The Cytochromes of Mitochondria from *Tetrahymena pyriformis* Strain ST

By D. LLOYD and B. CHANCE

Department of Microbiology, University College, Newport Road, Cardiff CF1 1TA, U.K.,
and Johnson Research Foundation, Department of Biophysics and Physical Biochemistry,
University of Pennsylvania, Philadelphia, Pa. 19104, U.S.A.

(Received 14 February 1972)

1. Mitochondria of the obligately aerobic ciliate protozoon, *Tetrahymena pyriformis* strain ST, are unusual in that they possess a cytochrome oxidase system that does not react with reduced mammalian cytochrome *c*; the presence of cytochromes $a_{603} + a_3$ is masked in the α -band region of spectra by the broad absorption band of cytochrome a_{620} . 2. Other haemoproteins present include cytochromes b_{560} , b_{556} , c_{553} and c_{549} . 3. The reaction of reduced cytochrome a_3 with CO is reversed by flash photolysis, and in the presence of O_2 the subsequent oxidation of this cytochrome is followed by that of cytochrome a_{603} . 4. Cytochromes a_{620} and b_{560} also react with CO and with KCN; the latter cytochrome corresponds with that designated cytochrome *o* by other workers. 5. The contribution of cytochrome a_{603} to difference spectra is revealed by making use of the fact that it does not react with KCN. 6. Cytochrome a_{620} is unstable, and its α -absorption band is lost from spectra of mitochondria which have been aged or treated with ultrasound, detergents or organic solvents. 7. Possible pathways of electron transport via the several different terminal oxidases in *Tetrahymena* mitochondria are proposed.

The presence of unusual *a*-type cytochromes in the ciliate protozoon *Tetrahymena pyriformis* was first noted by Baker & Baumberger (1941), who identified absorption maxima at 587–590 nm and 616–618 nm as cytochromes a_1 and a_2 respectively. In subsequent investigations (Ryley, 1952; Keilin & Ryley, 1953; Eichel, 1954; Kobayashi, 1965; Van de Vijver, 1966), it was reported that cell-free homogenates or mitochondria were unable to oxidize reduced mammalian cytochrome *c* despite the sensitivity of substrate oxidations to azide, CO and CN^- , the classical inhibitors of cytochrome oxidase. The absence of a cytochrome oxidase α -band at 604 nm from absorption spectra of whole cells (Ryley, 1952) was apparently confirmed by examination of isolated mitochondria (Kobayashi, 1965), and detailed spectrophotometric analysis of cytochrome components have been reported (Turner & Lloyd, 1968; Turner *et al.*, 1969; Perlsh & Eichel, 1968, 1969). The work of Turner *et al.* (1971) with phosphorylating mitochondria clearly indicated that haem *a* is present, that the absorption maximum at 448 nm represents the γ -band of a cytochrome oxidase, and that the haemoprotein with a broad absorption band at around 620 nm (cytochrome a_{620}) undergoes reduction when mitochondrial suspensions become anaerobic. The re-oxidation of cytochrome a_{620} by O_2 is more rapid than that of cytochrome c_{553} or cytochrome b_{560} , and it thus reacts fast enough to be a terminal oxidase.

The present paper details a more intensive investigation of *Tetrahymena* mitochondrial cyto-

chromes, and concentrates on the terminal sectors of the respiratory chains of this obligately aerobic organism with a view to elucidating the components reacting with O_2 .

Experimental

Methods

Maintenance, growth and harvesting of the organism. *Tetrahymena pyriformis* strain ST (from Dr. Y. Suyama, Department of Biology, University of Pennsylvania, U.S.A.) was maintained, grown and harvested as described previously (Lloyd *et al.*, 1971). Volumes of culture up to 20 litres were used for the preparation of mitochondria, and harvesting was in the late-exponential phase of growth (1×10^5 – 4×10^5 organisms/ml).

Disruption of organisms and isolation of mitochondrial fractions. Procedures involved in the breakage of cells and the isolation of mitochondria have been detailed previously (Turner *et al.*, 1971).

Partial purification of cytochromes from isolated mitochondria. Attempts to purify cytochrome a_{620} proved unsuccessful, owing to the instability of this haemoprotein. However, several of the methods attempted achieved partial separation of some of the other cytochromes. Mitochondria were first treated with ultrasound [500 W generator (MSE Ltd., Crawley, Sussex, U.K.), power setting 5, four periods of 15 s each] to release cytochromes c_{553} and b_{560} . Subsequent extraction with cholate and fractionation

Fig. 1. Difference spectrum of *Tetrahymena* mitochondria at 77°K

The mitochondrial suspension contained 9.0mg of protein/ml. Reduction was done by adding excess of $\text{Na}_2\text{S}_2\text{O}_4$ to the sample cuvette contents; oxidation of the reference suspension was by aeration; both cuvettes were then immersed in liquid N_2 . Cuvettes had a light-path of 2mm and the spectral band-width was 2nm. Cytochromes are represented by small italic letters.

with $(\text{NH}_4)_2\text{SO}_4$ by the exact procedure detailed by Yonetani (1961) yielded a fraction (S_2) rich in cytochrome c_{549} . Extraction of sonicated mitochondria by using deoxycholate and cholate as described by Tzagoloff (1969) gave a fraction (S_2) rich in cytochrome b_{558} . These separations aided the interpretation of cytochrome spectra of intact mitochondria (Figs. 1 and 2).

Analytical methods

Cytochrome spectra. Difference spectra were traced at room temperature by using a Cary model 14 spectrophotometer fitted with a 0–0.1 extinction slide wire (in the α -region) or a 0–1.0 slide wire (in the γ -region). The spectral band-width was set at 3.7nm at 650nm. Alternatively a split-beam spectrophotometer (Yang & Legallais, 1954) with a liquid- N_2 attachment (Chance, 1957) was employed. Intact mitochondria were suspended in the isolation buffer [0.35M-mannitol, 0.1% (w/v) bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.), 0.1mM-EGTA*, 10mM-tris-HCl buffer, pH7.2] for all spectrophotometric analyses.

Protein determinations. Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Kinetics of the cytochrome *c* oxidase–oxygen reaction. The apparatus used for measuring rates of reoxidation of reduced cytochrome *c* oxidase has

* Abbreviation: EGTA, ethanedioxybis(ethylamine)-tetra-acetate.

Fig. 2. Difference spectra of cytochromes purified from *Tetrahymena*

(a–c) Cytochrome b_{560} and (d) cytochrome c_{553} . Traces were obtained by graphical subtraction of appropriate absolute spectra published by Yamanaka *et al.* (1968). The straight lines represent base-lines obtained by joining isosbestic points. (a) Resultant difference spectrum produced by subtraction of oxidized absolute spectrum from dithionite-reduced absolute spectrum. Concentration of cytochrome b_{560} was $6.4\mu\text{M}$. (b) Resultant difference spectrum produced by subtraction of dithionite-reduced absolute spectrum from absolute spectrum of CO-treated dithionite-reduced preparation. Concentration of cytochrome b_{560} was $6\mu\text{M}$. (c) Resultant difference spectrum produced by subtraction of dithionite-reduced absolute spectrum from spectrum of KCN-treated dithionite-reduced preparation. Concentration of cytochrome b_{560} was $5.8\mu\text{M}$. (d) Resultant difference spectrum produced by subtraction of oxidized absolute spectrum from dithionite-reduced absolute spectrum. Concentration of cytochrome c_{553} was $5.4\mu\text{M}$. (e) Dithionite-reduced–oxidized difference spectrum of cytochrome c_{549} (0.93mg of protein/ml). (f) Dithionite-reduced–oxidized difference spectrum of cytochrome b_{558} (5.5mg of protein/ml). (g) Dithionite-reduced–oxidized difference spectrum of supernatant from mitochondria after treatment with ultrasound (500W MSE generator; power setting 5; four periods of 15s each) (3mg of protein/ml). All spectra were obtained at room temperature in cuvettes of light-path 10mm.

Fig. 3. Kinetics of photolytic decomposition of CO-liganded cytochrome oxidase and the subsequent reactions with O₂ in mitochondria from *Tetrahymena pyriformis*

(a) Diagram of idealized trace indicating sequence of reactions producing changes of extinction in experiments shown in (b) and (c). Addition of ascorbate+*NNN'**N'*-tetramethyl-*p*-phenylenediamine to a mitochondrial suspension gives a steady-state reduction of cytochromes *a*+*a*₃ corresponding to the trace at position (1). Attainment of anaerobiosis gives an extinction increase (2) caused by cytochrome reduction. Mixing with CO leads to a 50% decrease in extinction (3) resulting from formation of reduced cytochrome *a*₃-CO complex. Flash photolysis dissociates this complex and restores the extinction to the value (4) obtaining before CO addition; in the absence of O₂ recombination of cytochrome *a*₃ and CO occurs (5). Mixing with O₂ 100ms before photolysis allows both cytochromes *a*₃ and *a*₆₀₃ to become oxidized after the flash, but resolution of the very fast initial reaction is not attained. Better time resolution indicates an extinction increase (6), resulting from the dissociation of the reduced cytochrome *a*₃-CO complex, followed by oxidation of released reduced cytochrome *a*₃, during which extinction decreases rapidly to a value (7) corresponding to that before photolysis. This reaction is followed by a further slower extinction decrease (8), which corresponds with that expected for the reoxidation of reduced cytochrome *a*₆₀₃. (b) Trace 1, extinction changes produced during anaerobic photolysis; trace 2, flow-velocity trace (downward displacement indicates period of mixing with oxygenated buffer); trace 3, extinction changes produced on mixing with oxygenated buffer followed by photolysis. Only overall reactions are shown; this scale is not adequate for satisfactory resolution of kinetics. (c) Trace 1, position of trace after anaerobic photolysis; trace 2, extinction changes produced on mixing with oxygenated buffer followed by photolysis. All cuvettes (light-path 6mm) contained mitochondrial protein (3mg/ml), respiratory substrate 10mM-ascorbate+1mM-*NNN'**N'*-tetramethyl-*p*-phenylenediamine, 300μM-CO and 17μM-O₂. The temperature of incubation was 24°C. TMPD represents *NNN'**N'*-tetramethyl-*p*-phenylenediamine.

Fig. 4. Difference spectra of *Tetrahymena* mitochondria produced in the presence of ascorbate+NNN'N'-tetramethyl-*p*-phenylenediamine and CO

(a) The sample cuvette contained a mitochondrial suspension allowed to become anaerobic after the addition of 1 mM-ascorbate+0.1 mM-NNN'N'-tetramethyl-*p*-phenylenediamine; the reference cuvette contained an aerated mitochondrial suspension. (b) Both cuvettes initially contained anaerobic mitochondrial suspensions, produced as in (a). The sample cuvette contents were bubbled with CO for 2 min before the difference spectrum was recorded. Cuvettes (light-path 10 mm) contained protein (8 mg/ml); spectra were recorded at room temperature.

been fully described by Chance & Erecińska (1971). Flash photolysis of a mixture of CO-liganded cytochrome *c* oxidase and O₂ was achieved during flow through the 1 ml observation chamber of the regenerative-flow apparatus. A liquid dye laser (designed and constructed by Mr. J. Bunkenberg) giving a 0.2 J flash of about 0.4 μs duration provided the energy necessary for over 90% photolysis with 585 nm light. Time-shared measurements of the dual-wavelength extinction changes employed a 60 Hz chopping frequency.

Results

Cytochromes in low-temperature spectra

A mitochondrial fraction was further purified by centrifugation for 1 h at 20000 rev./min in the 3 × 50 ml swing-out rotor of a Sorvall RC 2B centrifuge through a gradient containing 0.6–2.0 M-sucrose–10 mM–potassium phosphate–0.1% bovine serum albumin, pH 7.2. The band containing mitochondria ($\rho = 1.21$) was examined at the temperature of liquid N₂ in the split-beam spectrophotometer (Fig. 1). The interpretation of the dithionite-reduced–oxidized spectrum is aided by considering the spectra of

cytochromes purified from *Tetrahymena* (Fig. 2). Cytochrome *b*₅₆₀ shows absorption maxima at 560, 529 and 424 nm (Fig. 2a), and cytochrome *c*₅₅₃ has maxima at 553, 523 and 414 nm (Fig. 2d) [results of Yamanaka *et al.* (1968) replotted as difference spectra]. Treatment of mitochondrial suspensions with ultrasound released all the detectable cytochromes *c*₅₅₃ and *b*₅₆₀ present into a fraction not sedimentable by centrifugation for 6 × 10⁶ g·min (Fig. 2g); further fractionation of the residual membranes revealed the presence of cytochrome *c*₅₄₉ (absorption maxima at 549, 520 and 418 nm; Fig. 2e) and cytochrome *b*₅₅₈ (absorption maxima at 558, 524 and 432 nm; Fig. 2f). The spectrum shown in Fig. 1 also indicated the presence of *a*-type cytochromes; the major absorption maximum in the α -region was at 617 nm with a shoulder at 604 nm, and the Soret maximum was at 447 nm. Other components absorbing at 545, 574 and 587 nm were also revealed.

Kinetics of the cytochrome *c* oxidase–oxygen reaction

A suspension of mitochondria was allowed to become anaerobic in the presence of 1 mM-ascorbate–0.1 mM-NNN'N'-tetramethyl-*p*-phenylenediamine.

Addition of $300\mu\text{M}$ -CO resulted in the loss of about 50% of the extinction observed at 448–460nm (Fig. 3a). Photolysis induced by the laser flash in the absence of O_2 led to the dissociation of CO from the cytochrome *c* oxidase–CO complex (Fig. 3b, trace 1), which was accompanied by an increased extinction owing to the production of reduced cytochrome *c* oxidase. Under anaerobic conditions only recombination of CO with the enzyme could occur, and the trace returned within 200ms almost to its original 'pre-flash' value. When the laser was discharged 100ms after mixing with $17\mu\text{M}$ - O_2 (flow-velocity trace, Fig. 3b, trace 2) the reduced cytochrome *c* oxidase liberated from its liganded form can react with O_2 as indicated by the decrease in extinction (Fig. 3b, trace 3). On this time-scale only the oxidation of cytochrome a_{603} (which provides about 50% of the extinction change at 448–460nm, but which does not itself react with CO) is seen; time resolution of the early part of the reaction is inadequate to assess reaction half-times. To permit the recording of very rapid extinction changes over the first 8ms after the laser flash, a special form of the dual-wavelength spectrophotometric technique termed 'cycle selection' has been devised (Chance & Erecińska, 1971), which is compatible with the 60Hz light-modulation usually employed. By using this technique the initial few ms of the reaction are resolved (Fig. 3c, trace 2) and the very-rapid initial reoxidation reaction accounting for the oxidase (cytochrome a_3) itself is revealed. The reaction half-times for the terminal oxidase and cytochrome a_{603} were 0.6ms and about 2.0ms respectively at $17\mu\text{M}$ - O_2 and 24°C . Corresponding values for these cytochromes in mitochondria from pigeon heart are 1.3ms and 10ms (Chance & Erecińska, 1971) for similar conditions. The slower reaction of cytochrome a_3 with CO causes little interference with these measurements. Another minor component was also present which reacted slowly with O_2 in the presence of CO during the flow-time ($t_{\frac{1}{2}} \approx 50\text{ms}$) and led to the slight displacement of trace 3 from trace 1 before photolysis (Fig. 3b). Interference at this wavelength pair (448–460nm) from *b*-type cytochromes and flavoprotein components is not possible, as these components react much more slowly (half-times 100–200ms; Turner *et al.*, 1971); *c*-type cytochromes do not absorb at this wavelength.

Cytochromes reacting with CO

Mitochondrial suspensions made anaerobic in the presence of 1mM-ascorbate–0.1mM-*NNN'*-tetramethyl-*p*-phenylenediamine showed a broad absorption band with a maximum in the region 614–618nm and a Soret band at 448nm; cytochrome c_{553} was also reduced (maxima at 553, 522 and 415nm; Fig. 4a). CO reacted with the reduced cytochromes leaving troughs at 619, 583, 550–560,

522–528, 448–450 and 424nm, and producing absorption maxima at 597, 572, 542, 472 and 434nm (Fig. 4b). The trough at 619nm and peak at 597nm are largely produced by the reaction of an *a*-type cytochrome (referred to hereafter as cytochrome a_{620}) with CO. These broad absorption bands may mask any contribution from a classical type of cytochrome $a+a_3$, which would give a trough at 605 and a peak at 590nm (Yonetani, 1960). However, the trough at 448–450nm is characteristic of such an oxidase (cytochrome a_3), but these features may also have a contribution from cytochrome a_{620} . Comparison of this spectrum with that plotted (Fig. 2b) for a similar difference spectrum of purified cytochrome b_{560} (Yamanaka *et al.*, 1968) strongly suggests that this autoxidizable cytochrome also contributes to the reduced CO spectrum of intact mitochondria, and may account for the absorption maxima at 572, 542 and 472nm, and the minima at 560 and 528nm. The trough at 556nm (Fig. 4b) indicates the presence of another CO-reacting haemoprotein, which has not been further investigated.

Difference spectra in the presence of cyanide

Mammalian cytochrome a_3 reacts in the reduced or oxidized state with CN^- ; the reduced CN^- complex is easily autoxidized, whereas the oxidized CN^-

Fig. 5. Effects of cyanide on difference spectra of *Tetrahymena* mitochondria

(a) Mitochondrial suspensions were reduced in both cuvettes by the addition of 1mg of $\text{Na}_2\text{S}_2\text{O}_4$. The spectrum was recorded after addition of KCN to the sample cuvette. (b) Both cuvettes initially contained aerated mitochondrial suspensions. The spectrum was recorded after addition of KCN to the sample cuvette. (c) Both cuvettes contained mitochondrial suspensions aerated in the presence of KCN. The spectrum was recorded after $\text{Na}_2\text{S}_2\text{O}_4$ was added to the sample cuvette. (d) Both cuvettes initially contained aerated mitochondria. KCN was added to the reference cuvette only, and then $\text{Na}_2\text{S}_2\text{O}_4$ was added to both cuvettes before the spectrum was recorded. (e) Trace obtained by subtraction of spectrum obtained in (b) from that obtained in (d). (f) Trace obtained by subtraction of spectrum obtained in (c) from a dithionite-reduced–oxidized spectrum. (g) The mitochondrial suspension in the sample cuvette was treated with KCN after addition of 2.5% (v/v) methanol, and then $\text{Na}_2\text{S}_2\text{O}_4$ was added. The spectrum was recorded with aerated mitochondrial suspension as reference. Spectra were recorded at room temperature in 10mm light-path cuvettes; final concentration of protein was 8mg/ml and of KCN was 6mM.

complex cannot be easily reduced (Keilin & Hartree, 1939). Cytochrome *a* does not react with CN^- . Spectrophotometric separation of cytochromes *a* and *a*₃ have been achieved by using these properties (Yonetani, 1960), although other interpretations of these results are also possible (Wanio *et al.*, 1968). The effect of KCN on spectra of *Tetrahymena* mitochondria was tested in an attempt to resolve the apparent complexity of cytochrome *a* components. Reaction of KCN with the dithionite-reduced mitochondria (Fig. 5*a*) gave troughs at 622–628 nm and 450 nm, a similar effect to that produced by reaction with CO under similar conditions (Fig. 4*b*). The addition of CN^- to the positive of two cuvettes containing aerated mitochondria also produced a complex difference spectrum (Fig. 5*b*), with troughs at 620 and 559 nm, and peaks at 590, 578, 570, 550 and 475 nm. Reaction of both positive and reference cuvette contents with CN^- , followed by dithionite reduction of the positive cuvette contents (Fig. 5*c*), revealed the presence of cytochrome *a*₆₀₃ (which does not react with KCN). The assignment of the 603 nm band to cytochrome *a*₆₀₃ assumes that the oxidized CN^- complexes of both *a*₆₂₀ and *a*₃ cannot be rapidly reduced by dithionite. Addition of cyanide to the reference cuvette, followed by dithionite reduction of both cuvette contents, produced the complex spectrum shown in Fig. 5*d*), which, when corrected for the contribution of the oxidized CN^- complexes, gives the absorption due to cytochromes *a*₆₂₀+*a*₃ in a reduced–oxidized spectrum (Fig. 5*e*). The absorption maximum at 620 nm can also be seen (Fig. 5*f*) if the spectrum in Fig. 5*c*) is subtracted graphically from the dithionite-reduced–oxidized spectrum (not shown, but identical with the ascorb-

Fig. 6. Lability of α -bands of *a*-type cytochromes in dithionite-reduced–oxidized difference spectra of *Tetrahymena* mitochondria

(a) Freshly isolated mitochondria (8mg of protein/ml). (b) Spectrum produced immediately after addition of 0.2% KCl and 11% (v/v) 3-methylbutan-1-ol to mitochondria (5.3mg of protein/ml). (c) Spectrum obtained 1 h after addition of Triton X-100 (0.07%, v/v) to mitochondria (26mg of protein/ml). (d) Spectrum of mitochondrial membranes sedimentable at $6 \times 10^6 g$ -min after treatment with ultrasound (500 W MSE generator, power setting 5, four periods of 15 s each) (4mg of protein/ml). (e) Membranes treated as in (d), but dissolved in 7% (w/v) KCl and 10% (w/v) sodium deoxycholate (8mg of protein/ml). (f) Mitochondria suspended in 10mM-potassium phosphate buffer (pH 7.4) at 20°C for 18 h (21.5mg of protein/ml). Spectra were recorded at room temperature, in 10mm light-path cuvettes.

ate + *NNN'*-tetramethyl-*p*-phenylenediamine-reduced-oxidized spectrum, Fig. 4a). The CN^- complexes of cytochrome b_{560} also contribute to these spectra (compare with Fig. 2c). Mitochondria reduced with dithionite after the addition of 2.5 mM-KCN in the presence of 2.5% (v/v) methanol gave absorption maxima at 600, 604 and 617 nm (Fig. 5g), as methanol shifts the α -band of cytochrome a_{603} , permits reduction of the $a_3\text{-CN}^-$ complex (600 nm band?) (Epel & Butler, 1969), and thus exposes the 617 nm band.

Lability of α -type cytochromes

A marked instability of the α -bands of cytochromes a was observed during experiments with *Tetrahymena* mitochondria. Thus the addition of organic solvents or detergents, or treatment with ultrasound, resulted in loss of the absorption bands at wavelengths greater than 610 nm (Fig. 6). Thus it was not possible to preserve the spectrophotometric properties of *Tetrahymena* cytochrome oxidase during purification by the methods of Yonetani (1961), Greenwood (1963), Griffiths & Wharton (1961) or Tzagoloff (1969). An absorption maximum at 590–595 nm was always observed when the 618 nm maximum disappeared; sonication, or aging at -14°C , did not remove the absorption band at 600–610 nm. Mitochondria stored

Fig. 7. Difference spectra of aged *Tetrahymena* mitochondria

Mitochondrial suspension was stored for 2 days at -14°C . Spectra were recorded at room temperature, in 10 mm light-path cuvettes. The final concentration of protein was 8 mg/ml, and of KCN was 6 mM. (a) Both cuvettes initially contained aerated mitochondrial suspensions. The spectrum was recorded after addition of KCN to the sample cuvette. (b) Both cuvettes initially contained aerated mitochondrial suspensions. The spectrum was recorded after addition of $\text{Na}_2\text{S}_2\text{O}_4$ to the sample cuvette. (c) Both cuvettes initially contained mitochondrial suspensions aerated in the presence of KCN. The spectrum was recorded after addition of $\text{Na}_2\text{S}_2\text{O}_4$ to the sample cuvette. (d) Both cuvettes initially contained aerated mitochondria. KCN was added to the reference cuvette only, then $\text{Na}_2\text{S}_2\text{O}_4$ was added to both cuvettes immediately before the spectrum was recorded. (e) Mitochondrial suspensions were reduced in both cuvettes by the addition of $\text{Na}_2\text{S}_2\text{O}_4$. The spectrum was recorded after addition of KCN to the sample cuvette. (f) Mitochondrial suspensions were reduced in both cuvettes by the addition of $\text{Na}_2\text{S}_2\text{O}_4$. The spectrum was recorded after bubbling CO through the sample cuvette for 2 min.

at -14°C for 2 days showed similar but not identical difference spectra in the α -region irrespective of the absence or the presence of CN^- before reduction with dithionite (Figs. 7b and 7c). The contribution from cytochromes reacting with CN^- in their oxidized forms was now negligible at wavelengths longer than 600 nm (Fig. 7a) and the contribution of cytochromes a_{603} and a_3 could be assessed by Yonetani's (1960) method (Figs. 7c and 7d). There were still several cytochromes present which react in their reduced forms with CN^- (Fig. 7e). A marked change in the dithionite+CO–dithionite-reduced spectrum (Fig. 7f) also resulted from the loss of the cytochrome a_{620} . The Soret trough at 446 nm confirms the presence of cytochrome a_3 ; all other absorption maxima and minima, except the 590 nm trough, may be attributed to the presence of cytochrome b_{560} (Yamanaka *et al.*, 1968; cf. Fig. 2b).

Discussion

The mitochondria of the ciliate protozoon *Tetrahymena pyriformis* have several unusual features. The apparent lack of a conventional cytochrome oxidase system accepting electrons from reduced cytochrome c from mammalian (Riley, 1952; Eichel, 1954) or bacterial (Eichel, 1956) sources, but reacting slowly with yeast cytochrome c (Kobayashi, 1965), and their anomalous absorption spectra (Riley, 1952) have posed the problem of the identity of the terminal oxidase. Several other protozoa including an amoeba, *Hartmannella castellanii* (Lloyd & Griffiths, 1968), a ciliate, *Paramecium aurelia* (Kung, 1970), and a flagellate, *Polytomella caeca* (Lloyd & Chance, 1968), have mitochondria which show conventional cytochrome $a+a_3$ spectra and oxidize reduced mammalian cytochrome c .

The present investigation confirms the presence of a variety of terminal mitochondrial oxidases in *Tetrahymena*. Thus cytochrome a_{620} , a haemoprotein

rapidly oxidized by O_2 in anaerobic mitochondrial suspensions, and showing a high affinity for O_2 (Turner *et al.*, 1971), resembles cytochrome a_3 in that it reacts in the reduced form with CO and in both oxidized and reduced states with CN^- . The broad absorption band in the α -region caused by this component largely obscures the presence of absorption maxima at 600–610 nm characteristic of cytochromes $a+a_3$, but these latter species can be demonstrated in the Soret region as two kinetically distinct species contributing equally to the total absorption at around 448 nm. The contribution of cytochrome a_{620} in this region has not been precisely ascertained, but conditions leading to complete loss of this unstable species do not diminish the Soret absorption band; this can be clearly demonstrated in CO difference spectra.

During the course of this investigation Perlsh & Eichel (1971) have reported the presence of cytochrome o in particulate preparations obtained from several strains of *Tetrahymena* after disruption of the organisms by treatment for 15 s with ultrasound. Although their difference spectra (e.g. ascorbate+ $\text{NNN}'\text{N}'$ -tetramethyl- p -phenylenediamine-reduced–oxidized) show a weak absorption band at around 613 nm, the CO difference spectra do not show the 620 nm absorption minimum and 597 nm maximum characteristic of the CO–cytochrome a_{620} complex of our freshly prepared mitochondria. Whether this discrepancy results from different growth conditions, modification of the cytochrome during cell breakage by ultrasound, or is a characteristic of the strain (S) of organism used by Perlsh & Eichel (1971), is not clear. The spectra published by these workers closely resemble those presented here for aged mitochondria which have lost cytochrome a_{620} .

We propose that the cytochrome o described by Perlsh & Eichel (1971) is identical with the slowly autooxidizable, CN^- - and CO-reacting cytochrome b_{560} , previously purified from sonicated or acetone-

Table 1. Comparison of the difference spectra of cytochromes b_{560} and o in *Tetrahymena pyriformis*

Values for wavelengths of absorption maxima and minima refer to difference spectra obtained after dividing a dithionite-reduced suspension between two cuvettes and bubbling the sample cuvette with CO. Values for the purified cytochrome b_{560} were obtained by graphical subtraction of the appropriate absolute spectra.

Preparation	Characteristics of difference spectrum ($\lambda_{\text{max.}}$, nm)					
	Maxima	...	Minima	...	Maxima	Minima
Purified cytochrome b_{560} (Yamanaka <i>et al.</i> , 1968)	572		537		470	417
		560		529		434
Cytochrome o (Perlsh & Eichel, 1971)	572		537		470	418
		558		520		437
Cytochrome b_{560} (the present paper)	572		542		470	418
		560		522

Scheme 1. Possible pathways of terminal oxidation in *Tetrahymena* mitochondria

Reaction half-times and pseudo-first-order kinetic constants for cytochromes a_{620} and c_{553} and flavoprotein oxidase are from the results of Turner *et al.* (1971). All kinetic results refer to oxidation rates at 24°C and 17 μM -O₂: calculations of kinetic constants assume irreversible reactions. TMPD represents *NNN'*-tetra-methyl-*p*-phenylenediamine. Fp represents flavoprotein. Cytochromes are represented by small italic letters.

dried preparations of *Tetrahymena* by Yamanaka *et al.* (1968). This cytochrome is atypical of mitochondrial *b*-type cytochromes, in that it is easily removed from homogenates without the need for extraction methods with detergents or organic solvents (Yamanaka *et al.*, 1968) and it can be preferentially released from isolated mitochondria by rather intensive treatment with ultrasound.

The properties of cytochrome b_{560} and cytochrome *o* are compared in Table 1. It is distinct from the CO-reacting microsomal oxidases (cytochromes *P*-420 and *P*-450) or cytochrome b_5 of *Tetrahymena* (Poole *et al.*, 1971), but we cannot discount the possibility that it represents a modified haemoprotein resulting from cytochrome degradation. Possible confusion with a haemoglobin (Perlish & Eichel, 1971) is ruled out, as strain ST grown under highly aerobic conditions has none of the haemoglobin detected in other strains by Keilin & Ryley (1953). Cytochrome *o* (Chance, 1953) is widely distributed in prokaryotic systems both in bacteria (Kamen & Horio, 1970) and blue-green algae (Webster & Hackett, 1966). In some bacteria (e.g. *Acetobacter suboxydans*) it serves as the sole oxidase (Daniel, 1970). It has also been detected in yeast (Mok *et al.*, 1969), and in a protozoon, *Crithidia fasciculata* (Hill & White, 1968). A similar CO-reacting haemoprotein was also detected in extracts of the colourless alga, *Polytoma uella*, and in mitochondria from *Astasia longa*, a colourless species related to *Euglena* (Webster & Hackett, 1965). These latter mitochondria also resembled those from *Tetrahymena* in that they would not oxidize reduced mammalian cytochrome *c*, although they did exhibit

an α -band at 605 nm. Mitochondria isolated from higher-plant tissues sometimes have a peroxidase which reacts with CO associated with them; however, careful fractionation studies have indicated that this enzyme is not truly mitochondrial (Plesnicar *et al.*, 1967).

Evidence for another class of mitochondrial oxidases has previously been presented (Turner *et al.*, 1971). Thus flavoproteins, which are reoxidized more rapidly than cytochrome *b*, must provide yet another alternative route of electrons to O₂ and may be responsible for the bypass of the antimycin A-sensitive site revealed in the steady-state spectra. It should be stressed, however, that peroxisomal contamination of the crude mitochondrial fractions used in these studies may be partly responsible for the presence of flavoprotein oxidases, as these organelles are known to contain D-amino acid oxidase and α -hydroxy acid oxidases (Müller, 1969). Possible alternative terminal electron-transport systems in *Tetrahymena* mitochondria are represented in Scheme 1.

D. L. thanks the Wellcome Trust for a travel grant, Dr. W. D. Bonner Jr., Dr. D. Wilson, Dr. R. Douce, Dr. M. Erecińska and Dr. P. Kusel for valuable discussions, and Mrs. L. Howells and Miss Keiko Sera for technical assistance.

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