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Inhibition of Respiration in *Prototheca zopfii* by Light[1]

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ABSTRACT

Irradiation of cells of *Prototheca zopfii* with blue light inhibited the respiratory capacity of the cells. The inhibition of respiration was correlated with a photodestruction of cytochrome *c*(551), cytochrome *b*(559), and cytochrome a_3. Cytochrome *c*(549), cytochrome *b*(555), and cytochrome *b*(564) were unaffected by the irradiation treatment. The α-band of reduced cytochrome *a* was shifted from 599 to 603 nm by irradiation, an effect similar to that observed when methanol was added to nonirradiated cells. The presence of oxygen was required during irradiation for both photoinhibition of respiration and photodestruction of the cytochromes. Cytochrome a_3 was protected against photodestruction by cyanide. Photodestruction of these same cytochromes also occurred when washed mitochondria of *P. zopfii* were irradiated.

Blue and near-ultraviolet radiation are inhibitory or lethal to a wide variety of prokaryotic and eukaryotic microorganisms and to a number of tissues of higher plants and animals. Among the prokaryotes visible radiation and near-ultraviolet radiation have been reported to kill or inhibit the growth of the heterotropic bacteria *Streptococcus salivarius* (4), *Escherichia coli* (17, 19, 20), *Bacterium prodigiosum* (38), *Pseudomonas abruginosa* (20), and *Haemophilus infiuenzae* (18), and a number of carotenoidless mutants of such normally carotenoid-containing bacteria as *Sarcina lutea* (23), *Mycobacterium* sp. (43), *Halobacterium salinarium* (9), and *Myxococcus xanthus* (5, 6) and the chemoatotrophic bacteria *Nitrosomonas europaea* (1, 36) and *Nitrobacter winogradskyi* and the denitrifying bacterium *Micrococcus denitrificans* (16, 26).

The growth, division, and respiration of such eukaryotic microorganisms as the green algae *Chlorella pyrenodiosa* (37) and *Euglena gracilis* (8, 31), the colorless algae *Prototheca zopfii* (14) and *Astasia longa* (8), and the yeast *Saccharomyces cerevisiae* (10, 15, 24, 25, 34), have also been reported to be inhibited by blue light. Various processes in cells of higher plants and animals have also been reported to be inhibited by blue or near-ultraviolet light, such as cell division (21, 42), respiration (35, 33) and motility of sperm (41, 28, 29).

In spite of such a large literature on the inhibitory effects of blue and near-ultraviolet radiations, there have been few definitive investigations focused on molecular mechanisms, with the exception of a few studies made with a limited number of bacteria. Most studies, especially those concerned with eukaryotic organisms, have been of a more qualitative nature, with little attempt made to define the photoreceptor or the mechanisms of action. The point of departure for this investigation was the previous report (14) that blue light inhibited the growth of the colorless eukaryotic alga *Prototheca zopfii*. It was the purpose of this study to localize the primary physiological system inhibited by light and to identify the molecular site of the photoaction. An abridged report of this work has appeared previously (11).

MATERIAL AND METHODS

Growth Conditions. The alga *Prototheca zopfii* Kruger, obtained as a pure culture (ACC328) from the Culture Collection at Indiana University, was grown on a defined medium (Table I), which had been sterilized by autoclaving all nutrients together. For studies of the effects of light on protein and nucleic acid synthesis, respiration, and cell division, cultures were grown in 38- × 200-mm Kimax culture tubes containing 100 ml of medium. The tubes were fitted with cotton plugs through which bubbler tubes were passed to supply the culture with air and to keep the cells in suspension. The air was prefiltered through cotton and humidified before entering the culture medium. The cultures were grown in a constant temperature glass-walled water bath maintained at 28 ± 0.5 C. Growing cells were irradiated by means of two banks of fluorescent lamps placed on opposite sides of the bath. Each bank consisted of four 40-w GE-F40 cool white fluorescent lamps. Dark control cultures were grown under identical conditions in the same bath but were wrapped in aluminum foil. The intensity of light used in all these experiments was 1500 ft-c (summed from both directions) as measured with a Weston illumination meter or 2.9×10^4 ergs/cm^2·sec as measured with a Yellow Springs Instrument Co. radiometer, model 65. The cultures were always innoculated with cells from a log phase culture. For studies in which starved cultures were required, the cells were first grown for 24 hr in 2-liter Erlenmeyer flasks containing 333 ml of growth medium at 30 ± 1 C on a New Brunswick rotary shaker.

Starvation Procedure. When shaker cultures reached a concentration of 2 to 3 × 10^6 cells/ml, the flasks were cooled on ice, and the cells were aseptically transferred to sterile 250-ml centrifuge bottles and pelleted by centrifuging (525*g*, 5 min) in a Servall refrigerated centrifuge. The cells were suspended to one-fifth of their original volume in 0.015 M phosphate buffer, pH 6.9 (the starvation medium) and repelleted at 525*g* for 5 min; the resultant pellet was resuspended in the starvation medium to approximately the original concentration of 2 to 3 × 10^6 cells/ml and either divided into 100-ml fractions to be starved in culture tubes in the constant temperature bath or divided into 333-ml fractions in 2-liter Erlenmeyer flasks for starvation on the rotary shaker.

Isolation of Mitochondria. Mitochondria were prepared by a modification of the method of Bonner (3). Cells grown in a New Brunswick fermentor in 100 liters of standard glycerol medium

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Table I. *Medium for the Culture of Prototheca zopfii*

Compound	Concn
	g/liter
Sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)	1.0
NH_4Cl	1.0
K_2HPO_4	1.0
KH_2PO_4	1.0
$MgSO_4 \cdot 7H_2O$	0.25
Glycerol	5.0
Micronutrient stock solution (pH adjusted to 6.9)	
$Ca(NO_3)_2 \cdot 4H_2O$	2.0
$CoCl_2 \cdot 6H_2O$	0.04
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.22
$CuSO_4$	0.079
MoO_3	0.015
H_3BO_3	2.86
Fe-EDTA stock solution	
Na_2EDTA	42.0
$FeSO_4 \cdot 7H_2O$	24.9
Thiamine·HCl	0.0003

supplemented with Union Carbide antifoam SAG471 were harvested with a Sharples centrifuge and resuspended in 4 liters of phosphate buffer (0.025 M, pH 6.9). The cells, while being starved overnight at 4 C, were aerated by blowing humidified air across the stirred surface of the suspension. The starved cells, harvested by centrifugation (525*g*, 15 min), were washed and resuspended to three times their packed volume in a medium containing 0.3 M manitol, 1 mM EDTA, 0.1% bovine serum albumin, 0.05% cysteine, pH 7.2. The pH of the cell suspension was adjusted to 7.2 by the addition of KOH before the cells were broken in a Ribi press at a pressure of 10,000 p.s.i. The pH of the effluent was always kept between 6.8 and 7.2 by the addition of KOH. The brei was centifuged at 480*g* for 15 min to remove whole cells and cell wall debris, and the resultant supernatant fraction was centrifuged at 14,000*g* for 15 min. The mitochondrial pellet was suspended in a wash medium containing 0.3 M mannitol 1 mM EDTA, and 0.1% bovine serum albumin and homogenized with a loose fitting Teflon homogenizer. After a low speed centrifugation at 250*g* for 15 min, the mitochondria were sedimented at 5,900*g* for 15 min. The supernatant was aspirated off, and the mitochondrial pellet was resuspended in an equal volume of wash medium.

Protein and Nucleic Acid Determination. Protein and nucleic acid concentrations of the growing cultures were determined by means of a spectrophotometric assay *in vivo* based on the Warburg-Christian method for determination of protein and nucleic acid concentration in cell-free extract (40). The spectrophotometric assay on cell suspensions was found to be linear in the absorbance range from 0 to 0.3. Since the growth medium changed its absorbance character in the ultraviolet during the growth of the culture, the reference cuvette was always filled with growth medium obtained from a second aliquot of the growing culture cleared of cells by a high speed centrifugation. Protein and nucleic acid concentrations were calculated from formulas A and B, respectively:

$$\text{Protein concentration} = (1.560\ A_{280} - 0.763\ A_{260})d \quad \text{(A)}$$

$$\text{Nucleic acid concentration} = (0.0627\ A_{260} - 0.0358\ A_{280})d \quad \text{(B)}$$

where d = the dilution factor needed to bring the absorbance of the sample within the linear range, A_{280} = measured absorbance of diluted cell suspension at 280 nm, and A_{260} = measured absorbance of diluted cell suspension at 260 nm.

The spectrophotometric assay for protein on whole cell suspensions was in good agreement with control experiments in whoch the Lowry method (22) was used to determine protein concentration.

Determination of Cell Number. Cell counts were made with a Coulter electronic cell counter, model B. Cells were diluted with Millipore-filtered standard saline solution (0–9% NaCl) containing 1% formaldehyde to a concentration between 50,000 and 80,000 cells/ml. Coincidence corrections were calculated from correction tables supplied by the Coulter Co.

Oxygen Uptake Measurements. Rates of oxygen uptake were measured polarographically with a Yellow Springs Instrument Co. Clark-type oxygen electron with 0.001-inch Teflon film. The electrode was used with a specially constructed 3.3-ml Lucite cuvette immersed in a constant temperature bath (28 C). Additions of either cells or inhibitors were made to the equilibrated buffer through a movable well with a small opening. Potassium cyanide (reagent grade) and sodium azide were obtained from Matheson, Coleman and Bell; methanol C (reagent grade) from Allied Chemical, and antimycin A, type III, from Sigma.

High Intensity Irradiation of Cells and Mitochondria. Starved cells were irradiated in a Kimax tissue culture flask with a 1-cm path length with filtered light from an AH-6 super pressure mercury lamp. The light passed through a glass condensing lens, the plate glass window of the constant temperature water bath, and one or more Corning glass filters placed within the bath in front of the irradiation cell. In the experiments with the Corning filter 9782 the intensity of the irradiation at the surface of the irradiation vessel was 2×10^6 ergs/cm^2·sec, while in experiments with Corning filters 5433 plus 3850, the intensity was about 2.5×10^5 ergs/cm^2·sec as measured with a Yellow Springs Instrument Co. radiometer, model 65. Cells were kept in suspension either by stirring with a magnetic stirrer or by bubbling the suspension with humidified air.

In anaerobic irradiation experiments, concentrated starved cells were injected with a syringe through a silicon stopper into a 1-cm cuvette containing 5 ml of deoxygenated buffer. The diluted cell suspension was further deoxygenated by bubbling with purified nitrogen for 5 min before the cuvette was finally sealed. The cell suspension was irradiated for 1 hr with Corning filters 5433 plus 3850. Aerobic control suspensions were similarly exposed, but the cell suspensions were left open to the air.

Mitochondria were irradiated for 1 hr at 0 C without stirring in a Kimax tissue culture flask with filtered light from the AH-6 super pressure mercury lamp. The light, condensed by means of a glass lens, passed through two Pyrex windows in a water tank, 18 cm of water and the Corning 9782 glass filter before being reflected down onto a thin (1.7-mm) layer of mitochondria.

Spectroscopy. Absolute spectra were measured with a specially constructed recording single beam spectrophotometer similar to the instrument described previously by Norris and Butler (30). Monochromatic light (0.8-mm half-band width) from a Bausch and Lomb 500-mm grating monochromator was reflected by a front surface mirror vertically down through the sample cell to a 2-inch end window phototube with an S20 response (EMI 9558C) placed immediately below the sample cell. The sample cell and Dewar for measurement of spectra at liquid nitrogen temperature have been described previously (30). A linear base line correction system was employed to compensate partially for the system response of the spectrophotometer and the light scatter characteristics of the sample. The linear correction system was applied over the spectral range of 500 to 580 nm for the measurement of the absorption spectra of the α and β absorption bands of the *b*- and *c*-type cytochromes and over the range of 570 to 630 nm for the spectrum of the α-band of cytochrome oxidase.

Absolute spectra were measured with a light-scattering agent, Al_2O_3 (1 μ high purity alumina abrasive, Linde Division, Union Carbide), added to the sample in order to increase the optical path length and, thereby, increase the magnitude of the absorption bands (7). Addition of 0.5 g of Al_2O_3 to 1 ml of cell suspension (6 mm sample depth in cuvette) gave a thick white slurry in which the absorption bands of the cells were intensified 40- to 50-fold. Freezing the sample with Al_2O_3 to 77 K increased the light scatter further, and the absorption band intensification increased an additional 1.2-fold. The light-scattering agent was used in these experiments to permit spectral measurements on relatively dilute suspensions of cells. In the absence of the scattering agent the concentration of cells needed to give the same degree of absorption would have been such that the cells would have gone anaerobic relatively rapidly, thus making measurements in aerated cell suspensions more difficult.

In practice 0.1 ml of a concentrated suspension of starved cells was added to 0.9 ml of aerated medium in the sample cell. Substrate (10% ethanol) or inhibitors were added in 25-μl aliquots and were allowed to incubate 30 to 60 sec before addition of 0.5 g of Al_2O_3. Reduced cells were obtained by stirring a small amount of solid dithionite into the slurry after addition of Al_2O_3 and incubating for 2 min or by allowing the cells to respire for 5 to 15 min until the slurry became anaerobic. The samples were frozen to 77 K before mesurement. The concentrations of cells, substrates, and inhibitors used in each experiment are given in the figure legends.

Difference spectra were measured with a split beam scanning spectrophotometer constructed at the Johnson Foundation, University of Pennsylvania. The instrument employed a Bausch and Lomb 500-mm grating monochromator blazed at 300 nm and an EMI 9558C photomultiplier. The half-band width of the monochromatic light was set at 0.8 nm in the spectral region 500 to 630 nm and 1.6 nm in the spectral region 360 to 500 nm. The sample and reference cuvettes (4 mm sample thickness) and the Dewar system have been described previously (2). A multitapped potentiometer geared to the wave length drive was used to obtain a flat base line at a senstivity of 0.1 absorbance full scale. Oxidized cells plus scatter agent, $CaCO_3$, were frozen into both cuvettes to set the base line correction system. Difference spectra were measured in the presence of $CaCO_3$ (J. T. Baker Cat. No. 1288) as an external light-scattering agent.

RESULTS

Initial studies made with exponentially growing cultures of *Prototheca zopfii* showed that irradiation of these cells with light from cool white fluorescent lamps resulted in an inhibition of protein synthesis, nucleic acid synthesis, respiration, and cell division. The growth curves and computed growth rate constants, K_L and K_D, for irradiated and dark control cultures are presented in Figure 1. All four physiological parameters were inhibited by 45 to 50% during the irradiation period. The inhibition constants, $K_I = K_D - K_I$, were all in the range 0.07 to 0.08 per hr.

Subsequent studies on respiration were made with starved cultures of *P. zopfii* to eliminate growth and cell division during the experiment. In these experiments, the respiratory capacity of an aliquot of cells was determined by measuring the rate of oxygen uptake after the addition of substrate. Cells left in the dark for as long as 7 days showed no loss in their capacity to respire on added substrate. Continuous irradiation with 3×10^4 ergs/cm^2·sec of white light from fluorescent lamps, however, exponentially inhibited the respiratory capacity of the starved cells after a lag phase of about 4 hr (Fig. 2). The degree of inhibition was found to be independent of the time of previous dark starvation from 0 to 36 hr. The inhibition rate constants were approximately 0.04 per hr (17 hr for 50% inhibition). The starved cultures used in these

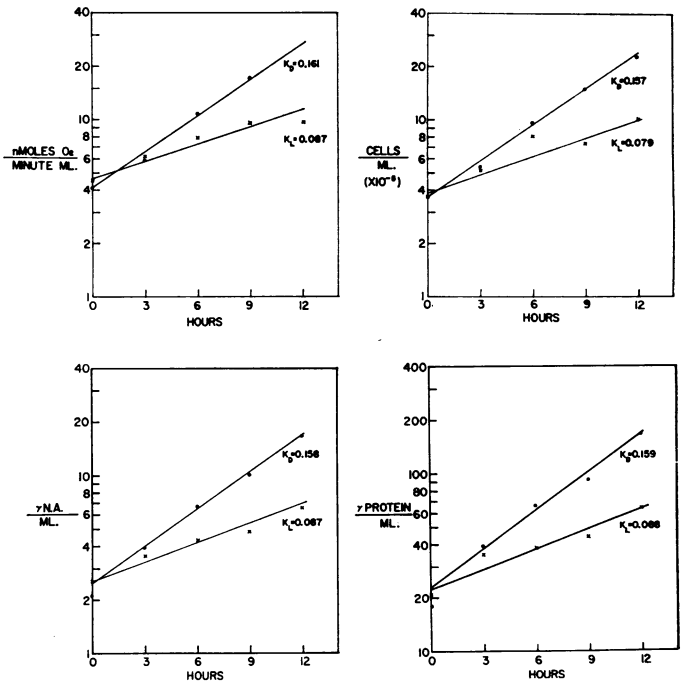

FIG. 1. Inhibition of respiration, cell division, nucleic acid synthesis, and protein synthesis in growing cultures by white light from fluorescent lamps ($I = 2.9 \times 10^4$ ergs/cm^2·sec). Upper left: Rate of oxygen uptake per ml of culture *versus* hours of growth in light (×) and dark (○); upper right: number of cells per ml of culture *versus* hours of growth in light (×) and dark (○); lower left: concentration of nucleic acid in μg/ml of culture *versus* hours of growing in light (×) and dark (○); lower right: concentration of protein in μg/ml of culture *versus* hours of growth in light (×) and dark (○). K_D and K_L are the growth rate constants for the various parameters for dark-grown and light-grown cultures, respectively.

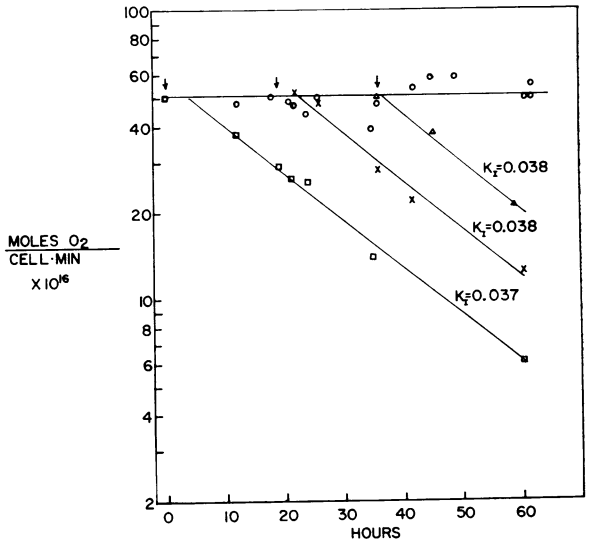

FIG. 2. Inhibition of capacity of starved cells (3×10^6 cells/ml) to respire on added substrate (0.25% ethanol) by white light from fluorescent lamps ($I = 2.9 \times 10^4$ ergs/cm^2·sec). Rate of oxygen uptake per cell after the addition of substrate *versus* hours starved. ○: Non-irradiated control; □: cells irradiated continuously from start of starvation; ×: cells irradiated after 19 hr of dark starvation; △: cells irradiated after 36 hr of dark starvation. Arrows indicate start of irradiation periods.

experiments were fairly turbid because of the relatively high cell concentration (2–3 × 10^6 cells/ml) employed so that the lower K_I may have been due to the less efficient irradiation of the cells.

Viability measurements were made on irradiated starved cells to determine if the light-induced inhibition of respiration might be due to a lethal action of the light. Viability was measured by comparing the colony-forming ability of cells irradiated 89 hr to that of dark control cells. Table II presents the results from such an experiment. While the viabilities of the irradiated and dark control cells did not differ significantly, the rate of respiration of the irradiated cells was about $\frac{1}{30}$ the rate of the dark control cells. It was concluded that the light-induced inhibition of respiration could not be ascribed to a killing of cells.

The reduced minus oxidized difference spectrum of *P. zopfii* cells at 77 K showed a cytochrome content similar to that found in higher plant tissue. Examination of the absorption spectra of these cells in the accompanying paper resolved seven cytochromes two *c*-type with maxima at 549 and 551 nm; three *b*-type with maxima at 555, 559, and 564 nm; and two *a*-type, *a* and a_3 (12).

Comparison of the difference spectrum of cells irradiated 2 hr with 2 × 10^5 ergs/cm²·sec of blue light (Fig. 3, curve A) with the difference spectrum of nonirradiated cells (Fig. 3, curve B) shows a large loss of absorbance in the Soret region of cytochrome oxidase and a shift in the α-band of cytochrome oxidase from 598 to 601 nm. Marked differences between the spectra of irradiated and nonirradiated cells also occurred in the α-band region of cytochrome oxidase when the spectra were measured under conditions which resolved the *a* and a_3 components. The reduced minus oxidized difference spectra of Figure 4 demonstrate the spectral resolution of cytochromes *a* and a_3 with nonirradiated cells. The α-band of the reduced cytochrome oxidase was at 598 nm when the cells were reduced with dithionite (curve A). The presence of KCN during reduction had little effect on the α-band of cytochrome oxidase even though the Soret band showed a marked

Table II. *Viability and Respiratory Capacity of Starved Cells Irradiated 89 hr with 1500 ft-c from Fluorescent Lamps*

Parameter	Irradiated	Nonirradiated
No. of cells/ml	1.52 × 10^6	1.42 × 10^6
No. of colony formers/ml	1.21 × 10^6	1.23 × 10^6
Viability	80%	87%
$\frac{\text{Viability of light cells}}{\text{Viability of dark cells}}$	0.92	
$\frac{\text{Respiratory capacity of light cells}}{\text{Respiratory capacity of dark cells}}$	0.03	

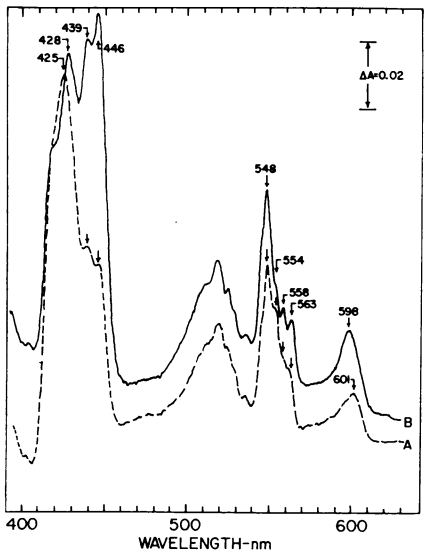

FIG. 3. Low temperature difference spectra of cells (8.7 × 10^6 cells/ml) suspended in starvation medium, pH 5.5, with added light scatter agent (0.33 g of $CaCO_3$/ml). Curve A: Cells irradiated 2 hr (AH-6 lamp, Corning filters 5433 + 3850, I = 2.5 × 10^5 ergs/cm²-sec); dithionite reduced *versus* substrate (0.25% ethanol) respiring aerated cells; curve B: dark control cells; dithionite *versus* substrate-respiring aerated cells.

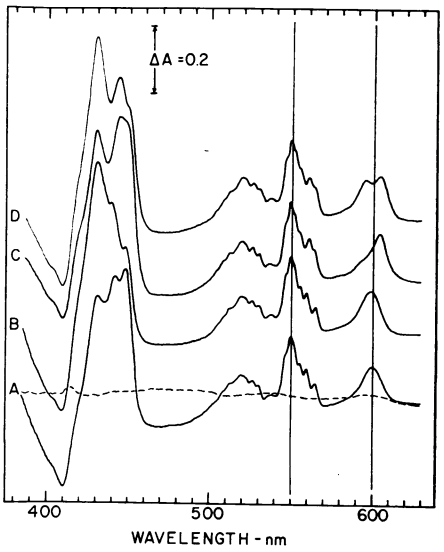

FIG. 4. Low temperature difference spectra of nonirradiated cells (2.4 × 10^6 cells/ml) in starvation medium, pH 6.9, with 0.4 g of $CaCO_3$/ml. Curve A: Dithionite *versus* aerated; curve B, KCN (2.5 × 10^{-4} M), dithionite *versus* aerated; curve C: methanol (2.5%), dithionite *versus* aerated; curve D: methanol (2.5%), KCN (2.5 × 10^{-4} M), dithionite *versus* aerated.

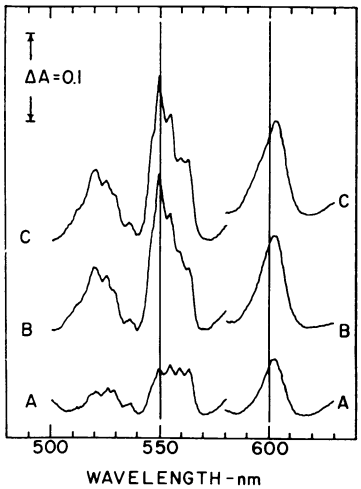

FIG. 5. Low temperature absolute spectra of starved cells irradiated 1 hr (AH-6 lamp, Corning filter 9782, I = 2 × 10^6 ergs/cm²-sec) suspended in starvation medium, pH 6.9, with 0.5 g of Al_2O_3/ml. Curve A: Endogenously respiring aerated cells; curve B: dithionite-reduced cells; curve C: cells treated with methanol (2.5%), KCN (2.5 × 10^{-4} M), and dithionite.

effect (curve B). The reduction in the presence of methanol caused the reduced cytochrome oxidase band to shift to 603 nm (curve C). When reduction by dithionite was carried out in the presence of both KCN and methanol, the α-band of cytochrome oxidase split into two bands with maxima at 595 and 603 nm (curve D). It is shown in the accompanying paper (12) that methanol shifted the α-band of cytochrome *a* to 603 nm (the effect shown in curve C of Fig. 4) and permitted dithionite to reduce the cyanide-cytochrome a_3 complex to give the 595 nm band. In the absence of methanol dithionite does not reduce the complex (44).

Similar spectra measured with irradiated cells in the presence of methanol, cyanide, and dithionite did not show splitting of the α-band (Fig. 5, curve C). The reduced oxidase band of the irradiated cells appeared at 603 nm even without methanol (curves A and B). Apparently cytochrome a_3 was destroyed by the irradiation treatment. Comparison of the spectra of the dithionite-reduced samples of nonirradiated cells (Fig. 4, curve A) and irradiated cells (Fig. 5, curve B) also indicates that cytochrome *c*(551) and cytochrome *b*(559) were partially destroyed by the irradiation.

Confirmation that the cytochrome a_3 was destroyed by light was obtained with CO-dissociation difference spectra (Fig. 6). Nonirradiated cells showed a normal difference spectrum for the CO-cytochrome a_3 complex while the difference spectrum of the irradiated cells gave essentially no indication of the complex.

The results obtained with the high intensity light from the AH-6 lamp were also found at more moderate light intensities. Cultures of cells, starved for 24 hr in the dark, were exposed to continuous irradiation from cool white fluorescent lamps of an intensity of about 3×10^4 ergs/cm^2·sec. At various time intervals, aliquots were assayed spectrophotometrically with the methanol-cyanide assay to resolve the α-bands of cytochromes *a* and a_3. Figure 7 graphically displays the results. The heights of the α-bands for the various cytochromes were normalized against the methanol-shifted α-band of cytochrome *a* at 603 nm to correct for differences in cell concentration in the concentrated aliquots. It should be recognized that the slopes do not give the true rate constants for the loss of individual cytochromes because the measured absorbance at a given wave length is a composite of the absorbances of two or more overlapping components. If proper corrections could be made, the slopes for the losses of the individual components would be greater. As can be seen from the figure, cytochromes *c*(549), *b*(555), and *b*(564) were unaffected after 72 hr of continuous irradiation, while cytochromes a_3 and *b*(559) were being destroyed. Cytochrome *c*(551) was also destroyed but at an apparent lower rate probably because of the large overlap from the α-bands of *c*(549) and *b*(555).

Irradiation of starved cells in the absence of oxygen had essentially no effect. The capacity to respire added substrate after the anaerobic irradiation was 93% of the unirradiated control while the same irradiation treatment (1 hr with a filtered AH-6 lamp) under aerobic conditions inhibited the exogenous rate of respiration to 12% of the control. The absorption spectrum (measured in the presence of methanol, cyanide, and dithionite) of the cells irradiated under anaerobic conditions (curve A, Fig. 8) showed no loss of cytochrome a_3 nor any of the other cytochromes, while cytochrome a_3 as well as *b*(559) and *c*(551) was substantially destroyed by the aerobic irradiation treatment (curve B, Fig. 8). Cytochrome a_3 was also protected against photodestruction by the presence of cyanide (2.5×10^{-4} M). Figure 9 shows the absorption spectra of cells treated with methanol, cyanide, and dithionite after the cells had been irradiated under aerobic conditions for 45 min with blue light (2×10^6 ergs/cm^2·sec) from an AH-6 lamp in the presence (curve B) or absence (curve C) of cyanide. The spectrum of nonirradiated control cells is presented as curve A. The presence of the 595 nm band in the spectrum of the cells irradiated in the presence of cyanide indicated that the cytochrome a_3 was protected against photodestruction when present as the a_3-cyanide complex. Cytochrome *b*(559) was, however, at least partially

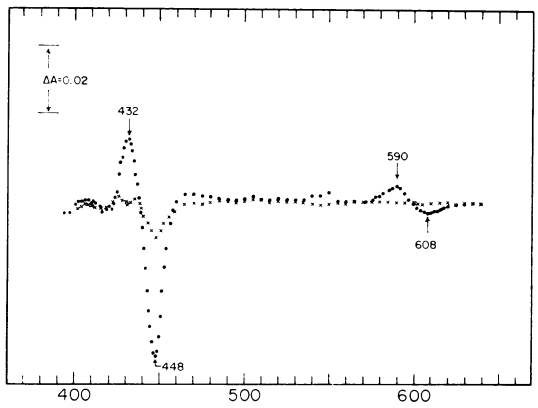

FIG. 6. Low temperature CO photodissociation difference spectra of nonirradiated cells (●) and cells irradiated 2 hr (AH-6 lamp, Corning filters 5433 + 3850, $I = 2.5 \times 10^5$ ergs/cm^2·sec) (×). Starved cells (8.7×10^7 cells/ml) suspended in starvation medium, pH 6.9, with 0.33 g of $CaCO_3$ per ml were reduced with dithionite and gassed with CO by blowing the humidified gas across the surface of the reduced suspension. Cells were frozen in both sample and reference cuvettes, and a base line was recorded. The reference cuvette was irradiated for 10 sec with white light from a Unitron LKR lamp to dissociate the CO-a_3 complex, and the spectrum was recorded. The spectra presented in the figure were computed by subtracting the base line from the recorded spectra.

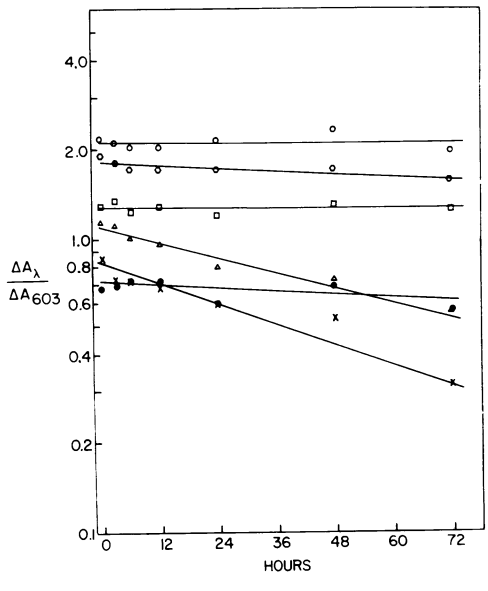

FIG. 7. The effects of moderate intensities (2.9×10^4 ergs/cm^2-sec) of white fluorescent light on starved cells ($2–3 \times 10^6$ cells/ml) suspended in starvation medium, pH 6.9. At various time intervals an aliquot of cells was removed from the light and the cells were concentrated to an approximate concentration of 1.7×10^7 cells/ml by centrifugation (525 *g* for 5 min). Low temperature absolute spectra (with 0.5 g of Al_2O_3/ml) were made of cells in starvation medium, pH 6.9, with methanol, (2.5%), cyanide (2.5×10^{-4} M), and dithionite. The heights of the α-bands of the various cytochromes were measured against the α-band of cytochrome *a* (at 603 nm) to correct for differences in cell concentration. The normalized heights, $\Delta A_\lambda / \Delta A_{603}$, of the various α-peaks of the cytochromes, *c* (549) (○), *c* (551) (⬡), *b* (555) (□), *b* (559) (△), *b* (564) (●), and a_3 (×), are plotted *versus* time irradiated.

destroyed even in the presence of cyanide (curve B *versus* curve A).

The irradiation of mitochondria isolated from actively growing cells of *P. zopfii* resulted in a destruction of the same cytochromes that were photodestroyed in intact cells. Twice washed mitochondria were irradiated for 1 hr at 0 C with blue light from a filtered AH-6 lamp, and the cytochromes were spectrally assayed with the methanol-cyanide technique. Cytochrome *c*(549), which is soluble, was washed out of the mitochondria while cytochrome *c*(551) remained. A comparison of the absolute low temperature spectra of the irradiated (curve B, Fig. 10) and nonirradiated control mitochondria (curve A, Fig. 10) showed that cytochromes *c*(559), *b*(559), and a_3 were the cytochromes most affected by light.

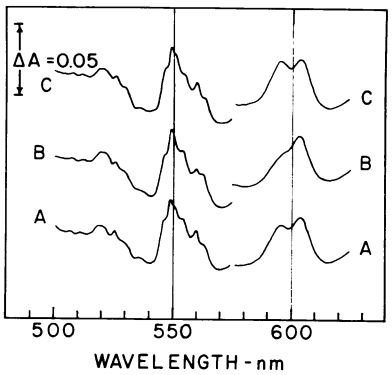

FIG. 8. Low temperature absolute spectra (with 0.5 g of Al_2O_3/ml) of cells (1.6×10^7 cells/ml) in starvation medium, pH 6.9, reduced with dithionite in the presence of methanol (2.5%) and KCN (2.5×10^{-4} M). Curve A: Cells irradiated anaerobically for 1 hr; curve B: cells irradiated aerobically for 1 hr; curve C: nonirradiated cells. Irradiation conditions: AH-6 lamp + Corning filters 5433 + 3850, $I = 2.5 \times 10^5$ ergs/cm²·sec.

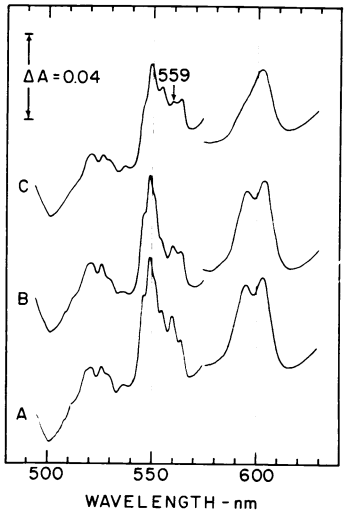

FIG. 9. Low temperature absolute spectra (with 0.5 g of Al_2O_3/ml) of cells (2.2×10^7 cells/ml) in starvation medium, pH 6.9, reduced with dithionite in the presence of methanol (2.5%) and KCN (2.5×10^{-4} M). Curve A: Nonirradiated cells aerated for 45 min in presence of 3.3×10^{-4} M KCN; curve B: aerated cells irradiated for 45 min in presence of 3.3×10^{-4} M KCN; curve C: aerated cells irradiated 45 min in the absence of cyanide. Irradiation conditions: AH-6 lamp + Corning filter 9782, $I = 2 \times 10^6$ ergs/cm²·sec.

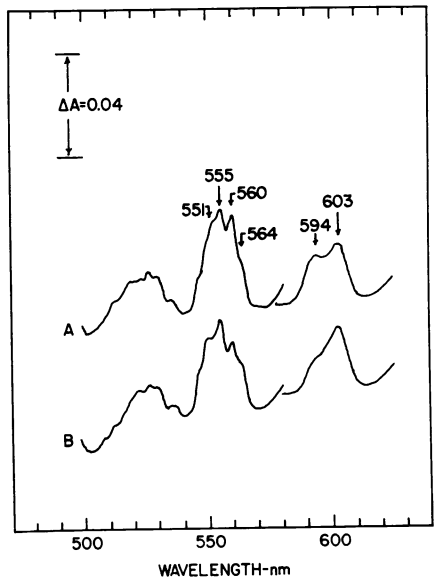

FIG. 10. Low temperature absolute spectra (with 0.5 g of Al_2O_3/ml) of twice washed mitochondria reduced with dithionite in the presence of methanol (2.5%) and KCN (2.5×10^{-4} M). Curve A: Nonirradiated mitochondria; curve B: mitochondria irradiated for 1 hr (AH-6 lamp, Corning filter 9782, $I = 10^6$ ergs/cm²·sec) at 0 C.

DISCUSSION

Light was found to inhibit the respiration of both growing and starved cultures of *P. zopfii*. The photoinhibition of growth (as determined by cell number, or the amount of nucleic acid or of protein) may be assumed to be a consequence of the inhibition of respiration. Spectroscopic studies revealed that, of the seven cytochromes associated with the respiratory electron transport chain, three—*c*(551), *b*(559) and a_3—were photolabile. Cytochromes *c*(549), *b*(555), and *b*(564) did not appear to be affected. The photodestruction of cytochrome a_3 was ascertained by the disappearance of the reduced Soret band in reduced minus oxidized difference spectra as well as by the loss of the capacity to bind photoreversibly with CO and by the loss of the capacity to bind with cyanide to produce the 595 nm absorption band observed in the presence of cyanide, methanol, and dithionite. The spectral properties of cytochrome *a* were also affected by irradiation. The height of the cytochrome *a* α-band was not significantly changed by an irradiation sufficient to destroy cytochrome a_3, but the peak was shifted 3 to 5 nm to the red. A similar shift in the cytochrome *a* band was observed on addition of methanol to nonirradiated cells. This spectral shift may reflect a physical change in the environment of cytochrome *a*. The same changes in the pigments of the electron transport chain were found to occur whether the cells were irradiated with high intensities from a filtered super-pressure mercury lamp for short periods or with moderate light intensities from fluorescent lamps for proportionately longer periods.

Irradiation of isolated twice-washed mitochondria resulted in a qualitatively similar destruction of those cytochromes which were destroyed in intact cells, thus indicating that the photoreceptor was a bound component of the mitochondria and that soluble components were not required. The photodestruction of cytochrome *c*(551) was seen most clearly in the studies with the isolated mitochondria from which most of the *c*(549) had been removed in the isolation procedure.

Starved *P. zopfii* cells irradiated for up to 4 days with fluorescent light remained viable although their capacity to respire on added substrates was inhibited by more than 95%. The ability of these cells to recover and grow normally indicated that the capacity for growth had not been impaired and that repair was possible.

Whether this recovery required a complete synthesis *de novo* of the whole respiratory apparatus remains to be determined.

If *P. zopfii* cultures were irradiated under anaerobic conditions, respiration upon the reintroduction of oxygen remained relatively unimpaired, and essentially no photodestruction of any of the cytochromes was noted. This lack of photoinhibition in the absence of oxygen is highly suggestive of a photooxidative type of mechanism. However, if the photoreceptor were a redox type pigment, such as a cytochrome, it is conceivable that only the oxidized form of the pigment would be photolabile, in which case the apparent oxygen requirement would be indirect. The data available at present do not allow a differentaition between these two alternative explanations. Protection against photodestruction of cytochrome a_3 under aerobic conditions was also afforded by the presence of cyanide.

The extent to which the photodestruction of respiratory pigments by visible light occurs in the biological world is difficult to estimate since little definitive work has been done other than with a few scattered organisms. However, our finding that the terminal oxidase in such diverse sources as *P. zopfii*, a plant; *S. cerevisiae*, a yeast; and the mitochondria from a higher animal (11) is destroyed by light, strongly suggests that this phenomenon may be of broad significance. The intensity of sunlight (14) should be sufficient on most days to inhibit appreciably respiration and growth of light-sensitive organisms such as *P. zopfii* and *S. cerevisiae*. Whether a photoinhibition phenomenon such as was observed in *P. zopfii* and *S. cerevisiae* serves as the photocontrol mechanism for regulating the phasing of light-sensitive circadian clocks, or for inducing growth synchrony in certain classes of photosynthetic algae, as has been proposed (13, 32, 39), remains to be determined.

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