THE OXIDATION-REDUCTION POTENTIAL OF THE LUCIFERIN-OXYLUCIFERIN SYSTEM.

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Oxygen is necessary for the luminescence of the ostracod crustacean, Cypridina, and most luminous animals. A substance, luciferin, is oxidized to oxyluciferin in the presence of a catalyst, luciferase. The oxyluciferin can be reduced to luciferin again, so that one is justified in speaking of a luciferin \( \Rightarrow \) oxyluciferin system. When the words luciferin, oxyluciferin, and luciferase are used in this paper, I refer only to the crude impure solutions from Cypridina hilgendorfii. For the preparation and isolation of these substances the reader is referred to my book (1920) or earlier papers (1919, b).

We should like to know more about the nature of the oxidation of luciferin. First, it must be understood that the word oxidation is used for a number of quite different processes. We may recognize at least two distinct types of reversible oxidations.

(1). The oxidation of hemoglobin to oxyhemoglobin, in which the oxygen is actually bound, but very loosely. This well known change depends on the concentration or pressure of the oxygen and is more properly called an oxygenation than an oxidation. Such oxygenations do not possess an oxidation-reduction potential. Luciferin is not to be compared with hemoglobin because removal of the oxygen from an oxyluciferin solution will not bring about the formation of luciferin, as it will the formation of hemoglobin from oxyhemoglobin.

(2). The oxidation of hemoglobin to methemoglobin, in which no oxygen is actually bound in the methemoglobin molecule although oxygen may be used up in its formation. However, the reaction may go on in complete absence of oxygen, by oxidation with \( \text{K}_2\text{Fe} (\text{CN})_4 \). Conant (1923–25) and his coworkers have thrown new light on this system, which indicates that it has a definite oxidation-reduction po-
If hemoglobin in acid solution is represented by $H_2Hb^{--}$, then oxyhemoglobin is $H_3HbO_2^{--}$ and methemoglobin is $H_2Hb^{=}$. A change in valence is the essential difference in the hemoglobin-methemoglobin transformation.

Other oxidations in which the essential change is one of valence necessitate also the removal of hydrogen, such as the oxidation of a leuco dye to the dye itself. Oxygen plays the rôle of hydrogen acceptor, forming water. Thus, methylene white (leucomethylene blue), $MH_2$, and safranin white (leucosafranin) oxidize to methylene blue, $M$, or safranin spontaneously in the air by loss of hydrogen; $MH_2 + O = M + H_2O$. As Clark and his coworkers (1923–26) have shown, these reactions have a definite oxidation-reduction potential also.

I have previously pointed out (1918–19, a) that the oxidation of luciferin bears some resemblance to the oxidation of a leuco dye and presumably has an oxidation-reduction potential. The equation might be represented thus:

$$LH_2 \ (\text{luciferin}) \rightleftharpoons L \ (\text{oxyluciferin}) + H_2 \quad H_2 + O = H_2O.$$

For a more complete discussion of oxidation-reduction potential the reader is referred to the papers of Clark and collaborators (1923–26), Conant (1926), and Conant and Cutter (1924). Suffice it to say here that oxidizing and reducing agents can be arranged in a series of varying strength, with strongest reducing agents at one end and strongest oxidizing agents at the other. The strength is conveniently measured by the potential which the oxidizing or reducing agent gives in contact with some noble indifferent metal, like gold or platinum. Reproducible potentials are observed only if the reaction (reductant $\rightleftharpoons$ oxidant) is reversible, and the exact value varies with the $H$ ion concentration and the ratio of oxidized to reduced substance. The symbol, $E'_o$, is used for the potential of such an oxidizing agent as methylene blue at a definite $pH$ and with chemically equivalent amounts ([red.]/[oxid.] = 1) of methylene blue (oxidant) and methylene white (reductant) in solution. The accompanying table gives the $E'_o$ values for a number of oxidizing or reducing agents at a $pH = 7.7$, a favorable value for luminescence of luciferin.
The H electrode represents the reducing power of hydrogen gas under one atmosphere pressure in presence of a catalyst like finely divided platinum. Many reducing agents like sulfides and chromous chloride stand above the H electrode and hydrosulfites and titanous salts are near it. The oxygen electrode represents the oxidizing power of oxygen under conditions similar to those of the H electrode. Many oxidizing agents like permanganates and dichromates come below the oxygen electrode.

<table>
<thead>
<tr>
<th>Substances</th>
<th>$E'$ Value at pH = 7.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H_2$ electrode</td>
<td>-0.46</td>
</tr>
<tr>
<td>$Na_2S_2O_3$ (Na hydrosulfite or hyposulfite)</td>
<td>-0.32 (approximately)</td>
</tr>
<tr>
<td>Safranin</td>
<td>-0.30</td>
</tr>
<tr>
<td>Anthraquinone $\beta$ Na sulfonate</td>
<td>-0.26</td>
</tr>
<tr>
<td>Anthraquinone 2-6-di Na sulfonate</td>
<td>-0.22</td>
</tr>
<tr>
<td>Indigo monosulfonate</td>
<td>-0.20</td>
</tr>
<tr>
<td>&quot; disulfonate (indigo carmine)</td>
<td>-0.15</td>
</tr>
<tr>
<td>&quot; trisulfonate</td>
<td>-0.115</td>
</tr>
<tr>
<td>&quot; tetrasulfonate</td>
<td>-0.075</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>-0.01</td>
</tr>
<tr>
<td>1-4-naphthoquinone</td>
<td>+0.03</td>
</tr>
<tr>
<td>Lauth's violet</td>
<td>+0.04</td>
</tr>
<tr>
<td>Methemoglobin (anaerobic)</td>
<td>+0.08 (approximately)</td>
</tr>
<tr>
<td>1-2-naphthoquinone</td>
<td>+0.13</td>
</tr>
<tr>
<td>2-6-dichlorindophenol</td>
<td>+0.20</td>
</tr>
<tr>
<td>Quinhydrone (equimolecular hydrochinone and quinone)</td>
<td>+0.24</td>
</tr>
<tr>
<td>K ferricyanide</td>
<td>+0.43</td>
</tr>
<tr>
<td>$O_2$ electrode</td>
<td>+0.76</td>
</tr>
</tbody>
</table>

Where does the luciferin-oxy luciferin system stand in this scale? One may gain some idea of the position of the luminescent system in the potential scale by finding what substances, whose potentials are known, will oxidize luciferin or reduce oxy luciferin in absence of oxygen. We know that oxygen and platinum will oxidize luciferin and that hydrogen and platinum will reduce oxy luciferin (Harvey, 1923), so that the position of luciferin-oxy luciferin must lie between the oxygen and hydrogen electrodes. Some years ago (January, 1922) I attempted to oxidize luciferin with light production in absence of oxygen by cystine, methylene blue, quinone, potassium ferricyanide,
and other substances, but always with negative results, as far as the appearance of luminescence is concerned.

Recently I have confirmed these results. I have also carried out a more systematic study to limit the potential of the luciferin-oxyluciferin system. It appears that luciferin may be readily oxidized in absence of oxygen although no luminescence occurs. It is all important to distinguish between the oxidation of luciferin with luminescence and without luminescence. Oxidation without luminescence may occur in absence of gaseous oxygen or in absence of luciferase (with gaseous oxygen) \(^1\) but oxidation with luminescence never occurs without oxygen and without luciferase. I shall return to this point later.

Rapid oxidation of luciferin without luminescence can be demonstrated very clearly and simply by adding to a luminescent mixture of luciferin and luciferase in phosphate buffer (pH = 7.7), weak \(K_3Fe(CN)_6\) solution drop by drop. On sufficient addition of \(K_3Fe(CN)_6\) the luminescence will disappear without any previous increase in brightness. All the luciferin has been oxidized by the ferricyanide. On now adding weak sodium hydrosulfite (\(Na_2S_2O_4\)) solution to the mixture and then shaking with air, the luminescence will return. \(Na_2S_2O_4\) has reduced the oxyluciferin formed by the ferricyanide, and with luciferase and oxygen present light appears. The oxidation and reduction can be brought about many times by successive additions of ferricyanide and hydrosulfite, so there can be no possibility that these reagents injure the luciferase.

It is obvious that one might titrate luciferin quantitatively with ferricyanide, using luminescence with luciferase as the end-point indicator, but some experiments along these lines show that there is no sharp end-point and lead me to believe that little significance is to be attached to such figures until luciferin can be obtained in the

\(^1\) The autoxidation of luciferin by oxygen in absence of luciferase might be compared with the autoxidation of benzaldehyde by oxygen. It is interesting to note in this connection that small amounts of hydrochinone or diphenylamine, which so markedly prevent (negative catalysis) the oxidation of benzaldehyde, have practically no inhibiting effect on the oxidation of luciferin, as judged by the luminescence. Quinone in the hydrochinone accelerates the spontaneous oxidation of luciferin. \(KCN\), which inhibits respiratory oxidations, does not affect the luminescence of luciferin.
pure state. There are probably other easily oxidizable substances in my solutions besides luciferin.

The experiment with ferricyanide and hydrosulfite shows at once that the potential of luciferin-oxyluciferin lies between these two substances. Since the oxidized form of any substance lower in the series of Table I will oxidize the reduced form of anything above, while the reduced form of anything above will reduce the oxidized form of anything below, we have only to test oxidation of luciferin and reduction of oxyluciferin by appropriate agents in the table.

There is only one point to guard against. If two substances lie close together in potential, the reaction between them will not be complete, for the potentials given are for equimolecular parts of oxidant and reductant, \([\text{red.}] / [\text{oxid.}] = 1\). For a ratio of \([\text{red.}] / [\text{oxid.}] = 1/99\) the potential would be shifted 50 millivolts toward the positive side, while for a ratio of \([\text{red.}] / [\text{oxid.}] = 99\), the potential would be shifted 50 millivolts toward the negative side. There is thus a certain band, about .1 volt wide, where oxidation and reduction will not be complete. By selecting substances in Table I fairly well separated from each other, we can limit the potential of luciferin. I have taken quinhydrone, 2-6-dichlorindophenol, 1-4-naphthoquinone, methylene blue, indigo tetrasulfonate, indigo disulfonate, anthraquinone \(\beta\) Na sulfonate, anthraquinone 2-6-di Na sulfonate, and safranin and carried out the experiments in the following manner.\(^2\)

Two test-tubes, \(A\) and \(B\), are connected as shown in Fig. 1 by glass tubes through rubber stoppers. The tube, \(C\), has an enlargement at \(D\) holding a small perforated platinum disk above which asbestos may be packed to serve as a filter. In testing for reduction of oxyluciferin, oxyluciferin + luciferase in \(M/10\) phosphate (pH = 7.7) buffer is placed in \(B\) and the reducing substance, say dilute safranin in \(M/10\) phosphate (pH = 7.7) buffer + some platinized asbestos in \(A\). A stream of pure hydrogen freed of oxygen by passage over red hot platinized asbestos in a quartz tube, is then passed through the tubes, \(A\) and \(B\), for \(\frac{1}{2}\) hour or more. The safranin is reduced to colorless safranin white by the hydrogen-platinum and all oxygen driven

\(^2\)I am deeply indebted to Dr. Keith Cannan for samples of quinhydrone, dichlorindophenol, and indigo tetrasulfonate and to Dr. J. B. Conant for samples of the naphthoquinones and the anthraquinone sulfonates.
from the system. Then Tube A is inverted and its contents filter into B, the platinized asbestos being kept back by the asbestos filter. This is important since oxyluciferin is reduced by hydrogen-platinum.

After mixing, oxidation of safranin will be indicated by reddening of the safranin white in the hydrogen atmosphere and reduction of oxyluciferin will be indicated by luminescence when air is admitted to the tube. There is undoubtedly some reduction of oxyluciferin by reduced safranin (which becomes red in the hydrogen atmosphere after mixing) and the anthraquinones, since luminescence is obtained when air is admitted to the mixture in B. A control tube of oxyluciferin and luciferase, but without addition of safranin white, or reduced anthraquinones in a hydrogen atmosphere gives no luminescence when air is admitted. Of the remaining dyes, indigo disulfonate, tetrasulfonate, methylene blue, 1,4-napthoquinone, and dichlorindophenol bring about practically no reduction of oxyluciferin. This is the case when the solutions have been in contact for 5 minutes before admitting air. If allowed to stand 90 minutes before testing, reduced indigo tetrasulfonate may reduce slightly, as judged by a very faint luminescence. However, the reduced yellowish indigo tetrasulfonate does not become appreciably colored (as would occur if it was oxidized by oxyluciferin) even after 90 minutes contact, so that the slight reduction of oxyluciferin can be detected only because a very small amount of luciferin gives a sufficiently bright luminescence to be detected by the eye. In other words the luminescent test is an extremely delicate
one. I am somewhat in doubt as to the meaning of very faint luminescences since a few bacteria may have brought about the slight reduction on standing 90 minutes. Relying on experiments which show undoubted reduction, I am inclined to place the luciferin-oxyluciferin system in the region between anthraquinone 2-6-di Na sulfonate (-.22) and indigo disulfonate (-.15).

Attacking the problem from the other side, that of oxidation, we place in Tube B luciferin in phosphate buffer (pH = 7.7) and in Tube A oxidized dye in phosphate buffer (pH = 7.7). Tube C need not be a filter in this case. After a stream of pure hydrogen has been passed for ½ to 1 hour, A is mixed with B. If oxidation of luciferin has occurred, the dye should become colorless and the luciferin give no more light on mixing with luciferase in air. The latter change is the best test since other reducing substances than luciferin may be present in crude luciferin solution. Passing up the list of substances in Table I from the oxidized end, there is no doubt that quinhydrone will oxidize luciferin in absence of oxygen but dichlorindophenol has practically no action and 1-4-napthoquinone or methylene blue none. Judging from these experiments on oxidation of luciferin, the potential of the luciferin-oxyluciferin system would be somewhere between dichlorindophenol ($E'_o = + .2$) and quinhydrone ($E'_o = + .24$).

The attempt to approach the potential from the oxidation and reduction side gives rather divergent values. There is thus no very sharp potential that can be assigned to luciferin. By this method of "bracketing" we find the limits for a pH = 7.7 somewhere between −.22 and + .24 volt. Systems between these values neither reduce oxyluciferin nor oxidize luciferin. Perhaps that is due to poising action in a mixture of oxidizable and protein substances, for it must not be forgotten that luciferin solutions contain many other substances from the luminous animal besides luciferin.

If impurities are not obscuring the result, the luciferin-oxyluciferin system behaves like some of those described by Conant, which are irreversible. I say irreversible, although it is quite certain that the stronger reducing agents which lie near the hydrogen electrode will reduce readily. These include hydrosulfites, sulfides, chromium chloride (CrCl₂), titanous chloride (TiCl₃), and hydrogen formed at

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3 Similar to buffer action in acid-alkaline solutions (see Clark, 1923–26).
cathodes or from metals (aluminum amalgam and magnesium in ammonium salts are especially good methods) or in contact with Pt or Pd. At the same time it is just as certain that quinone, K ferricyanide, Br water, and strong oxidizing agents will oxidize luciferin. We are not dealing with such irreversible oxidations, as in the case of aliphatic aldehydes to acids, where the acid cannot be reduced to aldehyde no matter how strong the reducing agent; or such irreversible reductions, as in the case of dibenzoylethylene to dibenzoylethane, where the latter cannot be oxidized no matter how strong the oxidizing agent (Conant, 1926).

There is a certain range in which neither oxidation of luciferin nor reduction of oxyluciferin occurs. Perhaps we are justified in speaking of both apparent oxidation and apparent reduction potentials, the “apparent reduction potential” differing from the “apparent oxidation potential” (in Conant’s sense, 1926) by about .5 volt.

The preceding experiments also prove that oxidation of luciferin may occur without oxygen (and without luminescence). What are the conditions for oxidation with luminescence? We know that luciferase is necessary for luminescence. Are both luciferase and oxygen necessary, or will luminescence appear in presence of luciferase alone, if oxidation of luciferin is brought about by K₄Fe(CN)₆? It is found that both luciferase and oxygen are necessary, for on adding potassium ferricyanide solution free of oxygen to a mixture of luciferin and luciferase in absence of oxygen (and of course dark) no luminescence will appear, although the luciferin will be oxidized, as is proved by the absence of light when oxygen is later admitted to the vessel.

We may suppose that the oxidation of luciferin by oxygen at the surface of colloidal particles of luciferase results in luminescence. The light is characteristic of the luciferase rather than the luciferin, since the color of the luminescence may be shown to be dependent on the kind of luciferase used and not on the luciferin (Harvey, 1917, 1924, a). Will any other easily oxidizable compounds luminesce when oxidized by oxygen in presence of luciferase? All my attempts to find such substances have failed, although I have tested many hydroxy- and aminophenols, leuco dyes, cysteine, and reduced bodies of unknown composition formed by reduction in tissue extracts of various animals, yeast and bacterial cultures.
For luminescence of *Cypridina* it is therefore necessary to have luciferin, luciferase, and free oxygen dissolved in water. It makes no difference how rapidly luciferin is oxidized by oxygen (for instance at high temperatures), luminescence never appears unless luciferase is also present, nor will luminescence appear in presence of luciferase unless oxygen is the oxidizing agent. Therefore, high reaction velocity *per se* is not a necessity for luminescence. But if luciferase and oxygen are present, then the greater the reaction velocity, the brighter will be the luminescence (Amberson, 1922).

**SUMMARY.**

The oxidation-reduction potential of the *Cypridina* luciferin-oxyluciferin system determined by a method of “bracketing” lies somewhere between that of anthraquinone 2-6-di Na sulfonate ($E'_a$ at pH of 7.7 = -.22) which reduces luciferin, and quinhydrone ($E'_a$ at pH of 7.7 = +.24), which oxidizes luciferin. Systems having an $E'_a$ value between -.22 and +.24 volt neither reduce oxyluciferin nor oxidize luciferin. If the luciferin-oxyluciferin system were truly reversible considerable reduction and oxidation should occur between -.22 and +.24. The system appears to be an irreversible one, with both “apparent oxidation” and “apparent reduction potentials” in Conant’s sense. Hydrosulphites, sulfides, CrCl$_3$, TiCl$_3$, and nascent hydrogen reduce oxyluciferin readily in absence of oxygen but without luminescence.

Luminescence only appears in water solution if luciferin is oxidized by dissolved oxygen in presence of luciferase. *Rapid* oxidation of luciferin by oxygen without luciferase or oxidation by K$_3$Fe(CN)$_6$ in presence of luciferase but without oxygen never gives luminescence.

**BIBLIOGRAPHY.**