Respiratory Chain of Colorless Algae
I. Chlorophyta and Euglenophyta[1]

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Introduction

Although it has been known for years that higher plants and yeast possess cytochromes of the a, b, and c type (18), there is a paucity of information on the respiratory chain of the algae. In particular, nothing is known about the terminal oxidase in this large group of plants (11). There have been some inhibitor studies on the respiration of algae with the classical inhibitors, carbon monoxide and cyanide: by Emerson on *Chlorella, Anabaena,* and *Oscillatoria* (8, 9); by Genevois and independently by Syrett on *Chlorella* (10, 30); and by Webster and Frenkel on *Anabaena* (32). Chance and Sager, working with a pale-green *Chlamydomonas* mutant, were able to demonstrate the presence of b- and c-type cytochromes by spectrophotometric methods, but they found no evidence for cytochrome oxidase, nor could they find evidence for a CO-combining pigment (6). However, Perini, Kamen, and Schiff did find spectral evidence for the existence of both a- and b-type cytochromes in an acetone powder for *Euglena* (23). In addition they purified 2 c-type cytochromes from the same organism. Cytochromes of the c-type have also been purified from the *Rhodophyta* (17, 33), and from the *Cyanophyta* (15, 16). Nonetheless, in no report has the entire respiratory chain of an alga been studied, and, except perhaps for *Euglena*, the terminal oxidase identified. Neither has there been any report of the preparation of mitochondria from the higher algae (11, 22, 28).

This paper is concerned with the following question: what is the nature of the respiratory chain in algae, and specifically, what is the terminal oxidase, if any, that is present in different groups of algae? Students of plant respiration have classically used etiolated tissue to avoid the presence of interfering photosynthetic pigments. The same approach has been used in this study by using colorless members of 3 algal divisions. This paper reports the results obtained for 2 of them: *Chlorophyta* and *Euglenophyta.*

Materials and Methods

All algae used in this study were obtained from the Culture Collection at Indiana University.

Prototheca zopfii was grown on the medium described by Anderson (1). If tap water were used to supply trace elements, greater growth was obtained. The cultures were grown at 30° on a shaker in 2.8 liter Fernbach flasks, each containing 500 ml. Cells were harvested at the end of log phase by suction filtration (Whatman No. 1 filter paper) and washed with water. *Polytoma uvella, Polytomella agilis,* and *Astasia longa* were grown on *Polytomella* medium (29) (2 g sodium acetate, 1 g yeast extract, and 1 g tryptone per liter of water), 500 ml in each Fernbach flask, without shaking and at room temperature. When the cells were harvested by low speed centrifugation they were routinely examined microscopically to determine what percentage were in motile forms: for most experiments this was greater than 90 %. When larger amounts of cells were needed (as for the disintegration experiments described below) *Polytoma* and *Astasia*, were grown in 9-liter bottles, each containing 6 liters of medium. Purified, sterile air was bubbled continuously through the medium. The cells were harvested as described above or with the Sharples super centrifuge. Cells were normally washed with the appropriate buffer and recentrifuged before use.

Respirometric experiments on *P. zopfii*, other than CO inhibition, were performed using standard procedures and equipment. Cells were harvested as described and suspended in 0.15 M phosphate, pH 7.0. An aliquot of this cell suspension was added to the Warburg flask together with water and phosphate buffer to bring the phosphate to a final concentration of 0.05 M. Glucose, when used, was present at a final concentration of 1 %. The final volume in each flask was 3.2 ml including 0.2 ml of 10 % KOH in each center well. For the cyanide inhibition studies the KOH center well solutions were made up according to Robbie (25). The temperature of the bath was 30°.

All CO inhibition and light reversal respirometric experiments were performed using the Gilson Model GRP 14 differential respirometer. The gas mixtures were flushed through the respirometer and out of the venting plugs of the flasks until at least 20 times the volume of the flasks had been flushed through. Control flasks were flushed with N_2-O_2 mixtures of the same O_2 content as the experimental flasks.

Spectra of whole cells and mitochondria, prepared as described below, were taken using the Cary

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Model 14 with the scattered transmission accessory and expanded scale. Liquid nitrogen difference spectra were taken using an unsilvered Dewar flask built into the scattered transmission accessory; 3-mm plastic cuvettes were used for these spectra. Suspensions of cells and cell fractions were oxidized by either bubbling O_2 into the cuvette for about 5 to 10 minutes, or by adding a small drop of 3 % H_2O_2 to the cuvette. Cells were reduced by slowly bubbling nitrogen into the cuvette (endogenously reduced) or by adding a small amount of dithionite. CO difference spectra were performed by slowly bubbling CO into the sample cuvette and nitrogen into the reference cuvette. In all types of difference spectra a base line was first obtained with suspensions in the sample and control cuvettes in the same redox state. The concentrations of cytochromes were determined from the spectra using the methods described by Hackett (12).

The various types of algae were disintegrated by blending an approximately 20 % cell suspension (wet wt/volume) with an equal volume of glass beads (type 090–5005 of the Minnesota Mining and Manufacturing Company) in the Servall Omnimixer at 0°. The suspending medium was 0.4 M mannitol, 0.02 M potassium phosphate (pH 7), 0.005 M EDTA; this will be referred to as mannitol medium. Optimum disintegration time was determined microscopically, enzymatically (cytochrome oxidase and NADH oxidase activities), and by the amount of protein solubilized. The extract was sedimented for about 10 minutes at 500 to 1000 × *g*, the precipitate discarded and the supernatant sedimented at 10,000 × *g* for 20 minutes, then at 80,000 for 30 minutes. The 2 precipitates were washed once with mannitol medium and recentrifuged.

The cytochrome oxidase assay was performed as described by Hackett (12). The cytochrome c used was Sigma Type III. The NADH oxidase assay was performed either in the mannitol medium or in the medium described by Wiskich and Bonner (31). The final concentration of NADH (Boehringer) was 1.67×10^{-4} M; the reaction was started by adding the NADH to the reaction cuvette. Assays were performed at room temperature using either the Beckman DU Spectrophotometer or the Cary Model 14 by following the decrease in OD with time at 340 mμ. Antimycin A, amytal, rotenone, and diphenylamine were dissolved in ethanol; when these inhibitors were used, the corresponding ethanol control was also performed.

Protein determinations were performed according to the method of Lowry et al (21). Since mannitol interferes with this determination, appropriate corrections were made.

Dry weights were determined by either centrifuging and washing the cells with water, transferring them to small tared flasks and drying overnight at 90° or by simply drying the suspension at 90° and correcting for the weight contributed by the salts.

Results

Prototheca zopfii. Respirometric Experiments. The endogenous respiration of *P. zopfii* is normally 2 to 6 μl O_2/mg dry wt per hour while glucose supported respiration is 28 to 30 μl O_2/mg dry wt per hour. The effects of the classical inhibitors, cyanide and CO on the glucose supported respiration are given in table I. Both are effective inhibitors of glucose oxidation by *P. zopfii*. The inhibition by CO is light reversible. The effects of these same 2 inhibitors on the endogenous respiration of *Prototheca* differs from the effects on glucose oxidation. An experiment with 10^{-5} M cyanide gave a 48 % stimulation of the control endogenous respiration. CO, which contains 5 % O_2 (i.e. CO/O_2 = 19/1), has only a slight and variable effect on the endogenous respiration.

Table I. *Effects of Cyanide and Carbon Monoxide on* Prototheca zopfii *Respiration*

	Qo_2	% Inhibition
Endogenous	5.0	...
Control (with glucose)	30	...
10^{-5} M HCN	20	33
10^{-4} M HCN	17	45
19/1 N_2/O_2	30	...
19/1 CO/O_2	20	33

The respiration of *Prototheca* in the presence of glucose is also inhibited by low concentrations of antimycin A (fig 1). Since antimycin A is added as an ethanolic solution, it is necessary to perform the ethanol control; ethanol gives an increased O_2 uptake over the glucose control. Figure 1 shows that antimycin A at a concentration of 3 μg/ml (5.5×10^{-6} M) reduces glucose oxidation by *Prototheca* to 5 % of the ethanol control value.

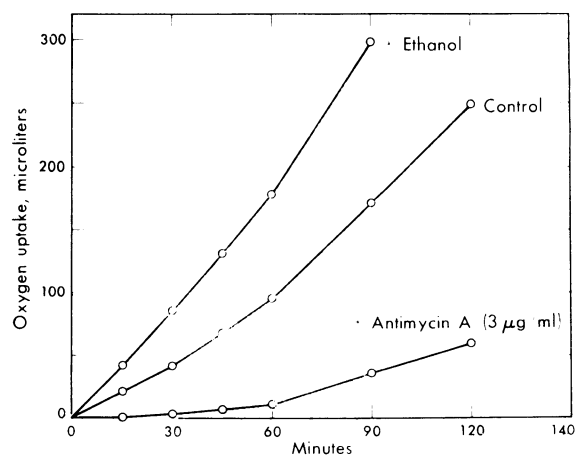

FIG. 1. The effect of antimycin A on glucose supported respiration of *P. zopfii*. Experimental conditions given in text. The antimycin and ethanol flasks contained 50 μl ethanol.

Table II. *Effects of 2, 4-DNP on the Endogenous and Glucose Respiration of* P. zopfii

	DNP conc (M)	Qo_2	% Inhibition (↓) or stimulation (↑)
Endogenous	0	3.4	...
	+ 1.0×10^{-5}	4.4	29 ↑
	+ 3.0×10^{-5}	6.9	103 ↑
	+ 1.0×10^{-4}	8.9	162 ↑
	+ 3.0×10^{-4}	6.6	95 ↑
Glucose	0	28	...
	+ 1.0×10^{-5}	31	10 ↑
	+ 3.0×10^{-5}	43	53 ↑
	+ 1.0×10^{-4}	12	59 ↓
	+ 2.7×10^{-4}	5	81 ↓

The results of the effects of 2,4-DNP (2,4-dinitrophenol) on the endogenous and glucose respiration are given in table II. In these experiments the final buffer concentration was 0.05 M phosphate + 0.03 M potassium phthalate, pH 5.0.

Difference Spectra of Whole Cells. An endogenously reduced minus oxidized difference spectrum of starved cells (aerated 3 days in 0.05 M phosphate, pH 7.0, at 30° after harvesting) is shown in figure 2. This difference spectrum has the same appearance as difference spectra of higher plants; the absorption bands for cytochromes b, c and a-a_3 are definitely present. CO difference spectra of cell suspensions show positive peaks at 591, 540, and 430 mμ and the Soret trough at 444 mμ; these are the same bands observed in higher plants and animals, in both of which cytochrome oxidase is the terminal oxidase. There is also, however, a shoulder at about 416 mμ on the 430 mμ CO Soret band which will be discussed later.

The complexity of the b-region of the spectrum can be shown by antimycin difference spectra, which reveal α-bands at 562 and 558 mμ, and by low temperature difference spectra. Figure 3 is one of the latter. Two bands are visible in the α-region for b-type cytochromes in this spectrum (554 and 558 mμ); further, the Soret band for cytochrome oxidase has split into 2 bands (440 and 446 mμ). Figure 3 is for dithionite reduced cells; endogenously reduced cells show the same bands but with the 547, 554, and 558 mμ bands of approximately the same intensity.

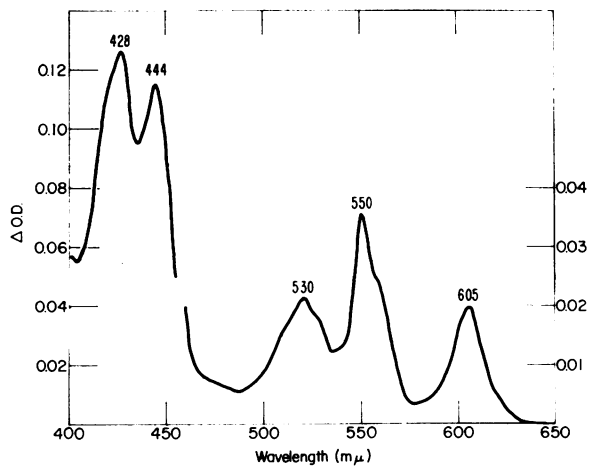

FIG. 2. Difference spectrum of P. *zopfii* (endogenously reduced)-(oxidized). The cell concentration was 200 mg wet wt/ml.

Disintegration and Sedimentation Experiments. Many methods were employed in an attempt to obtain mitochondria from *P. zopfii*. Such methods included hand grinding with alumina, freezing-thawing, use of the Hughes press, sonication both with and without abrasives present with the Raytheon (10KC) and with the MSE ultrasonic disintegrator. Finally it was discovered that the cells could be broken and

Table III. *Oxidase Activities of Disintegrated and Sedimented* P. zopfii *Cells*

Fraction	Total protein mg	Cytochrome oxidase* activity in 0.1 % digitonin	Total cytochrome oxidase activity	Cytochrome oxidase activity in digitonin/ cytochrome oxidase activity without digitonin	NADH** oxidase activity
10,000 × *g* precipitate	2.4	207	500	7.7	72
80,000 × *g* precipitate	2.5	40	100	4.5	8
80,000 × *g* supernatant	180	0.8	150	1.5	0

* Expressed as the first order rate constant, k, in ml/min per mg protein.
** mμmoles/min per mg protein.

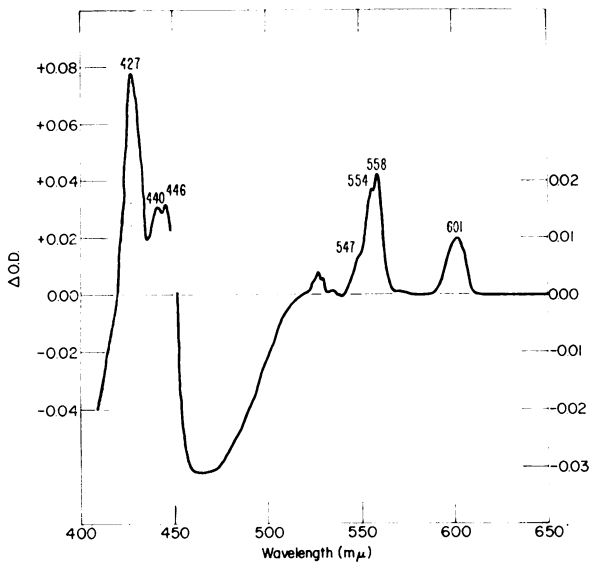

FIG. 3. Difference spectrum of *P. zopfii* at −190° (dithionite reduced)-(oxidized). The cell concentration was 41 mg dry wt/ml.

particles isolated by shaking the cell suspension with glass beads in a Mickle shaker. The Mickle shaker was superseded by the Servall Omnimixer which has the advantage of convenience and much larger capacity. Harvesting *P. zopfii* cells 24 hours before use and storing them in the refrigerator as the cell paste resulted in better breakage. Table III gives the results for 8.5 g of cells (wet wt) disintegrated for 15 minutes at one-quarter maximum speed. The ratio of cytochrome oxidase activity in the presence of 0.1 % digitonin to the cytochrome oxidase activity without digitonin is used as a rough quantitative measure of the integrity of the particles (12, 24). The effects of inhibitors on the NADH oxidase activity of the 10,000 × *g* particles are given in table IV. A considerable apparent NADH oxidase was present in the supernatant; this activity was independent of O_2 (as determined using an anaerobic cuvette flushed with helium) and is thus not a true

Table IV. *Effects of Inhibitors on the NADH Oxidase Activities of the 10,000 × g Particles from* P. zopfii

Control rate = 72 mμmoles/min per mg protein.

Inhibitor	Conc	% Inhibition
Antimycin A	1.1×10^{-6}	100
KCN	1.0×10^{-3}	95
Rotenone	4.0×10^{-6}	42
Rotenone	4.0×10^{-5}	62
Amytal	1.0×10^{-3}	40

oxidase activity but a NADH dehydrogenating activity. It was also insensitive to cyanide and antimycin A. The oxidase activities of the particulate fractions were totally dependent on the presence of O_2.

The yields of the particulate fraction in the experiment described above were extremely low. One could get much larger yields of both total protein and total activity by disintegrating the cells for longer times, usually 30 minutes. However, longer disintegration times changed the distribution of activities so that the 80,000 × *g* particulate fraction often had more cytochrome oxidase and NADH oxidase activity (specific and total) than the 10,000 × *g* particulate fraction. The following experiment demonstrates that the particles derived from 30-minute disintegrated cells are polydisperse: The extract after disintegration was centrifuged at 1000 × *g* for 10 minutes and the precipitate discarded. The supernatant fraction was then centrifuged successively at 5000 × *g*, 10,000 × *g*, and 20,000 × *g* for 20 minutes, and at 41,000 × *g* and 80,000 × *g* for 30 minutes. Each precipitate was drained, suspended in mannitol medium, and assayed for cytochrome oxidase activity and NADH oxidase activity. The results are given in table V.

Difference spectra of particulate preparations from *Prototheca* were very similar to whole cell difference spectra. An estimation of the amounts of cytochromes present in a 10,000 × *g* particulate fraction as calculated from NADH and $Na_2S_2O_4$ reduced difference spectra is given in table VI.

Table V. *Distribution of Oxidase Activities in 30-Minute Disintegrated* P. zopfii *Cells*

Fraction	Cytochrome oxidase activity* in 0.1 % digitonin	Ratio: activity in digitonin / activity without digitonin	NADH** oxidase activity	% Inhibition by 1.1×10^{-6} M Antimycin A
5,000 × *g* precipitate	207	8.5	16	86
10,000 × *g* precipitate	234	5.5	25	87
20,000 × *g* precipitate	240	4.5	31	93
40,000 × *g* precipitate	237	5.2	39	94
80,000 × *g* precipitate	39	3.9	32	92
80,000 × *g* supernatant	5.4	2.4	3.1	***

* k in ml/min per mg protein.
** mμmoles/min per mg.
*** Control rate was too low to test for inhibition.

Table VI. *Amounts of Cytochromes Present in 10,000 × g Particles from* P. zopfii

Method	Cyto-chrome	mμmoles/mg protein	Relative amounts
NADH-Oxidized	a-a_3	0.088	1.00
	b	0.043	0.49
	c	0.071	0.81
$Na_2S_2O_4$-Oxidized	a-a_3	0.113	1.00
	b	0.215	1.90
	c	0.170	1.50

Polytoma uvella. Difference Spectra of Whole Cells. Although *P. uvella* lacks chlorophyll and is nonphotosynthetic, carotenoids are present in large enough concentration to give a suspension of cells a bright orange color. The absorption of the carotenoids extends from below 400 mμ to above 560 mμ with an essentially flat absorption maximum from 450 to 490 mμ. There is, therefore, considerable overlap with absorption bands of the cytochromes, which makes their observation and determination of relative amounts rather difficult. Nonetheless, whole cell difference spectra of *P. uvella* reveal the presence of cytochromes b, c, and a-a_3 as manifested by α-bands at 604, 560, 550 mμ, and Soret bands at 444 and 426 mμ.

Difference Spectra of Acetone-Extracted Cells. It was found that 100% acetone at 0° would extract completely the carotenoids from *P. uvella.* The amounts of b and c-type cytochromes calculated from difference spectra of such cells—in 0.06 M phosphate, pH 6.0, and 33 % glycerol—agree well with the amounts estimated from spectra of unextracted cells. However, the α-band for cytochrome oxidase has shifted from 604 mμ to 595 mμ in the extracted cells. CO difference spectra of extracted cells are very puzzling (fig 4); there are absorption maxima at 570, 540, 430 (shoulder), and 414 mμ and a trough at 444 mμ.

Disintegration and Sedimentation Experiments. Cells were disintegrated and particulate fractions and a supernatant material were obtained as for *Prototheca.* Table VII summarizes the results of enzymatic assays on these 3 fractions, which were obtained by starting with 800 mg (dry wt) of cells in 22 ml mannitol medium and using a disintegration

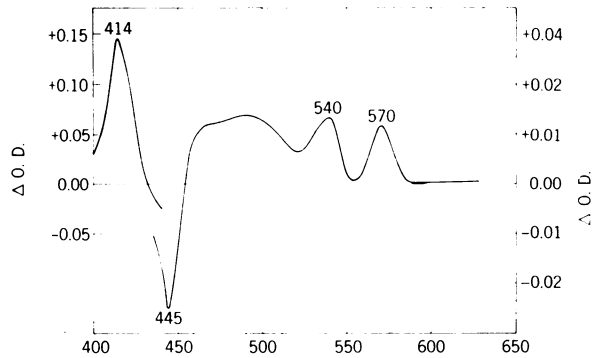

FIG. 4. CO difference spectrum of acetone-extracted *P. uvella.* Dithionite reduced cells, 12.5 mg dry wt/ml.

time of 5 minutes at one-quarter maximum speed. Like the results for *Prototheca* there was a NADH dehydrogenating activity in the supernatant which was independent of the presence of O_2, insensitive to 2.2×10^{-6} M antimycin A, and inhibited only 32 % by 10^{-3} M cyanide. The cytochrome oxidase activity of the 10,000 × *g* particles was inhibited completely by 10^{-5} M cyanide and 47 % when tested in an anaerobic cuvette containing a CO/O_2 = 9/1 mixture. Wiskich and Bonner (31) have described an assay medium for higher plant mitochondria (sweet potato) which gives higher oxidation rates and better inhibitor response. In agreement with their work, 10,000 × *g* particles from *P. uvella,* which had a NADH oxidase activity of 40 mμmoles/min per mg protein, had an activity of 98 mμmoles/min per mg protein when assayed in the assay medium described by them. The effects of inhibitors on this activity as assayed in this medium are given in table VIII.

Table VIII. *Effects of Inhibitors on NADH Oxidase Activity of* Polytoma uvella *Mitochondria*

Control rate = 98 mμmoles/min per mg protein.

Inhibitor	Conc (M)	% Inhibition
KCN	1.0×10^{-3}	97
Antimycin A	1.1×10^{-6}	97
Rotenone	3.0×10^{-5}	97
Diphenylamine	3.3×10^{-4}	97
Amytal	1.0×10^{-3}	84

Table VII. *Localization of NADH Oxidase and Cytochrome c Oxidase in* Polytoma uvella *Homogenates*

	Total protein (mg)	NADH oxidase mμmoles/min per mg	% of total	Cytochrome c oxidase* k(ml/min per mg)	% of total
10,000 × *g* particles	27	43	90	27	92
80,000 × *g* particles	16	8	10	4.2	8
80,000 × *g* supernatant	69	0	0	0	0

* Assayed in 0.1 % digitonin.

Table IX. *Amounts of Cytochromes Present in 10,000 × g Particles from* P. uvella

Method	Cytochrome	mμmoles/mg protein	Relative amounts
NADH-Oxidized	a-a_3	0.16	1.00
	b	0.16	1.00
	c	0.17	1.06
$Na_2S_2O_4$-Oxidized	a-a_3	0.12	1.00
	b	0.38	3.16
	c	0.13	1.08

The amounts of a, b, and c type cytochromes present in these 10,000 × g particles as calculated from difference spectra is given in table IX. A CO difference spectrum of these particles is given in figure 5; 2 reduced CO Soret bands are clearly present in this spectrum.

Polytomella agilis Aragao. Difference Spectra of Whole Cells. Cells were grown as described from a 1% innoculum for 2 days. At the end of this time more than 98 % of the cells were in motile form.

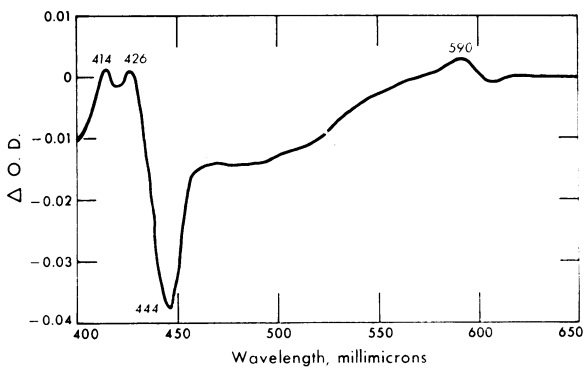

FIG. 5. CO difference spectrum of *P. uvella* mitochondria (dithionite + CO)-(dithionite). The final protein concentration was 3 mg/ml.

After centrifuging and washing, they were suspended in 0.1 M phosphate, pH 6.0 and glycerol was added to a final concentration of 33 %. In all 4 spectra presented the final cell concentration was 16.7 mg dry weight per ml. Figure 6 is a dithionite reduced minus oxygenated difference spectrum. This spectrum shows a marked increase in the amount of b-type cytochromes as compared to an endogenously reduced minus oxygenated difference spectrum. Although this spectrum shows 2 bands in the b-region, they are more clearly visible in the antimycin difference spectrum given in figure 7. It should be noted here that the dithionite reduced minus endogenous reduced difference spectrum appears essentially identical to figure 7. Two CO difference spectra are given in figure 8; one is an endogenously reduced CO difference spectrum and resembles the spectrum obtained with yeast and animal tissues. The second was performed on dithionite reduced cells and shows distinct differences from the first spectrum: the peak at 590 mμ has broadened to 570 to 590 mμ, the (OD

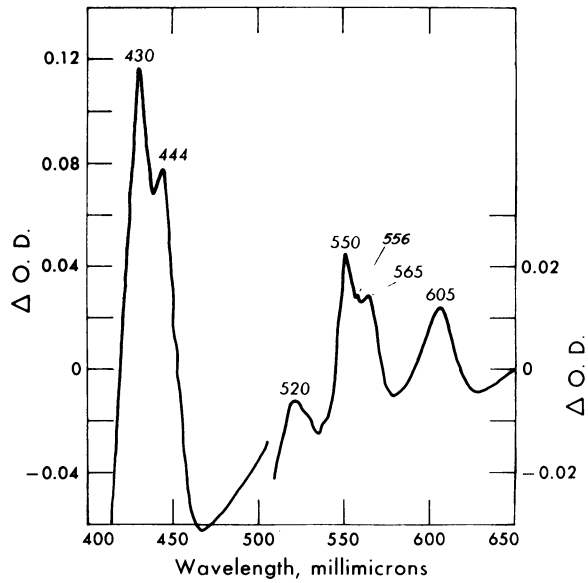

FIG. 6. Difference spectrum of *P. agilis*. (dithionite reduced)-(oxidized). See text for cell concentration.

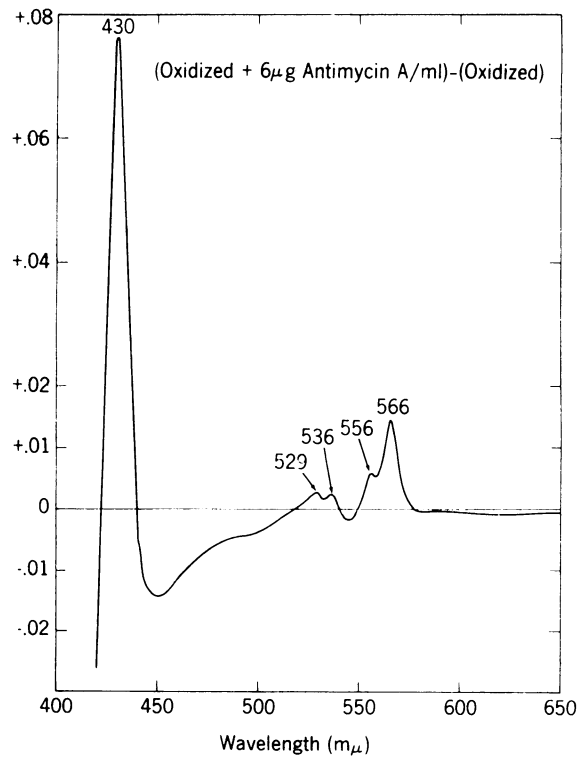

FIG. 7. Antimycin difference spectrum of *P. agilis* (oxidized + 6 μg antimycin A/ml)-(oxidized). Cell concentration given in text.

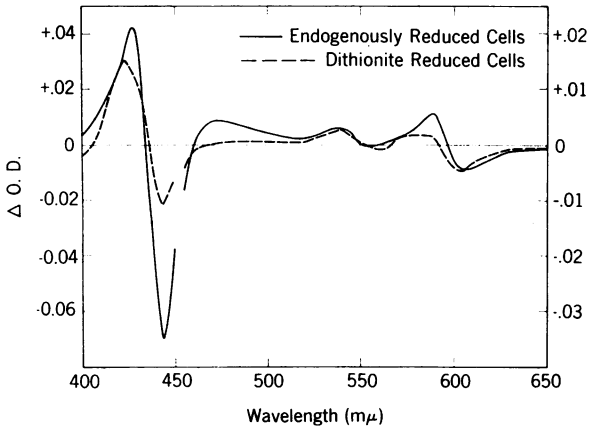

FIG. 8. CO difference spectra of *P. agilis*. Cell concentration given in text.

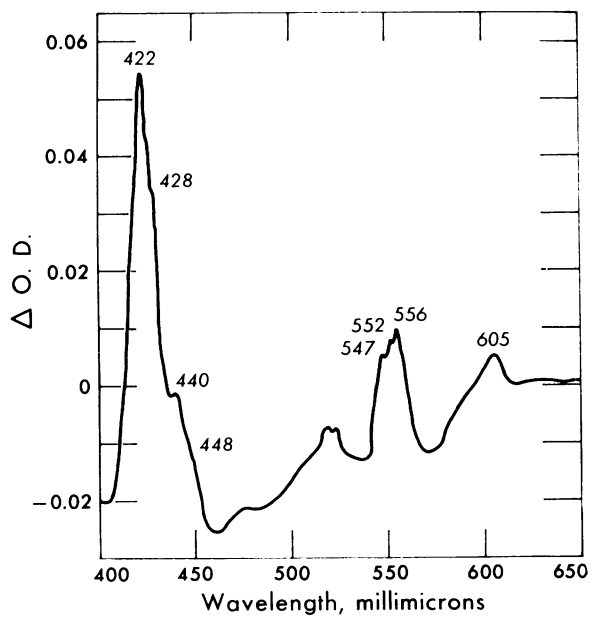

FIG. 9. Difference spectrum of *A. longa* at −190° (N_2)-(oxidized). Cell concentration was 37 mg dry wt/ml.

at 430 mμ) minus (OD at 444 mμ) difference has decreased, and the Soret maximum has shifted from 428 to 423 mμ.

Astasia longa Pringsheim. Difference Spectra of Whole Cells and Mitochondria. Room temperature difference spectra of whole cells show a Soret band at 424 mμ, a large broad band at 560 mμ, and an α-band for an a-type cytochrome at 608 mμ. Liquid nitrogen difference spectra are more revealing. Figure 9 is such a difference spectrum of endogenously reduced cells. The position and shape of the α-band for the a-type cytochrome in this spectrum differ from the position and shape of the α-band of cytochrome oxidase in similar spectra of higher plants, yeast, and the colorless green algae. An excess of b-type cytochromes is manifested in dithionite reduced difference spectra as figure 10 shows; this figure also shows the splitting of the 556 mμ band into a 554 and a 557 mμ band. Mitchondria were isolated by blending 1.52 g (dry wt) of washed cells in 22 ml of mannitol medium as described for 2 minutes at one-quarter maximum speed. The 10,000 × *g* precipitate was washed and homogenized in 3 ml of mannitol medium, each ml containing 2.9 mg protein.

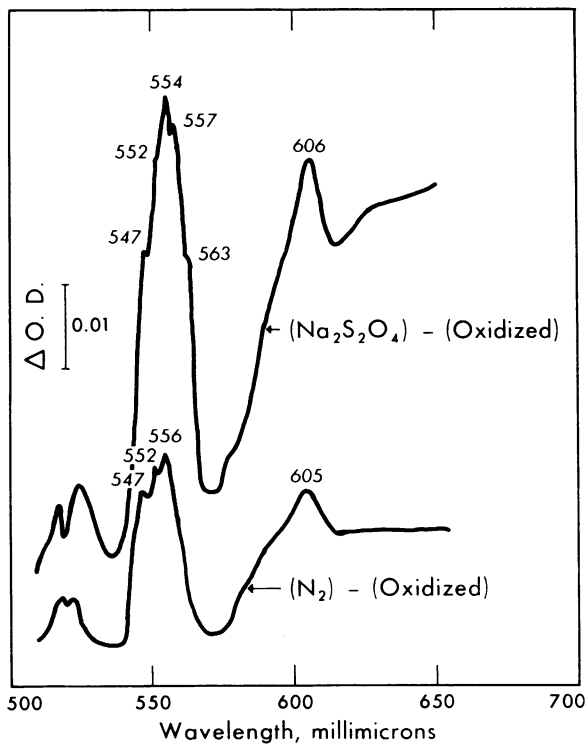

FIG. 10. Difference spectra of *A. longa* at −190°. Cell concentration was 37 mg dry wt/ml.

The shoulder visible at 448 mμ on the 440 mμ reduced Soret band for the a-type cytochrome in the endogenously reduced difference spectrum (fig 9) is more clearly seen in the NADH reduced minus oxidized difference spectrum of these mitochondria (fig 11). Like whole cells, reduction of the mitochondria with dithionite reveals the present of b-type cytochromes which remain oxidized in the endogenously reduced state. CO difference spectra of whole cells revealed only a small peak near 420 mμ and a small broad trough near 440 mμ. A CO difference spectrum of these mitochondria, shown in figure 12, shows a trough at 444 mμ and a peak at 431 mμ which are characteristic for cytochrome a-a_3, but the (OD at 430 mμ) minus (OD at 444 mμ) difference is much too small. The presence of an additional CO combining pigment is manifested by bands at 417, 540, and 568 mμ.

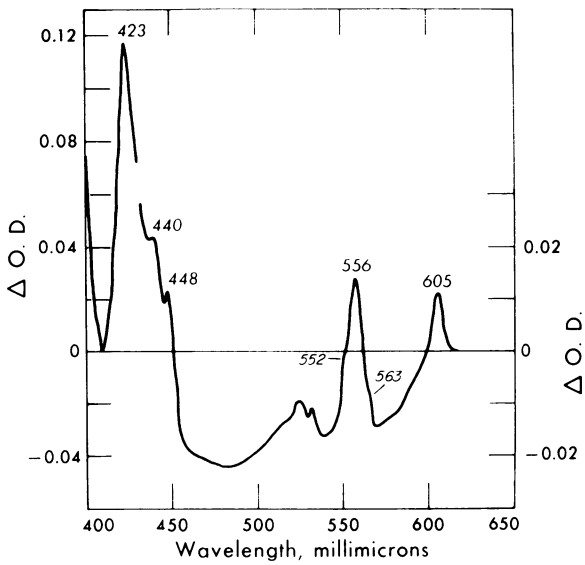

FIG. 11. Difference spectrum of *A. longa* mitochondria at −190°. Protein concentration given in text.

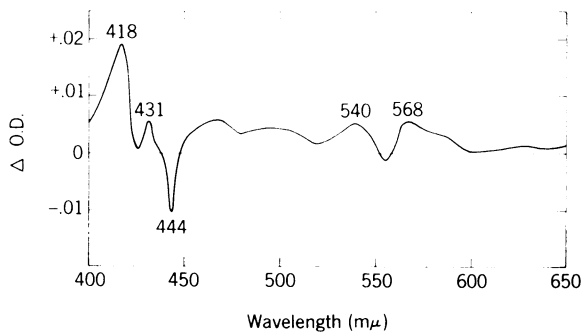

FIG. 12. CO difference spectrum of *A. longa* mitochondria at −190°. Protein concentration given in text.

A cytochrome oxidase assay on a crude cell extract from *A. longa* gave negative results. Since it is possible that an inhibitor of cytochrome oxidase was present in the extract, the assay was repeated on the purified mitochondria described above. Again there was no activity.

Discussion

Chlorophyta. The Qo_2's found for the endogenous and glucose supported respiration of *Prototheca* are very similar to those found by Genevois (10) for *Chlorella*. Also, the effects of cyanide and CO on the respiration of *Chlorella* (8, 9) are the same as those observed in this report for *Prototheca*. The responses of the endogenous respiration of *Prototheca* and *Chlorella* to these 2 inhibitors lead one to the conclusion that either the endogenous respiration is mediated by a cyanide-insensitive pathway or that the terminal oxidase is not the rate limiting component. Besides combining with iron-containing terminal oxidases, cyanide and CO are known to act as mild uncoupling agents (14). If the endogenous respiration were uncoupled so that the uncoupled rate were approximately the same as the simultaneously inhibited terminal oxidation, this could explain the results with these 2 inhibitors. In agreement with this, it was observed that although CO had little effect on the endogenous respiration of *Prototheca*, the rate in the light was usually slightly higher than the dark rate. Thus, light should remove the inhibition of the terminal oxidation allowing the respiration to increase slightly until the partially uncoupled oxidative phosphorylations become rate limiting again. The system is more complicated than this, however, as the results with the uncoupling reagent, 2,4-DNP, show. If oxidative phosphorylation were always completely rate-limiting, one would expect the uncoupled endogenous respiration to approach glucose respiration, but this does not occur (table II). This suggests that perhaps some other step in endogenous substrate utilization becomes rate limiting.

The effects of 2,4-DNP on the O_2 uptake of *Prototheca* are similar to those observed for higher plants and animals (27) in which one sees an increase in respiration with increasing DNP concentration until a maximum is reached, then the respiration decreases as the DNP concentration is increased further. The most informative respirometric experiment, however, is the inhibition by antimycin A. Since the inhibition is more than 90 %, essentially all of the electrons are passing through the respiratory chain for the oxidation of glucose by *Prototheca*.

Difference spectra of the 3 *Chlorophyta* studied in this report resemble difference spectra of higher plant preparations, especially with regard to the presence of excess b-type cytochromes (3). The splitting of the Soret band of cytochrome oxidase in low temperature difference spectra of *Prototheca* has also been observed by Bonner (4) in low temperature spectra of higher plant mitochondria.

The properties of the 10,000 × *g* particles allow their being called mitochondria. Their sedimentability, the amounts of cytochromes present, their high NADH and cytochrome c oxidase activities, and the responses of these 2 activities to inhibitors are almost identical to the same properties of mitochondria isolated from higher plants (7, 13, 24). The NADH dehydrogenating activity observed in the supernatants obtained after high speed centrifugation is also often observed in similar preparations of higher plants (D. P. Hackett, unpublished results).

The CO difference spectra of all 3 *Chlorophyta* studied show the presence of an additional CO combining pigment with a Soret band for the CO complex near 420 mμ. This pigment is usually more prominent in dithionite reduced preparations where its absorption bands overlap those of cytochrome oxidase, causing a distortion in the appearance of the latter

(fig 8). The CO difference spectrum of *Polytoma* mitochondria (fig 5) shows a distinct band at 414 mμ which corroborates the results observed in *Prototheca* and *Polytomella* and gives some validity to the CO difference spectrum of acetone-extracted *Polytoma* cells (fig 4). However, this latter spectrum must be interpreted with caution because of the fact that cytochrome a-a_3 has definitely undergone some spectral change as evidenced by the shift of its α-band from 604 to 595 mμ in difference spectra of acetone treated cells and also by the fact that b-type cytochromes are known to combine with CO when denatured (2). Nevertheless, similar CO binding pigments have been recently found in mung bean mitochondria (5), potato mitochondria (D. P. Hackett, unpublished results) and anaerobically grown yeast (20).

It was suspected that the additional CO-binding pigment of *Polytoma* might be a peroxidase since the CO-Soret for horseradish peroxidase is at 423 mμ (19), but there is good evidence against this. When assayed for peroxidase activity with the *o*-dianisidine assay (26) there was no correlation between the amount of pigment present, based on its spectrum, and the peroxidase activity of the preparation; the activity was always less than 1 % of what one would expect if it were as active as horseradish peroxidase. Also, no difference spectrum was observed with fluoride on a fraction which was known to contain the 415 mμ CO band.

Euglenophyta. Although *Astasia* possesses a, b, and c-type cytochromes, the respiratory chain is quite unlike that of higher plants and green algae. The most obvious difference is in the position and shape of the α-band of the a-type cytochrome. Another significant difference is that unlike mitochondria from the green algae, *Astasia* mitochondria are unable to oxidize reduced mammalian cytochrome c. *Astasia* exhibits a very complex spectrum in the α-region for b and c-type cytochrome. A low temperature difference spectrum of NADH reduced *Astasia* mitochondria (fig 11) looks quite unlike similar spectra of higher plant mitochondria (3). Although higher plants do possess a pigment with an α-band near 555 mμ, it does not predominate in such difference spectra as does the 556 mμ band in *Astasia*.

Perini, Kamen, and Schiff (23) have isolated 2 cytochromes of the c-type from *Euglena*. Their cytochrome c (556) has absorption maxima at 558 and 554 mμ in the purified state and is presumably the same pigment observed in this report in *Astasia* which has an absorption maximum at 556 mμ, which sometimes splits into 2 bands (554 and 557 mμ) at −190° (fig 10). Their cytochrome c (552) probably corresponds to the 552 mμ band observed in *Astasia*. These same workers (23a) have also shown that the O_2 uptake of sonicated cells of *Euglena* is inhibited only slightly by CO-O_2 mixtures while cyanide is a more potent inhibitor.

The 430 mμ CO Soret band and the corresponding 444 mμ trough which are characteristic for cytochrome oxidase are present in CO difference spectra of *Astasia* (fig 12), but both peak and trough are extremely small. As in the green algae, there appears to be another CO-binding pigment present. Either an overlap of the CO absorption maxima and minima of this second pigment with the CO absorption maxima and minima of the a-type cytochrome or a reduced binding capacity for CO of the a-type cytochrome could account for the small Soret maxima and minima observed in the CO difference spectra.

Summary

Whole cell difference spectra of *Prototheca zopfii*, *Polytoma uvella*, and *Polytomella agilis* reveal the presence of cytochromes b, c, and a-a_3. A excess of b-type cytochromes is revealed by low temperature difference spectra (−190°) and by antimycin difference spectra. Particles sedimenting at 10,000 × *g* and possessing high cytochrome c oxidase and NADH oxidase activities have been isolated from *P. zopfii* and *P. uvella*. These properties and the presence of cytochromes in amounts characteristic of higher plant mitochondria indicate that these particles are algal mitochondria. Carbon monoxide difference spectra of both the whole cells and the particles reveal the presence of an additional carbon monoxide-binding pigment besides cytochrome oxidase. All the data suggest that the green algae have a respiratory chain essentially identical to that of higher plants.

Low temperature difference spectra of *Astasia longa* are very complex in the α-region for b- and c-type cytochromes. The appearance of these spectra is quite unlike similar spectra of higher plants. Also, the position and shape of the α-band for the a-type cytochrome differs from similar spectra of the green algae and higher plants. Furthermore mitochondria isolated from *Astasia* are unable to oxidize reduced mammalian cytochrome c.

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Literature Cited

1. Anderson, E. H. 1945. Nature of the growth factor for the colorless alga *Prototheca zopfii*. J. Gen. Physiol. 28: 287–327.
2. Ben-Gershom, E. 1961. Use of carbon monoxide for detecting denaturation in haemoproteins. Biochem. J. 78: 218–23.
3. Bonner, W. D., Jr. 1961. Haematin Enzymes. J. E. Falk, R. Lemberg, and R. K. Morton, eds. Pergamon Press, Oxford, Part II, p 479–97.

4. BONNER, W. D., JR. 1963. Proceedings of the Fifth International Congress of Biochemistry. Pergamon Press, Oxford, Vol. II, p 50–62.
5. BONNER, W. D., JR. 1964. Interactions between electron transport and the reactions of plant mitochondria with oxygen. Plant Physiol. 39: ix.
6. CHANCE, B. AND R. SAGER. 1957. Oxygen and light induced oxidations of cytochome, flavoprotein, and pyridine nucleotide in a *Chlamydomas* mutant. Plant Physiol. 32: 548–61.
7. CRANE, F. L. 1961. Structure and function of mitochondria. Ann. Rev. Plant Physiol. 12: 13–34.
8. EMERSON, R. 1927. The effect of certain respiratory inhibitors on the respiration of *Chlorella*. J. Gen. Physiol. 10: 469–77.
9. EMERSON, R. 1927. Über die Wirkung von Blausäure, Schwefelwasserstoff und Kohlenoxyd auf die Atmung verschiedener Algen. Doctoral Dissertation, Friedrich-Wilhelms Universität, Berlin.
10. GENEVOIS, L. 1927. Über Atmung und Gärung in grünen Pflanzen. Biochem. Z. 186: 461–73.
11. GIBBS, M. 1962. Physiology and Biochemistry of Algae. R. A. Lewin, ed. Academic Press, New York, p 61–90.
12. HACKETT, D. P. 1964. Modern Methods of Plant Analysis. Springer-Verlag, Berlin, Vol. VII, p 647–94.
13. HACKETT, D. P. 1955. Recent studies on plant mitochondria. Intern. Rev. Cytol. IV: 143–96.
14. HACKETT, D. P., B. RICE, AND C. SCHMID. 1960. The partial dissociation of phosphorylation from oxidation in plant mitochondria by respiratory chain inhibitors. J. Biol. Chem. 235: 2140–44.
15. HOLTON, R. W. AND J. MYERS. 1963. Cytochromes of a blue-green alga: extraction of a c-type with a strongly negative redox potential. Science 142: 234–35.
16. KATOH, S. 1959. Studies on the algal cytochrome of c-type. J. Biochem. (Tokyo) 46: 629–32.
17. KATOH, S. 1960. Crystallization of an algal cytochrome. *Porphyra tenera* cytochrome 553. Nature 186: 138–39.
18. KEILIN, D. 1925. On cytochrome, a respiratory pigment common to animals, yeast, and higher plants. Proc. Roy. Soc. London B 98: 312–39.
19. KEILIN, D. AND E. F. HARTREE. 1951. Purification of horseradish peroxidase and comparison of its properties with those of catalase and methaemoglobin. Biochem. J. 49: 88–104.
20. LINDENMAYER, A. AND L. SMITH. 1957. Some oxidative enzymes of anaerobically grown yeast. Federation Proc. 16: 212.
21. LOWRY, O. H., N. S. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–75.
22. MILLBANK, J. W. 1957. Studies on the preparation of a respiratory cell-free preparation of the alga *Chlorella*. J. Exptl Botany 8: 96–104.
23. PERINI, F., M. D. KAMEN, AND J. A. SCHIFF. 1964. Iron-containing proteins in *Euglena*. I. Detection and characterization. Biochim. Biophys. Acta 88: 74–90.

23a. PERINI, F., J. A. SCHIFF, AND M. D. KAMEN. 1964. Iron-containing proteins in *Euglena*. II. Functional localization. Biochim. Biophys. Acta 88: 91–98.

24. RAGLAND, T. E. AND D. P. HACKETT. 1961. The intracellular localization of some oxidative activities in etiolated pea stems. Biochim. Biophys. Acta 54: 577–80.
25. ROBBIE, W. A. 1948. Use of cyanide in tissue respiration studies. Methods Med. Res. I: 307–16.
26. SCHICHI, H. AND D. P. HACKETT. 1962. Studies on the b-type cytochromes from mung bean seedlings. J. Biol. Chem. 237: 2955–58.
27. SIMON, E. W. 1953. Mechanisms of dinitrophenol toxicity. Biol. Revs. Cambridge Philos. Soc. 28: 453–79.
28. SPEER, H. L. AND R. F. JONES. 1964. Studies on the respiration of whole cells and cell-free preparations of the red alga *Porphyridium cruentum*. Physiol. Plantarum 17: 287–98.
29. STARR, R. C. 1964. The culture collection of algae at Indiana University. Am. J. Botany 51: 1013–44.
30. SYRETT, P. J. 1951. The effect of cyanide on the respiration and the oxidative assimilation of glucose by *Chlorella vulgaris*. Ann. Botany 15: 473–82.
31. WISKICH, J. T. AND W. D. BONNER, JR. 1963. Preparation and properties of sweet potato mitochondria. Plant Physiol. 38: 594–604.
32. WEBSTER, G. C. AND A. W. FRENKEL. 1953. Some respiratory characteristics of the blue-green alga, *Anabaena*. Plant Physiol. 28: 63–69.
33. YAKUSHIJI, E., Y. SUGIMURA, I. SEKUZU, I. MORIKAWA, AND K. OKUNUKI. 1960. Properties of crystalline cytochrome 553 from *Porphyra tenera*. Nature 185: 105–06.