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The Respiratory Chain of *Chlorella protothecoides*

I. INHIBITOR RESPONSES AND CYTOCHROME COMPONENTS OF WHOLE CELLS[1]

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ABSTRACT

The respiration and cytochrome properties of "glucose-bleached" *Chlorella protothecoides* Krüger, Indiana strain 25, were studied. This organism, when grown heterotrophically with high glucose and a low organic nitrogen source, has no chlorophyll, little carotenoid, and diminished chloroplast structure—factors which make it suitable for respiration studies.

Whole cell endogenous oxygen uptake rates are either stimulated or only slightly inhibited by cyanide, azide, CO, and antimycin. When these inhibitors are used with *m*-chlorobenzhydroxamic acid (*m*CLAM), an inhibitor of higher plant mitochondrial alternate oxidase, O_2 uptake is inhibited. There is little effect of *m*CLAM by itself on the rate of oxygen uptake. The inhibition by CO of O_2 uptake in the presence of *m*CLAM is reversed by light.

The cytochrome chain of *C. protothecoides* consists of cytochromes aa_3, *b*, and *c*, as revealed by room temperature difference spectra. In common with mitochondria of higher plants, there is a further reduction of cytochrome *b* with dithionite. In the presence of antimycin, the cytochromes aa_3 and *c* are oxidized and cytochrome *b* is reduced. Cyanide causes a partial reduction of cytochromes aa_3 and *c* while cytochrome *b* remains oxidized. This general response is characteristic of higher plant mitochrondria having large amounts of cyanide-resistant respiration. Carbon monoxide spectra reveal one CO-combining pigment. The cytochrome *b* region differs from that of higher plants in that the typical complex spectrum does not appear at low temperature (−190 C).

The concentration of cytochrome aa_3 per cell volume was observed during the greening of "glucose-bleached" cells. The concentration of these cytochromes nearly tripled during the 24 hours of the initial stages of greening.

The respiration of unicellular algae has commanded the attention of prominent biologists almost since the beginning of modern investigations of intracellular respiratory metabolism. Warburg (30) introduced *Chlorella* as an object of photosynthesis research in 1919. The classical investigation of Emerson, working in Warburg's laboratory, on the effects of respiratory inhibitors on *Chlorella* (8), established that they are virtually inactive. Chance and Sager (5) and Hiyama, Nishimura, and Chance (12) have examined the respiratory system of a pale green mutant of *Chlamydomonas reinhardi* which lacks spectrophotometrically detectable cytochromes *a* and a_3. *Euglena* has been studied by Sharpless and Butow (26, 27). Many aspects of its respiration and cytochrome behavior are unusual compared with higher plant and mammalian systems. Webster and Hackett (33, 34) surveyed some of the permanently colorless, non-photosynthetic unicellular and filamentous algae. Two members of this group have been treated more extensively in recent studies: the flagellate *Polytomella caeca* by Lloyd and Chance (20) and *Prototheca zopfii* by Lloyd (17–19) and by Epel and Butler (9), which is considered by some to be a colorless counterpart to *Chlorella*.

Although numerous studies have shown that inhibitor-resistant respiration is widespread among the algae, little is known about the pathway of electron transport or the properties of their respiratory pigments. This investigation examines the respiration and cytochrome components in whole cells of *Chlorella protothecoides*, an inhibitor-resistant species which when grown heterotrophically on high concentrations of glucose and low concentrations of a nitrogen source has no Chl, a trace of carotenoid, and diminished chloroplast structure (21).

MATERIALS AND METHODS

The Organism and Its Culture. A culture of *Chlorella protothecoides* Krüger, strain 25 was obtained from the Culture Collection of Algae at Indiana University. It was maintained on Petri dishes containing peptone agar and exposed to room light. A loop of the culture was used to inoculate 250-ml Erlenmeyer flasks containing 100 ml of culture medium. These were shaken in the dark in an incubator at 22 C for 5 days and used to inoculate 2.8-liter low form flasks containing 1 liter of culture medium. The cultures were harvested by centrifugation when they had reached the late log or early stationary phase of growth; they were washed once with buffer and recentrifuged. Yield after about 5 days in the 2.8-liter flasks was from 5 to 8 ml packed cell volume/liter.

For the routine preparation of "glucose-bleached" cells for oxygen uptake and cytochrome analyses the culture medium of Shihira-Ishikawa and Hase (28) was used. It contained, per liter: KH_2PO_4, 0.7 g; K_2HPO_4, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; $FeSO_4 \cdot 7H_2O$, 3 mg; thiamine hydrochloride, 10 μg; glucose, 10 g; glycine, 0.1 g; Arnon's A_5 solution, 1 ml; pH 6.3. Arnon's A_5 solution contained, per liter: H_3BO_3, 2.9 g; $MnCl_2 \cdot 4H_2O$, 1.8 g; $ZnSO_4 \cdot 7H_2O$, 0.22 g; $CuSO_4 \cdot 5H_2O$, 0.08 g, MoO_3, 0.018 g. The culture medium was autoclaved for 15 min at 15 p.s.i.

In greening experiments, the yellow cells were centrifuged aseptically and resuspended in a greening medium which dif-

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fered from the culture medium by having no glucose and by the addition of 5 g of glycine per liter. The culture was shaken in 2.8-liter low form flasks at about 22 C, bubbled with 5% CO_2 in air which had been filtered through cotton wool and a Millipore 0.3-μm pore size filter. The culture was illuminated with a battery of General Electric Cool White fluorescent lamps placed 30 cm above the surface of the culture. The intensity was 2,000 lux measured with a Weston light meter.

Cell densities were determined either as packed cell volume measured by hematocrit or as mg dry weight per ml determined by filtering 1 ml of cell suspension through a 0.45-μm Millipore filter, rinsing with cold water, and weighing after drying overnight at 95 C.

Measuring Oxygen Uptake Rates. Whole cell O_2 uptake measurements were made using a modification of the technique of Estabrook (10). The semiclosed vessel had a capacity of 1.30 ml and was thermostatted at 25 C.

Oxygen uptake rates were measured from the slope of the recorder trace of O_2 tension with respect to time. After a control rate had been established, an inhibitor or other chemical was added and after a time a new linear rate established. The convention of expressing O_2 uptake rates as $Q_{O_2} = \mu l/mg$ dry wt·hr was followed. The rate of O_2 uptake after the addition of chemicals was usually calculated as a percentage of the initial or control rate. During the titrations of inhibitor response, a new cell suspension was used for each determination, the vessel was washed thoroughly, and a new membrane was installed on the electrode. These precautions were necessary to preclude the carryover of inhibitors from one experiment to the next.

Carbon monoxide inhibition of O_2 uptake was studied in the same apparatus. Mixtures of O_2 in CO were made by displacing water from a graduated cylinder. The O_2 electrode was standardized with air-saturated buffer and compared with buffer which had been boiled and then equilibrated with the gas mixture in a large syringe thermostatted at 25 C. Control O_2 uptake rates were measured in air-equilibrated buffer, and, after the O_2 concentration had been reduced to zero, the contents of the vessel were drawn into a 50-ml syringe nearly filled with CO/O_2. The syringe and contents were shaken at 25 C for 2 min. The contents were returned to the O_2 electrode vessel, more gas mixture was bubbled through, the vessel was sealed and covered with black polyethylene film, and the O_2 uptake rate was measured in the dark. The concentration of CO was assumed to remain constant throughout the experiment.

Light reversal of CO inhibition of O_2 uptake was tested with a microscope illuminator shining through 10 cm of a 1% $CuSO_4$ heat filter. Light intensity measured at the surface of the vessel with a Weston light meter was 2,000 lux. No rise in temperature was observed in a dummy experiment.

Cytochrome Difference Spectra. Absolute absorption spectra of whole cells were obtained in a Cary model 14 recording spectrophotometer equipped with a scattered transmission accessory. The light path was 1 mm.

Cytochrome difference spectra were measured at room temperature with the same instrument provided with a 0 to 0.1 absorbance slide wire. A high intensity light source was used in combination with a specially constructed light mask around the cuvettes to reduce interference from stray light. Cells were suspended in 0.25 M sucrose buffer at about 30% packed cell volume, and 3-ml aliquots of the suspension were pipetted into quartz cuvettes having a 10-mm light path.

Anaerobiosis was achieved by allowing the cell suspension to use up the O_2 or by bubbling with a fine stream of purified N_2 for about 5 min. Additional reduction was caused by adding a small amount of dry sodium dithionite. The cells in the reference cuvette were made aerobic by bubbling with O_2. The difference spectrum was rapidly scanned from the longer to shorter wavelengths.

Antimycin difference spectra were taken by adding an ethanolic solution to the sample cuvette and bubbling with O_2 for 5 min. An identical amount of ethanol was added as a control to the reference cuvette, which was also bubbled with O_2.

Carbon monoxide difference spectra were obtained by electronically balancing two cell suspensions made anaerobic with N_2 and then bubbling CO through the sample cuvette for about 3 min.

Liquid N_2 temperature difference spectra were taken using an unsilvered Dewar flask placed into the scattered transmission accessory. The sample was housed in a specially made brass sample holder with acrylic windows and a light path of 3 mm suspended in the Dewar and was treated according to the quick freeze technique of Chance and Schoener (6). The brass sample holder was precooled in liquid N_2. The reference and sample were pretreated with N_2, sodium dithionite, or O_2 and then rapidly injected from a syringe into the sample holder, where they were quickly frozen. The apparatus was plunged into a Dewar flask full of liquid N_2 and, after active boiling had ceased, was removed and placed in the spectrophotometer where the level of liquid N_2 in the Dewar flask was kept just below the cuvette windows.

Steady state reduction levels of cytochromes were measured in an Aminco-Chance dual wavelength spectrophotometer. The slit width was set at 0.3 mm and the full scale deflection of the instrument was set to either 2 or 1% transmission. Cell aliquots were diluted to about 10% packed cell volume in 0.25 M sucrose buffer and the cell suspension was pipetted into a 10-mm light path cuvette and inserted into the apparatus.

The suspension was bubbled with O_2 for a short while, the spectrophotometer was balanced, and the recorder deflection upon anaerobiosis was measured. After this procedure was tested a few times for reproducibility, the sample was bubbled with O_2 and balanced rapidly, an aliquot of potassium cyanide was added, and the deflection due to the reduction of the cytochrome by the cyanide and the final deflection due to anaerobiosis were measured.

m-Chlorobenzhydroxamic acid was synthesized from freshly synthesized hydroxylamine and *m*-chlorobenzoyl chloride obtained from Aldrich Chemical Co. The product melted at 181 to 183 C and was compared in melting point and inhibitory activity with a sample generously provided by G. R. Schonbaum.

Antimycin was purchased from the Sigma Chemical Co. Concentration was determined optically at 320 nm using the millimolar extinction coefficient of 4.8.

RESULTS

Effects of Cyanide, Azide, and *m*CLAM[3] on Oxygen Uptake in Whole Cells. A typical pattern of oxygen uptake of whole cells is shown in Figure 1a. When cyanide to a final concentration of 1.0 mM is added to a suspension of cells, there is an immediate increase in the rate of O_2 uptake (Fig. 1b). The rate is linear to a very low concentration of O_2 in the presence of KCN as it is in the control. A high oxygen affinity, cyanide-resistant terminal oxidase has been described in higher plants. Schonbaum *et al.* (25) found that the aromatic hydroxamic acids are specific inhibitors of this alternate oxidase. We tested one of the more effective of these, *m*CLAM,[3] on the cyanide-stimulated respiration of *Chlorella* (Fig. 1c). When *m*CLAM was added after cyanide, O_2 uptake was completely inhibited

[3] Abbreviation: *m*CLAM: *m*-chlorobenzhydroxamic acid.

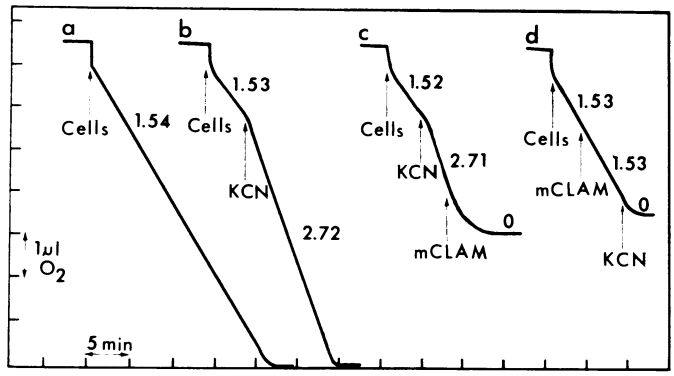

FIG. 1. Pattern of oxygen uptake of whole cells in response to added cyanide and *m*CLAM. The cells were added at the point indicated to a final concentration of 16.0 mg dry wt/ml in a volume of 1.30 ml; additions are expressed as final concentrations; the numbers beside the traces are the Q_{O_2}'s. Curve a: control; curve b: effect of 1 mM KCN; curve c: effect of serial additions of 1 mM KCN followed by 1 mM *m*CLAM; curve d: effect of serial additions of 1 mM *m*CLAM followed by 1 mM KCN.

after a short time. The converse experiment is shown in Figure 1d. There is usually no effect of *m*CLAM alone on the O_2 uptake rate, but when cyanide is subsequently added there is a complete and rapid inhibition. These experiments were repeated on many batches of cells, and similar patterns were observed. Sometimes the inhibition of O_2 uptake by cyanide and *m*CLAM was not complete, and sometimes *m*CLAM alone slightly stimulated or inhibited O_2 uptake.

The reversibility of cyanide and *m*CLAM inhibition was tested by serial addition of cyanide and *m*CLAM, removing the cell suspension from the vessel, washing the cells three times in fresh buffer, and then returning them to the vessel and adding the inhibitors again in the same order. The response of the washed cells was the same as that of fresh ones, proving that both cyanide and *m*CLAM inhibitions are reversible. Similar experiments proved sodium azide and antimycin to be reversible inhibitors of O_2 uptake when added in combination with *m*CLAM.

A series of titrations of O_2 uptake rates against inhibitor concentration were done. The results for cyanide without and with 1.0 mM *m*CLAM are plotted in Figure 2a. The relationship of O_2 uptake rate to concentration of cyanide is a complicated one. There is an initial inhibition at a concentration of 1 μM, but at higher concentrations the inhibition is reversed and eventually a stimulation is seen. The plot of O_2 uptake after the addition of both cyanide and *m*CLAM shows a peak of slight stimulation at a concentration of 1 mM *m*CLAM and 10 μM cyanide. Thereafter the rate falls off, and 50% inhibition of O_2 uptake corresponds to a cyanide concentration of 30 μM.

A similar experiment done with sodium azide and sodium azide plus *m*CLAM is plotted in Figure 2b. Again there was an inhibition of O_2 uptake at 1 μM and a subsequent stimulation at higher concentrations, with a peak stimulation at 20 μM and a slight stimulation at higher concentrations. There is a considerable stimulation of O_2 uptake by azide in the range from 10 to 100 μM in the presence of 1 mM *m*CLAM. Higher concentrations inhibited O_2 uptake with the inhibition 50% at 0.3 mM sodium azide and 1 mM *m*CLAM.

Titrations of O_2 uptake with varying concentrations of *m*CLAM showed a 50% inhibition at 0.5 mM *m*CLAM and 1 mM cyanide.

Carbon Monoxide and *m*CLAM. The light-reversible inhibition of O_2 uptake by CO is the classical test for the presence of cytochrome oxidase acting as the terminal oxidase (33). An experiment was conducted to test the role of cytochrome oxidase in the uptake of O_2 by *Chlorella protothecoides* using an initial mixture of 15% O_2 in CO (Fig. 3a, left). There was a slight stimulation of O_2 uptake over the control. The rate of O_2 uptake was constant until the flask contents reached very high ratios of CO/O_2.

When the same experiment was run with the addition of 1 mM *m*CLAM, there was an increasing inhibition of the O_2 uptake rate with higher CO/O_2 ratios as the O_2 was depleted (Fig. 3a, right). Oxygen uptake rate as a function of the CO/O_2 is plotted in Figure 3b. The apparent affinity coefficient, k, for the terminal oxidase is calculated by the following equation

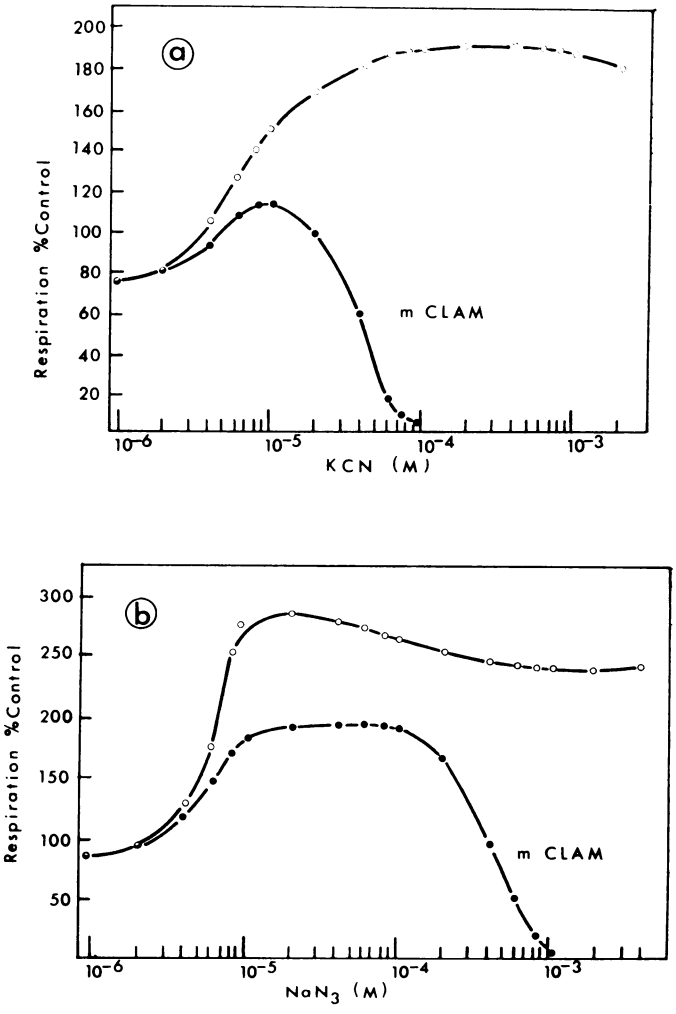

FIG. 2. a: O_2 uptake of cells as a function of the concentration of KCN and KCN plus 1 mM *m*CLAM. Final cell density was 12.2 mg dry wt/ml; the control Q_{O_2} was 1.85. Rate in KCN (○); rate in KCN plus 1 mM *m*CLAM (●). For each point the endogenous control rate was first measured, then KCN was added and the rate measured again, and finally 1 mM *m*CLAM was added and a final rate was obtained. b: O_2 uptake of cells as a function of the concentration of NaN_3 and NaN_3 plus 1 mM *m*CLAM. Final cell density was 15.3 mg dry wt/ml; the control Q_{O_2} was 1.69. Rate in NaN_3, (○); rate in NaN_3 plus 1 mM *m*CLAM (●). For each point the endogenous control rate was first measured, then NaN_3 was added and the rate measured again, and finally, 1 mM *m*CLAM was added and a final rate was obtained.

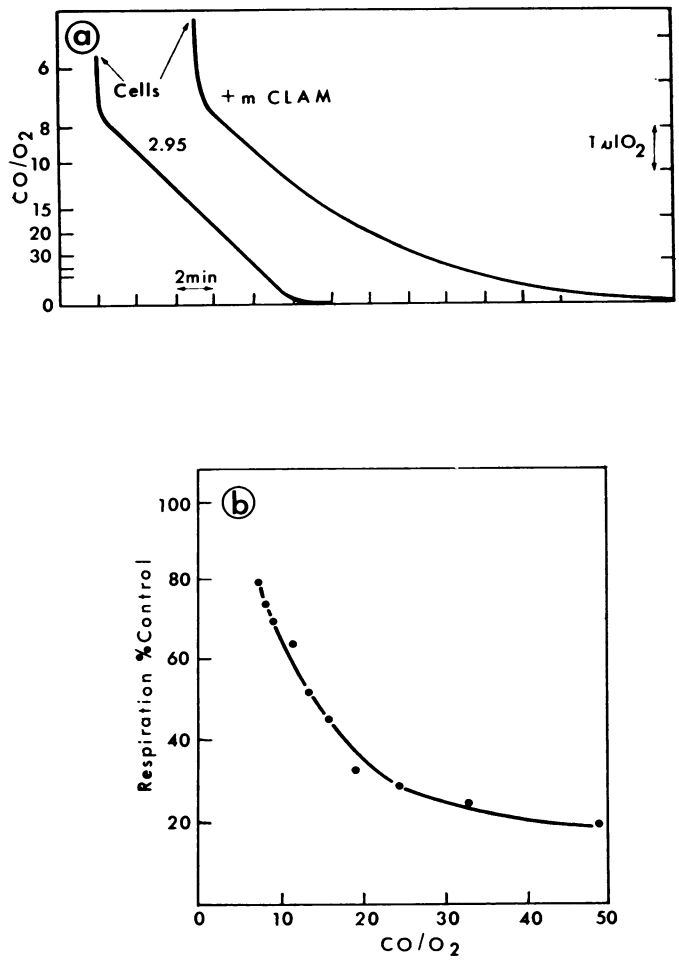

FIG. 3. Effect of CO and CO plus *m*CLAM on O_2 uptake in the dark. a: Traces of O_2 uptake in the presence of CO (left) and CO plus 1 mM *m*CLAM (right). Cell concentration was 11.1 mg dry wt/ml in a volume of 1.30 ml; room and vessel temperature 25 C. The cells were incubated in buffer gassed with 15% O_2 in CO, added to the empty vessel, and sealed off from the atmosphere at the start of the run. The number along the trace is the Q_{O_2}. b: Inhibition of O_2 uptake as a function of the ratio of CO to O_2 in the presence of 1 mM *m*CLAM. This is a plot of the tangents to the trace in a (right) at selected gas ratios.

(31)

$$k = \frac{n}{1-n} \times \frac{CO}{O_2}$$

where n = the fraction of respiration not inhibited in the CO/O_2 mixture. The partition coefficient varies with the CO/O_2; however, for purposes of comparison, the ratio which inhibits respiration by 50% is 12 under these conditions. This was taken as the partition coefficient, k.

Light reversibility of the CO inhibition observed in the presence of *m*CLAM was tested by shining a light through a copper sulfate heat filter at the O_2 electrode vessel. A recorder trace of this experiment is shown in Figure 4. Carbon monoxide inhibition of O_2 uptake in the presence of *m*CLAM is reversible by light.

Antimycin and *m*CLAM. The electron transport inhibitor antimycin caused a slight inhibition of O_2 uptake or rarely a stimulation with most batches of *Chlorella* cells tested. When both antimycin and *m*CLAM were used, there was a strong inhibition of O_2 uptake with a residual O_2 uptake of about 5 to 15% of the control value. A typical titration curve of O_2 uptake with respect to concentration of antimycin is shown in Figure 5. Antimycin by itself at the highest concentration inhibited O_2 uptake by 5%. In conjunction with 1 mM *m*CLAM, inhibition was 95% at 40 nM antimycin. Half-maximal inhibition was obtained at 6 nM antimycin.

Cytochrome Spectra. The anaerobic-aerobic difference spectrum in *Chlorella prototheocoides* (Fig. 6a) shows a broad peak with a maximum at 607 nm, another narrower peak at 551 nm, and a shoulder at 562 nm. These are interpreted to be the peaks

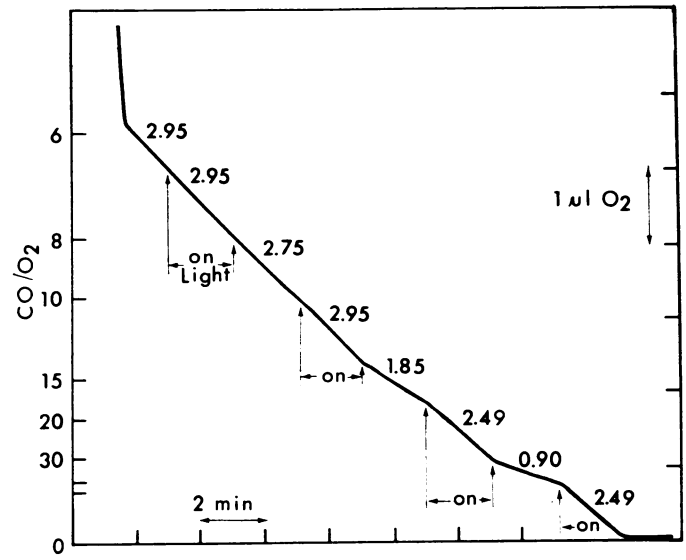

FIG. 4. Reversibility by light of the inhibition by CO of O_2 uptake in the presence of *m*CLAM. Cell concentration was 11.1 mg dry wt/ml; room and vessel temperature 25 C. The cells were incubated in a buffer containing 1 mM *m*CLAM and gassed with 15% O_2 in CO, added to the empty vessel and sealed off from the atmosphere at the start of the run. At the times indicated the cell suspension was illuminated by light of an intensity of 2 klux. The numbers along the trace are the Q_{O_2}'s.

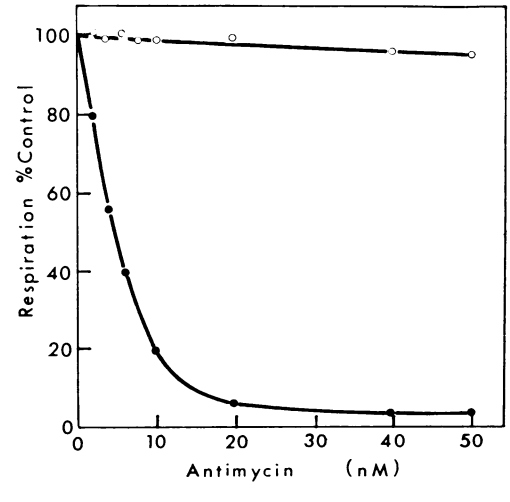

FIG. 5. Response of O_2 uptake of cells as a function of concentration of antimycin and antimycin plus 1 mM *m*CLAM. Concentration of cells was 10.0 mg dry wt/ml in a volume of 1.30 ml; the control Q_{O_2} was 2.10. Rate in antimycin, (○); rate in antimycin plus 1 mM *m*CLAM (●). For each point the endogenous control rate was first measured, then antimycin in ethanolic solution was added and the rate measured again and finally, 1 mM *m*CLAM was added and a final rate was obtained.

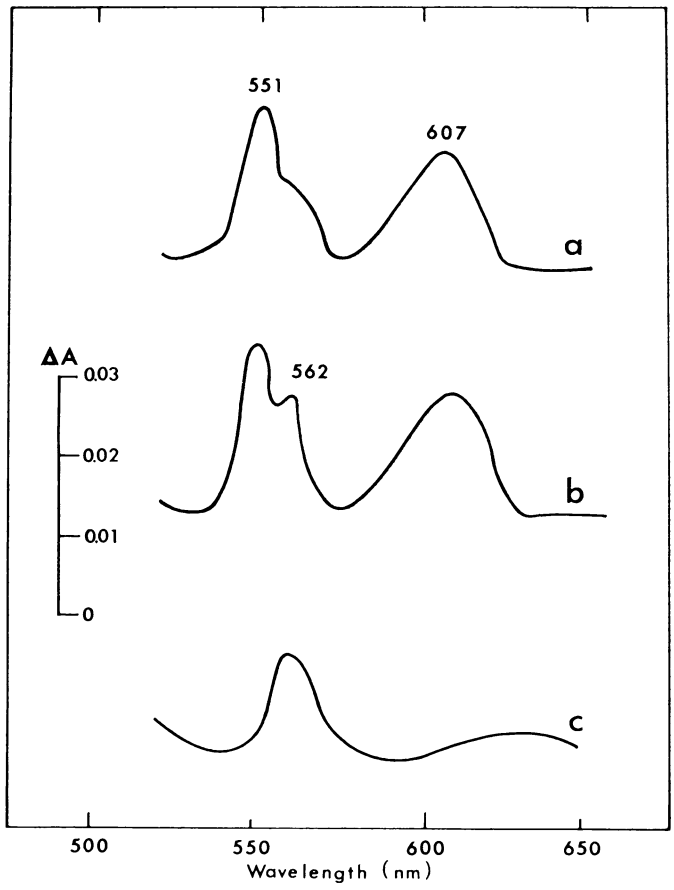

FIG. 6. Difference spectra of the cytochromes of whole cells. Concentration of cells was 73.1 mg dry wt/ml in 3 ml; light path 10 mm; spectral band width less than 0.8 nm. Curve a: anaerobic (N_2) minus aerobic (O_2); curve b: anaerobic (dithionite) minus aerobic (O_2); curve c: aerobic plus 5×10^{-5} M antimycin minus aerobic.

Table I. *Concentrations of Cytochromes in Whole Cells*
The reference wavelength was 575 nm in all cases

Cytochrome	Measuring Wavelength	Extinction Coefficient	Concentration	
			−Dithionite	+Dithionite
	nm	*mM/cm*	*nmoles/g dry wt*	
aa_3	607	16	13	13
b	562	20	6.5	9.1
c	551	19	14.5	16

corresponding to cytochrome aa_3, *c*, and *b*. Upon the addition of sodium dithionite the shoulder at 562 nm becomes a distinct peak, and the peak at 551 nm increases slightly in height (Fig. 6b). The concentrations of cytochromes were calculated using the extinction coefficients and procedures of Hackett (11) except that the absorption maxima of the cytochromes of *Chlorella* were slightly different (Table I).

When 50 μM antimycin is added to the sample cuvette and it is bubbled with O_2 for 10 min, the difference spectrum reveals only the peak at 562 nm (Fig. 6c). This indicates that electron transport is blocked before cytochrome *c*. A CO difference spectrum is shown in Figure 7. It reveals peaks at 430, 540, and 590 nm, with troughs at 445 and 605 nm.

Advantage was taken of the increased resolution and amplification properties of the liquid N_2 temperature technique to obtain more information about the cytochromes of *Chlorella*. The endogenously reduced minus oxidized spectrum (Fig. 8a) reveals that the cytochrome aa_3 region has a sharp peak at 607 nm with a shoulder at 595 nm. Cytochrome *b* appears as a low peak at 561 nm, while cytochrome *c* shows a peak at 549 nm and a shoulder at 545 nm. Additional reduction with dithionite (Fig. 8b) increases the height and sharpness of the cytochrome *b* peak at 561 nm.

The kinetics of the response to cyanide of the cytochrome system of *Chlorella* cells were examined by the technique of dual wavelength spectroscopy. After the addition of cyanide to the aerobic cell suspension, cytochrome aa_3 is reduced to 82% of the full oxidized-reduced difference spectrum (Fig. 9a). The 18% of oxidized cytochrome aa_3 remaining becomes reduced upon anaerobiosis and can be oxidized with O_2. Cytochrome *c* was reduced to a maximum of 75% under the same conditions (Fig. 9b). Cytochrome *b* remained oxidized upon the addition of cyanide (Fig. 9c) and reacted to anaerobiosis and aerobiosis in the same manner as the control.

Increase of Cytochrome Concentration during Greening. Matsuka and Hase (22) reported that the respiration of the strain of *C. protothecoides* used in these studies increases markedly during greening. To test the hypothesis that this increase may be due to an increase in the electron transport capacity, the concentrations of respiratory cytochromes during the early stages of greening were investigated.

Bleached cells were grown in greening medium, and samples were taken at 6-hr intervals and placed directly into the Cary spectrophotometer to record absorption spectra and into the Aminco-Chance dual beam spectrophotometer to measure the cytochrome content. The packed cell volume remained constant throughout the experiment while the dry weight decreased. The concentration of cytochrome was measured as a function of the change in absorption in the cytochrome aa_3 region resulting from aerobic-anaerobic transitions and calculated as nmoles of cytochrome per ml of packed cells.

The cells showed no Chl absorption until 24 hr, when there was a trace, and at 48 hr the absorption spectrum was normal for green cells. These are virtually the same results reported by Shihira-Ishikawa and Hase (28). Cytochrome aa_3 increases 2.58 times from time 0 to 24 hr (Fig. 10). It was difficult to measure cytochrome spectra after 24 hr because of the presence of an appreciable amount of Chl. These observations, correlated with the work of Matsuka and Hase (22) on respiration

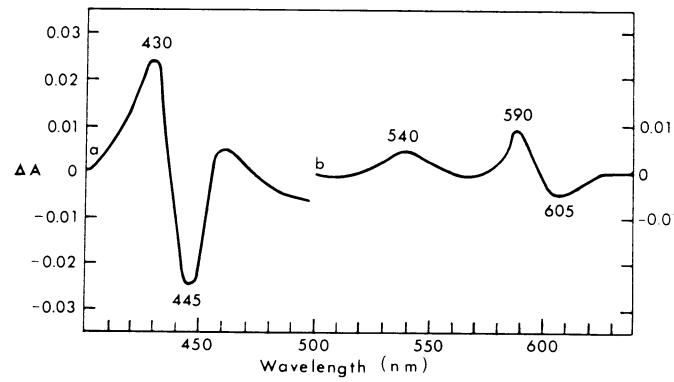

FIG. 7. Carbon monoxide difference spectrum of whole cells. Concentration of cells was: curve a: 34.7 mg dry wt/ml; curve b: 102 mg dry wt/ml. The light path was 10 mm; spectral band width: curve a, less than 0.7 nm; curve b, less than 1.0 nm. The reference and sample cuvettes were made anaerobic with N_2 and then the sample cuvette was bubbled with CO for 5 min and the spectrum scanned.

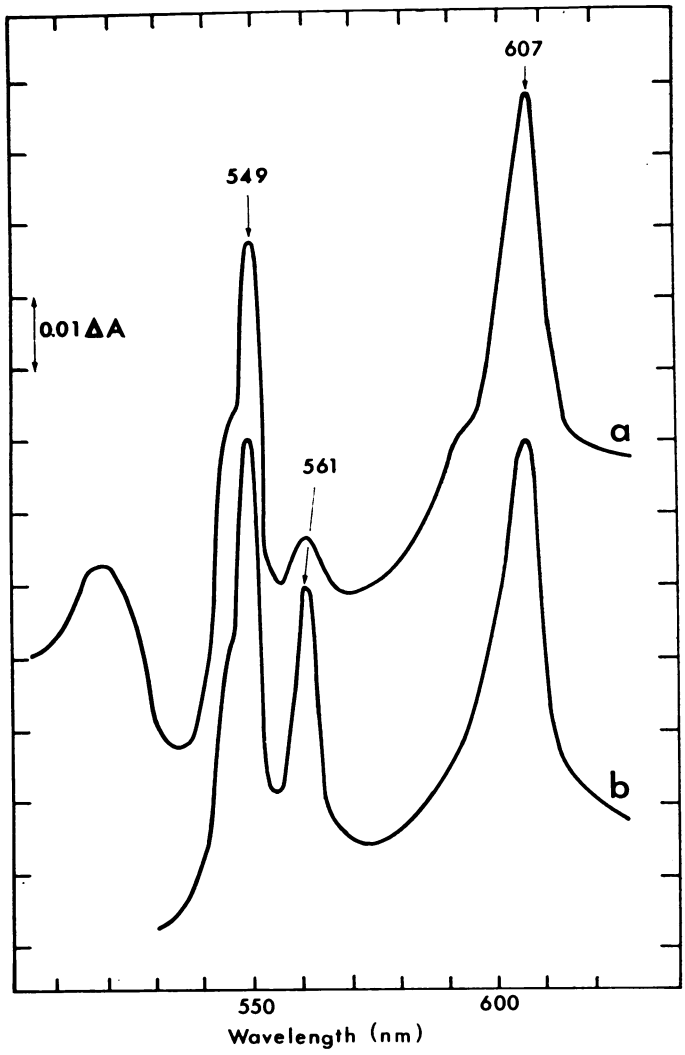

FIG. 8. Difference spectra of the cytochromes of whole cells at the temperature of liquid nitrogen (77K). Concentration of cells was 64 mg dry wt/ml; optical path 3 mm with acrylic windows in the cuvettes; spectral band width less than 0.9 nm. Curve a: anaerobic (N_2) minus aerobic (O_2); curve b: anaerobic (dithionite) minus aerobic (O_2).

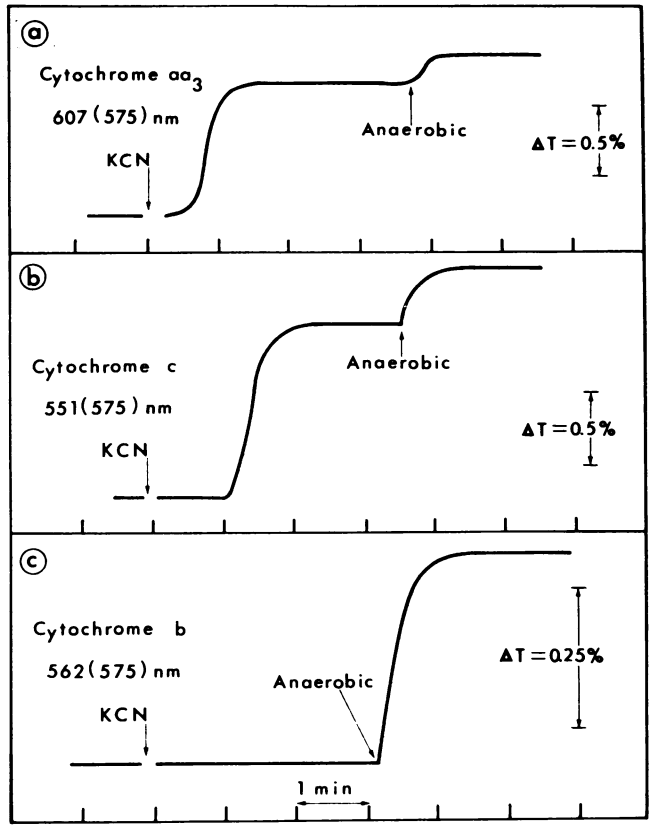

FIG. 9. a: Effect of cyanide on the oxidation-reduction state of cytochromes aa_3; b: effect of cyanide on the oxidation-reduction state of cytochrome *c*; c: effect of cyanide on the oxidation-reduction state of cytochrome *b*. Dual wavelength spectrophotometer tracing; full scale deflection 2%T in a and b and 1% T in c; slit width 0.3 mm; light path 10 mm; concentration of cells, 20.3 mg dry wt/ml. At the point indicated, 1 mM final concentration KCN was added to an aerobic suspension of cells.

rates, indicate that an early step in the conversion of the cell from the bleached, nonphotosynthetic form to the green photosynthetic one is the building up of the mitochondrial complement.

DISCUSSION

The respiratory chain of *Chlorella protothecoides* resembles that of higher plants and certain microorganisms in possessing two pathways to O_2: a cytochrome pathway and a cyanide- and antimycin-insensitive pathway alternate to the cytochrome chain before the cytochrome *c* region and which is inhibited by substituted benzhydroxamic acids. The *Chlorella* cytochrome pathway is similar to the higher plant one with the exception of having only one cytochrome *b* revealed in low temperature spectra (4, 16). The amount of alternate oxidase in higher plant mitochondria has been associated by Bendall and Bonner (3) with tissues that are rapidly growing or which liberate large quantities of heat. *Neurospora* alternate oxidase has been found in large amounts only when there has been a

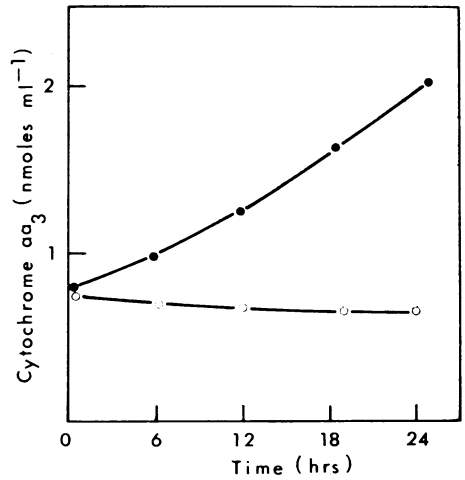

FIG. 10. Increase in concentration of cytochromes aa_3 during greening. Samples were removed from the culture flask and placed directly in the dual wavelength spectrophotometer. Measuring wavelength, 607 nm; reference wavelength, 575 nm; 10 mm light path; full scale recorder deflection, 2% T; concentration of cells, 10% packed cell volume. The concentration of cytochrome was obtained by measuring the magnitude of the anaerobic to aerobic oxidation-reduction change. Cells incubated in greening medium (●); cells incubated in bleaching medium (○).

defective cytochrome chain either as a result of mutation (13, 14) or by treatment of cells with chloramphenicol (15), an inhibitor of mitochondrial protein synthesis. A similar situation occurs in yeast where the alternate oxidase arises during growth on a copper-limited medium which causes a deficient cytochrome oxidase (7). The situation in yeast and *Neurospora* suggests that the alternate oxidase in these organisms functions to take the place of an inoperative or diminished cytochrome system. In *C. protothecoides* the alternate oxidase seems to be a stable feature of cells at all stages of their growth and development. This may be a reflection of the great nutritional versatility of this organism.

The control of the activity of the alternate pathway in higher plant mitochondria is by the activity of the cytochrome pathway (1, 2). There is no direct stimulation of the alternate pathway by cyanide. It appears that the stimulation of oxygen uptake by cyanide and azide in *Chlorella* cells is not due to a stimulation of the alternate oxidase directly. The stimulation is the result of an increase of the electron flux into the electron transport chain, perhaps by releasing constraints on glycolysis by lowering the level of ATP in the cell, as suggested by Sargent and Taylor (24).

The increase in the amount of respiratory cytochromes in *Chlorella* during greening is similar to results obtained in studies of *Euglena* (29), where it has been suggested that there is an energy requirement for the synthesis of the photosynthetic apparatus and the increase in respiratory capability is a response to this need. In *C. protothecoides,* however, a great deal of work by Hase (22) and his co-workers has indicated that many inhibitors of respiration and uncouplers of oxidative phosphorylation accelerate the greening process, and it was concluded that the ATP necessary for greening was obtained from nonrespiratory sources. Mitochondria have many functions. The Krebs cycle in particular is important not only in the synthesis of ATP but in heme biosynthesis, fatty acid metabolism, amino acid synthesis, etc. (23). The increase in respiration accompanying greening in *Chlorella* may not be a high respiratory rate in order to obtain ATP, but a response to a need for building blocks for the chloroplast.

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