Phosphorylation Sites, Cytochrome Complement, and Alternate Pathways of Coupled Electron Transport in Euglena gracilis Mitochondria*

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SUMMARY

Mitochondria from Euglena gracilis have been examined for the possible absence of a phosphorylation coupling site and of b type cytochromes. Mitochondria from cells grown on glutamate + malate as carbon source are shown by site-specific assays to contain coupling sites analogous to Sites I, II, and III of the mammalian respiratory chain. An alternate pathway, relatively specific for oxidation of lactate and NADH, is able to bypass the antimycin-sensitive site. The lowered phosphorylation efficiency associated with this pathway suggests that it involves one less than the normal number of coupling sites. In the absence of antimycin, oxidation of lactate involves at least two coupling sites in these mitochondria.

Low temperature difference spectra provide evidence for the presence of seven substrate-reducible cytochrome components in Euglena mitochondria, including components analogous to a and a3 of mammalian cytochrome oxidase, two c type cytochromes, and three b types. One of these may bind CO and cyanide; another resembles mammalian cytochrome b.

Oxidative phosphorylation in mitochondria isolated from Euglena gracilis has been described by Buetow and Buchanan (1) who showed a number of similarities to mammalian mitochondria. Based on their observation of P:O ratios approaching one less than the theoretical values for various substrates, and on the reported inability of Perini, Schiff, and Kamen (2) to detect mitochondrial b type cytochromes in Euglena, these authors suggested that Euglena mitochondria might lack one of the three coupling sites found in mitochondria of higher organisms or be deficient in some component (such as a b type cytochrome) essential for the activity of one such site.

Mitochondria lacking a specific coupling site are of interest because they offer a possible approach to identifying the components that participate in the initial, site-specific reactions of oxidative phosphorylation. The discovery that the mitochondria of Saccharomyces cerevisiae lack coupling site I, rotenone-sensitive NADH oxidase, and an electron paramagnetic resonance active component in the NADH dehydrogenase region of the electron transport chain (3, 4) has stimulated a good deal of work in this area. It would evidently be useful to identify other cases in which phosphorylation sites are missing from the mitochondrial electron transport chain.

The present investigations were undertaken to establish the number and location of coupling sites in Euglena mitochondria and to characterize the respiratory chain further. Results obtained under one set of growth conditions are described in this paper. These indicate the presence of three coupling sites, but suggest that one of these can be bypassed selectively by an alternate pathway of electron transport. We present spectroscopic evidence that the respiratory chain involves at least three b type cytochromes in addition to the c and a types already identified (2, 5).

These results serve as a basis for comparing biochemical properties of the mitochondria under different conditions of growth. In some instances, profound modifications of the biochemical patterns described here occur. One of these is the subject of an accompanying paper (6).

METHODS

Organisms and Culture—Permanently bleached E. gracilis, strain Z, and E. gracilis var. bacillaris were obtained as single clones from agar plates after maintaining liquid cultures at 33° for 7 to 10 days. Except as noted all experiments were performed with a bleached Z strain cell line, designated Bg. Mass cultures were grown semicontinuously at 27 ± 2° in 4-liter aspirator bottles fitted with magnetic stirrers and nonreturn air outlets; filter sterilized air was supplied at a moderate rate through a single 8-mm diameter tube near the bottom of each bottle. All of the experiments reported here used a defined medium based on

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a citrate plus salts mixture modified from a medium described by Hutner et al. (7) and containing 6.0 g per liter of dl-malic acid + 6.6 g per liter of sodium glutamate as carbon source. Initial pH was adjusted to 4.1 ± 0.1 with NaOH. Thiamine HCl and Vitamin B12 (Nutritional Biochemicals) were added as filter sterilized solutions just before each growth cycle, to final concentrations of 1 mg and 5 μg per liter, respectively. The growth conditions gave generation times of about 12 hours and cultures densities in late log phase of approximately 0.5 × 10^6 cells per ml, corresponding to 3 to 4 g, wet cell weight, per liter. Stocks were maintained at 27°C on a semidefined liquid medium and transferred at 2- to 3-week intervals.

The exact compositions of the media used were as follows; all quantities are expressed in milligrams per liter.

**Stock Culture Medium** —Na$_2$citrate·2H$_2$O, 72; Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O, 14; MgSO$_4$·7H$_2$O, 50; KH$_2$PO$_4$, 40; tryptone (Difco) 1200; yeast autolysate (Difco) 800; sodium acetate, 1200; thiamine·HCl, 1; B$_2$; 0.001; pH 6.2 to 6.6.

**Base Mixture for Mass Culture Medium** —Na$_2$citrate·2H$_2$O, 185; CaCl$_2$·2H$_2$O, 46; MgSO$_4$·7H$_2$O, 185; (NH$_4$)$_2$SO$_4$, 230; KH$_2$PO$_4$, 460; Fe(NH$_4$)$_2$(SO$_4$)$_2$·6H$_2$O, 45; MnSO$_4$·H$_2$O, 17; ZnSO$_4$·7H$_2$O, 24; CuSO$_4$·5H$_2$O, 0.51; H$_3$BO$_3$, 0.01; this mixture was stored dry and adjusted to pH 4.0 ± 0.2 with H$_2$SO$_4$ after solution.

**Preparation of Mitochondria** —Mid to late log phase cultures were filtered through several layers of cheesecloth to remove slime and the cells were harvested by centrifugation at 12000 × g for 5 min. From this point all operations were carried out at 0-4°C. The cells were washed once in a medium containing 0.15 M sorbitol, 1 mM EDTA, and 25 mM Tris chloride (pH 7.5), washed again in isolation medium (0.3 M sorbitol, 0.5 mM EDTA, and 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.0)), and suspended at a concentration of approximately 30% (w/v) in the same medium. Twenty to 40 ml of this suspension was shaken with 50 g of acid-washed glass beads (0.5-mm diameter) for 20 set at maximum speed in an MSK cell homogenizer (Bronwill Scientific Company). The homogenate was decanted, the beads were rinsed two or three times, and the volume was adjusted to a 10% (w/v) suspension. The diluted homogenate was centrifuged at 12000 × g for 10 min, yielding a large, firm pellet of starch, unbroken cells, and debris. The supernatant suspension was centrifuged at 8000 × g for 15 min and the crude mitochondrial pellet was resuspended by swirling and careful pipetting to separate it from a small starch pellet usually present. The mitochondria were washed once or twice in 20 to 30 ml of isolation medium and resuspended at a concentration of 0.2 to 30 mg protein per ml. When the mitochondria were to be used for phosphorylation experiments the washing and resuspending medium was supplemented with crystalline bovine serum albumin (Sigma) at 0.1 g/100 ml. Final yields of mitochondrial protein determined according to Lowry et al. (8) or by the biuret procedure (9) were 1.5 to 2.5 mg per g of wet cells. Mitochondria were prepared on the day of each experiment since oxidative phosphorylation was found to decay rather rapidly on storage, either at 4°C or frozen at -20°C or -90°C. The data presented in Tables I and II were obtained with mitochondria prepared by the procedure of Buetow and Buchanan (10), in which the cells were broken by hand grinding in a mortar with glass beads (0.2-mm diameter). In this case the isolation medium contained 0.25 M sucrose, 0.5 mM EDTA, and 25 mM Tris chloride (pH 7.4). Less consistent results were obtained by this procedure, probably because of the difficulty of reproducing the conditions of breakage.

**Measurement of Respiration and Phosphorylation** —Rates of oxygen consumption were measured in sorbitol-EDTA-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer at 30°C in a water-jacketed 1.5-ml glass chamber (Gilson Medical Electronics) containing a Clark type polarographic oxygen electrode. Oxidative phosphorylation was assayed either in the same apparatus with $^32$P, (New England Nuclear) at specific activities between 2 × 10^6 and 10^7 cpm per μmole or in the Warburg respirometer with $^{32}$P; at specific activities of 0.5 to 2.5 × 10^6 cpm per μmole. In either case the assay mixtures contained 16.7 or 20 mM Pi, 5 mM Mg$^{2+}$, 2 mM ATP, 33 mM glucose, 1 mg per ml of bovine serum albumin, and 3 to 5 units per ml of hexokinase (Boehringer, dialyzed). In the Warburg experiments, flask center wells contained filter paper strips moistened with 10% KOH or with 1 M KCN in 0.1 M KOH when cyanide was added to the medium. Incubations were terminated after exhaustion of dissolved O$_2$ in the polarographic experiments or after 30 to 45 min in Warburg experiments, by adding 0.1 volume of 50% (w/v) trichloroacetic acid, and the precipitated proteins were removed by centrifugation. Inorganic phosphate was precipitated from 0.2-ml aliquots of the deproteinized solutions by the triethylammonium molybdate method of Sugino and Miyoshi (11); after centrifugation the supernatant solutions containing esterified $^{32}$P were filtered through small plugs of nonabsorbent cotton in Pasteur pipettes to assure removal of traces of the precipitate. This procedure reliably removed more than 99.9% of the radioactivity from blank mixtures, provided that the $^{32}$P was of high radiochemical purity. Esterified phosphate was determined by counting samples and suitably diluted standards on aluminum planchets in a Nuclear-Chicago low background gas flow counter.

**Site-specific Phosphorylation Assays** —Phosphorylation corresponding to coupling Site I was assayed by the method of Schatz and Racker (12) with coenzyme Q$_1$ as acceptor for electron transfer from NADH. Phosphorylation in the span from NADH to oxygen was assayed in the same experiments by omitting coenzyme Q$_1$ and cyanide from the mixture. The reactions were followed by recording absorbance changes due to the disappearance of NADH at 340 nm with a Gilford automatic spectrophotometer, with an adjusted extinction coefficient of 0.8 mm$^{-1}$ cm$^{-1}$ when coenzyme Q$_1$ was present (12). Site II phosphorylation was assayed by a modification of the method described by Lee, Sottocasa, and Ernster (13). Reduction of ferricyanide was determined spectrophotometrically at 410 nm, with an extinction coefficient of 1.0 mm$^{-1}$ cm$^{-1}$, and phosphorylation was measured as described above. P/μ mole ratios were calculated on the basis of half of the number of moles of ferricyanide reduced. (Details are given in Table II and the legend to Fig. 1.) Site III assays were carried out with ascorbate plus N-methylphenalmine methyl sulfate to donate electrons at the level of cytochrome c. Oxygen consumption was measured polarographically, and oxygen uptake due to the cytochrome oxidase reaction was estimated in control mixtures containing cyanide. Experimental values were corrected by subtracting the control O$_2$ uptakes occurring during the same time interval.
started by adding 0.13 rmole of NADH, and the disappearance of 
temperature 30°. Final concentrations of additions were: KCN, 
KCN + CO₂, rotenone. 157 90 0.4  
KCN + Co₂l. 442 90 14.8  
KCN.. 21 35 1.2  

cyanide control (second line) during the same time interval. 

terminated upon exhaustion of NADH by adding 0.1 
NADH consumed are corrected for the NADH consumed in the 
NADH was followed spectrophotometrically at 340 nm. REac-
bovine serum albumin, 0.25 M sucrose, and approximately 0.5 mg 
chloride, 10 mM 32P; (4.5 X 10⁵ cpm per pmole), 2 mg per ml of 
ATP, 32 mM glucose, 10 units per ml of hexokinase, 5 mM Tris 
ethanesulfonic acid, adjusted to pH 7.0 with KOH.

**Table I**

**Assay of phosphorylation at coupling Site I**

Reaction mixtures contained 0.5 mM EDTA, 2 mM MgCl₂, 1 mM 
ATP, 32 mM glucose, 10 units per ml of hexokinase, 5 mM Tris 
chloride, 10 mM 32P, (4.5 X 10⁵ cpm per µmole), 2 mg per ml of 
bovine serum albumin, 0.25 M sucrose, and approximately 0.5 mg 
per ml of mitochondrial protein. Final volume 1.0 ml, pH 7.2, 
temperature 30°. Final concentrations of additions were: KCN, 
2.8 mM; coenzyme Q₁₀, 0.12 mM; rotenone, 40 µM. Reactions were 
started by adding 0.13 µmole of NADH, and the disappearance of 
NADH was followed spectrophotometrically at 340 µm. Reactions 
terminated upon exhaustion of NADH by adding 0.1 
ml of 50% trichloracetic acid, and esterified phosphate was deter-
ted as described under “Methods.” The reported values of 
mixed activity are corrected for the NADH consumed in the 
cytrate control (second line) during the same time interval.

trophotometer modified to allow measurements at liquid nitrogen 
temperature. Relative concentrations of the various absorbing 
species were calculated by reference to the intensity of the fully 
reduced cytochrome oxidase a-band at 607 µm (reference wave 
length, 625 µm). For spectra at liquid nitrogen temperature, 
reduction of ferricyanide by succinate in the presence of rotenone 
was carried out in the polarograph in a volume succinate, 16 mM; ferricyanide, 1.0 mM; antimycin, 10 mM; KCN, 13 mM; ascorbate, 6.7 mM; N-methylphenazinium methyl 
sulfate, 0.67 µM; antimycin, 13 µM; KCN, 1 mM; mitochondrial 
protein, 1.2 mg per ml. When ferricyanide was the acceptor the 
volume was 1.0 ml; final concentrations in these experiments were: 
succinate, 16 mM; ferricyanide, 1.0 mM; antimycin, 10 µM; KCN, 1.2 mM; mitochondrial protein, 1.45 mg per ml. The P/2e⁻ values 
given in parentheses are corrected for electron transfer and Pi 
esterification in the corresponding controls.

![Image](http://www.jbc.org/Downloadedfrom)

**Table II**

**Assay of phosphorylation at coupling Sites II and III**

All assay mixtures contained 3.3 µM rotenone and were other-
wise as described under “Methods.” The experiments with 
oxygen as acceptor were carried out in the polarograph in a volume of 
1.5 ml. Final concentrations of added materials were: succ-
cinate, 13 mM; ascorbate, 6.7 mM; N-methylphenazinium methyl 
sulfate, 0.67 µM; antimycin, 13 µM; KCN, 1 mM; mitochondrial 
protein, 1.2 mg per ml. When ferricyanide was the acceptor the 
volume was 1.0 ml; final concentrations in these experiments were: 
succinate, 16 mM; ferricyanide, 1.0 mM; antimycin, 10 µM; KCN, 1.2 mM; mitochondrial protein, 1.45 mg per ml. The P/2e⁻ values 
given in parentheses are corrected for electron transfer and Pi 
esterification in the corresponding controls.
FIG. 1. Simultaneous assay of coupling Sites II and III. The reaction mixtures contained 0.25 M sucrose, 25 mM Tris chloride (pH 7.4), 13.3 mM 32P1 (specific activity 1.6 × 10⁴ cpm per amole), phosphorylation cofactors, and bovine serum albumin as described under “Methods,” 6.7 mM succinate, 3.5 μM rotenone, and 1.7 mg per ml of mitochondrial protein. Ferricyanide was present at 0, 0.28, 0.84, and 0.84 mM in the order plotted; in addition, the last sample contained 0.4 mM KCN. Total rates of electron transfer in the four samples were 95, 77, 72, and 60 mFeq min⁻¹ mg⁻¹, respectively. Oxygen consumption was measured polarographically; ferricyanide remaining was determined spectrophotometrically after deproteinization of the reaction mixtures. Control experiments confirmed that ferrocyanide was not oxidized under the conditions used.

succinate in the polarograph, O₂ consumption slowed by more than 50%, returning to its original rate after all of the ferricyanide had been reduced as judged by the disappearance of its yellow color. In the experiment illustrated by Fig. 1, mitochondria were allowed to oxidize measured amounts of ferriyanide and of oxygen, present together, and the fraction of the total electron flux reaching each acceptor was varied by varying the initial concentration of ferricyanide and the time of incubation, and by addition to one sample of a low concentration of cyanide, sufficient to inhibit the oxygen reaction by about 60%. If reducing equivalents must traverse two independent coupling sites between succinate and oxygen, and one of these is between succinate and ferricyanide, then one expects the P/2e⁻ ratio (P esterified/total equivalents transferred) to decrease linearly with a decrease in the fraction of the flux reaching oxygen. The P/2e⁻ ratio extrapolated to zero reduction of oxygen should be equal to the P/2e⁻ ratio for Site II alone. The data of Fig. 1 conform to the prediction of a linear variation of the P/2e⁻ ratio, providing strong evidence for the presence of two separate phosphorylation sites in the span between succinate and oxygen. The extrapolated values for the ferricyanide reaction (0.24) are somewhat less than half of the P/2e⁻ ratio from succinate to oxygen (0.66). If it is assumed that only 75% of the ferricyanide was reduced via the phosphorylating pathway, in agreement with the observed sensitivity of the reaction to antimony (Table II, and the text above), then the corrected P/2e⁻ value for Site II becomes 0.32. It should be noted that the total rates of electron transfer (see legend to Fig. 1) differed by less than a factor of 1.5 between the various samples. Experiments of this type have yielded consistent results even when the succinate P:O ratio was as low as 0.2.

In addition, we have confirmed that the phosphorylation observed at both sites is completely inhibited by 3.3 μM carbonyl cyanide-m-chlorophenylhydrazone. Similar results were also obtained with mitochondria from E. gracilis var. bacillaris.

Antimycin-resistant respiration—Mitochondria prepared under the present conditions have, as a rule, shown a high degree of inhibition of succinate oxidation by antimony. On the other hand, we have consistently observed that a significant fraction of the NADH oxidase activity (corrected for cyanide-insensitive NADH oxidase, see “Discussion”) was sensitive to antimony. Fig. 2 shows titrations of succinoxidase and NADH oxidase activities with antimony, in a preparation low in cyanide-insensitive NADH oxidase activity. The succinoxidase titration curve shows the sigmoid shape and sharp end point characteristic of antimycin inhibition in mammalian mitochondria (14); the end point lies at about 0.15 mmoles of antimony per mg of protein, a value comparable to the estimated cytochrome a content of these mitochondria (20). While the shape and transition midpoint of the curve for NADH oxidase are identical, it displays about 25% residual activity which is still sensitive to cyanide; this activity persisted up to more than 100-fold higher concentrations of antimycin. All preparations tested, both from Z strain and var. bacillaris cells, have given similar results. In other experiments, oxidation of malate showed a partial antimycin resistance similar to NADH, and oxidation of D- and L-lactate were even more resistant to antimycin, maximum in-
Each flask contained 1.95 mg of mitochondrial protein in a final volume of 2.0 ml. The P:O ratios shown were calculated from phorylation values shown on the last line of the table.

KCN (2 mM)

None ................... 1.25 mM NAD+, described under "Methods" with the addition of 82 mM ethanol, 100% cyanide-insensitive respiration. Because a large correction was necessary, little significance can be attached to the absolute values of the P:O ratios obtained.

Table IV compares results obtained with lactate and succinate. The data have not been corrected for cyanide-resistant activities, which were found to be low and similar for the three substrates. It can be seen that, with succinate as substrate, oxidation and phosphorylation are strongly inhibited by antimycin. The rate of D-lactate oxidation, on the other hand, is unaffected by antimycin, while the P:O ratio is decreased by almost 50%. Oxidation of L-lactate is moderately inhibited by antimycin and the P:O ratio is diminished by about one-third. The finding of P:O ratios significantly greater than 1.0 with both lactate isomers distinguishes Euglena from yeast, whose mitochondrial lactate oxidase system includes only the single phosphorylation site associated with cytochrome oxidase (3). The depression of P:O ratios by antimycin may reflect some uncoupling by the inhibitor at the relatively high concentration used (2.4 mmoles per mg of protein). Significant uncoupling by antimycin at concentrations greater than 0.8 mmole per mg of protein has been observed in other systems (15, 16). However, in another experiment antimycin at only 0.52 mmole per mg of protein depressed the P:O ratios for both D- and L-lactate by more than 50%; note also in Table III that antimycin apparently had a similar effect on the P:O ratio whether present at 0.09 or at 0.47 mmole per mg of protein.

**Cytocromes**—Fig. 3 shows a difference spectrum recorded at liquid nitrogen temperature between aerobically oxidized mitochondria and anaerobic, succinate-reduced mitochondria and aerobic, oxidized mitochondria. A large a-band complex (Fig. 3B), which at room temperature appears as a perfectly symmetrical peak at 607 µm, is resolved into at least four components, with shoulders at 552 and 565 µm and maxima at 558 and 561 µm. The a-band of cytochrome oxidase at 607 µm is smaller and more symmetrical, with a broad shoulder centered at 594 µm. In the Soret region (Fig. 3A) a maximum at 443 µm and a shoulder at 453 µm suggest the presence of two cytochrome a type components analogous to the a and a₃ of mammalian cytochrome oxidase (17).
Fig. 3 and also Fig. 7 of the accompanying paper (6)). Curve b type cytochrome at 561 μm has been clearly resolved (compare cytochromes (21) allows further resolution of the overlapping at least two b type cytochromes. In other experiments a third trough at 422 and 433 μm, respectively.

Material systems (19, 20). Fig. 4 have been reported for Astasia longa (5) and for some bac-

cyanide-binding cytochrome appears as a symmetrical peak and component (data not shown). In this case the Soret feature of the difference spectrum induced by succinate plus antimycin, re-

induces a difference spectrum in substrate-reduced, anaerobic absorption band originally centered near 560 μm. Cyanide induces a difference spectrum in substrate-reduced, anaerobic mitochondria which is similar to this slower reacting CO component (data not shown). In this case the Soret feature of the cyani-bonding cytochrome appears as a symmetrical peak and trough at 422 and 433 μm, respectively.

Carbon monoxide difference spectra similar to those shown in Fig. 4 have been reported for Astasia longa (5) and for some bacterial systems (19, 20).

The use of antimycin for selective reduction of the b type cytochromes (21) allows further resolution of the overlapping α-bands in the region around 558 μm. Fig. 5, Curve A, shows a difference spectrum induced by succinate plus antimycin, re-

corded at liquid nitrogen temperature. The maximum at 558 μm and the pronounced shoulder at 555 μm indicate the presence of at least two b type cytochromes. In other experiments a third b type cytochrome at 561 μm has been clearly resolved (compare Fig. 3 and also Fig. 7 of the accompanying paper (6)). Curve B of Fig. 5 is the difference (succinate + cyanide) - (eucinate + antimycin), recorded directly, and shows those components which were not reduced in the presence of antimycin. Here a shoulder at 552 μm and a peak at 555 μm indicate the presence of two c type cytochromes. The features in the region between 555 and 565 μm are complex, and may be due in part to a shift in the a-band of the cyanide-binding cytochrome mentioned above. It is possible, however, that part of the strong shoulder at 560 μm is due to a b type cytochrome which did not become fully reduced in the presence of antimycin, this would be consis-
tent with the finding of an alternate respiratory pathway in these mitochondria which bypasses the antimycin block.

The small trough at 565 μm in Fig. 5, Curve B, almost certainly results from the fact that the corresponding b type cytochrome, like mammalian cytochrome b shows a greater absorbance in the presence of antimycin (22) or in dithionite-reduced mitochondria (23) than under anaerobiosis or in the presence of cytochrome oxidase inhibitors. The effect may be verified by comparing the relative prominence of this band in Fig. 5, Curve C (dithionite, oxidized), and in Fig. 3 (anaerobic - oxidized).

Table V summarizes spectral data for the cytochromes which we have been able to detect in Euglena mitochondria. These data have been verified by comparison of difference spectra made under a variety of conditions, and we believe that they describe the minimum number of components necessary to account consistently for the spectral features observed. The types of cytochromes present and their relative proportions, as judged from difference spectra, have been very consistent between different lots of mitochondria as well as in mitochondria isolated under different conditions of growth (6). The content of cytochrome oxidase in all of these preparations has averaged 0.25 mmole per mg of mitochondrial protein (range: 0.12 to 0.56), based on room temperature measurements of the reduced α-band at 508 μm, with 625 μm as a reference wave length and assuming an extinction coefficient difference of 10 mm³ cm⁻¹ (24).

Comparison of whole cell difference spectra suggests that the c type component at 555 μm (Soret, 423 μm) is partially lost
Table V
Spectral characteristics of Euglena mitochondrial cytochromes

| Type | α-Band maxima | Intensity | Spectra maxima | Other features |
|------|---------------|-----------|----------------|---------------|
|      | mμ            |           |                |               |
| a    | 607           | 1.0       | 453            | Binds CO (rapidly) |
| b    | (593? )       | (0.3)     | 444            | Antimycin enhancement |
|      | 555           | 1.0       | 431            | (One of these may bind CO (slowly) and cytochrome oxidase) |
|      | 561           | 1.9       | (428?)         |                   |
|      | 558           | 2.2       | (457?)         |                   |
| c    | 555           | 1.8       | 422            | Soluble c type |
| c    | 551           | 1.0       | (419?)         |                   |

The data are for reduced-oxidized difference spectra at liquid nitrogen temperature. Intensities of absorbance were measured from base lines drawn through the 543, 575, and 625 mμ points in dithionite-reduced spectra, and are given relative to the intensity of the cytochrome α α-band at 590 mμ (mean of five determinations).

During preparation of mitochondria, and this component shows the most variability in mitochondrial spectra. For these reasons as well as its spectral properties, we identify this component with the soluble c type cytochrome isolated and characterized by Perini, Kamen, and Schiff (25) and designated as "Euglena cytochrome 556." In the pure state this cytochrome was shown to have a double α-band (reduced) with maxima at 554.5 and 558 mμ (25). If it retains this property in situ then part of the absorbance at 558 mμ must be contributed by this cytochrome; however, it is not possible to account for all of the differences that we have observed at both 555 and 558 mμ in terms of a single component.

We have not detected any qualitative difference between spectra induced by succinate and by d-lactate. In particular, the same group of three b type cytochromes is reduced to about the same extent in the steady state either substrate. There is, however, a quantitative difference in the degree of further reduction of the b type cytochromes brought about by addition of antimycin, the increase being 3 to 4 times larger for succinate than for lactate. With either substrates the three b components respond to antimycin as a group; none of them shows large absorbance changes relative to the others. The 565 mμ b type shows the largest percentage change, however.

Discussion

It is apparent from the results presented in this paper that three phosphorylation sites corresponding to mammalian Sites I, II, and III, lying in the regions of the electron transport chain sensitive to rotenone, antimycin, and cyanide, respectively, are present in Euglena mitochondria. Moreover, these mitochondria appear to have a full complement of cytochromes but differ from mammalian mitochondria in that at least three b type cytochromes can be visualized. It is of interest to note that this feature is characteristic of mitochondria of higher plants (21, 26). Furthermore, one of the Euglena b type cytochromes shows enhanced absorption upon addition of antimycin, similar to the mammalian mitochondrial b cytochrome (22, 23) which is closely related to coupling Site II.

It is evident that the reasons for the low P:O ratios observed in Euglena mitochondria cannot include the complete absence of a particular coupling site or a limiting amount of a b cytochrome as suggested by Buettow and Buchanan (1). This conclusion is confirmed by the results of the site-specific assays, the simultaneous assay of Sites II and III, and the spectroscopic data. One obvious explanation for low P:O ratios is that the present methods of preparation yield damaged, partly uncoupled mitochondria. This was certainly the case in our early experiments, in which P:O ratios less than 1.0 were regularly observed with all substrates, and there was no indication of respiratory control by ADP. In more recent preparations we have observed stimulations of respiration of the order of 2-fold by phosphate acceptor, and P:O ratios significantly higher than 1.0 with succinate and lactate, suggesting that with improved methods of preparation theoretical P:O ratios might be obtained consistently. The elimination of a nonphosphorylating, cyanide-insensitive NADH oxidase activity (compare Table III), which fractionation studies (not reported in this paper) indicate to be a cytoplasmic contaminant in the mitochondrial preparations, and lead to substantial increases in the apparent P:O ratios for oxidation of NADH and of NAD-linked substrates.

Under certain conditions the efficiency of oxidative phosphorylation may be affected by the presence of alternate pathways for electron transport, which bypass one or more coupling sites. Inhibitor-resistant respiration has been observed frequently in mitochondria from plant tissues (27-29) and in some cases the resistant pathways show considerably altered phosphorylation efficiencies. Hackett, Rice, and Schmid (29) observed that P:O ratios in sweet potato mitochondria with citrate and succinate as substrates were specifically depressed by several respiratory chain inhibitors including carbon monoxide, cyanide, and antimycin. They suggested that the normal respiratory chain between cytochrome b and oxygen could be bypassed by an alternate pathway containing fewer phosphorylation sites. We have always observed phosphorylating respiration in Euglena mitochondria to be cyanide-sensitive, suggesting that cytochrome oxidase participates in the alternate as well as the normal pathway. Levels of CO sufficient to inhibit succinate oxidation by more than 40% have no effect on the P:O ratio.

An extreme example, in which all phosphorylation sites between succinate and oxygen are bypassed by an alternate pathway, occurs in Euglena under different growth conditions from those used here, and is described in the following paper (6).

The effects of antimycin on the P:O ratios with NADH, lactate, and even succinate (Table IV) suggest that in the presence of antimycin electron transport bypasses a single phosphorylation site, presumably Site II, thus lowering the P:O ratio by about one-third for NADH and about one-half for succinate or lactate. It must further be supposed that the alternate pathway carries only a minor fraction of the electron flow in the absence of antimycin. It is not difficult to imagine how this might be the case for succinate, NADH, or L-lactate, since antimycin inhibits the total rate of oxidation of these substrates by at least 40%. On the other hand, d-lactate is oxidized at nearly the same rate in the presence or the absence of the inhibitor, only the P:O ratio changing.

One possible explanation of these results is that oxidation of d-lactate (and to a lesser extent of L-lactate or NADH) normally proceeds largely via an alternate electron transport chain containing two phosphorylation sites, i.e. Site III and a site similar to but not identical with Site II of the succinate chain. The

1 Unpublished experiments.
depression of P:O ratios with other substrates could result from a combination of diversion of electron flow into this alternate pathway and of site specific uncoupling by antimycin. This uncoupling must be specific since, over a wide range of antimycin concentrations, P:O ratios are depressed to a similar extent. Another possibility is that antimycin somehow activates an alternate pathway specific for lactate oxidation, which bypasses the normal antimycin-sensitive locus and coupling Site II, so that net electron flux remains nearly the same in the presence of the inhibitor while the P:O ratio decreases.

In Euglena it is nevertheless clear that in the absence of antimycin the oxidation of lactate must involve at least two coupling sites. This is consistent with the observation of Rutner and Price (30) that the mitochondrial lactic dehydrogenase of yeast normally donates electrons at the level of cytochrome c (3). Euglena reacts preferentially with coenzyme Q, while the mitochondrial lactic dehydrogenase of yeast normally donates electrons at the level of cytochrome c (3).

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