Responses of Heterotrophic Cultures of Chlorella vulgaris Beyerinck to Darkness and Light. II. Action Spectrum for and Mechanism of the Light Requirement for Heterotrophic Growth 1,2

Edward P. Karlander and Robert W. Krauss
Department of Botany, University of Maryland, College Park, Maryland

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Summary. Chlorella vulgaris Beyerinck (Emerson's strain), fails to grow in the dark even when sugars are provided. This phenomenon was clearly demonstrated in the alga. C. vulgaris, for which the growth rate in darkness on a glucose medium remained constant for 2 days and then declined to approach zero. Pigment concentrations also declined in darkness. Changes in flow rate of 1% CO₂-in-air from zero to 7 ml per minute caused a progressive increase in the dark growth rate over a 5-day period, but did not maintain growth in the dark. Rates above 7 ml per minute produced no changes in growth rates.

White light intensities below the compensation point of the alga maintained heterotrophic growth. The saturation value for this response was 0.8 μW/cm². White light also initiated growth in non-growing cultures transferred from darkness to light.

The action spectrum for heterotrophic growth indicated a porphyrin as the active pigment. Light in the 425 μ region was 4 times as effective as white light in stimulating heterotrophic growth. A secondary peak of growth stimulation occurred in the 575 μ region.

The respiration of glucose by the alga was stimulated by low intensities of white light. This response was not immediate, but was clearly present after the third day of incubation.

Malonate and cyanide were inhibitory to growth of C. vulgaris on inorganic medium or glucose medium under 300 ft-c of white light. These data suggested that succinic dehydrogenase and cytochrome oxidase systems were present.

Substances inhibitory to growth were excreted into the medium under dark-growth conditions, and 2 of these substances were identified as formic and acetic acids.

The evidence suggested that respiration of glucose cannot proceed for an extended period of time in darkness. The reason for this is postulated to be the lack of a cytochrome or a cytochrome precursor.

The responses of organisms to light have generally been recorded in terms of their morphology and physiology. In green plants, photosynthesis has been the major concern since the time of De Saussure and Priestley.

However, nonphotosynthetic responses of organisms to light have received increasing attention in recent years. Of special interest to students of photosynthesis, as well as those interested in regulatory effects of radiation, is the inability of certain plants to grow in the absence of light. Such obligate phototrophy is found in a number of species of algae. At least one species of Chlorella (5, 9, 12, 13, 14, 15) has been well documented for its inability to grow on any reduced carbon source in total darkness. This alga of particular interest is Chlorella vulgaris (Emerson's strain), which Finkle et al. reported would not grow on glucose in the dark (5). This observation was confirmed by Killam and Myers (14, 15) who attempted to overcome the "dark-block" by adding various substances to the medium. The inability of the organism to grow for an extended period of time on glucose in the dark was also confirmed by Griffith (9) and Karlander and Krauss (13).

The studies in this paper were designed to determine the action spectrum for counteracting the "dark-block" in Chlorella vulgaris and to find the cause for the cessation of growth in the dark.

Materials and Methods

Pure cultures of Chlorella vulgaris Beyerinck (Emerson's strain), from Dr. Meyers' laboratory, obtained originally from Dr. Appleman, were employed in this study. This strain has a maximum growth rate in the light of 1.8 doublings per day.

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Two media were employed. The inorganic medium contained the following nutrients in g per liter: KNO₃, 1.00; MgSO₄·7H₂O, 0.25; K₂HPO₄, 1.00; KH₂PO₄, 1.00; H₂BO₃, 0.10; EDTA₃NaFe, 0.0385; EDTA Na₂Mn, 0.0071; EDTA Na₂Ca, 0.0077; EDTA Na₂Co, 0.0093; EDTA Na₂Cu, 0.0077; and EDTA Na₂Zn, 0.0067. The organic medium was of the same composition as the inorganic medium plus 10 g of glucose per liter. The media were prepared with distilled water from a glass still. The initial pH was 6.8. The experiments dealing with inhibitors involved the inorganic or glucose medium with either KCN or malonate at various concentrations.

Cultures used to determine the rate of growth in darkness, the loss of pigments, and the effects of bubble rate were contained in Coleman cuvettes, 19 × 150 mm, supplied with the glucose medium, and aerated at 25°C. The cuvettes were closed with cotton stoppers through which cotton-plugged bubbler tubes were inserted to deliver 1% CO₂ in-air. This aeration mixture served as a gas supply and kept the cells suspended. The rate of aeration was controlled by an external monitoring system, as described by Karlander and Krauss (13). Cuvettes were removed daily from the dark chamber, and the OD absorption spectra, and dry weights of the cells were obtained. The bubble rates of cultures, except where bubble-rate was being studied, were maintained at 10 ± 3 ml per minute. Inocula were cultured under the optimum conditions of white light (275 ft-c) and temperature (25°C) on the glucose medium. Absorption spectra were measured on a Bausch and Lomb 505 recording spectrophotometer using 5 μm slit widths and opal glass diffusing plates, and equipped with an EMI-6255 photomultiplier. The OD at 440 μm and at 680 μm were divided by the OD at 550 nm for each culture and the ratio minus one plotted against time.

The effects of inhibitors were studied in 25 × 200 mm tightly stoppered pyrex test tubes, each containing 20 ml of culture. The cultures were shaken on a Labline shaker bath at 25°C under a 100-w tungsten bulb positioned to give an illumination of 300 ft-c at the culture surface. Both the organic (glucose) and inorganic media were used. OD's were read on a Coleman Jr. spectrophotometer over a 48-hour period and plotted against time.

The growth rate response to white light was obtained using unilateral illumination from a tungsten lamp. Coleman cuvettes, 19 × 150 mm, were fitted with cotton plugs and equipped with cotton-plugged bubbler tubes. Cells were grown on glucose medium at 25°C for 5 days and their dry weights obtained. Irradiance was measured with a calibrated Reeder thermopile inserted in a cuvette in the water bath.

The inocula for the experiments, designed to test the utilization of glucose in the light and in the dark, were cultured on the inorganic medium in 3-liter Erlenmeyer flasks fitted with cotton stoppers and cotton-plugged bubbler tubes. The flasks were aerated with 1% CO₂ in-air and shaken on a Labline shaker bath at 25°C under white light with an illuminance of 300 ft-c at the culture surface. Each flask contained one and a half liters of culture. When these had attained a cell concentration of 5 mg per ml, they were centrifuged and the supernatant decanted. They were then resuspended in inorganic medium and centrifuged, the supernatant decanted, and the cells resuspended in inorganic medium. The cell suspension was then split into 2 parts of 100 ml each and placed in 2.50 × 13.3 mm test tubes. Each of these tubes was closed with a rubber stopper through which were fitted an internal cooling coil, a cotton-stoppered bubbler tube, a cotton-stoppered exhaust tube, and a port for introducing glucose. The cultures were then placed in individual dark boxes and agitated by magnetic stirrers and internal stirring bars. The cultures were kept in the dark for 24 hours to reduce the carbohydrate in the cells. Then the exhaust port of each culture was fed into the DC 100 gas flow chamber installed on a Model 6000 Dynaod made by Nuclear Chicago. One hundred and fifty μC of uniformly-labeled C¹⁴ glucose, in 1% nonradioactive glucose solution, were then added through the glucose port. The evolved radioactive CO₂ was recorded as voltage. Activity was obtained for one-half hour each hour from each culture. At the end of 48 hours, one culture was irradiated with white light from a 150-w tungsten lamp at the rate of 10 μw per cm² at the flask surface while the control culture remained in the dark.

Further studies on the respiration of glucose by algae in light and darkness were conducted with C¹⁴ labeled CO₂ collected in NaOH. Two 1-liter Erlenmeyer flasks, each containing 500 ml of glucose medium with 100 μC of uniformly-labeled C¹⁴ glucose, were each inoculated with 10 ml of cell suspension, giving an initial cell concentration of 0.028 mg per ml. The inoculum was from an optimally growing culture. One flask (the control) was in complete darkness and the other was unilaterally irradiated with a 100-w tungsten lamp supplying 1.0 μw per cm² of white light at the flask surface. Agitation was by internal stirring bars. The flasks were fitted with rubber stoppers with 2 glass tubes plugged with cotton. The inlet tube opened below the culture surface and the exhaust tube above the surface. CO₂-free air was bubbled through the cultures. The exhaust tube was connected to a sintered glass aerator which was submerged in 10 ml of 1 N NaOH to collect the C¹⁴O₂. The NaOH was changed every 24 hours and an aliquot was assayed by placing 0.5 ml of the solution containing the trapped C¹⁴O₂ in a planchet and adding 0.05 ml of saturated BaCl₂ solution and 0.05 ml of a 1.0 M NH₄OH solution. The planchet was dried for 85°C and then counted with a Packard automatic scaler, series 250 A, with a model 220-A proportional counting amplifier and a model 210 flow-window counter.

The experiments with monochromatic light were performed in a dark room with apparatus as described by Karlander and Krauss (13). This apparatus con-
sisted of 2 water baths, each containing a test tube with a thermopile and a test tube of algal suspension. The test tubes in each water bath were illuminated by 1 monochromator. The test tube with the thermopile could be placed in the light path periodically for measurements of irradiance, while the cells were being irradiated for long periods at controlled temperatures. A tungsten lamp was used as a light source for each of the monochromators. The water baths in these experiments were 86 mm in diameter by 12 cm long. Energy measurements were made with a Reeder thermopile calibrated with a standard lamp from the National Bureau of Standards.

In the experiments with monochromatic light, 25 \( \times \) 119 mm test tubes, fitted with cotton stoppers and bubbling tubes for supplying 1% CO\(_2\)-in-air, contained the cultures on the glucose medium. The medium was inoculated from optimally-growing cultures on the glucose medium under 275 ft-c white light at 25°. Experiments employed various wavelengths of monochromatic light using a 20.8 \( \mu \)m halfband width and an equal light intensity of 0.2 \( \mu \)w per cm\(^2\). Controls were placed in darkness. The cultures were incubated under the various spectral bands for 5 days, after which their OD's, absorption spectra, and dry weights were measured.

For gas chromatographic analysis of supernatant media, algae were cultured in the dark in 3-liter Erlemeyer flasks with cotton plugs. Each flask contained one and a half liters of glucose medium. The cultures were grown in the dark for 12 days. The initial cell concentration was 0.2 mg per ml dry weight and the initial pH was 6.8. The final pH was 6.5. The cells were removed by centrifugation and the supernatant acidified to pH 1.6 with H\(_2\)SO\(_4\) prior to extraction with ether. One-tenth ml of octyl alcohol, 0.05 ml of concentrated H\(_2\)SO\(_4\), and 4 g of anhydrous CaSO\(_4\) were then added to each extract of 1.5 liter of supernatant. The extract was then refluxed for 22 hours at 43° to bring about esterification of the octanol-1 with any organic acids present. Standards of formic, acetic and lactic acids were esterified in the same manner as the supernatant extracts. Ether extracts of the glucose medium, as well as the glucose medium on which cells had been grown in the light, were subjected to the esterification procedure. The ether extracts of these solutions were analyzed by gas chromatography.

A Glowal Chromalab, model A-110, dual-column gas chromatograph served to analyze for esterified organic acids. The 2-meter, 3.4-mm ID glass columns were packed with a commercial preparation of 1% Dow Corning Qf-1 silicone on a filler of 100 to 120 mesh gas-chrom P. The chromatograph employed a hydrogen detector supplied with hydrogen at the rate of 25 ml per minute from an Aerograph model 650 hydrogen generator. The tracing was recorded on a Leeds and Northrup model H speedomax recorder with a range of 0 to 50 mv. The column temperature was 105° and the detector and flash temperature was 150°. A range attenuation of 3 \( \times \) 10\(^{-1}\) amp was used. The carrier gas was argon at 5 psi. Three-\( \mu \)l samples were delivered from a Hamilton 10 \( \mu \)l syringe.

A formic acid color test was used to confirm its presence. The ether extract of the supernatant was reduced to 1 ml and placed in a test tube. Any formic acid present was then converted to formaldehyde by nascent hydrogen produced by magnesium powder and dilute HCl. Three ml of 12 N sulfuric acid and 0.3 g of chromotropic acid (1,8-dihydroxynaphthalene-3,6 disulfonic acid) were added to the test tube. The tube was then placed in a 60° water bath for 10 minutes. A violet pink color indicated the presence of formic acid (4).

All experiments were conducted with cells grown in bacteria-free, unialgal cultures. All experiments and all data are the results of at least 2 experiments with replication in each.

**Results and Discussion**

When an organism such as *C. vulgaris* is cultured under various qualities and quantities of light, OD as a measure of growth may be misleading. The reasons for this are that pigments may be formed or depleted under various light regimes, and that the ratios of pigments may change as shown previously by Karlander and Krauss (13). Better growth criteria, for comparing results of different light regimes, are cell numbers as used by Griffiths (8, 9, 10) or dry weights. Growth and growth rates presented herein, except for the inhibitor studies, are therefore reported in terms of dry weight. The differences in OD's in the inhibitor experiments were large enough to indicate a decrease in growth with increased inhibitor concentration.

The data of figure 1 characterize the growth of *C. vulgaris* when cultured in the dark on the glucose medium with adequate aeration. The rate of growth remained nearly constant for the first 2 days and then dropped off sharply during the third day. After the third day, the rate declined more slowly.

![Fig. 1. The growth rates of *Chlorella vulgaris* (Emerson's strain), after transfer into darkness, in 19 \( \times \) 150 mm cuvettes containing 11 ml of glucose medium bubbled with 1% CO\(_2\)-in-air.](image-url)
and approached zero. These data show that a history of being cultured in the light enables the cells to grow for 2 days in the dark at 90% of their maximum rate. After this 2-day period whatever was carried over from the light was rapidly lost or depleted, as evidenced by the diminishing growth rate. These data appear to support the suggestion of Danforth (2) that the “dark-block” could be the failure of the organism to couple the oxidative release of energy with substrate assimilation. However, Killam and Meyers (14, 15) reported that carbon was assimilated in the dark and that iodine staining indicated a greater starch content per cell for dark-grown than for light-grown cells. Therefore, the locus of the “dark-block” does not seem to be either the permeability of the cells to the substrate or substrate assimilation.

In order to have some measure of pigment loss in cultures in darkness, the absorption spectra of the whole cells, used in the experiment demonstrating the progressive reduction in growth presented in figure 1, were used to obtain the pigment decay curves presented in figure 2. The 680 m\(\mu\) peak represents predominantly chlorophyll, but the 440 m\(\mu\) peak is the result of absorption of chlorophyll plus carotenoid. It appears that the carotenoid content per cell mass increased during the first day of darkness while the chlorophyll content per cell mass decreased. During the second day of dark growth the carotenoids decreased rapidly in relation to chlorophyll. After the second day, chlorophyll and carotenoids decreased in such a manner as to make the 2 curves nearly parallel. This is more clearly shown in the difference curve.

**Fig. 2.** Ratios of absorption at 440 m\(\mu\) and 680 m\(\mu\) to absorption at 550 m\(\mu\) in cultures of *Chlorella vulgaris* (Emerson’s strain), after being transferred from light to darkness. The cells were incubated in 19 \(\times\) 150 mm cuvettes on glucose medium at 25\(^\circ\) and bubbled with 1% CO\(_2\)-in-air.

**Fig. 3.** The effect of bubbling rate on the growth rate of *Chlorella vulgaris* (Emerson’s strain) grown in darkness on glucose medium at 25\(^\circ\) for 5 days. The algae were incubated in 19 \(\times\) 150 mm cuvettes containing 11 ml of medium and had an initial dry weight of 0.06 mg/ml.

**Fig. 4.** The effect of KCN on the growth of *Chlorella vulgaris* (Emerson’s strain) in 20 ml of glucose medium in sealed 25 \(\times\) 200 mm test tubes at 25\(^\circ\). The cultures were agitated on a shaker bath and irradiated with 300 ft-c white light. OD’s were read at 550 m\(\mu\).
Earlier studies indicated that the results of growth experiments in the dark were strongly influenced by the rate of bubbling. The importance of adequate aeration is illustrated in figure 3. The growth for a 5-day period did not become optimal until an aeration rate of 6 to 7 ml per minute of 1% CO₂-in-air was attained. This increase in dark growth of C. vulgaris on a glucose medium was also noted by Griffiths (8) for another strain of C. vulgaris.

To shed further light on the nature of the respiratory pathway and terminal oxidase system of C. vulgaris, its responses to cyanide and malonate on both inorganic and glucose media were plotted (fig 4, 5, 6, and 7). These inhibitors were added to the medium at pH 6.8, which is well below the pK value of HCN, but far above the pK values of malonic acid. Since malonic acid would be nearly all in the ionic form, it might not be expected to be inhibitory (11). However, both substances inhibited growth in the inorganic medium and the glucose medium. The reduced growth in the inorganic cultures, relative to the glucose cultures, is attributed to the lack of CO₂ for photosynthesis in sealed culture vessels. If one assumes that respiration is a pre-

![Fig. 5. The effect of sodium malonate on the growth of Chlorella vulgaris (Emerson's strain) in 20 ml of glucose medium in sealed 25 x 200 mm test tubes at 25°. The cultures were agitated on a shaker bath and irradiated with 300 ft-c of white light. OD's were read at 550 μ.](image)

![Fig. 6. The effect of KCN on the growth of Chlorella vulgaris (Emerson's strain) in 20 ml of inorganic medium in sealed 25 x 200 mm test tubes at 25°. The cultures were agitated on a shaker bath and irradiated with 300 ft-c of white light. OD's were read at 550 μ.](image)

requisite for growth, then the cyanide data indicate a respiratory oxidase containing a heavy metal and the malonate data indicate that succinic dehydrogenase is present (6, 16). Hence it appears that C. vulgaris has at least some of the usual metabolic pathways. Gibbs (7) believes that the presence of the Embden-Meyerhof pathway, the pentose phosphate pathway, and Krebs’ cycle can be a reasonable assumption for the algae. Fogg (6) noted that the evidence points to a respirational pattern in the Chlorophyceae similar to that of other organisms, and Devlin (3) has shown that many of the usual glycolytic enzymes are present in C. pyrenoidosa. However, C. vulgaris and C. pyrenoidosa differ with respect to their growth rates in darkness on glucose media, in compensating light with or without glucose, and in continuous saturating light with or without glucose (15). Furthermore, C. pyrenoidosa can grow on glucose in the dark and does not lose its chlorophyll.

Once the O₂ requirements for dark growth were well understood, it became possible to do experiments to measure accurately the responses of growth to various light intensities. The growth response curve
of *C. vulgaris* to white light is presented in figure 8. The maximum rate of growth was reached at a saturation value of 0.8 μw/cm². This value is well below the photosynthetic compensation point. These data, as well as those from experiments where CO₂ was excluded from the aeration supply, indicate that photosynthetic fixation of CO₂ is not the reason for the stimulation of heterotrophic growth.

The presence of a fairly high O₂ requirement for growth in the dark suggested that the stimulation of growth by light might be located in the respiratory mechanism. Therefore, the rate of respiration of exogenous substrate under dark and light conditions was obtained by measuring the rate of C¹⁴O₂ production by cells of *C. vulgaris* which were first starved and then supplied uniformly-labeled C¹⁴ glucose. The cells were incubated in the dark on uniformly-labeled C¹⁴ glucose for 48 hours, and then 1 culture was irradiated with white light. No difference in C¹⁴O₂ production was detected between the cultures in the dark or in the light over a 16-hour period. Light did not give a response immediately detectable in respiration. These data correspond well with the data concerning the dark growth of *C. vulgaris* presented in figure 1, where growth was shown not to stop immediately in the dark, but actually to remain almost constant for the first 2 days.

![Graph](image-url)

**Fig. 7.** The effect of sodium malonate on the growth of *Chlorella vulgaris* (Emerson's strain) in 20 ml of inorganic medium in sealed 25 × 200 mm test tubes at 25°C. The cultures were agitated on a shaker bath and irradiated with 300 ft-c of white light. OD's were read at 550 nm.

![Graph](image-url)

**Fig. 8.** The growth response of *Chlorella vulgaris* (Emerson's strain) to various levels of continuous unilateral irradiation of white light. Cultures were grown for 5 days on glucose medium in 19 × 150-mm cuvettes and bubbled with 1% CO₂-in-air.

![Graph](image-url)

**Fig. 9.** Comparison of the accumulative amounts of C¹⁴O₂ evolved by two 500-ml cultures of *Chlorella vulgaris* (Emerson's strain). The culture in the light received 1.0 μw/cm² of white light at the surface of the 1-liter culture flask. Both culture vessels contained 100 μw of n-C¹⁴-glucose in addition to the normal glucose medium. The initial cell concentration was 0.078 mg dry weight/ml.
The apparent lag in the effect of light on respiration is shown in the data in figure 9. The cells were not starved, but were transferred from cultures growing under optimal conditions. The difference between the cultures in the light and those in the dark did not become readily apparent until the third day when the difference was apparent. The rate of respiration of the dark culture remained constant for 6 days, whereas the respiration rate of the irradiated culture continued to rise. The constant slope of the light curve in figure 9 after the sixth day is attributed to the age of the culture, since the cell concentration had exceeded that optimal for good growth.

The long period of low-level respiration in the dark shown in figure 9 suggests that glucose is taken up in the dark as the result of the cells being illuminated previously. However, the rate of respiration did not increase with time as in the light culture but continued at a constant rate for 9 days.

The action spectrum plotted in figure 10 indicates that the photoreceptor is a porphyrin compound, since the peak at 425 µµ is in the Soret region. Porphyrins are also known to have 4 absorption bands in the 500 to 700 µµ region, their relative heights depending on their structures (18). Chlorophyll was ruled out as the active pigment, since there was no red peak and also because the 575 µµ peak was in the region of minimum absorption of chlorophyll (17, 18). Carotenoids were also ruled out by the 575 µµ peak, since the carotenoids found in *Chlorella* did not have absorption peaks in this region (6). It should be noted that the energy at 425 µµ was 4 times as effective in overcoming the “dark-block” as that for white light. A further action spectrum was found by Bjorn, Suzuki, and Nilsson (1) for the light response of excised wheat roots.

The possible interference in the respiratory mechanism, in the absence of light, would lead to the expectation that some respiratory intermediate might accumulate and be secreted by the cells. The drop in pH, which was observed earlier in cultures grown in darkness (13), further supported this logic. Therefore, the supernatant fractions were examined for the presence of organic acids. (Figure 11 indicates that.) Compounds were liberated from cultures kept in the dark on glucose. These were shown in other chromatographs not to be released from light-grown cultures. Two of the compounds liberated in the dark were identified as formic and acetic acids by the use of gas chromatographic analysis. Since formic acid was unexpected, confirmatory spot tests were made. The chromatropic acid test produced a weak violet to pink color, supporting the data from the gas chromatograph for the presence of formic acid (4). These acidic products have also been shown to be inhibitory to *C. vulgaris* (Bramon No. 1 strain) cultured in darkness by Griffiths (8). It can be assumed that an inoperative terminal oxidase system would adjust metabolism toward anaerobiosis. In this regard it should be noted that Weiss and Mukerjee (19) found acetic acid as the major acid product of fermentation in *C. vulgaris*.

Experiments, aimed at supporting the view that formic and acetic acids were the direct cause of the
inhibition of growth in the dark, were performed. When the supernatant medium containing these acids was replaced by fresh medium, growth in the dark accelerated for a time. However, it was observed that the amount of acceleration of growth depended on the amount of illumination employed during the replacement of media. When illumination was eliminated entirely during replacement, no growth was observed during the subsequent dark period. It was apparent that the secreted acids were symptoms of the inhibition of growth and not the direct cause of it.

Considering the evidence at hand — the continuing growth at 90% of the maximum rate for 2 days in darkness, the declining rate after the second day in darkness, the decline in pigment content, the effect of aeration on cultures grown in darkness, the effect of inhibitors on growth, the results of low intensities of white light on growth and respiration, the action spectra, and the production of fatty acids in darkness — there is ample reason for considering that the terminal oxidase system of C. vulgaris is the locus of the “dark-block” to heterotrophic growth on glucose.

In conclusion, the experiments strongly support the view that light affects the exogenous respiration of glucose through its action on one of the porphyrins which is a precursor to, or is one of, the cytochrome pigments in the terminal oxidase chain. Just how the porphyrin is affected can only be postulated from the foregoing data. Perhaps the particular cytochrome is synthesized through a light-mediated reaction analogous to the conversion of protochlorophyll to chlorophyll (17). If the formation of cytochrome or one of its precursors is mediated by light, the gradual decline in growth rate noted in the dark would be explained by the gradual reduction of cytochrome, ultimately to a critical level. This would also explain the effect of O₂ pressure and aeration rate on the dark growth of this particular organism, since the diminishing amount of cytochrome would be compensated by a higher O₂ supply.