FURTHER STUDIES ON THE INHIBITION OF CYPRIDINA LUMINESCENCE BY LIGHT, WITH SOME OBSERVATIONS ON METHYLENE BLUE.

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In a previous paper (1924-25) I have shown that a filtered luminescent mixture of Cypridina luciferin and luciferase has its luminescence suppressed (inhibited) by light from a carbon arc in 2 or 3 seconds with 15,000 foot candles illumination. The action of the light is upon the luciferin and not upon the luciferase. I have reported the suppression as partially reversible, the luminescence returning slightly in the dark, but am now inclined to regard this effect as apparent, due to better dark adaptation of the eyes. The inhibiting wave-lengths are in the blue-violet region, $\lambda = .46 \mu$ to $\lambda = .38 \mu$. Consequently one finds no suppression of luminescence by red, orange, yellow, or green light even after prolonged exposure.

The present paper deals with three further aspects of inhibition of luminescence by light, namely (1) photodynamic action of dyes; (2) influence of oxygen; (3) influence of H ion concentration on the inhibition.

1. Photodynamic Action of Dyes.—As the dye sensitization of many photochemical reactions is well known—notably that of the photographic plate by dicyanin, pinacyanol, erythrosin, etc., and the photodynamic action of acridine, eosin, etc., on living tissues, enzymes, and antibodies—it is not surprising to find a similar effect of dyes on the inhibitory action of light on Cypridina luminescence.

I have said that inhibition is brought about by blue (.46 to .38\(\mu\)) light but not by red, orange, yellow, or green light. However, if we add to the luciferin–luciferase mixture one of a number of dyes, then we find that red, orange, yellow, or green light will inhibit the luminescence in a few seconds. The wave-length of light which will inhibit
Cypridina luminescence in the presence of sensitizing dye depends, of course, on the position of the absorption band of the dye, only those wave-lengths inhibiting which are absorbed. The converse is not necessarily true, that if a dye possesses an absorption band it is a photosensitizer with respect to that light. There may be or may not be sensitization.

A number of dyes have been tested by a student of mine, Mr. A. Hunsberger, Jr. The method is this. In a dark room, light from a carbon arc in a dark house passes through 6 cm. of water and is condensed to a small beam which strikes the middle portion of a narrow test-tube containing the luminescent mixture of Cypridina luciferin and luciferase. The light beam can be colored by light filters and cut off instantly by a photographic shutter. The luminescence of the exposed area of the test-tube is then compared with the non-exposed regions above and below.

Wratten gelatin filters were used to obtain light of a known range of wave-lengths. As the percentage transmission of the filters varies, some being much denser than others, it is impossible to select filters that will permit equal amounts of nearly monochromatic light to pass. In fact I have selected filters of high transmission which begin to absorb strongly at some definite wave-length (No. 15, 22, 29, 88) or those with broad transmission bands (No. 61) rather than the denser monochromatic filters, in order that the exposure need not be too long. 15 seconds was selected as a convenient time.

The filters are:

- No. 61 green; over 10 per cent transmission .50 to .57μ.
- “ 15 yellow; “ 10 “ “ .52 “ .70μ and beyond.
- “ 22 orange; “ 10 “ “ .55 “ .70μ “ “
- “ 29 red; “ 10 “ “ .62 “ .70μ “ “
- “ 88 infra-red; no visible transmitted except 5 per cent at .70μ.

The dyes tested are given in Table I.

Allowing for the unequal transmission of the filters and the widening of the absorption bands of the dye with increase in concentration, there is undoubtedly an agreement with the rule that the dyes sensitize only for that wave-length of light which they absorb.

It can also be shown that the inhibiting action of green light in pres-
ence of eosin is upon the luciferin and not the luciferase, just as in the case of violet wave-lengths acting without sensitizer.

I think we may predict with fair certainty that Ctenophores, the inhibition of whose luminescence by light is so well known, will also be sensitized by photodynamic dyes.

2. Influence of Oxygen.—It must be recalled that luminescence only occurs if luciferin, luciferase, and oxygen are together in solution, but that luciferin will oxidize without luminescence if luciferase is absent.

It seems most probable, therefore, that light acts by causing rapid oxidation of luciferin without luminescence. Consequently the area exposed to light, of a luminescent mixture of luciferin and luciferase in a test-tube, will not luminesce so brightly because some of the luciferin has been photochemically oxidized. This view can be tested by completely exposing luciferin solutions to light in absence of oxygen (by evacuation or bubbling of pure hydrogen), with a control tube illuminated in presence of oxygen, and then mixing both tubes with luciferase. The luciferin exposed in absence of oxygen gives a bright light, while the control in presence of oxygen gives no luminescence or a very faint one. There is no inhibition in absence of oxygen.

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<td>Fluoresceine</td>
<td>K tetrabromfluoresceine.</td>
<td>μμ 475-502 450-505</td>
<td>Negative.</td>
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<tr>
<td>Eosin</td>
<td>K or Na tetralodofluoresceine.</td>
<td>500-530 480-540</td>
<td>Green, yellow.</td>
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<tr>
<td>Erythrosin</td>
<td>K or Na 4 iodo 2 chlorfluoresceine.</td>
<td>500-530 480-560</td>
<td>“ “</td>
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<tr>
<td>Rose bengale</td>
<td>K methyl ether of 4 Br 2 Cl fluoresceine.</td>
<td>530-550 480-560</td>
<td>“ “</td>
</tr>
<tr>
<td>Cyanosin</td>
<td>Tetramethyl thionin HCl.</td>
<td>500-545</td>
<td>“ “</td>
</tr>
<tr>
<td>Acridine or aniline red E 103.</td>
<td></td>
<td>480-530</td>
<td>Yellow, orange, red.</td>
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I find also that the photosensitive dyes will not sensitize the inhibition of luminescence from luciferin in green or yellow light in absence of oxygen. In this respect the phenomenon agrees with photodynamic dye action in tissues. There is no poisonous action of eosin on enzymes or organisms in light in absence of oxygen (von Tappeiner, 1909).

The dye simply acts by making wave-lengths effective which would not be effective in its absence. The light acts by rapidly oxidizing luciferin.

The manner in which the photosensitizers make wave-lengths photochemically effective is not well understood. Perhaps the first question to be asked is whether the sensitizer undergoes any change. We may suppose the eosin to oxidize the luciferin in presence of light, itself undergoing reduction to a leuco body. Accordingly I have exposed mixtures of luciferin and eosin to white light (8600 foot candles) and also to green light, for from 4 to 7 minutes, but have never observed any indication of the decolorization of eosin, although it is known that eosin is affected by light (Gros, 1901). There is also no indication of the decolorization of methylene blue and luciferin exposed to white light (8600 foot candles) for 4 minutes, although the oxidative action of the light on luciferin is greatly increased by the presence of these dyes. If any change occurs in the dye it involves no color change or is momentary. Under proper conditions, however, methylene blue is affected by light, as described below.

3. Influence of H Ion Concentration.—I have often compared luciferin to leuco methylene blue and its oxidation to the oxidation of leuco methylene blue with formation of the blue dye. Expressed as a reaction the change would be

\[ \text{MH}_2 (\text{leuco methylene blue or methylene white}) \rightleftharpoons \text{M} (\text{methylene blue}) + \text{H}_2 \]

\[ \text{LH}_2 (\text{luciferin}) \rightleftharpoons \text{L} (\text{oxyluciferin}) + \text{H}_2 \]

\[ \text{H}_2 + \text{O} = \text{H}_2\text{O} \]

In view of the effect of light on luciferin oxidation it is interesting to note that methylene white oxidation is affected by light also. This was observed by Clark (1925), and the effect can be very nicely seen by reducing methylene blue with Zn dust and dilute acid, pouring the
colorless solution into a narrow test-tube and exposing the middle portion of the colorless solution to the condensed beam from a carbon arc lamp, first passing the beam through a water layer to remove its heat. Blueing will occur in the illuminated region in a few seconds. Oxygen is of course present in this experiment, but I can confirm Clark's observation that blueing will occur in absence of oxygen after reduction by platinized asbestos and hydrogen. However, the solution must be acid. After removal of oxygen and reduction by Na₂S₂O₄, blueing of methylene white in light will also occur, provided the solution is acid enough and that not too much Na₂S₂O₄ has been added. Neutral and alkaline solutions will not turn blue in light under the same conditions. There seems to be a shift in equilibrium of the methylene blue ⇔ methylene white system toward the side of oxidation in the light.

If methylene blue is reduced by Pt asbestos and hydrogen in two tubes, one of m/50 HCl, the other of m/50 NaOH, and the tubes shaken slightly to dissolve a little oxygen it can be easily observed that the methylene white oxidizes much more quickly in the alkaline tube, which becomes blue as compared to the acid tube, only faintly bluish—a well known phenomenon. On now exposing the two tubes to a beam of light, there results a blue band in the acid medium and a colorless band in the alkaline medium. We have acceleration of oxidation in acid and of reduction in alkali in light. I have observed the same thing when NH₂SH or H₂S is used as reducing agent and also when Na₂S₂O₄ is employed.

If just enough Na₂S₂O₄ is added to decolorize methylene blue in m/50 HCl and the colorless tube is exposed to light, blueing will occur; but if a little more Na₂S₂O₄ is added no blue band occurs in light. However, upon shaking with air until some of the Na₂S₂O₄ is removed by oxidation, a blue band now appears in light. Reducing with Na₂S₂O₄ in an alkaline medium (Clark and Lubs buffer, m/20 H₂BO₃, KCl, NaOH, pH = 10) and exposing to light, we observe no change; but if shaken with air till partly blue and then exposing to light, a colorless band appears.

This colorless substance is methylene white and not a colorless oxidation product of methylene blue, because by thorough shaking with air the blue color will return again completely.
We may sum up the behavior of methylene blue in light as follows: Methylene white in presence of reducing agents will turn blue in absence of oxygen if the solution is acid enough but not in neutral or alkaline solutions. In presence of some oxygen and reducing agent, ¹ acidity favors the change to blue (oxidation) while alkalinity favors the change to colorless (reduction). Without reducing agent, methylene blue will be rendered colorless by light slowly in fairly alkaline solution (m/50 NaOH) but not in m/10 Na₂HPO₄ (pH = 9) or in distilled water.

Indigo carmine does not behave like methylene blue under the influence of illumination.

If the luciferin–oxyluciferin system is to behave like methylene blue, we should expect inhibition of luminescence in light in acid medium containing some oxygen and reducing agent (like Na₂S₂O₄). Under these conditions oxidation to oxyluciferin would be favored and less luciferin remain to luminesce with luciferase. Hence a dark band should appear in a luminescent tube after illumination. On the other hand, in alkaline medium a luminescent band should appear after illumination, since reduction would be favored and more luciferin accumulate in the previously illuminated region.

However, I have been unable to observe a more luminescent band after illumination in solutions of any reaction. If luciferin is prepared in a series of buffer solutions and a little luciferase added we get the following results in light-exposed and dark regions of the tube. No reducing agent is present.

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<td>m/20 K H phthalate, NaOH = 5.6</td>
<td>Faint.</td>
<td>“ “ “</td>
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It will be noted that inhibition, indicating oxidation of luciferin, always occurs no matter what the reaction, acid or alkaline.

If Na₂S₂O₄ is now added to the above tubes to remove the oxygen

¹ Or its oxidation product.
the luminescence disappears and exposure to light in absence of oxygen never causes luminescence to return. If the tubes are shaken slightly to dissolve oxygen, luminescence will return and then exposure to light gives the same results as recorded in the table in the absence of any reducing agent. Light exposure always results in inhibition which is more rapid the more alkaline the medium and is also more rapid in presence of the Na$_2$S$_2$O$_4$ (or its oxidation products) than previously. It is as if the oxidation products of Na$_2$S$_2$O$_4$ accelerated the effects of light, as they do in the case of methylene blue. I have never observed a more luminescent band in the region previously exposed to light.

We see that the behavior of luciferin in light is only in part similar to that of methylene blue. One always obtains acceleration of oxidation of luciferin by light and not acceleration of reduction under the same conditions (alkaline medium) necessary for the phenomenon in methylene blue. Perhaps it is pushing the analogy too far to expect that the methylene white—methylene blue and the luciferin—oxyluciferin systems will behave in exactly the same way after exposure to light.

**SUMMARY.**

1. Eosin, erythrosin, rose bengale, cyanosin, acridine, and methylene blue act photodynamically on the luminescence of a *Cypridina* luciferin—luciferase solution. In presence of these dyes inhibition of luminescence, which without the dye occurs only in blue-violet light, takes place in green, yellow, orange, or red light, depending on the position of the absorption bands of the dye.

2. Inhibition of *Cypridina* luminescence without photosensitive dye in blue-violet light, or with photosensitive dye in longer wavelengths, does not occur in absence of oxygen. Light acts by accelerating the oxidation of luciferin without luminescence. Eosin or methylene blue act by making longer wavelengths effective, but there is no evidence that these dyes become reduced in the process.

3. The luciferin—oxyluciferin system is similar to the methylene white—methylene blue system in many ways but not exactly similar in respect to photochemical change. Oxidation of the dye is favored in acid solution, reduction in alkaline solution. However, oxidation
of luciferin is favored in all pH ranges from 4 to 10 but is much more rapid in alkaline solution, either in light or darkness. There is no evidence that reduction of oxyluciferin is favored in alkaline solution. Clark's observation that oxidation (blueing) of methylene white occurs in complete absence of oxygen has been confirmed for acid solutions. I observed no blueing in light in alkaline solution.

BIBLIOGRAPHY.