Electron Transport in Mitochondria Isolated from the Flagellate *Polytomella caeca*

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1. Mitochondria isolated from *Polytomella caeca* contain cytochromes *b*, *c* + *c*₁ and *a* + *a*₃ and several flavoprotein species. 2. Electron transport is inhibited by antimycin A, rotenone, piericidin A and cyanide. 3. Spectral data indicate that antimycin A inhibits the reoxidation of reduced cytochrome *b*. 4. Various types of flavoprotein are characterized by simultaneous spectrophotometric and fluorimetric measurements on antimycin A-inhibited preparations and also by their absorption and fluorescence-emission spectra. 5. The rotenone-sensitive site lies between the two flavoproteins of the respiratory chain, designated FpD1 and FpD2. 6. Other flavoprotein species detected include those involved in the oxidation of succinate and externally added NADH; a large proportion of mitochondrial flavine is reduced by dithionite but not by known respiratory substrates. 7. The kinetics of flavoprotein and cytochrome reactions were studied.

The flagellate *Polytomella caeca* may be regarded either as a protozoon or as a colourless alga of the Order Volvocales (Wise, 1959). When grown aerobically in a mineral salts-thiamine medium, with acetate as sole carbon source, this organism has highly organized mitochondria, which can be easily isolated after gentle mechanical disruption of the fragile cell membranes (Evans & Lloyd, 1966). The mitochondria so obtained seem to be reasonably intact, as they appear structurally whole in electron micrographs, oxidize NADH and various tricarboxylic acid-cycle intermediates with a degree of respiratory control and show the phenomenon of latent enzyme activities (D. Lloyd, D. A. Evans & S. E. Venables, unpublished work). The present paper describes an investigation of electron transport in *Polytomella* mitochondria with particular reference to the flavoprotein and cytochrome components of the system.

METHODS AND MATERIALS

Maintenance, growth and harvesting of the organism. *Polytomella caeca* Ehrenberg was obtained in pure culture from the Culture Collection of Algae, Department of Botany, Indiana University, Bloomington, Ind., U.S.A. (catalogue no. 19). The organism was grown with gentle forced aeration at 25° in the acetate (or propionate)-mineral salts-thiamine medium of Wise (1959) at pH 6.0. The conditions of aeration (flow rate 1.5 l./min. through a plain 4 mm.-diam. delivery tube) were critical, as higher

flow rates or fine bubbles produced by spargers caused disruption of the fragile cells. The organism was maintained in liquid culture on this medium and subcultured every 4 days. Small-volume cultures (100 ml.) were grown in 500 ml. conical flasks without shaking and used as inocula for 20 l. force-aerated cultures.

Cells were harvested in late exponential growth phase (48 hr.) at a cell density of about 0.15 mg. dry wt./ml. by centrifuging for 10 min. at 1000g. Cells were washed once by resuspension in 3 vol. of 20 mM-potassium phosphate buffer, pH 7.0, and reharvested.

Preparation of extracts and isolation of mitochondria. The cells were suspended in 4 vol. of a medium consisting of 0.32 M-sucrose in 4 mM-EDTA-20 mM-tris-HCl buffer, pH 7.4, and broken, either by passage through an ice-cold 3 × 3 glass sinter (A. Gallenkamp and Co. Ltd., London, E.C. 2) under reduced pressure or by careful homogenization by hand in a glass homogenizer with a Teflon pestle (A. H. Thomas Co., Philadelphia, Pa., U.S.A.).

These extracts were fractionated at 4° in the 8 × 50 ml. head of a Sorvall RC2 centrifuge as follows. The crude homogenate was centrifuged at 1000g for 8 min. and the supernatant recentrifuged at this speed. The pellets so obtained consisted of a mixture of intact cells, membrane and nuclear fragments, and large amounts of starch. The supernatant obtained after the second centrifugation was further centrifuged at 10000g for 15 min. to give a crude mitochondrial pellet, which, after one washing by resuspension in 4 vol. of the isolation medium and recentrifuging at 10000g for 10 min., was used without further treatment.

Analytical methods. Rates of O₂ uptake were measured polarographically at 25° as described by Lloyd (1965b). Mitochondrial protein was assayed by the biuret method (Layne, 1957). Changes of flavoprotein extinction and fluorescence (Chance & Schoener, 1966) were followed simultaneously with a double-beam spectrophotometer (Chance, 1951a,b,c) and an attached fluorimeter. The

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double-beam spectrophotometer was used with the wavelength pair 475–510 μ , and fluorimetric measurements were made by excitation with the 436 μ line of a 1000 w water-cooled mercury arc with detection at 570 μ . Appropriate filters were used to guard each of the two photomultipliers from interference by the opposite system. The measurements of flavoprotein absorption may include some contribution from non-haem iron, though there is some evidence that this species does not exist in optically measurable concentrations in the steady state of the mitochondrial electron-transport chain (see discussion of Chance & Schoener, 1966).

Cytochrome and flavoprotein spectra were obtained by using a split-beam spectrophotometer (Yang & Legallais, 1954) with a liquid-N₂ attachment (Chance, 1957). Techniques for the measurement of flavoprotein fluorescence-emission spectra were essentially as described by Chance & Schoener (1966). Rapid-reaction kinetics were followed in the regenerative-flow apparatus (Chance, 1954). Extraction and determination of flavine were by the method of Rao, Felton & Huennekens (1967).

Materials. Antimycin A, rotenone and NADH were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. β -Hydroxypropionic acid was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Carnitine derivatives were kindly provided by Dr P. B. Garland, and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and piericidin A were gifts from Dr C.-P. Lee and Dr K. Folkers respectively. All other reagents were of the highest grade available commercially. Malonic semialdehyde was prepared as described by Lloyd (1965a). Solutions of substrates were neutralized where necessary with tris to pH 7.2. Antimycin A, rotenone, piericidin A, pentachlorophenol and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were all used as solutions in methanol; not more than 20 μ l. of methanol solution was added/ml. of total reaction volume.

RESULTS

Cytochrome components. Fig. 1 shows a difference spectrum (dithionite-reduced – oxidized) of *Polytomella* mitochondria at the temperature of liquid nitrogen. Peaks at 605, 562 and 549 μ correspond to the α -bands of cytochromes *a*, *b* and *c* respectively, and absorption maxima at 534, 524 and 517 μ represent the β -bands of cytochromes *b*, *c*₁ and *c*. The Soret region (not shown) was characterized by peaks of cytochromes *a*₃ and *b* at 440 and 427 μ respectively. The band of cytochrome *c* was seen as a shoulder below 420 μ .

Inhibition of electron transport by antimycin A, rotenone, piericidin A and cyanide. Inhibition curves for antimycin A and rotenone are shown in Fig. 2. The oxidation of succinate and of exogenous NADH is 80–90% inhibited at 4 μ moles of antimycin A/mg. of protein; further increase in inhibitor concentration gives virtually complete inhibition of succinoxidase activity, but more than 90% inhibition of NADH oxidase was not achieved. Rotenone (2 μ moles/mg. of protein) produced 70% inhibition of citrate oxidation, and again much higher concentrations of the inhibitor failed to give

Fig. 1. Difference spectrum (dithionite-reduced – oxidized) of a mitochondrial suspension (20 mg. of protein/ml.) at the temperature of liquid N₂. The path length was 3 mm.

Fig. 2. Inhibition of NADH and succinate oxidation by antimycin A and of NADH and citrate oxidation by rotenone. Mitochondria (1–4 mg. of protein in 0.2 ml. of sucrose-tris-EDTA isolation medium) were added to 0.8 ml. of medium consisting of 0.25 M-sucrose in 5 mM-EDTA–14 mM-potassium phosphate buffer, pH 7.4, containing bovine serum albumin (0.15%), and brought to state 3 by the addition of 200 μ moles of ADP and substrate. $\circ$, Rotenone inhibition of citrate (10 mM) oxidation; $\blacktriangle$, antimycin A inhibition of NADH (1 mM) oxidation; $\triangle$, antimycin A inhibition of succinate (10 mM) oxidation; $\bullet$, rotenone inhibition of NADH (1 mM) oxidation; $\square$, rotenone inhibition of NADH oxidation in partially disrupted (homogenized) mitochondria.

more than 90% inhibition. The oxidation of exogenous NADH by intact mitochondria was markedly less sensitive to rotenone inhibition, but mild disruption (e.g. by freezing and thawing) produced a mitochondrial preparation that had the same sensitivity to rotenone with regard to the oxidation of exogenous or endogenous NADH. Piericidin A at a concentration of 5 μ moles/mg.

Fig. 3. Spectra of antimycin A-inhibited mitochondrial suspension (20mg. of protein/ml.) at the temperature of liquid N_2 . The reference cuvette contained mitochondria that had been aerated before being frozen. The sample cuvette contained either 10mM-succinate (curve 1) or 1mM-NADH (curve 2) together with 3 μ M-pentachlorophenol and antimycin A (4 μ moles/mg. of protein). The path length was 3mm.

of protein gave 88% inhibition of the NADH oxidase activity of a broken mitochondrial preparation; this concentration of the inhibitor did not affect succinoxidase activity. Cyanide at 10mM gave 94% inhibition of succinoxidase but only 66% inhibition of NADH oxidase.

Spectra of antimycin A-inhibited mitochondria. The addition of succinate to pentachlorophenol (3 μ M)-uncoupled antimycin A (4 μ moles/mg. of protein)-inhibited mitochondrial suspensions at 22° resulted in an extensive reduction of cytochrome *b* (peaks at 567, 533 and 448 $m\mu$) and flavoprotein (trough at about 465 $m\mu$). After 10min. anaerobiosis (state 5; Chance & Williams, 1955) was attained accompanied by the reduction of cytochromes *a*+*a*₃ and *c* and a further reduction of cytochrome *b* and flavoprotein. In the inhibited state 3 condition little reduction of cytochromes *a*+*a*₃ and *c* was detectable in room-temperature spectra, but on freezing in liquid nitrogen a small extent of reduction of these cytochromes was revealed (Fig. 3), presumably due to the 'leak' of electrons through the incompletely inhibited antimycin A-sensitive site.

Similar spectral changes were noted with NADH as reductant in place of succinate except that the extent of cytochrome *b* reduction was less with this substrate. The peak at 585 $m\mu$ cannot be accounted for by any known cytochrome pigment.

Reduction and oxidation of flavoproteins. A rapid change in the redox state of flavoprotein of mitochondrial suspensions was observed both spectrophotometrically and fluorimetrically during the transition to anaerobiosis on exhaustion of oxygen

Fig. 4. Reduction and oxidation of flavoproteins. Simultaneous traces of fluorescence (excitation at 436 $m\mu$, measurement at 570 $m\mu$) and extinction changes (475–510 $m\mu$) of a suspension of mitochondria (5mg. of protein/ml.) in 100mM-KCl–5mM-MgCl₂–50mM-tris-HCl buffer, pH 7.4. The mitochondria were pretreated with 3 μ M-pentachlorophenol followed by 10 μ g. of antimycin A. Other additions were made as follows: A, ethanol (3mM); B, β -hydroxybutyrate (3mM); C, glutamate (3mM); D, malate (3mM); E, rotenone (15 μ g.); F, succinate (3mM); G, malonate (10mM); H, NADH (1mM); I, dithionite (excess). The total volume was 3.3ml.

in the cuvette. In all experiments involving the characterization of flavoproteins mitochondria were uncoupled by the addition of $3\mu\text{M}$ -penta-chlorophenol and electron transport was inhibited with antimycin A. Little flavoprotein reduction was observed on adding antimycin A in the absence of substrate, as the endogenous respiration of these mitochondrial suspensions was low ($4\mu\text{moles}$ of oxygen/mg. of protein). Decreases in both flavoprotein extinction and fluorescence were observed on adding the following substrates: ethanol, β -hydroxybutyrate, glutamate, malate, succinate, citrate and NADH. No responses were detected on adding α -oxoglutarate (even after the prior addition of 1mM -arsenite), pyruvate, butyrylcarnitine, octanoylcarnitine or palmitoylcarnitine. Typical responses are shown in Fig. 4. The extent of flavoprotein reduction observed on separately adding each of three NAD-linked substrates (ethanol, β -hydroxybutyrate and malate) was characteristic of the particular substrate added and independent of the order of addition. These separate responses were not abolished in the presence of oligomycin, but after freezing and thawing the overall reduction of flavoprotein linked to NAD-dependent substrates was accomplished by the addition of a single substrate. From the absorption traces approximate concentrations of various flavoprotein species were calculated (on the basis of $\epsilon=11\,000$) and these values are shown in Table 1. Comparison of simultaneous extinction and fluorescence changes gave values for the relative fluorescence yields of the various flavoprotein redox reactions. Table 2 summarizes these values relative to the yield for the succinate dehydrogenase flavoprotein. It is seen that a large excess of the flavoprotein of these mitochondria that was not reduced by any of the substrates tested was dithionite-reducible, as measured both by extinction and fluorescence changes. An investigation of spectral changes over the 430 – $530\text{m}\mu$ range indicated that, under the conditions employed in these experiments, interference to measurements of flavoprotein absorption at 475 – $510\text{m}\mu$ due to changes in the redox states of the cytochromes was minimal (Fig. 6).

NAD-linked substrates reduced highly fluorescent flavoproteins whereas externally added NADH reduced flavoprotein with a low fluorescent yield. The addition of rotenone ($2\mu\text{moles}$ /mg. of protein) after the reduction of flavoprotein by NAD-linked substrates led to the reoxidation of a fluorescent flavoprotein (Fig. 4). A similar observation was reported by Chance *et al.* (1967) with mammalian mitochondria and in that system it was evident that the NADH-dehydrogenase portion of the respiratory chain contains two flavoproteins operating in sequence, with both the rotenone-sensitive site and the coupling site I between them.

Table 1. *Mitochondrial content of various flavoproteins*

The values in section (a) were measured by extinction changes (475 – $510\text{m}\mu$) on the addition of substrates (or dithionite) to antimycin A-inhibited suspensions. Experimental conditions are given in the caption to Fig. 4. The value for flavoprotein reduced in the transition to anaerobiosis was obtained by using succinate as substrate in the absence of inhibitor. The values in section (b) were obtained after chemical extraction (Rao *et al.* 1967).

Section (a) Substrate added	Flavoprotein reduced (μmole /mg. of protein)
Succinate	0.046
NADH	0.038
Malate	0.046
Ethanol	0.026
β -Hydroxybutyrate	0.008
(Dithionite)	0.304
Succinate (state 3 $\rightarrow$ 5 transition)	0.08
Section (b)	
Total mitochondrial flavine (trypsin + acid extraction)	0.245
Peptide-bound flavine	0.049

Table 2. *Relative fluorescence yields for redox reactions of flavoproteins*

Experimental details are given in the caption to Fig. 4. Average values from six experiments with different mitochondrial suspensions are given.

Reaction of flavoprotein	Ratio fluorescence/ extinction change (arbitrary units)
Reoxidation with malonate after reduction with succinate	1.0
Reduction with succinate	0.7
Reduction with NADH	0.8
Reduction with malate	2.5
Reduction with ethanol	2.0
Reduction with β -hydroxybutyrate	1.7
Reoxidation with rotenone after reduction with NAD-linked substrate	4.0
Reoxidation with acetaldehyde after reduction with ethanol	4.0
Reduction with excess of dithionite	0.4

Malonate produced a partial inhibition of the reduction of the succinate dehydrogenase flavoprotein, the extent of which increased with increasing ratios of malonate to succinate. Flavoprotein reduction by ethanol was reversed by acetaldehyde (Fig. 5); this may not have been a redox effect but rather a steady-state effect. Thus the rate of electron flux through the 'leak' at the antimycin

Fig. 5. Reduction of flavoprotein by ethanol and reoxidation by acetaldehyde. Fluorimetric and spectrophotometric measurements and experimental conditions were as described in Fig. 4. Additions were made as follows: A, antimycin A ($10\mu\text{g.}$); B, ethanol (3mm); C, acetaldehyde (3mm).

Fig. 6. Flavoprotein absorption spectra. Difference spectra between sample and reference cuvettes (1 cm. light-path) at 23° are shown. A base line (1) was obtained with both suspensions (30 mg. of protein/ml.) in the fully oxidized state. Then successive additions were made, first to sample and then to reference cuvettes alternately (as follows) and a difference spectrum characteristic of each substrate was immediately recorded: curve 2, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone ($3\mu\text{M}$) + antimycin A ($15\mu\text{g.}$); curve 3, NADH (1mm); curve 4, succinate (3mm); curve 5, malate (3mm); curve 6, α -oxoglutarate (3mm); curve 7, dithionite (excess).

Fig. 7. Flavoprotein fluorescence-emission spectra. 'Single-ended' traces with base line representing residual emission (and light-scattering) after dithionite reduction of the mitochondrial suspension (30 mg. of protein/ml.) are shown. The excitation wavelength was $436\text{m}\mu$. The curves are uncorrected for energy-sensitivity of the detector. Curve 1, aerated mitochondria + pentachlorophenol ($3\mu\text{M}$) + antimycin A ($15\mu\text{g.}$); curve 2, spectrum after addition of malate (3mm), then succinate (3mm) (curve 3) and finally NADH (1mm) (curve 5). The emission spectrum of riboflavin solution is also shown (curve 4).

A-inhibited site may have been sufficiently great that, as the concentration of NADH was depleted by the alcohol-aldehyde equilibrium, the steady state rather than the equilibrium state became more oxidized. In cells grown with acetate as sole carbon source the extent of flavoprotein reduction obtained with β -hydroxypropionate, malonic semialdehyde or β -hydroxybutyrate was small, whereas mitochondria from cells grown with propionate showed greater responses to these substrates. No reduction of flavoprotein by propionyl-CoA or propionylcarnitine was detectable in mitochondria from cells grown with either acetate or propionate as carbon source.

Spectral changes on flavoprotein reduction. Examination of flavoprotein absorption spectra of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone-uncoupled antimycin A-inhibited mitochondrial suspensions was performed in a split-beam spectrophotometer. By alternately adding substrates to test and reference cuvettes the absorption spectra of flavoproteins in various redox states characteristic of the changes observed in the double-beam spectrophotometer were distinguished (Fig. 6). Under these conditions there was no interference by changes of extinction due to the γ -band of cytochrome *b*. After a base line (oxidized-oxidized) had been drawn, uncoupler and inhibitor were added to the sample cuvette; little endogenous

reduction of flavoprotein occurred (curve 2). On uncoupling and inhibition of the reference suspension and addition of NADH to the sample cuvette,

Fig. 8. Flavoprotein fluorescence-emission spectra of mitochondria in different metabolic states. Conditions were as described in Fig. 7. Curve 1, aerated mitochondria + pentachlorophenol ($3\mu\text{M}$); curve 2, same suspension + succinate (3mM) (state 3); curve 3, same suspension after the exhaustion of dissolved O_2 (state 5).

a different spectrum of the flavoprotein reduced by this substrate was obtained (curve 3). Further reduction of flavoprotein linked to succinate and malate occurred on addition of these substrates (curves 4 and 5) whereas α -oxoglutarate addition gave a barely detectable reduction of flavoprotein (curve 6). Finally, addition of excess of dithionite produced a further trough of flavoprotein absorption at $460\text{--}465\text{m}\mu$ (curve 7). Non-haem iron species may contribute to the absorption of the dithionite-reduced suspension in this region (see discussion of Chance & Schoener, 1966). Corresponding responses in fluorescence-emission spectra were observed (Fig. 7). Successive flavoprotein reduction reactions were observed on adding malate, succinate and NADH to the uncoupled antimycin A-inhibited suspension. (In Fig. 7 the base line represents the emission spectrum of a dithionite-reduced mitochondrial suspension.) The wavelength of maximal emission ($520\text{m}\mu$) is slightly shorter than that for the riboflavine standard ($527\text{m}\mu$). Fig. 8 shows the emission spectrum of an oxidized uncoupled mitochondrial suspension (curve 1); on addition of succinate some reduction of flavoprotein occurred (state 3) and this increased further after the transition to anaerobiosis (state 5).

Kinetics of flavoprotein reduction. Fig. 9 shows the initial rates of flavoprotein reduction by NADH, succinate and malate in the absence and presence of rotenone. It is seen that NADH gave

Fig. 9. Kinetics of flavoprotein reduction. The mitochondrial suspension (5mg. of protein/ml.) in 100mM-KCl – 5mM-MgCl_2 – 50mM-tris-HCl buffer, pH 7.4, was incubated at 23° with pentachlorophenol ($3\mu\text{M}$) and antimycin A ($10\mu\text{g.}$) before the substrates were added as follows: A, malate (3mM) ($t_{\frac{1}{2}}=2\text{sec.}$ corresponds to $0.039\mu\text{M-flavoprotein/sec.}$); B, malate (3mM) in the presence of rotenone ($15\mu\text{g.}$) ($t_{\frac{1}{2}}=2\text{sec.}$ corresponds to $0.029\mu\text{M-flavoprotein/sec.}$); C, NADH (0.05mM) ($t_{\frac{1}{2}}=1\text{sec.}$ corresponds to $0.048\mu\text{M-flavoprotein/sec.}$); D, succinate (3mM) ($t_{\frac{1}{2}}=2\text{sec.}$ corresponds to $0.026\mu\text{M-flavoprotein/sec.}$).

Table 3. *Kinetics of cytochrome and flavoprotein responses*

The experiments were performed at 23° in the regenerative-flow apparatus. Further details are described in the Results section.

Redox reaction	Wavelength pair (m μ)	10 ⁻³ $\times$ ϵ value used in calculation	$t_{\frac{1}{2}}$ (msec.)	Initial reaction rate (M/sec.)
Reduction reactions (reductant in parentheses)				
Flavoprotein (NADH)	465—510	11	600	0.80
Cytochrome <i>b</i> (NADH)	430—410	160	100	0.35
Cytochrome <i>b</i> (succinate)	430—410	160	180	0.12
Oxidation reactions (3 μ M-oxygen)				
Flavoprotein	465—510	11	200	0.47
Cytochrome <i>b</i>	430—410	160	120	0.11
Cytochrome <i>c</i>	550—540	19	600	0.07
Cytochrome <i>a</i> (+ <i>a</i> ₃)	605—630	16	40	0.94
Cytochrome <i>a</i> ₃ (+ <i>a</i>)	445—455	91	35	1.14

the fastest flavoprotein reduction, and with this substrate, as with succinate, the response was essentially a monophasic one. With malate but without added rotenone a rapid initial phase of reduction ($t_{\frac{1}{2}}$ 2sec.) was followed by a slower response. In the presence of rotenone the extent of flavoprotein reduction was less and the kinetics were similar to those for the initial rapid response to malate in the absence of this inhibitor.

Kinetics of cytochrome and flavoprotein oxidation and reduction. In an experiment with the regenerative-flow apparatus it was possible to measure the kinetics of cytochrome and flavoprotein reduction and reoxidation in a mitochondrial suspension. The reductant used was NADH in amounts that were quickly oxidized to completion; cycles of reduction and oxidation of the NAD were followed and initial rates of reduction of flavoprotein and cytochrome *b* were measured. Succinate was then added and cytochrome *b* reduction again followed; the extent of cytochrome *b* reduction in this case was twice that produced by the addition of NADH. The transition to state 5 was accompanied by a further rapid reduction of cytochrome *b*. Rapid mixing of this anaerobic suspension with air-saturated buffer allowed a study of the kinetics of reoxidation of the cytochromes and flavoproteins. Half-times and reaction rates are summarized in Table 3. It is seen that the cytochrome *a*₃ (+*a*) oxidation reaction was very fast ($t_{\frac{1}{2}}$ 35 msec.) whereas that of cytochrome *c* was slow ($t_{\frac{1}{2}}$ 600 msec.).

DISCUSSION

Difference spectra show that mitochondria from *Polytomella caeca* have the classical *b-c-a* cytochrome pattern, and in this respect the organism is similar to the colourless alga *Prototheca zopfii* (Lloyd, 1966; Webster & Hackett, 1965) and the amoeba *Hartmanella castellanii* (Lloyd & Griffiths,

1968). The green flagellate *Euglena gracilis* has been shown to possess cytochromes *c* (556) and *a* (605) associated with its mitochondria (Perini, Schiff & Kamen, 1964), and its colourless counterpart *Astasia longa* has a very complex spectrum in the region for *b*- and *c*-type cytochromes (Webster & Hackett, 1965). The relative insensitivity of the respiration of *Euglena gracilis* to carbon monoxide suggests that there may be a non-participation of the *a*-type cytochrome in the main pathway of electron transport, and Chance & Sager (1957) failed to find the characteristic Soret band of cytochrome *a*₃ or the 605 m μ peak of cytochrome *a* in the pale-green mutant of *Chlamydomonas reinhardtii*. Thus the cytochromes of *Polytomella caeca* are quite different from those of several other flagellates, and even from organisms often regarded as its close relatives.

Spectral data indicate that antimycin A blocks the reoxidation of reduced cytochrome *b*. A 50% inhibition of succinate oxidation was observed at an inhibitor concentration of 1 μ mole/mg. of protein. This compares with the value of 0.05 μ mole/mg. of protein reported by Ernster, Dallner & Azzone (1963) for mammalian mitochondria. Low antimycin A-sensitivity has also been reported for mitochondria from *Saccharomyces carlsbergensis* (Ohnishi, Kawaguchi & Hagihara, 1966), from *Hartmanella castellanii* (Lloyd & Griffiths, 1968) and from *Tetrahymena pyriformis* (Kobayashi, 1965).

The oxidation of NAD-linked substrates in *Polytomella* mitochondria is rotenone-sensitive, and again mammalian mitochondria show a 30-fold higher sensitivity to this inhibitor (Ernster *et al.* 1963). As with the mammalian mitochondrial electron-transport system (Chance *et al.* 1967) the site of action of rotenone lies between the two flavoproteins of the NADH dehydrogenase segment of the respiratory chain. Thus on addition of

this inhibitor to antimycin A-inhibited suspensions in the presence of NAD-linked substrates a partial reoxidation of flavoprotein was noted, owing to a 'leak' of electrons through the antimycin A-sensitive site and the cytochrome chain to oxygen. The flavoprotein known as FpD1 remained reduced as it lies on the substrate side of the rotenone block, whereas the flavoprotein known as FpD2 became oxidized. Piericidin A, a ubiquinone analogue (Hall, Wu, Crane & Folkers, 1966) that is believed to act at the same site as rotenone (Horgan & Singer, 1967), also inhibits the oxidation of NAD-linked substrates by mitochondria from *Polytomella caeca*.

Simultaneous measurements of flavoprotein fluorescence and extinction changes indicate the presence of three types of flavoprotein other than those involved in the oxidation of endogenous NADH. These are the flavoproteins reducible by succinate, by exogenous NADH and by dithionite. These different species were distinguished by their fluorescence/extinction ratios and by the kinetics of their reduction. Thus, whereas NAD-linked substrates reduced highly fluorescent flavoproteins, externally added NADH reduced a less fluorescent species more rapidly. In mitochondria broken by freezing and thawing this distinction was lost. There would also appear to be some 'compartmentation' in intact mitochondria, either of flavoproteins themselves or of endogenous NAD, as the responses to different NAD-linked substrates were distinctive; again this phenomenon was abolished in partially disrupted mitochondria. The lack of response to added α -oxoglutarate in the presence of antimycin A may be explained by the observation that under these conditions the redox state of the endogenous NAD^+ -NADH couple (as observed fluorimetrically) shifted in the direction of oxidation.

Evans & Lloyd (1967) have shown that two NAD-linked enzymes, β -hydroxypropionate dehydrogenase and malonic semialdehyde dehydrogenase, are present in mitochondria from propionate-grown cells but are barely detectable in mitochondria from cells grown with acetate. This finding explains the pattern of flavoprotein responses obtained with the substrates of these enzymes. The identity of the dithionite-reducible flavoprotein that was not reducible by known substrates of the mitochondria remains unelucidated. In the mammalian system this has been shown to be largely due to flavoproteins involved in fatty acid oxidation (Garland, Chance, Ernster, Lee & Wong, 1967), but no such flavoprotein was identified in mitochondria from *Polytomella caeca* even when the organism was grown with propionate.

It has also been observed that, though succinate, β -hydroxypropionate (only in mitochondria from cells grown with propionate) and ethanol (all in the presence of antimycin A) reduced a non-haem iron

component yielding a ' $g=1.94$ signal' in electron-paramagnetic-resonance spectra, α -oxoglutarate did not produce a signal. The reduction of this component by external NADH was again dependent on the intactness of the mitochondria. In fresh mitochondrial preparations no ' $g=1.94$ signal' was obtained, whereas preparations aged for 2 days at -10° showed a reduction of non-haem iron by NADH (H. Schleyer, D. Lloyd, & B. Chance unpublished work). A further distinction between the pathways of oxidation of external and internally generated NADH lies in differences in rotenone-sensitivity of the two systems.

Both absorption and fluorescence-emission spectra confirm the validity of ascribing the changes observed in the double-beam spectrophotometer with an attached fluorimeter to flavoprotein.

Rapid-reaction kinetics confirm the involvement of the flavoprotein and cytochrome $a+a_3$ components of the electron-transport chain. The rather slow redox reactions of cytochromes b and c by comparison with the terminal oxidase step warrants further investigation. The rate of oxidation of reduced cytochrome a_3 observed was about one-tenth of that measured in comparable experiments with rat liver mitochondria.

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