PHYSICO-CHEMICAL STUDIES ON BIOLUMINESCENCE

II. THE PRODUCTION OF LIGHT BY CYPRIDINA HILGENDORFII IS NOT AN OXIDATION

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Received for publication November 13, 1919

INTRODUCTORY

That the production of light and oxidation are intimately related to each other is a very general phenomenon judged from common sense and considered from the viewpoints of physics and chemistry. It is, therefore, no wonder that we find in the literature on the subject of the relation of oxygen to light production in living organisms, the statement that the production of light by bacteria, Noctiluca, Pholus, fire-flies and others, is an oxidation (4, p. 353–358).

Harvey (2, p. 321) who has studied the action of oxygen on the production of light by Cypridina hilgendorfii, concludes:

Oxygen is necessary for light production as may be seen by placing the crushed animals in an hydrogen atmosphere, or by bubbling hydrogen through a glowing extract of the animals. The light never completely disappears even after a long time, but remains dim so that very little oxygen (as no special precautions were taken to remove the last traces of oxygen from the hydrogen, prepared in a Kipp generator) is sufficient to give light. Upon readmitting oxygen, however, a brilliant glow results. Every other species of animal investigated likewise requires oxygen for phosphorescence.

The writer also imagined that “oxygen and water are necessary for the production of light by Cypridina hilgendorfii” and fancied that luciferase “is an oxidizing enzyme” (3, pp. 321 and 448). But the results of his recent work, which was carried out under more careful experimental conditions, have contradicted his expectation. That is to say, he has found that the light production in Cypridina is by no means a phe-

1 This paper was published in the Japanese Journal of Zoology, xxxi.

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nomenon of oxidation. Oxygen is not necessary for the production of light by this animal as will be shown in this paper.

The following experiments were conducted in the Science Department of the Kyushu Imperial University, Fukuoka, Japan. The writer expresses his appreciation of the interest and suggestions of Dr. Tsuncya Marusawa, Professor of Physical Chemistry in the University, throughout the course of the work. To Prof. Ayao Kuwaki and all the members of the department, the writer acknowledges his gratitude for the privileges of the laboratory and their interest in the work. The work was supported by the private contribution of Mr. Jihachi Hamano.

MATERIAL

Cypridina hilgendorfii were thoroughly dried in the direct sunlight. The animals were then crushed and the shell and body separated by means of a sieve. The body material containing the maxillary glands was extracted with several changes of ether during the course of a few days "without impairing in the least their power to produce light when again moistened." For convenience's sake, the bodies thus prepared are hereafter called the "experimental material" or simply "material."

DESCRIPTION OF APPARATUS

Believing in Harvey's statements, the writer first tried "by bubbling hydrogen through a glowing extract" or a glowing mixture of distilled water and Cypridina to see whether the light disappears or not. Hydrogen gas was prepared in a Kipp generator. The method was, however, so crude that he could not obtain decisive results. At the suggestion and also under the direction of Doctor Marusawa, therefore, the writer conducted all his experiments with the following apparatus. The arrangement of the apparatus as shown in figure 1 is typical although it was variously modified for the different gases employed.

The apparatus consists of two wings, right and left. Each wing has an experiment bottle, $E$ or $E_1$, of a capacity about 60 cc. The bottle is fitted with a tight rubber stopper in which three glass tubes, $A$, $B$ and $C$, or $A_1$, $B_1$ and $C_1$, with one stop-cock for each, are inserted with the arm bent at 90 degrees. In the case of the bottle $E$, the glass tube $B$ with a stop-cock $b$ is connected to a T-shaped glass tube, $D$, with a stop-cock, $d$, by means of rubber tube. The second arm of the glass tube $D$ is connected to the flask $W$ by means of glass and rubber tubes,
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Fig. 1

MAP
and the third leads to the Kipp generator, $K$, through the gas wash bottle. The glass tube $C$, which has a stop-cock $c$, and is T-shaped, is connected to the flask $W$ by means of glass and rubber tubes, and a third arm has a stop-cock $g$ by means of which water from the flask $W$ is caused to flow. The glass tube $A$ with a stop-cock $a$ is connected to a T-shaped glass tube $J$, the second arm of which leads to a Gaede oil pump, $P$, through a manometer, $M$, and a safety bottle and the third to the glass tube $A_1$ of the experiment bottle $E_1$ at the left wing of the apparatus. The arrangement of the bottle $E_1$ is exactly similar to that of the bottle $E$, though sometimes a gas holder is used, depending on the gas under investigation. So the Kipp's generator is not in permanent use.

The apparatus was placed in a dark room, to facilitate the observation of the production of light.

THE METHOD OF EXPERIMENT

As the chief purpose of the following experiments was to determine whether the production of light by Cypridina hilgendorfii is an oxidation or not, a hydrogen experiment was always conducted together with any other gas experiment as a control, besides a control for which air was used. Special care was, of course, exercised when an oxygen experiment was performed. The principle of the method is that the production of light by the material can be observed in the water free from any gases or in any pure gas.

In the first place, therefore, the distilled water to be used should be thoroughly heated by boiling for a few hours. While the water is still boiling the flask $W$ or $W_1$ is fitted with a tight rubber stopper which carries two glass tubes, long $l$ and short $s$, tightly fitted. Each of these two tubes has a stop-cock. Steam comes out from the outer ends of these two tubes within a few minutes, since the water is still boiling. These tubes are then closed by the stop-cocks $e$ and $f$. The glass tube $s$ or $s_1$, as the case may be, is now ready to be connected to one arm of the glass tube $D$ or $D_1$ by means of glass and rubber tubes.

One of the most important procedures in this method is to have any desired gas for experiment prepared in the Kipp's generator or the gas holder connected with the gas wash bottles and with the glass tube $D$ or $D_1$ before the glass tube $a$ or $a_1$ of the flask $W$ or $W_1$ is to be connected to one arm of the glass tube $D$ or $D_1$. In other words, any desired gas should be ready to pass through the gas washers and the glass tube $D$. 

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or $D_1$ before the glass tube $s$ or $s_1$ of the flask $W$ or $W_1$ is connected to one arm of the latter, and the stop-cock of the glass tube $s$ or $s_1$ is opened. Because the water and also the space in the flask $W$ or $W_1$ should be filled up with the desired gas, while the water becomes cooler and cooler. Special care should, of course, be taken that any air in any glass and rubber tubes should be excluded by all possible means, whenever any connection is made. Before the glass tube $s$ or $s_1$ of the flask $W$ or $W_1$ is, therefore, to be connected to the tube $D$ or $D_1$, the washers should be evacuated and then filled with the gas to be used up to the stop-cock $h$ or $h_1$.

All being thus prepared, the tube $s$ or $s_1$ is connected to the tube $D$ or $D_1$ without any unnecessary delay, while the water is still boiling and then the stop-cocks $e$ or $e_1$, $h$ or $h_1$ and $d$ or $d_1$, in order, are opened. Hot steam and gas come out from the end of the arm, because the water is still boiling and the gas is slowly generating. All air is thus driven out from all spaces and the stop-cock $d$ or $d_1$ is closed. At the same time the flask $W$ or $W_1$ is removed from the flame. Everything thus arranged, the water and space in the flask $W$ or $W_1$ are gradually filled up by the gas until the temperature of the water becomes equal to that of the room. When the temperature equilibrium of the inside and outside of the flask $W$ or $W_1$ has been established, the glass tube $l$ or $l_1$ is pushed down into the water, and is now ready for its connection to one arm of the glass tube $C$ or $C_1$ of the experiment bottle $E$ or $E_1$.

Four bottles, two for the experiment and two for the control, of similar capacity and shape, are cleaned and dried thoroughly. In each of these bottles 0.2 gram of the experimental material is placed. The experiment bottles are then fitted with tight rubber stoppers each of which carries three glass tubes as previously described. One arm of the glass tube $C$ or $C_1$ which is T-shaped is now connected to the glass tube $l$ or $l_1$ of the flask $W$ or $W_1$. And then the gas-saturated water is introduced up to the stop-cock $c$ or $c_1$, by the management of one arm of the tube $C$ or $C_1$. The bottle $E$ or $E_1$ is fixed on an iron stand. Now the connections of the tube $B$ or $B_1$ through the tube $A$ or $A_1$ to the T-shaped tube $J$ and the third arm of the last to the pump, $P$, through the manometer, $M$, and also a safety bottle, are made by means of heavy-walled rubber tubes. All arrangements thus made, the apparatus is now ready for experiment.\(^2\)

In the first place, the stop-cocks $a_1$ and $b_1$ are opened and at the same time the pump is started to evacuate all air in the bottle $E_1$ and the

\(^2\) It is convenient to begin any experiment from the left wing first.
rest of the apparatus. A complete evacuation of the air, however, is impossible by one operation. In order to complete the evacuation of the air, therefore, it is necessary to fill up all the spaces again with the gas desired, which has been prepared for the experiment. The stop-cock \( d_1 \) is now opened, and the gas fills all the spaces. After this filling is complete, the stop-cock \( d_1 \) is closed and the pump is again started. This same procedure is repeated ten times, though five times are sufficient. After the last filling with the gas, the stop-cocks \( a_1 \) and \( b_1 \) are closed. The last procedure is simply to pour a necessary amount of water from the flask \( W_1 \) into the bottle \( E_1 \) for an observation of the production of light by the material in the bottle. But this should not be done until the bottle \( E \) has been prepared in the same way.

To do this the pump \( P \) is again started to evacuate the gas in all tubes and spaces from the pump and up to the points of the stop-cocks \( a \) and \( a_1 \). After this evacuation, the stop-cocks \( a, b \) and \( d \) are opened. In so doing, another gas is introduced to fill the bottle \( E \) and the rest of the apparatus. After this filling the stop-cock \( d \) is closed and the pump is again started. After this evacuation, the stop-cock \( d \) is opened and fresh gas is permitted to enter. This procedure is repeated ten times as before. After the gas has filled bottle \( E \) and the rest of the apparatus, all the stop-cocks, \( a, b \) and \( d \), are closed. The bottle \( E \) is now also ready to receive water.

Now the stop-cock \( c \) is opened and about 20 cc. of the water are poured from the flask \( W \) into the bottle \( E \). At the same time, about 20 cc. of the ordinary distilled water are also poured in one of the control bottles which is fitted with a tight stopper. The time should be recorded because it is a very important factor in the production of light. Then the stop-cock \( c_1 \) is opened and about 20 cc. of the water are poured from the flask \( W_1 \) in the bottle \( E_1 \). At the same time the second control is prepared with water. These procedures take about 2 to 3 minutes. Then the bottles \( E \) and \( E_1 \) with the tubes, \( A, B \) and \( C \), and \( A_1, B_1 \) and \( C_1 \) are freed from all the connections of the rest of the apparatus. At the same time the room is to be darkened. The production of light by the material in the bottles \( E \) and \( E_1 \) is thus to be observed together. As already stated, special care should be taken that the controls are to be set up at the same time when the water is poured in the bottles \( E \) and \( E_1 \), since time is a very important factor for the observation of the intensity of the light produced by the material. At the time of pouring water, therefore, at least three persons are to be ready for the work, to save time.
The writer is convinced that the method and apparatus described above are satisfactory to determine the effect of any particular gas on the material.

THE PREPARATION AND USE OF GASES

The gases used for these experiments were hydrogen, oxygen, nitrogen, carbon dioxide and carbon monoxide. Of these gases, hydrogen, nitrogen, carbon dioxide and carbon monoxide were prepared in the laboratory, and oxygen in bomb was used. The methods of preparing these four gases were those in common use. The writer thinks, however, that brief statements about the methods of preparing them may not be out of place, because all the procedures carried out by the writer need to be open for free discussion.

1. The preparation of hydrogen: Hydrogen was prepared with a Kipp's apparatus in which zinc and about 50 per cent H₂SO₄ were placed. The liberated gas was washed by passing it through four wash bottles. Distilled water was placed in the first, and saturated KMNO₄ solution in the second, a solution of 30 grams of KOH + 10 grams of C₆H₅(OH)₃ in 100 cc. of distilled water in the third, and concentrated H₂SO₄ in the last. As hydrogen was always used as a control in order to compare the action of this gas with that of any other gas, this Kipp's apparatus was always fixed in the right wing. The last wash bottle of this apparatus, therefore, was always connected to the tube, D. The solutions in the wash bottles were renewed from time to time.

2. The preparation of carbon dioxide: Carbon dioxide was also prepared with a Kipp's generator in which pieces of marble and about 15 per cent HCl were placed. The gas when liberated was washed by passing it through three wash bottles. Distilled water was placed in the first and second, and concentrated H₂SO₄ in the third. This last wash bottle was connected to the tube, D₁, when used for experiment.

3. The preparation of nitrogen: In the first place, 200 grams of NaNO₂, 300 grams of (NH₄)₂SO₄ and 200 grams of K₂CrO₇ were barely dissolved in separate beakers. The solutions were placed together in a 5-liter flask and 1500 cc. of distilled water were added to it. A condenser was connected to the stopper of the flask in order to cool the nitrogen gas. To this condenser, a heavy Erlenmeyer flask as a safety bottle and two wash bottles were connected. In each of these bottles 30 cc. of concentrated H₂SO₄, 20 grams of K₂Cr₂O₇ and 100 cc. of distilled water were placed.
The mixture contained in the flask was slowly heated on a low flame. When enough N₂ gas was liberated, the last wash bottle was connected to a gas holder which was already prepared to receive the gas. Special care was taken to have no air in any space. When the gas in the gas holder was used for experiment, it was again washed by passing through the same wash bottles as mentioned above.

4. The preparation of carbon monoxide: In a liter distillation flask, 500 cc. of 80 per cent H₂SO₄ were placed. In the rubber stopper of this flask, one long thermometer of 200°C. and one glass tube perforated on its sealed end of about 5 cm. with many small holes were inserted. The mercury part of the thermometer and the hole part of the tube were entirely dipped into the sulphuric acid of the flask. The outer end of the tube was bent about 90 degrees and a suitable rubber tube with a pinch-cocock was connected to it. The other end of the rubber tube was then connected to a separating funnel in which some formic acid was placed. To the distillation flask, a heavy Erlenmeyer flask as a safety bottle, and two wash bottles, in each of which 200 cc. of 20 per cent NaOH were placed, were connected. The sulphuric acid of the flask was slowly heated on a low flame to about 110°C., and the formic acid was added little by little. After enough gas was liberated, the gas was received in a gas holder.

When the carbon monoxide gas was used for experiment, it was again washed by passing through three gas wash bottles. Two hundred cubic centimeters of 20 per cent NaOH were placed in the first and second bottles, and 200 cc. of concentrated H₂SO₄ in the third, which was connected to the tube, D₁.

EXPERIMENTAL

With the methods and apparatus described in the previous section, the writer conducted his experiments with every possible care and repeated each series of experiments four or five times, even though no exception was found in any trial. The results of these experiments are summarized in table 1.

The figures of the table show a relative superiority and inferiority of the intensity³ of light produced by the material on which five independ-
ent gases and air were allowed to act. The figure "6," for example, means the highest degree of light intensity compared with all others. As repeatedly stated, the experiments on the action of hydrogen and of air were always made as controls of any other gases. But as the experiments on the other gases, carbon dioxide and carbon monoxide, for instance, were not carried out together, it was impossible to compare their light intensities at the same time. The time for observing the light intensity in each gas, however, was carefully recorded in comparison with that of light produced in hydrogen and in air. In this roundabout way, therefore, the action of each gas may be compared. Furthermore, it was enough if the actions of hydrogen and oxygen were

### TABLE 1

Comparative intensity of light produced by the material in various gas atmospheres and water

<table>
<thead>
<tr>
<th>TIME OBSERVED IN DARK ROOM</th>
<th>LIGHT INTENSITY OF THE MATERIAL OBSERVED AT A GIVEN TIME IN THE ATMOSPHERES AND WATER OF THE FOLLOWING</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H₂ (Special control)</td>
</tr>
<tr>
<td>1 m.</td>
<td>6</td>
</tr>
<tr>
<td>3-4 m.</td>
<td>6</td>
</tr>
<tr>
<td>5-8 m.</td>
<td>6</td>
</tr>
<tr>
<td>12-15 m.</td>
<td>6</td>
</tr>
<tr>
<td>20 m.</td>
<td>6</td>
</tr>
<tr>
<td>9-10 h.</td>
<td>5</td>
</tr>
<tr>
<td>13 h.</td>
<td>4</td>
</tr>
<tr>
<td>160 h.</td>
<td>1</td>
</tr>
<tr>
<td>200 h.</td>
<td>0?</td>
</tr>
</tbody>
</table>

accurately compared, since the essential problem of these experiments was to determine whether the production of light by the animal was an oxidation or not. There was an unmistakable difference of the light intensity between the actions of oxygen and air. The difference of the intensity of light produced in the hydrogen and oxygen atmospheres, as well as in the air, was so astonishingly marked that no one could

relation of V. Henri et Larguier des Bancels, however, the retina is very "sensitive to an amount of light energy as small as 5 times 10⁻¹⁸ ergs. This is about three thousand times as sensitive as the most rapid photographic plate" (1, p. 512). If so, although there is no quantitative means to decide the weak or strong intensity of light observed by the retina, there is no indicator superior to the qualitative judgment of the retina. It is believed, therefore, that the decision made by the retina is most accurate.
question it. And the action of the nitrogen gas in comparison with the action of carbon dioxide or of carbon monoxide was also very distinct. Superiority or inferiority between the actions of CO and CO₂ may be questioned, though the writer felt that the former was a little superior to the latter.

It will be worth while to describe some other facts than those shown in table 1. In the water saturated with air and other gases, the color of light produced by the material is bluish white, a few hours after the treatment, while in the water saturated with hydrogen, it is decidedly blue. This blue color in the latter lasts almost as long as the light continues. In the cases of air and other gases the light after shaking takes about three or four seconds to return to the state before shaking, while in the case of hydrogen it takes about eight seconds. In a perfectly dark room it is observed that the strong light after shaking lasts quite long. As the time ratio of durability, 1:2, however, is not altered, no deviation is involved in this observation. Generally speaking, the "steady homogeneous glow" of light is to be observed, as Harvey pointed out. But, the light in the hydrogen-saturated water is observed to be heterogeneous by the flowing and precipitating of extremely minute particles when shaken in a dark room. Harvey's claim of "complete proof of the truly soluble character of the light-producing substances" (2, p. 321) may not be true. The writer will try to make this point clear by using an ultramicroscope in the near future. In the resting state, the light in the case of hydrogen glows as if the solution glows itself but the glow of the individual points of the material is not so marked as it is in the cases of air and any other gases, while in the latter cases the solution is clear. And also the solution forms when shaken but not so much as in the former. In the cases of all gases, when the production of light is near to the end, the solution does not glow as a whole but only at the surface when shaken. This is markedly so in the case of hydrogen. But in the case of air, the solution glows as a whole till the end.

These are six characteristics of the effect of the hydrogen gas on the production of light by the material besides those shown in table 1. Whether these are of any significance regarding the light production or are simply to be overlooked as meaningless, can not be settled unless further facts are found.

After an experiment was over, a test was made with a lighted match to see whether the gas used was present or not. The results were always positive even after seventy-five days had passed. Each solution
was also tested with litmus paper to see whether it was alkaline or acid. In the cases of hydrogen, nitrogen, oxygen and air, the solution was found to be neutral, although the solution was slightly alkaline after about two hundred hours in the last case. In the case of carbon dioxide, the solution was always acid and was distinctly milky with white precipitation. In the case of carbon monoxide, the solution was also acid, though very faint; and the acidity seemed to increase a little after about two hundred hours. In the cases of oxygen, air and carbon monoxide, the solution and material became very black, while in the case of hydrogen and nitrogen the solution was clear and the material became reddish brown.

CONCLUSION AND DISCUSSION

That the less the quantity of oxygen contained in the experiment bottle the more intense is the light produced by the material after the first minute is quite obvious according to the results stated above. The light produced is more intense in the water saturated with air than in the water saturated with oxygen and is markedly more intense and durable in the water saturated with hydrogen or nitrogen than in the water saturated with oxygen or air. In other words, oxygen is not necessary for the production of light by the material. If so, there is no ground for the assumption that the production of light by the animal is an oxidation. If the production of light by the animal is due to an oxidation, as Harvey claims, the more intense light should be produced by the greater concentration of oxygen. This is not the case, and the writer therefore concludes that the production of light by the animal is not an oxidation.

A question arises whether the production of light by the material is a reduction or simply an hydrolysis. In expectation of getting some light on this question, the writer made experiments with carbon monoxide as a reducing substance. But the results were not so marked as expected. That is to say, the results were not as good as those in the case of hydrogen. If so, is this not a reduction? This question is not settled as yet. Carbon monoxide is a “poison gas.” It may, therefore, act as a poison just as it acts on the hemoglobin of the blood. At any rate, it may be necessary to take into such biological consideration some factors besides the action of carbon monoxide purely looked upon from the viewpoint of chemistry. If so, it is no wonder that the action of carbon monoxide on the production of light by the material is inferior
to that of hydrogen or nitrogen. Whatever the exact interpretation of the facts may be, no decisive conclusion can be made unless further facts are found.

As to the action of carbon dioxide, there is the same question as in the case of carbon monoxide. If the light production of the material is not an oxidation, the intensity of light produced should be the same in carbon dioxide as in hydrogen or nitrogen. But this is not the case. However, if the fact is considered that carbon dioxide is dissolved in water and carbonic acid is formed, the riddle may be readily solved. Because it is a well-known fact that acid is injurious to organisms while alkali is not.

Furthermore, there are other points in table 1 which require explanation. In the first place, the light-producing substances in the water saturated with oxygen disappear fastest in spite of the production of the poorest light. On the other hand, the production of light in the water saturated with hydrogen is most intense and most enduring. Such phenomena may be explained by the following assumptions. That the higher the oxygen tension is the faster the oxidation of any substance is obvious. Although the production of light in question is by no means an oxidation, the light-producing substances, especially the luciférine, may always be subjected to an oxidation and thus the disappearance of the substance is apparent. If it be true that the higher the oxygen tension is the more the light-producing substance or substances may be oxidized (though this oxidation of the substances has no bearing on the light production), it is no wonder that the light becomes weaker and disappears faster if the substance or substances become less and less by an oxidation. Such a consideration explains the facts that the light is weakest and lasts shortest in the water saturated with oxygen and on the other hand, that the light is strongest and lasts longest in the water saturated with hydrogen with no oxygen.

If so, the action of the water saturated with carbon monoxide, carbon dioxide and nitrogen might be the same as the action of the water saturated with hydrogen. But this is not the case. As to the action of the first two gases there may be possibly some physiological or biological factors involved, as already stated. So the mere fact that free oxygen is not present should not be looked upon as a complete explanation. Taking this as granted, then, how is it in the case of nitrogen? The difference in action of hydrogen and nitrogen is not very marked, but it is still easily distinguishable. It may be said, therefore, that the superiority of the action of hydrogen over any other gas is its own speci-
ficity. In brief, the writer should confess that he has not yet clear
knowledge with which he can explain these various difficulties. He
expects to make special efforts to gather all possible data based on
experiments. These difficulties may be of such a nature that they may
be explained when further facts are available.

SUMMARY

1. The intensity of light produced by the material is strongest and
   lasts longest in the water saturated with hydrogen.
2. The intensity of light produced by the material is weakest and
   lasts shortest in the water saturated with oxygen. Therefore the pro-
   duction of light by the material is not an oxidation.

BIBLIOGRAPHY

(2) Harvey: This Journal, 1917, xlii, 318.