

THE CHEMILUMESCENCE OF CHLOROPHYLL IN PHOTOCHEMICAL REACTIONS

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THE study of the luminescence of chlorophyll has made a substantial contribution to the modern conceptions of the primary physical processes which immediately follow the light-absorption process: the storing, migration, and expenditure of the energy of the absorbed quantum. However, the phenomenon of decisive significance in photosynthesis is the subsequent transformation of the energy of the light quantum into chemical energy, as takes place through the process of photochemical reactions. The study of the chemiluminescence taking place in the course of the reverse reactions of the photochemical reactions, is one of the most promising ways of determining the nature and mechanisms of the direct photochemical processes in photosynthesis. This chemiluminescence may be due to the products appearing at various stages in the transformation of the light energy into chemical energy. Thus, a many-sided study of the chemiluminescence will open new possibilities for study of the entire chain of successive processes linking the primary photophysical event with the biochemical enzymatic reactions.

The chemiluminescence of chlorophyll and magnesium phthalocyanine in solution has been known for a relatively long time. However, it has been possible to observe it only under severe conditions (in boiling tetralin containing peroxides) in which destruction of the pigment molecule takes place.^{1,2} Later, chemiluminescence of chlorophyll was found in alkaline solution containing aldehydes. This phenomenon was also accompanied by the violation of the intactness of the molecule. In the opinion of the authors themselves, this case is probably of interest only from the standpoint of chlorophyll chemistry.³

The interest of biologists and physicists was attracted to the phenomenon of chemiluminescence in photosynthesis by the papers of Strehler and Arnold et al., who discovered and studied a persistent afterglow in photosynthetic organisms and in suspensions of chloroplasts.^{4,5} A connection was convincingly demonstrated in these papers between the observed emission and photosynthesis. This permitted the authors to suggest that this emission was chemiluminescence.^{4,5} However, the mechanisms of the processes of the observed phenomenon, and above all, the role of chlorophyll itself, have not yet been elucidated. The basic question is not yet clear as to whether the chemiluminescence is due to chemical transformations of chlorophyll and its photoproducts, or the chlorophyll

merely emits quanta formed in the process of recombination of other products of photosynthesis.⁵ In this regard, we took up the problem of studying chemiluminescence in photochemical reactions in a model system containing chlorophyll.

The reaction of reversible photoreduction of chlorophyll was taken as an example of such a reaction. As is known, this reaction is the basis of the photosensitizing action of chlorophyll in solution.⁶ A study was also made of the chemiluminescence of chlorophyll during photooxidation in solution, and of the afterglow and "thermoluminescence" of pigment films and chloroplasts.

In the study of chemiluminescence, the sensitivity of the methods of measurement is of primary importance because of the exceedingly low intensity of the emission. Hence, the measurements have been made in an apparatus for the study of extremely weak radiation, built around a photomultiplier cooled with liquid nitrogen, and operating as a photon counter.⁷

CHEMILUMINESCENCE IN THE PHOTOREDUCTION OF CHLOROPHYLL

We have observed chemiluminescence in the reversible oxidation by atmospheric oxygen of the photoreduced forms of chlorophyll.

The photoreduction of chlorophyll (a+b) in pyridine solution in the presence of ascorbic acid was carried out in a Thunberg vacuum tube. The solution was evacuated with the aid of an oil pump, and was irradiated for five minutes at room temperature with focused light from a 500-watt incandescent lamp passing through a water (heat) filter and a red light filter (KS-2 or KS-10).

After irradiation the Thunberg tube was placed in front of the window of the Dewar flask containing the photomultiplier. Then, in darkness and with vigorous stirring, air was admitted and ammonia solution was added to accelerate the reverse reactions.⁸ The decay of the chemiluminescence was recorded over the period from five seconds to five minutes from the moment of admitting oxygen.

The decay curve of the chemiluminescence during the oxidation of the photoreduced forms is given in Fig. 1. Attention may be called to the similarity of this curve to that of the decay of chemiluminescence of a kidney bean leaf, as obtained under the same conditions of irradiation and measurement. Also to be

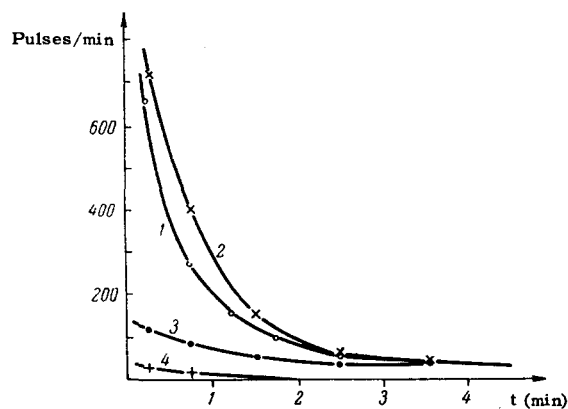


FIG. 1. The afterglow of leaves and the chemiluminescence of chlorophyll solution. 1 – green leaf of kidney bean, 2 – chemiluminescence during the oxidation of the photoreduced forms of chlorophyll [5 ml of a pyridine solution of chlorophyll ($D = 1$ at $668 \text{ m}\mu$) + 2.4 mg ascorbic acid, with admission of O_2 and addition of 0.1 ml 10% ammonia after the irradiation], 3 – the same, but with 0.1 mg ascorbic acid, 4 – the same, but without ascorbic acid. Ordinate – relative intensity of emission (number of pulses per minute). The scale for curve 1 is reduced by a factor of 4.

noted is the fact that the chemiluminescence of the solution is only four times less in intensity than the luminescence of the leaf (the lesser concentration of chlorophyll in the solution must be taken into account here).

The results of more detailed studies of the observed processes may be summarized as follows:

1) The chemiluminescence is due to the reverse reactions of oxidation of the photoreduced forms of chlorophyll. Each of the essential factors for photoreduction (chlorophyll, ascorbic acid, and irradiation of the solution in vacuo) is necessary for the appearance of the chemiluminescence. If photoreduction did not occur during the irradiation of the solutions in vacuo, the chemiluminescence was either very weak, or not observed at all. As the amount of ascorbic acid is increased, the luminescence becomes stronger. The chemiluminescence appears only upon admission of oxygen after photoreduction of chlorophyll, and becomes much stronger upon addition of ammonia, which accelerates the reverse reactions of the photoreduced forms of chlorophyll. The kinetic curves of the chemiluminescence under various conditions of performance of the photoreduction and the reverse reaction are shown in Figs. 1 and 2.

We have also observed chemiluminescence in the cases of the photoreduction of pure chlorophyll *a* and its analog pheophytin *a*.

An essential observation is that the effect remains when the ascorbic acid is replaced by another hydrogen donor, phenylhydrazine. When phenylhydrazine is used (0.2 ml per sample), the emission intensity is appreciably greater than in the effects with ascorbic acid described above.

The addition of dyes (methyl red and neutral red) to the solution leads to the suppression of the chemi-

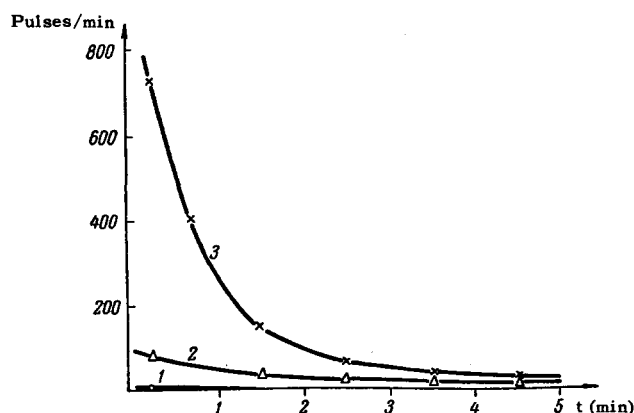


FIG. 2. The dependence of the chemiluminescence on oxygen and ammonia. 1 – The afterglow of the solution directly after photoreduction in the absence of O_2 , 2 – the same + O_2 , 3 – the same + O_2 + 0.1 ml ammonia. The other conditions are the same as for curve 2 in Fig. 1.

luminescence; since these dyes are hydrogen acceptors, they suppress the photoreduction.⁶

2) The quantum of chemiluminescence is apparently emitted by a molecule of chlorophyll. It has thus far not been possible to measure the chemiluminescence spectrum because of its low intensity. However, experiments with light filters have shown that the chemiluminescence spectrum lies in the spectral region of the luminescence of chlorophyll ($665 - 800 \text{ m}\mu$), which is transmitted by the light filter KS-13. The light filter SZS-10, transmitting in the broad spectral range from 400 to $660 \text{ m}\mu$, did not transmit more than 1.5% of the light of chemiluminescence.

3) The form of chlorophyll responsible for the chemiluminescence is apparently not the secondary "red" photoreduced form, but certain other intermediate compounds lying in the pathway of oxidation from the secondary form to chlorophyll. Above all, the observations have revealed a lack of proportionality between the degree of photoreduction and the intensity of the chemiluminescence. Chemiluminescence was observed even in experiments in which the photoreduction of pheophytin *a* was carried out at a temperature of -40°C , i.e., under conditions in which the secondary photoreduced form of the pigment is not produced at all. Finally, the results of comparison of the decay of chemiluminescence with measurements of the optical density of the solution give evidence in favor of the view that the chemiluminescence is due to the oxidation of intermediate photoreduced forms. These measurements during the course of the reverse reactions of oxidation of the photoreduced forms were carried out with the recording spectrophotometer SF-2M under experimental conditions identical to those of the chemiluminescence studies. The measurement of the optical density of the solution after addition of oxygen and ammonia was made at the absorption peak of the secondary reduced form at $525 \text{ m}\mu$ and at the absorption peak in the red for chlorophyll *a*.

It was found that upon addition of oxygen and ammonia, a practically instantaneous (< 5 sec) decrease in the absorption at $525\text{ m}\mu$ occurs without an accompanying burst of chemiluminescence. In the further course of the reverse reactions, in the time interval of interest to us (from 5 sec to 5 min), the absorption at $525\text{ m}\mu$ remains constant (with an accuracy of ± 0.002).

On the other hand, the increase in the optical density at the absorption maximum of chlorophyll during its regeneration from the photoreduced forms does not cease with the rapid stage (up to 5 sec). Rather, it continues at a lower rate over the entire time interval from 5 sec to 5 min. The rate curve of the change of optical density at the absorption maximum of chlorophyll and the decay curve of the chemiluminescence are very similar in form (Fig. 3). These data are evidence that the chemiluminescence is due to the oxidation of the intermediate forms of the pigment lying on the pathway from the secondary reduced form to chlorophyll.

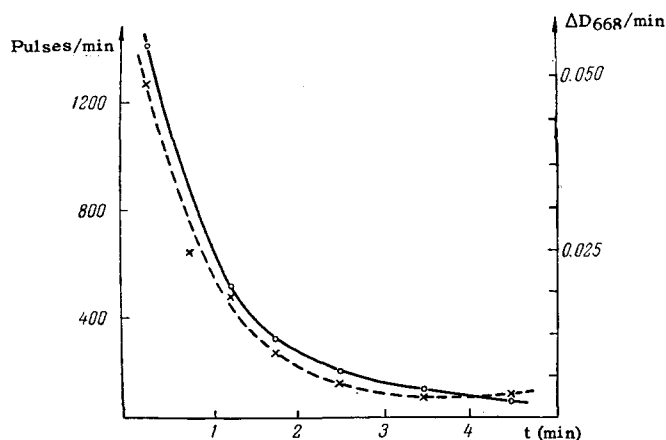


FIG. 3. The kinetics of chemiluminescence and the rate of change of the optical density ($D_{668\text{ m}\mu}$) in the oxidation of the photoreduced forms of chlorophyll. Solid curve - chemiluminescence (the conditions are the same as for curve 2 in Fig. 1). Dotted curve - rate of change in the optical density of the solution at $\lambda = 668\text{ m}\mu$ ($D_{668\text{ m}\mu}/\text{time}$).

CHEMILUMINESCENCE IN THE PHOTOOXIDATION OF CHLOROPHYLL IN SOLUTION

Pyridine solutions of mixtures of chlorophyll a and b, pure chlorophyll a, and pheophytin a were studied. Unevacuated solutions were irradiated in the same way as was described above, and then (after 5 sec) the emission transmitted by a red light filter KS-13 was measured. In Fig. 4 are shown the decay curves of the chemiluminescence following the irradiation of the chlorophyll solutions in the presence of air. The rapid decay of the luminescence during the first few minutes and the presence of a persistent slowly-decaying component are of interest.

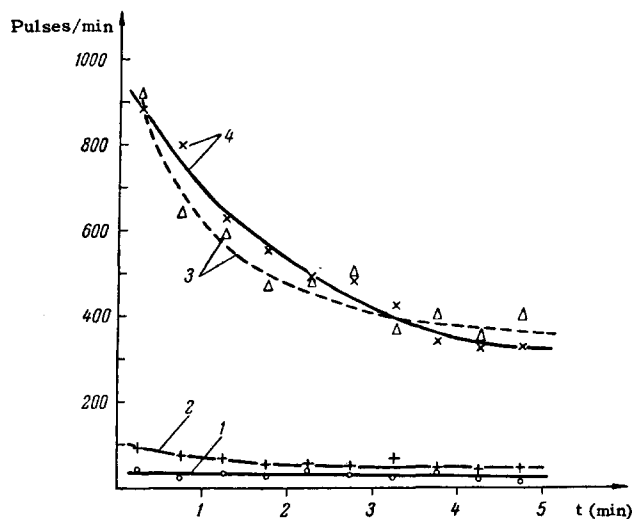


FIG. 4. The chemiluminescence of chlorophyll in pyridine solution. 1 - after irradiation of the solution in vacuo, 2 - after irradiation of the solution in vacuo, with subsequent admission of air, 3 - after irradiation of an unevacuated solution, 4 - the same, but with the addition of oleic acid before the irradiation.

The addition of oleic acid to the solution being irradiated did not produce an additional effect. Solutions irradiated in vacuo and non-irradiated solutions in the presence of air gave insignificant effects comparable with the background value. A weak chemiluminescence was observed when the solution was irradiated in vacuo and then air was admitted. It is interesting to note that in some cases the addition to the solution before irradiation of such substances as ascorbic acid, dyes, etc., intensified the chemiluminescence.

CHEMILUMINESCENCE OF CHLOROPHYLL IN FILMS AND IN CHLOROPLASTS

As is known, chlorophyll occurs in a different state in photosynthetic cells from that which it has in solution. The high concentration of the pigment, the ordered arrangement, and the presence of a many-component, chemically heterogeneous environment may all apparently affect the chemiluminescence of chlorophyll under these conditions. In line with this, it is of interest to study the chemiluminescence of chlorophyll in model systems. Such systems might be, e.g., films of the pure pigments, films obtained from extracts of leaves, and films of chloroplasts. Arnold and Sherwood have discovered a luminescence of chloroplast films; they observed this luminescence upon heating after irradiation of the films (thermoluminescence).⁹ The authors explained this phenomenon by the accumulation of electrons in semiconductor traps, with subsequent emission of the energy on heating. One of us has pointed out that this effect may be related to the chemiluminescence of chlorophyll in films.¹⁰ In a later paper, Arnold and Sherwood have also acknowl-

edged the possibility of explaining the thermoluminescence in terms of chemiluminescence.¹¹ In line with this, the observations of these authors are of interest, that in the presence of oxygen (but not in an atmosphere of nitrogen) films of chloroplasts acquire a charge, that is, they store energy.¹¹ With the aim of elucidating the nature of this phenomenon, it was of interest to study the thermoluminescence of films of chloroplasts and pigments, along with studying the afterglow.

Films of chlorophyll (a+b) were prepared by dissolving a crystalline preparation in ethyl alcohol, and then evaporating the solvent in vacuo in a Thunberg tube. When a sufficiently uniform film had been obtained on the inside of the Thunberg tube over a period of 15–20 min, an additional evacuation was performed to remove the remaining traces of the solvent. This method permitted us to study the emission of films in various environments (vacuum, oxygen, nitrogen) and at various temperatures. Alcoholic extracts were obtained from sugar-beet and kidney-bean leaves by grinding in a mortar with CaCO₃. Films were prepared from the extracts by the method described above.

In order to obtain films from homogenates, sugar-beet or kidney-bean leaves were ground in a mortar in the cold with the addition of a small amount of buffer solution ($\frac{1}{15}$ M Na₂HPO₄). The homogenate was pressed through cloth and transferred to a Thunberg tube for preparation of the film. Separate experiments were conducted with suspensions of chloroplasts washed with distilled water. The films which were obtained were irradiated through a water (heat) filter and a red (KS-2) light filter by the focused light of a 500-watt incandescent lamp. After 5–10 sec., the samples were placed in front of the photomultiplier window, and their emission at room temperature was recorded at ten-second intervals for three minutes. Then the Thunberg tube was heated by a special heater to 80°C. The sample was kept at this temperature for 15 sec, whereupon the thermoluminescence of the film was measured from the 5th to 12th minutes after the irradiation. The results of the experiments with pigment

films and chloroplasts may be summarized as follows:

1) All types of films showed both emission at room temperature decaying within five minutes after irradiation and thermoluminescence which can be detected by subsequent heating of the sample. This effect is insignificant in films of pure pigments, more marked in films prepared from extracts, and maximum in films of chloroplasts (see Table I and Fig. 5).

2) The afterglow at room temperature, and especially the thermoluminescence, depend essentially on the presence of oxygen during the irradiation. In films of chloroplasts and extracts which have been repeatedly irradiated in vacuo, the afterglow and the thermoluminescence fall to a background level. Upon irradiation in the presence of oxygen, intense afterglow and thermoluminescence appear in these films. This emission does not decrease after many repetitions of the experi-

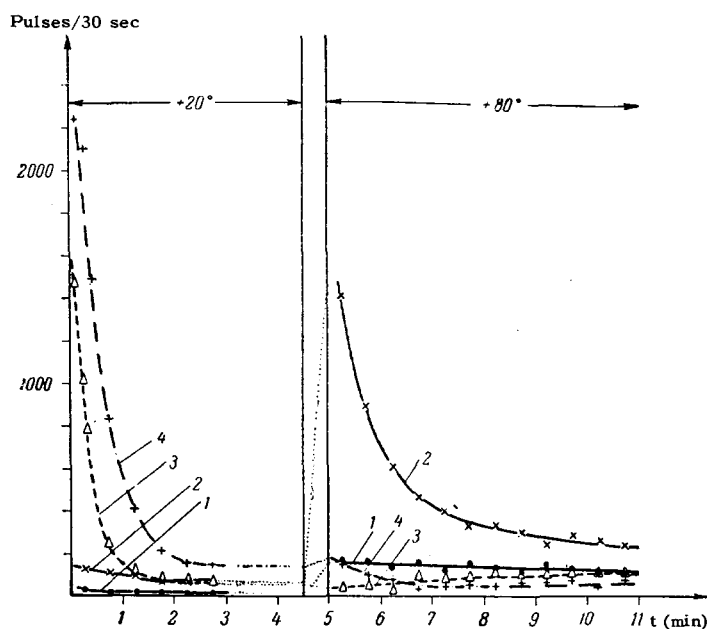


FIG. 5. The afterglow and thermoluminescence of films of homogenates of sugar beet leaves. 1—a film irradiated at room temperature in vacuo, 2—the same, with irradiation in oxygen, 3—a film irradiated in oxygen at -196°C , 4—a film irradiated in vacuo at -196°C .

TABLE I. Afterglow and thermoluminescence of films irradiated at room temperature

Material from which film was prepared	Gaseous environment during irradiation	Afterglow, pulses/3 min $\times 10^3$	Thermoluminescence, pulses/6 min $\times 10^3$
Fresh homogenate of sugar-beet leaves	vacuum	1.00	5.64
The same film after repeated irradiation	vacuum	0.09	0.71
The same film	oxygen	1.02	10.20
Alcoholic extract from sugar-beet leaves	vacuum	0.06	1.75
The same film	oxygen	1.30	4.87
Alcoholic solution of chlorophylls a+b	vacuum	0.06	0.96
The same film	oxygen	0.14	0.97

ment in oxygen. Subsequent evacuation of the tube or replacement of the oxygen by nitrogen results again in a fall of the intensity of emission to a background level which is small in magnitude and almost constant in time.

3) Freshly-prepared films of chloroplasts irradiated for the first time show, even in vacuo, intense afterglow at room temperature and thermoluminescence. However, these disappear in subsequent irradiations of the film in the absence of oxygen. Apparently, this phenomenon may be explained by the fact that freshly-prepared films contain a certain amount of oxidizing agents, the supply of which is exhausted upon repeated irradiations.

4) The thermoluminescence of chloroplast films was observed if the samples had been irradiated in the presence of oxygen at temperatures of $+20^{\circ}\text{C}$ or -55°C , but was absent when the film had been irradiated at liquid nitrogen temperature, in correspondence with the observations of Arnold and Sherwood.⁹

5) If chloroplast films are irradiated at liquid-nitrogen temperature, they show a very intense, quickly-decaying afterglow. In contrast with the afterglow at room temperature, this emission was not activated, but rather, was even suppressed by oxygen. It is highly probable that this low-temperature afterglow, which follows a first-order rate equation, is due to the emission of energy stored in triplet levels, a possibility which has been pointed out by A. N. Terenin.¹²

DISCUSSION

In their discussion of the phenomenon of afterglow in photosynthetic organisms, Strehler and his associates have proposed a scheme in which the emission of the light quantum takes place in the reverse reaction of the hypothetical primary reducing and oxidizing agents.⁵ Chlorophyll, in the opinion of these authors, is not responsible for the chemiluminescence, since it does not participate directly in the chemical reactions of photosynthesis, and plays only the role of a purely physical sensitizer which does not undergo chemical change. The fundamental argument which these authors advance to corroborate these views is based on the results of measurements of the differential spectra of photosynthetic organisms. In the irradiation of these organisms, it was not possible to detect a decrease in the absorption at the red maximum of chlorophyll which might be analogous to the well-known increase in the absorption in the region of $520\text{ m}\mu$.

However, in a recent paper, Coleman and Rabinowitch¹³ found the presence of such a parallelism between an increase in absorption in the region of $520\text{ m}\mu$ and a decrease at the chlorophyll maximum during the irradiation of photosynthetic cells (chlorella). The authors consider these effects to be the result of the photoreduction of chlorophyll taking place in the living cell.

From the viewpoint of the Strehler scheme, it is also difficult to explain without additional assumptions the following very essential fact: the persistent afterglow in photosynthesis coincides in spectrum with the fluorescence of chlorophyll. The chemiluminescence which we have observed upon oxidation of the photoreduced forms also apparently is due to chlorophyll.

The results which we have obtained in experiments on the photoreduction and photooxidation of chlorophyll in solution permit us to conclude, in our opinion, that the persistent afterglow of leaves, photosynthetic organisms, and suspensions of chloroplasts, which have been studied by Strehler, Arnold, and their associates, may be explained on the basis of the chemiluminescence of chlorophyll and its photoreduction products during their oxidation in the course of the reverse reactions of photosynthesis. This conclusion agrees with the conceptions of the direct chemical participation of chlorophyll molecules in the reactions associated with electron transfer in photosynthesis.

It was found in the experiments with films that the afterglow and the thermoluminescence of chloroplast films depend on the presence of oxygen during the irradiation. This circumstance, as well as the fact that there is no thermoluminescence when the sample is irradiated at liquid-nitrogen temperature, is evidence of the chemical nature of the processes bringing about the emission. Apparently, the observed facts agree better with the conception of the storing of the energy of the quanta by the chloroplasts, not in semiconductor electron traps,^{9,11} but rather, in the form of energy-rich products of photochemical reactions. The existence of several values of the activation energy of the process of thermoluminescence, in the opinion of Arnold and Sherwood, is evidence in favor of the semiconductor mechanism of emission (multiplicity of levels in traps). However, this fact may be explained from the viewpoint of chemiluminescence by the existence of several reactions having rates showing different temperature-dependences.

Thus, the persistent afterglow of photosynthetic cells and tissue homogenates, chloroplasts, and solutions of pigments, as well as the thermoluminescence of chloroplasts, probably have a common character. That is, they are due to the chemiluminescence of chlorophyll, although the concrete mechanisms of the processes are apparently different in different cases.

CONCLUSIONS

1. A red chemiluminescence during the reverse oxidation of the intermediate photoreduced forms of chlorophyll, and a chemiluminescence upon irradiation of chlorophyll solutions in the presence of air, have been discovered and studied.

2. A study was made of the afterglow and thermoluminescence of films of chloroplasts, extracts, and pure pigments, and of the relation of these effects to the temperature and the presence of oxygen during

the irradiation. Decaying thermoluminescence and afterglow are observed only when oxygen is present during the irradiation. Thermoluminescence does not take place if chloroplast films are irradiated at liquid nitrogen temperature. Under these conditions, an intense afterglow not activated by oxygen appears.

3. It may be assumed that the persistent afterglow of photosynthetic cells and tissues, chloroplast suspensions, and pigment solutions, as well as the thermoluminescence of chloroplasts have a common character, i.e., they are due to the chemiluminescence of the photoproducts of chlorophyll, although the concrete mechanisms obviously are different under different conditions.

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