, H$_2$O$_2$, etc. results in a reddish luminescence. The energy from the oxidation of some siloxene molecules is transferred to others, converting them to energy-rich molecules which luminesce on return to the normal state. Siloxene on exposure to ultra-violet light will also exhibit reddish luminescence (fluorescence). In this case the energy of the ultra-violet radiation goes to form energy-rich molecules.
Siloxene oxidizes in several steps, finally forming silicic acid. If it is so completely oxidized that its own luminescence is no longer excited, and rhodamine B adsorbed on the oxidized siloxene, treatment of the material with acid permanganate will result in a yellow luminescence characteristic of the yellow fluorescence spectrum of rhodamine B. The oxidized siloxene molecules have transferred some of their energy to rhodamine B, resulting in luminescence.

Can oxidized siloxene transfer some of its energy to luciferase (or luciferin), causing luminescence in these substances? To test this possibility I have mixed well oxidized siloxene hydroxide\(^1\) with luciferase (and also with luciferin) and then added acid permanganate solution to the mixture, but no luminescence resulted in either case. As this siloxene hydroxide added to rhodamine B gave good luminescence on mixing with acid permanganate,\(^2\) I conclude that neither luciferin nor luciferase molecules can be excited to luminesce in this way. Luciferin or luciferase adsorbed on Patrick’s silicic acid jel or permutit also give no luminescence when mixed with acid permanganate. It is rather disappointing to find that siloxene is unable to transfer its energy of oxidation to luciferase and cause this substance to luminesce.

Thinking that fluorescent\(^3\) dyes might be excited to luminesce in the presence of luciferin undergoing oxidation, I have mixed luciferin with rhodamine B, fluorescein, quinine sulfate, and eosin and shaken in air. No luminescence appeared and there was also no luminescence on adding acid permanganate. The above fluorescent dyes also show no luminescence when mixed with luciferase solution.

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\(^1\) The material was kindly prepared for me as the bromide by Dr. S. O. Miller and Mr. J. R. Bates of the Chemistry Department. When water is added to the bromide, siloxene OH is formed. It is then treated with successive small amounts of acid permanganate till no more luminescence appears.

\(^2\) Acid permanganate will give a faint luminescence on being mixed with aqueous rhodamine B, but if the rhodamine B is first mixed with oxidized siloxene OH, a bright yellow luminescence occurs when acid permanganate is added.

\(^3\) The fact that these dyes fluoresce is an indication that their molecules are easily excited by radiation and might be excited by the energy of chemical reactions.
We may inquire finally whether luciferase can be excited to luminesce (fluoresce) by radiation of any kind. While it is true that the luminous organs of some forms fluoresce in near ultra-violet light ($\lambda = 0.40$ to $\lambda = 0.30$) (9), Cypridina material shows no fluorescence on exposure to near ultra-violet, x-ray, or cathode rays.5

The only condition under which I have ever noted any luminescence of luciferin without luciferase is in non-aqueous solvents. If dry powered Cypridina are extracted with 95 to 99 per cent alcohol (in which luciferin but not luciferase is soluble), filtered through several layers of filter paper, and this alcoholic luciferin solution treated with ozonized turpentine or minute amounts of solid KMnO4, disuccinyl peroxide, K2Fe(CN)6, PtCl4, PdCl2, and Ca hypochlorite, a faint luminescence will result. The alcohol alone gives no luminescence with those reagents and an alcohol extract of dried Cypridina whose luciferin had been oxidized also gave no luminescence with KMnO4. Toluene and acetone extracts of dried Cypridina give no luminescence with KMnO4. Luciferase is insoluble in both solvents; luciferin is soluble in acetone but not in toluene.

Many oxidizing agents, like solid quinone, MnO2, PbO2, Na perborate, BaO2, KClO4, FeCl3, iodine, and bromine gave no luminescence with the alcoholic extract. Finally, it should be noted that the alcoholic luciferin extract will luminesce faintly if merely heated to about 70°, while the addition of oxidizing agents gives a much brighter luminescence. Aqueous solutions of luciferin, even if luciferase is also present, will not luminesce at this temperature. It is possible that in alcohol the oxidation proceeds at the surface of luciferase particles, which pass the filter paper, suspended in the liquid. The luminescence is not to be compared in brightness with that in aqueous solutions with luciferase and I mention these results merely as a matter of record, as the only cases where Cypridina luciferin alone will luminesce. I do not

4 Exposure of Cypridina powder to ultra-violet light at liquid air temperature results in no pronounced fluorescence.

5 A test kindly made for me by Dr. W. D. Coolidge of the General Electric Company. Cathode rays outside the tube from 1 milliamper at 200,000 volts for 10 seconds caused no luminescence of dried Cypridina powder or of luciferase or luciferin solution.
believe they are necessarily significant for the theory of bioluminescence because so many organic substances luminesce on oxidation with strong oxidizing agents.

To say that luminescence intensity depends on reaction velocity of oxidation of luciferin is not sufficient. Luciferin can be oxidized by oxygen very rapidly at high temperatures, yet no luminescence appears in the absence of luciferase; luciferin can also be oxidized rapidly by K3Fe(CN)6 in the presence of luciferase but absence of oxygen, yet no luminescence appears. Only if both oxygen and luciferase are present, is luminescence dependent on reaction velocity (10).

Luciferase apparently plays two roles: (1) to catalyze the oxidation of luciferin and (2) to supply easily excited molecules (A') which emit light (luminescence) on return to the normal state (A). Luciferin is a substance whose energy of oxidation excites the luciferase. We may imagine the steps in the process as follows:

\[
\text{Luciferin (LH}_2\text{)} \rightleftharpoons \text{oxyluciferin (L) + H}_3 = 15 \text{ calories} \\
\text{H}_3 + \frac{1}{2} \text{ O}_2 = \text{H}_2\text{O'} + 69 \text{ calories}
\]

where \(\text{H}_2\text{O'}\) represents an energy-rich water molecule.

\[
\text{H}_2\text{O'} + \text{luciferase (A)} = \text{H}_2\text{O} + \text{excited luciferase (A')}
\]

\[
\text{A'} = \text{A} + \text{hv}
\]

This view again leads to the question why other easily oxidized substances (like methylene white) cannot take the place of luciferin when oxidized in the presence of luciferase, provided their oxidation supplies sufficient energy. As a matter of fact it is impossible to obtain light on mixing luciferase with autooxidizable substances, although a large number has been tested. In reply we can only say that the excitation of luciferase is specific as so many other biological phenomena are specific where proteins (luciferase behaves like a protein) are concerned.

It is possible that specificity depends on adsorption. Oxyluciferin cannot be completely separated from luciferase after dialysis through parchment for 12 days, although much of the oxyluciferin will dialyze away. Luciferase cannot pass through parchment. Kautsky and Zocher found that fluorescent dyes adsorbed on siloxene surfaces were the ones that could be excited to luminesce. It is possible that the autooxidizable substances tested were not adsorbed by luciferase and so were unable to transfer their energy to the luciferase.
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SUMMARY.

Oxidation of luciferin at anodes of various metals by nascent oxygen or oxidation by colloidal platinum or palladium and oxygen or other oxidizing agents never results in luminescence in the absence of luciferase.

Luciferase solution electrically connected with luciferin solution (by platinum electrodes in metallic contact and a salt bridge) shows no luminescence.

Luciferin or luciferase mixed with oxidized siloxene hydroxide and oxidized with acid permanganate does not luminesce. Fluorescent dyes do not luminesce in the presence of oxidizing luciferin alone or of luciferase alone.

Luciferin in alcohol luminesces slightly on heating or on addition of KMnO₄, disuccinyl peroxide, ozonized turpentine, and some other oxidizing agents. These are the only conditions in which I have ever observed luciferin luminescence without luciferase in solution.

Luciferase rather than luciferin is the source of the light in bioluminescence. The most probable hypothesis is that luminescence appears as a result of the excitation of luciferase molecules by the energy of oxidation of the luciferin. Luciferase plays in addition the part of catalyst, increasing the velocity of oxidation of luciferin. This hypothesis cannot be considered as proved until some oxidizing substance is found other than luciferin which can transfer its energy to luciferase with the production of light.

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