is not strictly obeyed. The cause of this is not known. This kinetic behavior coupled with the progressive lowering of the transformation limit with lowering of the temperature suggests that the reaction is not strictly a photochemical, intramolecular process but involves intermolecular interactions. Because the reaction depends on the first power of the light intensity, it is unlikely that two photoactivated holochromes have to react.

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THE DEVELOPMENT OF CHLOROPHYLL AND OXYGEN-EVOLVING POWER IN ETIOLATED BARLEY LEAVES WHEN ILLUMINATED[1]

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When etiolated leaves are illuminated they produce chlorophyll and acquire the ability to carry on photosynthesis. By following simultaneously the development of chlorophyll and photosynthetic capacity in such leaves much may be learned concerning the participation of chlorophyll in photosynthesis. This approach has been used by several investigators, who have reached different conclusions concerning the simultaneity of chlorophyll formation and the onset of photosynthetic capacity as a brief survey of previous investigations will show.

Engelmann (4) found that all the chlorophyll-containing cells which he examined were capable of evolving oxygen when they were illuminated. Furthermore, he claimed that chlorophyll-free but etiolin-containing (protochlorophyll-containing (22)) cells of dark-grown seedlings gave off oxygen immediately upon being illuminated, which claim was verified by Ewart (5).

Irving (8) and later Willstätter and Stoll (23) examined the development of photosynthetic capacity in relation to chlorophyll formation by following the photosynthetic utilization of carbon dioxide during the greening of seedlings. Irving stated that "Etiolated leaves do not possess the power to photosynthesize CO_2 even when they have developed a large part of their chlorophyll. When the leaves have developed their full green color photosynthetic ability develops rapidly. Photosynthetic activity is not directly connected to the amount of chlorophyll produced. It must be some other component of the photosynthetic apparatus which controls the beginning of the com-

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plete functional activity." Willstätter and Stoll, however, found that etiolated leaves, while greening, possessed great assimilatory power based on chlorophyll. With the appearance of chlorophyll the assimilatory power set in and, while the chlorophyll content of the greening etiolated leaves was small, these leaves had much higher assimilation numbers than other young leaves which had not been grown in the dark. The difference in behavior of these two types of leaves was probably caused by the fact that in the dark, chlorophyll production was inhibited by the lack of light but the formation of the enzyme factors was not.

Briggs (2) attributed the discrepancy between Irving's and Willstätter and Stoll's results to the difference in the ages of the leaves they used, because "very young leaves, no matter what their chlorophyll-content cannot function as assimilating organs . . . ."

Briggs (2) studied the pigment-photosynthesis relation in young leaves brought to different degrees of greenness. He placed such leaves in an atmosphere of hydrogen, which contained carbon dioxide, and followed their oxygen evolution during illumination. Under these conditions the leaves increased in assimilatory power without noticeable change in chlorophyll content. He concludes that an internal photochemical factor besides chlorophyll is produced which works in conjunction with chlorophyll in the photosynthetic process. This assimilatory power is built up in the dark as well as in the light because leaves which have been illuminated and placed in the dark for three days possess "an assimilatory power of the same dimensions as that of similar leaves, which have been exposed to light in the apparatus during a considerable part of three days."

Inman (7) found that etiolated leaves when illuminated began to evolve oxygen about the time the green color was visually detectable in the leaf. Some chlorophyll was formed long before oxygen evolution began.

Davis (3) demonstrated that mutant strains of *Chlorella pyrenoidosa,* although containing normal quantities of the chlorophylls, varied in their capacity to photosynthesize; the wild type absorbed carbon dioxide and evolved oxygen; one mutant did not absorb carbon dioxide but evolved oxygen; two different mutants did neither. Thus photosynthesis failed for lack of factors besides chlorophyll.

Blaauw-Jansen, Komen, and Thomas (1) found that etiolated oat leaves possessed a slight capacity for photosynthesis even when the leaves were first illuminated. This capacity increased with time and at first to a greater extent than the chlorophyll a content of the leaves. They called attention to the fact that photosynthesis increased with increase in ratio of chlorophyll b to chlorophyll a until the ratio of these pigments approached its "normal" value.

The chief questions raised by this survey are: Do etiolated leaves when first illuminated have the ability to liberate oxygen? May photosynthetic organisms contain considerable quantities of chlorophyll without possessing photosynthetic capacity? Must factors besides chlorophyll be developed before photosynthetic power is acquired? May such factors be developed in the absence of light? And, is photosynthetic capacity related to the ratio of chlorophyll b to chlorophyll a?

With the sensitive analytical methods now available for the estimation of oxygen (14) and of individual pigments (9, 10) and with the sharpening of the concepts concerning pigment formation and pigment interrelationships (11, 16, 22), it seemed possible that at least a partial clarification of the involvement of chlorophyll in photosynthesis could be gotten by a quantitative re-examination of the development of pigments and of photosynthetic capacity in etiolated leaves.

Materials and Methods

Method for Measuring Oxygen: The phosphorescence-quenching method for measuring oxygen evolution (14) was adapted to the purpose of this investigation. Preliminary reports on the modified method have already been given (17–20) and a detailed description is in preparation (21). The leaves, after having been pre-treated in the desired manner, were placed in the darkened reaction chamber of the photosynthesis apparatus. All the oxygen was removed from the apparatus by evacuation and flushing with pure hydrogen. No carbon dioxide was added to the gas stream so that whatever carbon dioxide was present came from the leaves themselves. When the phosphor had come into equilibrium with the flowing hydrogen, which required approximately 40 minutes, the leaves were illuminated for 10 minutes with light from a 25-watt tungsten lamp at 10 cm distance, and the oxygen evolved by the leaves was measured. (The light intensity on the leaf chamber was about 230 fc.)

Barley Leaf Material: In these experiments, the tips of leaves, 5 to 7 cm long, were cut from dark-grown seedlings, 9 to 11 days old. The leaves were separated into lots of known weights and were placed in glass vials with their cut ends immersed in about 1 cm of water. They were held in the dark until required for experimentation sometime during the day. The treatment given each lot of leaves is described in the appropriate place.

Pigment Analysis: All pigment analyses were performed according to the method of Koski, French, and Smith (12), which is given here only in brief outline. After the leaves had been treated as desired, they were extracted by grinding them in a mortar with acetone and sand. The pigments were transferred from acetone to ether and the pigments estimated spectrophotometrically. From the optical densities measured at 663, 644, and 624 mμ, the quantities of chlorophylls a and b and protochlorophyll were determined (9, 10, 12).

Results and Discussion

Oxygen Evolution During the Transformation of Protochlorophyll to Chlorophyll a: The first stage in the greening of etiolated leaves to be examined in relation to oxygen evolution was the transformation of protochlorophyll to chlorophyll a. For this purpose, the oxygen evolved by dark-grown

leaves during illumination was measured and compared with the protochlorophyll-chlorophyll transformation of the same leaves. The results of these experiments (summarized in table I, experiments 2 and 3) show that at most only a minute quantity of oxygen was evolved during the transformation. A control experiment (table I, experiment 1) in which one lot of leaves was carried through similar operations except for illumination, demonstrated that the method of handling produced no significant quantity of chlorophyll.

The photochemical transformation of protochlorophyll to chlorophyll a is relatively complete (~ 85 %) under these strictly anaerobic conditions. No detectable quantity of chlorophyll b is produced under these circumstances. Although the protochlorophyll-chlorophyll transformation is supposed to be a hydrogenation (6) it does not take place in the absence of light in a pure hydrogen atmosphere.

TABLE I

OXYGEN EVOLUTION DURING THE TRANSFORMATION OF PROTOCHLOROPHYLL TO CHLOROPHYLL A

	EXPERIMENT NO.		
	1	2	3
Minutes of irradiation ...	0.0	10	10
Gm of plant material	0.50	0.48	0.50
μl of O_2 * evolved	...	0.0018	0.0011
Moles of O_2 evolved	...	0.75×10^{-10}	0.44×10^{-10}
Gm of chlorophyll a formed	0.0134×10^{-6}	4.61×10^{-6}	4.11×10^{-6}
Moles of chlorophyll a formed	0.0015×10^{-8}	0.516×10^{-8}	0.460×10^{-8}
Molar ratio of O_2 evolved to chlorophyll a formed	...	1.45×10^{-2}	0.95×10^{-2}
Sum of chlorophyll a + protochlorophyll (gm)	6.09×10^{-6}	5.44×10^{-6}	5.03×10^{-6}
Percent transformation to chlorophyll a	0.2	85	82

* At room temperature (~ 25°C) and one atmosphere of pressure.

The mechanism whereby protochlorophyll is transformed to chlorophyll in the plant during irradiation is not known. From chemical considerations, the possibility exists that protochlorophyll could act as a hydrogen acceptor in the splitting of water thereby being transformed to chlorophyll a. If this happened, oxygen might be liberated according to the equation:

$$\text{Protochlorophyll} + \text{water} \xrightarrow{\text{light}} \text{chlorophyll a} + \text{oxygen} \quad \text{(Eq. 1)}$$

Inasmuch as the oxygen liberated is less than 3 % of that required by this equation, it is unlikely that the transformation follows this path.

EVOLUTION OF OXYGEN BY LEAVES EXPOSED TO CONTINUOUS ILLUMINATION: Leaves harvested from dark-grown barley seedlings were placed in glass vials containing water, about 1 cm deep, and irradiated in air for different periods of time with two 40-watt no. 4500 White Westinghouse fluorescent lamps. The leaves were about a meter distant from the lamps where the light intensity was approximately 110 fc. Two samples were irradiated at a time: one, from 0.2 to 0.3 gm, was used for subsequent measurement of oxygen evolution; the other, from 0.2 to 0.8 gm, was used for pigment analysis. The average results of three experiments are shown in figures 1A and 1B (continuous lines).

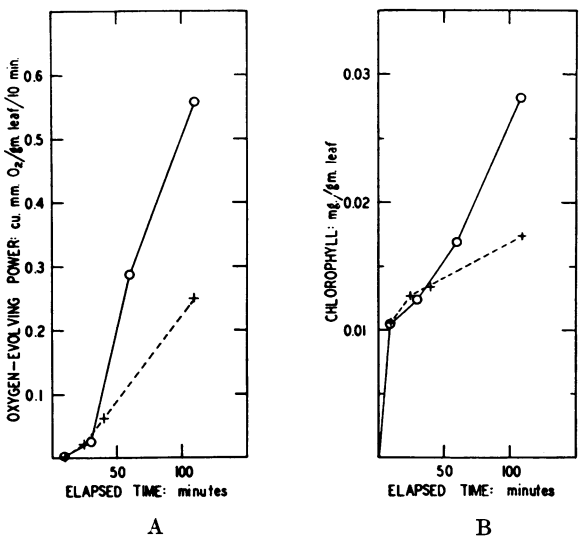

FIG. 1. Development of oxygen-evolving power (fig 1A) and of chlorophyll (fig 1B) in dark-grown barley leaves irradiated with various regimes of light and darkness. Continuous periods of illumination, ——○——; divided periods of illumination, - - - + - - -. In the experiments for the divided periods of illumination, the elapsed time is the sum of the sojourn in the dark plus 10 minutes of irradiation.

Figure 1 shows that as the period of irradiation, previous to the measurement of oxygen evolution, is increased, the development of oxygen-evolving capacity and of chlorophyll a both increase. During the initial period of exposure, the leaves acquire very little capacity to evolve oxygen although they form considerable chlorophyll. Exposures between 30 and 110 minutes bring about substantial increases in oxygen-evolving capacity, which are roughly proportional to the time (fig 1A). Chlorophyll formation is very rapid during the first minutes of irradiation; it then slows up for a short period after which it accelerates and approaches proportionality to the time of irradiation (fig 1B).

In figure 2 is shown the relationship between the chlorophyll a content of the leaves and their oxygen-

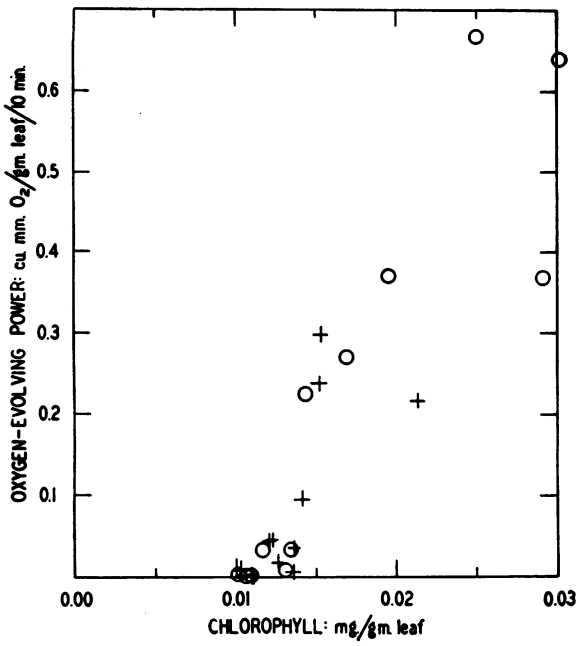

FIG. 2. Comparison of the oxygen-evolving power and the chlorophyll content of dark-grown barley leaves which had been irradiated with various regimes of light and darkness. Continuous periods of illumination, O; divided periods of illumination, +.

evolving capacity (circles). It is obvious that relatively large quantities of chlorophyll a are formed without imparting to the leaves a significant capacity to liberate oxygen. This initial chlorophyll, formed during a short period of illumination, is derived from the transformation of the protochlorophyll originally present in the dark-grown leaves and is apparently photosynthetically inactive. As additional chlorophyll is formed, the oxygen-evolving capacity increases and, at first, about in proportion to the increase in chlorophyll.

EFFECT OF DIFFERENT REGIMES OF LIGHT AND DARKNESS ON CHLOROPHYLL CONTENT AND OXYGEN-EVOLVING CAPACITY: The effects of different regimes of light and darkness on the development of chlorophyll and oxygen-evolving capacity were also studied.

In the first regime, three pairs of leaf samples were treated in various ways and the effect on chlorophyll content and oxygen-liberating capacity determined: one pair was irradiated with the fluorescent lamps for 10 minutes; a second pair was irradiated for 10 minutes and then placed in the dark at about 20°C for 110 minutes; a third pair was irradiated for 5 minutes, then placed in the dark for 110 minutes, and again irradiated for 5 minutes. All these operations were carried out in air. After these pretreatments, chlorophyll determinations were made on one sample of the pair and oxygen-evolution measurements were made on the other. The leaf samples used for chlorophyll determinations weighed 0.5 gm and for oxygen evolution, 0.2 gm.

The results of these experiments are given in table II. They demonstrate that illumination for 10 minutes produces chlorophyll, but little, if any, capacity to liberate oxygen. Addition of a dark period to the initial irradiation increases the chlorophyll very little but increases the oxygen-evolving power very considerably. When the sojourn in the dark is followed by a second brief irradiation in air, the quantity of chlorophyll is about doubled but the oxygen liberation is increased immensely.

A considerable quantity of protochlorophyll was produced in sample no. 2 during the sojourn in the dark. This protochlorophyll was undoubtedly converted to chlorophyll during the measurement of oxygen evolution. It it is assumed that the chlorophyll so produced accounted for the oxygen evolved in this experiment then it is remarkable that a final illumination in air, which would have produced little if any additional chlorophyll, should have enlarged the oxygen-liberating power so much (sample 3).

It may be concluded that once the dark-grown leaves have been illuminated they build up an agent in the dark by metabolic processes which enables them to liberate oxygen when they are again illuminated. This agent is quickly and considerably augmented by a second brief period of illumination in air following the sojourn in the dark. It seems clear that this agent cannot be entirely identified with chlorophyll because the increase in chlorophyll is about twofold whereas the increase in oxygen-liberating capacity is about 200-fold.

In the second regime, the effect of length of dark period interposed between two 5-minute light periods was examined. The average results of three experiments are graphed in figures 1A and 1B (broken lines). The abscissas represent the elapsed time, from the beginning of the first illumination period to the end of the second, that was used preparatory to measuring oxygen evolution; the ordinates show the corre-

TABLE II

THE EFFECT OF DIFFERENT REGIMES OF LIGHT AND DARKNESS ON THE CAPACITY OF DARK-GROWN BARLEY LEAVES TO EVOLVE OXYGEN DURING A TEN-MINUTE PERIOD OF PHOTOSYNTHESIS

	Sample no.		
	1	2	3
Regime for pretreatment			
Light	10	10	5
Dark	0	110	110
Light	0	0	5
Chlorophyll			
mg per gm leaf	0.0095	0.0099	0.0177
Protochlorophyll			
mg per gm leaf	0.0012	0.0049	0.0011
Oxygen evolution			
μl per gm leaf per 10 min	0.0021	0.088	0.425
Relative chlorophyll	1.00	1.04	1.87
Relative oxygen evolution	1.0	42	202

sponding quantities of oxygen liberated per gram of leaves during ten minutes of photosynthesis (fig 1A) and of chlorophyll produced (fig 1B).

It is evident that 10 minutes of continuous illumination (zero dark period) produced, at most, only a very small capacity to liberate oxygen. As the interposed dark period was increased (fig 1A), the power for oxygen liberation was increased almost in direct proportion to the length of the dark period, discounting a short period of induction.

The amounts of chlorophyll a produced under the different irradiation regimes just described are shown in figure 1B. With 10 minutes of continuous illumination (zero dark period), considerable chlorophyll was produced. The chlorophyll was increased by interposition of dark periods, but the proportionate increase in chlorophyll was very much less than the proportionate increase in oxygen evolution.

The relationship between oxygen-evolving capacity and chlorophyll content of leaves given divided periods of illumination is shown by the crosses in figure 2. The results are similar to those obtained with continuous illumination, as comparison with the circles demonstrate.

These experimental results confirm, by a different method, what has been found by others, that chlorophyll can be present in living organisms without affording the ability for photosynthesis. Formally, increase in the ability to liberate oxygen corresponds to the increase in chlorophyll content. This relationship, however, may be fortuitous since these two properties may increase simultaneously without being functionally connected.

The great enhancement of the power to liberate oxygen induced by subjecting dark-grown leaves, which have been briefly illuminated, to a period of darkness and subsequent illumination, demonstrates that this increased oxygen-evolving capacity results from both metabolic and photochemical reactions: metabolic, because of the influence of the dark period; photochemical, because of the effect of the second irradiation.

What causes the enhancement of the photosynthetic capacity is in doubt but three possibilities exist: activation of the initial chlorophyll formed and of all the chlorophyll formed thereafter; the formation of a chlorophyll different from that derived from the original protochlorophyll; or the elaboration of substances which work in conjunction with chlorophyll.

At present nothing can be said regarding the activation of inactive chlorophyll.

The observed difference in the abilities of the chlorophyll formed from protochlorophyll and of that formed subsequently to liberate oxygen suggests that these chlorophylls are different. This interpretation is supported by the observation of Krasnovskii and Kosobutskaya (13) that the spectrum of the natural chlorophyll first formed by irradiation of etiolated bean leaves differs from that of the natural chlorophyll formed afterwards.

Some of these experiments bring evidence that substances besides chlorophyll are formed which greatly increase photosynthetic ability. The treatment, in which leaves were given a final brief irradiation following an introductory exposure to light and darkness, increases the oxygen-liberating power so tremendously in comparison with the increase of chlorophyll that the greatly increased oxygen-evolving power can hardly be reconciled with the small additional quantity of chlorophyll produced. Rather, the results point to the elaboration of substances which act in conjunction with chlorophyll.

In many instances, chlorophyll b was not present in leaves from which oxygen was evolved. From this it may be concluded that chlorophyll b is not necessary for oxygen liberation.

Summary

The development of chlorophyll and of oxygen-evolving power was followed simultaneously in etiolated barley leaves that had been illuminated under various conditions. The pigments, protochlorophyll and chlorophyll, were estimated spectrophotometrically and the oxygen evolved was determined by means of a phosphorescence-quenching method.

The transformation of protochlorophyll to chlorophyll a took place in an anaerobic atmosphere. It was not accompanied by the evolution of oxygen.

Although the etiolated leaves that had been irradiated in air for a short time possessed chlorophyll that was derived from their protochlorophyll, they lacked the power to evolve oxygen. If, after this irradiation, these leaves were stored in air in darkness, they acquired a small capacity for oxygen production. This capacity was greatly augmented by a subsequent brief irradiation.

Chlorophyll b was not necessary for oxygen evolution.

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MEASUREMENT OF ALGAL GROWTH UNDER CONTROLLED STEADY-STATE CONDITIONS[1]

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For reasons set forth in a following paper it has become desirable to measure growth of a unicellular alga as a function of intensity and intermittency of illumination. Conventional methods cannot be used since growth of an algal culture is accompanied by increasing mutual shading of cells. Even though the incident illumination is held constant the intensity may vary greatly between the front and back surface of the culture and the average intensity per cell decreases continually during the course of an experiment. Some special means of measuring growth must be devised.

The present paper describes an apparatus for measuring the growth rate of an alga under conditions of constant population density and constant volume in a small chamber which may be illuminated under careful control in a focused optical system. As a means of measuring growth under steady-state conditions, the method is related to the *continuous-culture apparatus* of Myers and Clark (3) and the *chemostat* of Novick and Szilard (4).

The lucite growth chamber in its final form is diagrammed in figures 1 a and 1 b. The culture cell C, 5.7 cm I. D. × 1.0 cm thick, is sandwiched between two chambers, A, for temperature control by thermostated water circulated through ports, B. Complete details of the culture cell are shown in figure 1 b. The algal suspension is stirred by a one-fourth-inch stainless steel shaft, S, driven by a small air turbine. A 4 % carbon dioxide in air gas mixture is delivered continuously into the upper portion of the cell and fresh medium is added intermittently under control of a photometric device described below. The glass overflow tube, O, serves as an automatic siphon to maintain a constant volume of suspension in the chamber. Earlier arrangements of the overflow device shown in figures 1 c and d were abandoned for reasons which will be presented later.

The complete apparatus is shown in figure 2.

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