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RESPIRATORY CYTOCHROMES OF *EUGLENA GRACILIS*

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SUMMARY

1. Difference spectra of whole cells and of a particulate fraction of a streptomycin-bleached strain of *Euglena gracilis* showed the presence of a *b*-type cytochrome, cytochrome *b* (561 Euglena), and an *a*-type cytochrome, cytochrome *a*-type (609 Euglena). The cytochromes were characterized by pyridine hemochromogen formation and were found associated with a particulate fraction enriched with mitochondria.

2. Both *b*-type and *a*-type cytochromes were reduced by succinate, oxidized by oxygen and reacted with a soluble *c*-type cytochrome, cytochrome *c*-type (556 Euglena), in reversible oxidation–reduction reactions. The steady-state level of reduction for each cytochrome was 92, 22 and 5 % of the anaerobic level for the *b*-type, *c*-type and *a*-type cytochrome, respectively.

3. Oxidation of *c*-type and *a*-type cytochromes was completely inhibited by cyanide, although respiration of a particulate fraction was only 60 % inhibited by the same concentration of cyanide. Antimycin A inhibited respiration by up to 70 % but completely inhibited reduction of the *c*-type cytochrome.

4. The data suggest that electron transfer in the respiratory pathway of Euglena involves the *b*-, *c*- and *a*-type cytochrome in a direct sequence. The cyanide and antimycin A-insensitive oxidation pathway is considered to involve a more direct oxidation of the *b*-type cytochrome.

INTRODUCTION

The respiratory cytochromes of the Euglenophyta appear to differ markedly from those of other photosynthetic organisms. A typical *b*-type cytochrome was not detected in particles containing succinate dehydrogenase isolated from *Euglena gracilis*[1], and although difference spectra of whole cells and isolated mitochondria of the colorless euglenoid *Astasia longa* slightly indicated the presence of a *b*-type cytochrome, spectra were quite atypical of those given by algae belonging to Chlorophyta or by higher plants[2]. An *a*-type (maximum at 605 nm) and a *c*-type (α absorption peak centered at 556 nm) cytochrome have been identified in *E. gracilis*[1,3,4] and *A. longa*[2]. However cytochrome oxidase activity has been reported to be either low (*E. gracilis*)[5] or absent (*A. longa*),[2] and cellular respiration of *E. gracilis* is only marginally inhibited

Abbreviation: STE medium, 250 mM sucrose, 24 mM Tris–HCl buffer (pH 7.6) and 0.1 mM EDTA.

by high concentrations of CO (ref. 1). The oxidation–reduction potential of the *c*-type cytochrome at +310 mV (ref. 4) is higher than the potential reported for the cytochrome oxidase system of plants or animals[6].

In view of these results, a streptomycin-bleached mutant of *E. gracilis* was examined to determine both the types of cytochromes present and their role in mitochondrial electron transfer. Data show the presence of a *b*-type cytochrome in a mitochondria-rich fraction from Euglena as well as an unusual *a*-type cytochrome. The reduction of these cytochromes and of cytochrome *c* (556) in the presence of succinate and their oxidation by a cyanide-sensitive oxidase system are demonstrated.

MATERIALS AND METHODS

Euglena gracilis, Z strain (streptomycin-bleached), was grown at 27° in a medium containing in g/l: 0.5 Na_2EDTA; 0.3 KH_2PO_4; 0.5 $MgSO_4 \cdot 7H_2O$; 1.0 $(NH_4)_2SO_4$; 0.06 $CaCO_3$; 0.00063 thiamine–HCl; $5 \cdot 10^{-6}$ vitamin B_{12}; and 0.022 metals "45" (see ref. 7). 0.06 M succinate was included as the carbon growth substrate, and the pH was adjusted to 3.9. The cultures were aerated at a rate of 0.2 l/min per l of culture, and no special precautions were taken to exclude light. Cell-free extracts were prepared by passing washed cells suspended in 250 mM sucrose, 24 mM Tris–HCl buffer (pH 7.6) and 0.1 mM EDTA (STE medium) through a French pressure cell at 7200 lb/inch^2. Paramylon granules, cell debris and unbroken cells were removed by centrifuging at 800 × *g* for 15 min. The cell-free extract was further fractionated by differential centrifugation at 20000 × *g* for 30 min and at 105000 × *g* for 60 min. The precipitates obtained were resuspended in STE medium and are referred to as 20000 × *g* and 105000 × *g* particle preparations, respectively.

Spectra were recorded using a Shimadzu MPS-50L recording spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto, Japan). Samples for spectral analysis were diluted in STE medium to give an absolute absorbance of not more than 2.5 at 500 nm in 2 cm × 1 cm light path cuvettes. The photomultiplier voltage was adjusted in each case to keep the slit width below 0.5 mm in the 500 nm region and below 0.2 mm in the 550–660 nm region. Concentrations of the various cytochromes were determined from the reduced *minus* oxidized difference spectra recorded at room temperature. The wavelength pairs selected and the extinction coefficients used were: cytochrome *b*-type (561) 561 nm-575 nm, E_{mM} 20; cytochrome *c*-type (556) 558 nm-542 nm, E_{mM} 19.1; cytochrome *a*-type (609) 609 nm-630 nm, E_{mM} 16.5 (see ref. 8). Oxidation or reduction rates of cytochromes under various conditions were followed using an Aminco–Chance dual wavelength spectrophotometer (American Instrument Co. Md., U.S.A.) set at wavelengths indicated.

Oxygen uptake of suspensions of cells or of cell-free, particulate preparations was measured in a glass vessel containing 3 ml of stirred suspension using a Clark-type oxygen electrode. The suspending medium contained 250 mM sucrose, 24 mM Tris–HCl buffer (pH 7.6), 5 mM $MgCl_2$, 10 mM potassium phosphate buffer (pH 7.6) and 0.5 mM EDTA. Oxygen concentration in an air-saturated medium at 25° was taken as 240 mM. Anticymin A was added in 70 % (v/v) ethanol, and when used as an inhibitor it was compared to a corresponding control containing the equivalent amount of ethanol. Succinate dehydrogenase activity was estimated at 25° using a constant amount (3.0 mM) of phenazine methosulphate, as described by KING[9]. Protein nitrogen was estimated by titration after Kjeldahl digestion.

RESULTS

Difference spectra of cells and cell-free particle preparations

Fig. 1 shows difference spectra (reduced *minus* oxidized) of whole cells and a particle preparation enriched for mitochondria (the 20000 × *g* particle preparation).

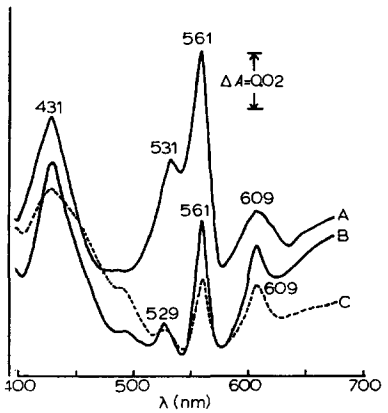

Fig. 1. Difference spectra (succinate-reduced *minus* oxidized) of whole cells (Curve A) and of a 20000 × g particle preparation (Curve C). In Curve B the 20000 × *g* particle preparation was reduced with dithionite.

In both spectra, absorption maxima were observed at 609 nm, 561 nm, 530 nm and 431 nm, indicating the reduction of *a*- and *b*-type cytochromes. With sodium dithionite as the reductant, both spectra showed a 2-fold intensification of the peak at 561 nm compared with the succinate-reduced samples. There was only a slight corresponding intensification in the absorption peak at 431 nm. The *c*-type cytochrome (556) reported to be present in Euglena[1,4] was not detected in whole cell difference spectra (Fig. 1, Curve A).

b-Type and a-type cytochromes

The absorption peaks at 561 nm and at 609 nm in the difference spectra (Fig. 1) were attributed to a *b*- and an *a*-type cytochrome, respectively. The following experiments were carried out to support this view. The pyridine hemochromogen formed by the heme extracted from a 20000 × *g* particle preparation with acetone, containing 5 % (v/v) HCl, showed absorption maxima at 557, 526 and 421 nm, characteristic of a protoheme-type prosthetic group. In difference spectra of the residue after extraction of the protoheme the α absorption peak at 609 nm shifted to 597 nm, the characteristic α absorption maxima of heme *a* protein hemochromogen[10,11]. The pyridine hemochromogen formed by the residue, after extracting the protoheme, showed α maximum at 586 nm typical of heme *a*-type prosthetic group.

Both the *b*- and the *a*-type cytochromes are firmly associated with cellular particulate matter. As shown in Table I, the ratio of the *b*-type to the *a*-type cytochrome in each isolated fraction is relatively constant. Relative to protein, the 20000 × *g* particle fraction contains the highest proportion of both cytochromes and succinate dehydrogenase activity.

Reversible oxidation and substrate reduction of the *b*- and *a*-type cytochromes of

a 20000 × *g* particle preparation are shown in Table II. With succinate as substrate, 47 % of the total dithionite-reducible cytochrome *b* is reduced rapidly, but the *a*-type cytochrome is completely reduced only after the system becomes anaerobic. Both cytochromes are completely oxidized by aeration for 2 min. In the presence of 1.0 mM KCN, both cytochromes are immediately reduced by succinate, and the reoxidation of *a*-type cytochrome is completely inhibited by KCN. However even in the presence of KCN, some of the reduced cytochrome *b* was oxidized. Further aeration almost completely oxidized the *b*-type cytochrome.

To further characterize the *a*-type cytochrome (609 nm) of Euglena, CO difference spectra (CO: succinate-reduced *minus* succinate-reduced) were recorded. After treating a 20000 × *g* particle preparation with CO for 30 sec, there was little forma-

TABLE I

THE DISTRIBUTION OF CYTOCHROMES AND OF SUCCINATE DEHYDROGENASE ACTIVITY IN FRACTIONS PREPARED FROM EUGLENA

The cells were broken and the particulate fractions were isolated by differential centrifugation (see MATERIALS AND METHODS). The amount of cytochrome, succinate dehydrogenase activity and protein content were determined by standard techniques.

Fraction	*Succinate dehydrogenase*			*Cytochromes*			
	Total protein (mg)	*Total activity (μmoles/min)*	*Specific activity (μmole/min per mg of protein)*	*b-Type (nmoles)*	*a-Type (nmoles)*	*Ratio b/a*	*Total (nmole/mg of protein)*
Broken cells	1030	10.4	0.011	408	131	3.2	0.522
800 × *g* ppt.	571	4.0	0.007	280	86	3.3	0.358
20000 × *g* ppt.	66	1.07	0.016	38	11	3.4	0.743
105000 × *g* pppt.	72	0.14	0.002	19	6	3.1	0.358
Soluble supernatant	425	0.19	0.0004	0	0	—	—

TABLE II

THE PERCENTAGE OF REDUCED CYTOCHROMES IN A 20000 × *g* PARTICLE PREPARATION AFTER VARIOUS TREATMENTS

The amount of each cytochrome reduced was obtained from difference spectra of the treated sample and of a 20000 × *g* particle sample preparation oxidized with 0.05 mM potassium ferricyanide. The sample contained 92 nmoles of *b*-type cytochrome and 33 nmoles of *a*-type cytochrome.

Treatment	*Cytochrome reduced* (%)	
	Cytochrome b-type (561)	*Cytochrome a-type (609)*
Aeration	0	0
Succinate 5 mM, 2 min	47	8
Succinate 5 mM, 5 min (anaerobic)	47	100
Aeration, 2 min	0	0
Succinate 5 mM + 1 mM KCN, 2 min	47	100
Aeration, 2 min	28	100
Aeration, 5 min	10	100
Sodium dithionite, 1 mg/ml	100	100

tion of a CO-complex (Curve A, Fig. 2). Successive treatments with CO showed a progressive increase in the formation of CO-complex. After 15 min, definite peaks at 420 nm, 466 nm, 544 nm and 576 nm and troughs at 447 nm, 522 nm and 561 m were visible (Fig. 2, Curve B). Similar peaks were found after prolonged treatment of broken cells with CO, but the peaks and troughs in the Soret region were less distinct.

c-Type cytochrome

The *c*-type cytochrome (556 nm) was readily extracted from the cells by successive freezing and thawing in 0.9% NaCl containing 0.3 M potassium phosphate buffer (pH 7.5). The spectrum of the *c*-type cytochrome extracted by this method and purified as described by PERINI *et al.*[4] is shown in Fig. 3. The reduced α band showed an asymmetric peak with a maximum at 558 nm and a shoulder at 554 nm; the peak was centered about 556 nm. The ratio $A_{558\ red}/A_{280\ ox}$ was 0.70, similar to the ratio obtained by PERINI *et al.*[4].

The amount of *c*-type cytochrome present in Euglena was found to vary between 1.5 and 2.5 nmoles/g wet weight of cells or 4.5–6.0 nmoles/10^9 cells. Similar quantities of the *c*-type cytochrome have been reported by COLMANO AND WOLKEN[12] and by PERINI *et al.*[1]. The quantity of each cytochrome present in Euglena was found to be in the ratio of 1 : 0.2 : 0.4 for the *b*-, *c*- and *a*-type cytochrome, respectively. PERINI *et al.*[1] have reported that a *c*-type and an *a*-type cytochrome (605 nm) are present in Euglena in the ratio of 1 : 1.

To determine whether the *c*-type cytochrome reacted with the *b*- and *a*-type cytochromes, a purified preparation of the *c*-type cytochrome was added to catalytic quantities of the 20000 × *g* particle preparation, and the oxidation and reduction of the *c*-type cytochrome were followed using the dual wavelength spectrophotometer. As shown in Fig. 4, Curve A, the *c*-type cytochrome is partially reduced by succinate (aerobic steady-state level of reduction of approx. 22 %) and is fully reduced almost

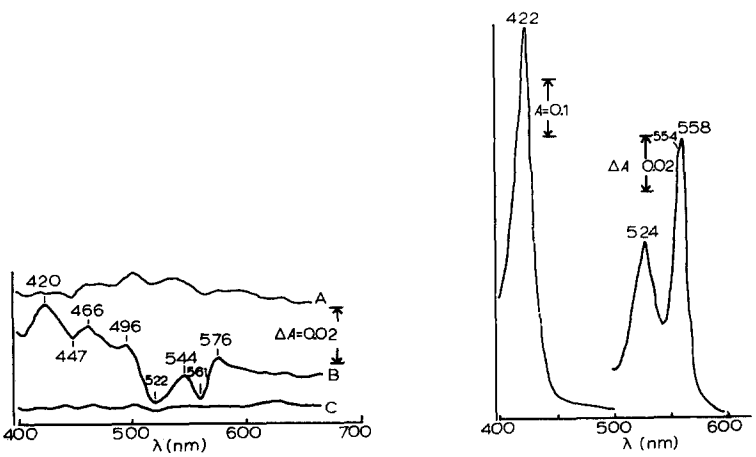

Fig. 2. CO-difference spectra (succinate-reduced with CO *minus* succinate-reduced) of a 20000 × *g* particle preparation. Both reference and test samples were reduced by 5 mM succinate and base line (C) was drawn after compensation at various wavelengths. CO was bubbled into the test cuvette for 30 sec (Curve A) and 15 min (Curve B).

Fig. 3. Absolute reduced spectrum of purified cytochrome *c*-type (556).

immediately after addition of KCN (Fig. 4, Curve B). However, the *c*-type cytochrome was not reduced by either succinate alone or by the 20000 × *g* particle preparation alone (Fig. 4, Curve C). 0.018 mM antimycin A completely inhibited reduction of the *c*-type cytochrome by succinate and the 20000 × *g* particle preparation, and as shown in Fig. 4, Curve A, when antimycin was added to the reaction after the system had reached the aerobic steady-state level of reduction, the *c*-type cytochrome was rapidly oxidized. Again, oxidation of the reduced *c*-type cytochrome was completely inhibited

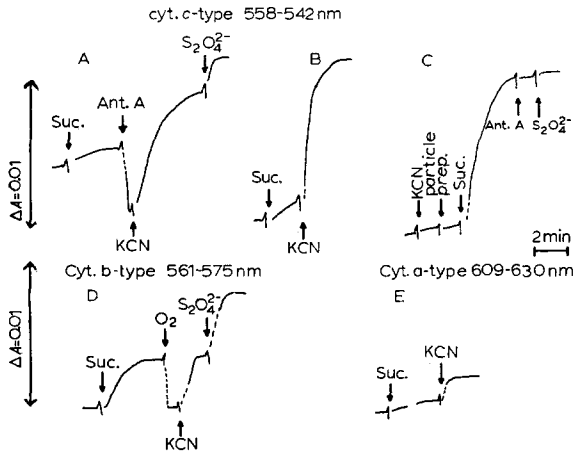

Fig. 4. The effect of various treatments on the steady-state redox level of cytochromes of Euglena. For Curves A, B, and C the reaction mixture consisted of 1.27 nmoles of cytochrome *c*-type (36% reduced) and 0.2 ml of 20000 × *g* particle preparation containing 0.15 nmole of cytochrome *b* (561) and 0.05 nmole of cytochrome *a*-type (609) in a volume of 2.0 ml. For Curves D and E, the 20000 × *g* particle preparation contained 1.5 nmoles of cytochrome *b* and 0.4 nmole of cytochrome *a*-type in a volume of 2.0 ml. The additions at the times indicated were: succinate pH 7.6 (Suc), 5 mM; KCN, 1 mM; antimycin A (Ant. A), 0.018 mM; sodium dithionite ($S_2O_4^{2-}$), 1 mg/ml. In E, KCN was added 12 min after the addition of succinate.

by 1 mM KCN. 2 mM sodium azide also effectively inhibited the oxidation. The aerobic steady-state level of reduction for each cytochrome was found to be: cytochrome *b*, 92% of the anaerobic level of reduction (Fig. 4, Curve D); cytochrome *c*-type 22% (Fig. 4, Curve A); cytochrome *a*-type, less than 5% (Fig. 4, Curve E). These figures were calculated by following the reduction of each cytochrome with the dual wavelength spectrophotometer in a mixture consisting of a 20000 × *g* particle preparation and purified *c*-type cytochrome, such that the cytochromes were in the same relative proportion as found in whole cells. The 20000 × *g* particle preparation catalyzed the reduction of mammalian cytochrome *c* by NADH or by succinate but did not catalyze the oxidation of reduced cytochrome *c*.

Cyanide-insensitive oxygen uptake

Although the experiments described above indicate that one route of succinate oxidation is *via b*- and *c*-type cytochromes and a cyanide-sensitive oxidase, this does not appear to be the only pathway available for the oxidation of succinate. Table III shows that almost half of the succinate-dependent uptake of oxygen by Euglena is insensitive to both cyanide and azide. The maximum inhibition of oxygen uptake by KCN varied slightly in different preparations but was regularly within the 50–60%

range. Antimycin A inhibited more strongly but, at concentrations showing effects near maximum, the further addition of KCN produced an additional inhibition (Table III). The inhibition with antimycin A increased with the concentration in a 'sigmoid' fashion as described by POTTER AND REIF[13]; although maximum inhibition did not exceed 72 %, the degree of inhibition was influenced by protein. In reaction mixtures containing 4.8, 3.8, and 1.9 mg protein, the concentration of antimycin A required for 50 % of maximum inhibition was 0.006, 0.005 and 0.003 mM, respectively.

TABLE III

EFFECT OF INHIBITORS ON THE RESPIRATION OF WHOLE CELLS AND THE 20000 × *g* PARTICLE PREPARATION

Oxygen uptake was measured polarographically at 25°, and the cells ($0.77 \cdot 10^6$) were suspended in growth medium (see MATERIALS AND METHODS). The 20000 × *g* particle preparation (containing 5.8 mg of protein) was suspended in STE medium containing 10 mM succinate.

Inhibitor	*Concn.* (*mM*)	*Inhibition* (%)	
		Whole cells	*20000 × g particle preparation*
KCN	0.1	49	28
	0.2	57	53
	0.3	57	60
	1.0	57	60
Sodium azide	0.8	52	55
	1.7	57	55
Antimycin A	0.004	27	15
	0.008	52	48
	0.016	60	63
	0.022	60	71
Antimycin A	0.008		
plus KCN	0.5	80	82
Antimycin A	0.022		
plus KCN	0.5	92	87

BUETOW AND BUCHANAN[14] have reported that respiration of Euglena mitochondria was inhibited 90 % with 0.003 mM antimycin A and 92 % with 0.4 mM KCN in reaction mixtures which contained approximately the same amount of protein as the 20000 × *g* particle preparation described in Table III.

DISCUSSION

The difference spectra of whole cells and of particulate preparations clearly indicate the presence of at least two particle-bound cytochromes in the streptomycin-bleached mutant of Euglena. From the absorption maximum of the pyridine hemochromes formed by these cytochromes, it is possible to designate them cytochrome *b*-type (561 Euglena) and cytochrome *a*-type (609 Euglena) in accordance with the recommendations of the International Commission on Enzymes[15]. Although a *b*-type

respiratory cytochrome has not been detected previously in euglenoids by difference spectra measured at room temperature, low temperature difference spectra of *Astasia longa* showed a minor inflection at 563 nm which could be attributed to a *b*-type cytochrome[2]. OLSON AND SMILLIE[16] and PERINI *et al.*[4] have described a cytochrome *b* (561 Euglena), but this cytochrome was detected in acetone powders obtained from Euglena grown in light and was shown to be associated with the chloroplasts. Thus this cytochrome could be termed cytochrome b_6 and should be distinguished from cytochrome *b* (561 Euglena) described here, which is associated with mitochondrial electron transport.

Both *b*- and *a*-type cytochromes are firmly bound to cellular particulate matter, and since the relative amounts present are the same in each of the isolated particle fractions (Table I), it is highly probable that both are associated with the same type of particle. Although it was not demonstrated that both the *b*- and the *a*-type cytochromes are exclusively located in a mitochondrial fraction, particles sedimented at $20000 \times g$ are considerably enriched in both cytochromes as well as in succinate dehydrogenase, suggesting that both cytochromes are associated with mitochondrial membranes. In addition both cytochromes are reduced by succinate and progressively reoxidized by air, indicating that they are involved in electron transport from succinate to oxygen. The cytochrome *c*-type (556) is most likely directly linked to the same electron transport system, since it is reduced by succinate in the presence of catalytic quantities of a $20000 \times g$ particle preparation, a reaction completely inhibited by antimycin A. Thus *b*-type (561), *c*-type (556) and *a*-type (609) cytochromes are considered to be associated with the mitochondrial electron transport.

Contrary to the findings of PERINI *et al.*[4] the cytochrome *c*-type (556), isolated and purified by the method described by these authors, was not rapidly autoxidized. Thus it was possible to show that the oxidation of cytochrome *c*-type (556) involves a cyanide-sensitive component of the particle preparation (Fig. 4, Curves A, B and C). The cyanide-sensitivity of the *c*-type cytochrome (556) oxidase system implies that the *a*-type cytochrome (609) functions as the terminal oxidase since oxidation of the *a*-type cytochrome is completely inhibited by 1 mM KCN (Table II and Fig. 4, Curve D). The oxidation–reduction potential (E_0) at pH 7.0 of the *c*-type cytochrome (556) has been estimated at +370 mV (ref. 3) and +307 mV (ref. 4); both values are substantially greater than the E'_0 of mammalian and plant cytochrome *c* (ref. 17) and are in excess of the E'_0 value of the cytochrome oxidase system (+278 mV) (ref. 6). Since the data presented here indicate that cytochrome *a*-type (609) is involved in the oxidation of the cytochrome *c*-type (556), the E'_0 potential of the *a*-type cytochrome is probably greater than +370 mV. It is not surprising that this cytochrome exhibits a number of unusual characteristics when compared with *a*-type cytochromes of other organisms. The maximum of the α band of reduced cytochrome *a*-type (609) is shifted 4 nm toward the red compared to the α band of cytochrome a-a_3 of plant and animal tissues and the *a*-type cytochrome of Euglena[4] grown in light. Soret absorption maxima at 444 nm of cytochrome a_3 were not detected in difference spectra of Euglena. In addition, the cytochrome *a*-type (609) does not combine with CO. The difference spectrum produced after prolonged bubbling of CO is not typical of any CO-complexes formed with *a*-type cytochromes[18], and as indicated by the development of the trough at 561 nm, it probably results from denaturation of the cytochromes in the particle preparation.

Based on evidence presented here, the mitochondrial electron transport system of Euglena can be depicted as a direct sequence from succinate through cytochrome *b*-type (561), cytochrome *c*-type (558) and cytochrome *a*-type (609) to oxygen. Such a scheme accounts for the observed interactions of the cytochromes but does not explain the large proportion of cyanide-insensitive respiration or the P:O ratio of 1 with succinate, reported by BUETOW AND BUCHANAN[14]. However, the latter no longer can be attributed to a deficiency of a *b*-type cytochrome. The inhibition of at least part of the cyanide-insensitive respiration by antimycin A (Table III) and the observed oxidation of cytochrome *b* in the presence of KCN (Table II) suggest that the cyanide-insensitive pathway for succinate oxidation could be linked to the electron transport pathway through cytochrome *b*. In this respect, the cytochrome *b*-type (561) of Euglena mitochondria may function in a similar manner to the cytochrome b_3 present in the microsomal fraction of wheat roots described by MARTIN AND MORTON[19]. The rate of autoxidation of purified preparations of the *c*-type cytochrome is too slow to account for the rate of oxygen uptake of Euglena in the presence of cyanide.

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