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MITOCHONDRIAL RESPIRATORY CHAIN OF *TETRAHYMENA PYRIFORMIS*

THE THERMODYNAMIC AND SPECTRAL PROPERTIES

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SUMMARY

The mitochondria isolated from the ciliate protozoon *Tetrahymena pyriformis* carry an oxidative phosphorylation with P/O ratio of 2 for succinate oxidation and P/O ratio of 3 for the oxidation of the NAD-linked substrates. The respiration is more than 90 % inhibited with 1 mM cyanide while antimycin A and rotenone inhibit at concentrations of 1000-fold higher than those effective in mammalian mitochondria.

Using a combination of spectral studies and potentiometric titrations, the components of the respiratory chain were identified and characterized with respect to the values of their half-reduction potentials. In the cytochrome bc_1 region of the chain a cytochrome *c* was present with an $E_{m7.2}$ of 0.225 V and two components with absorption maxima at 560 nm and the half-reduction potential values of −0.065 and −0.15 V at pH 7.2. The cytochrome with the more positive half-reduction potential was identified as the analogue of the cytochrome(s) *b* present in mitochondria of higher organisms, while the cytochrome with the more negative half-reduction potential was tentatively identified as cytochrome *o*. In addition ubiquinone was present at a concentration of approx. 4 nmol per mg mitochondrial protein.

In the spectral region where cytochromes *a* absorb at least three cytochromes were found. A cytochrome with an absorption maximum at 593 nm and a midpoint potential of −0.085 V at pH 7.2 was identified as cytochrome a_1. The absorption change at 615–640 nm, attributed usually to cytochrome a_2, was resolved into two components with $E_{m7.2}$ values of 0.245 and 0.345 V. It is concluded that the terminal oxidase in *Tetrahymena pyriformis* mitochondria is cytochrome a_2 which in its two-component structure resembles cytochrome aa_3.

INTRODUCTION

The mitochondria isolated from the ciliate protozoon *Tetrahymena pyriformis*

Abbreviations: HQNO, 2*n*-heptyl-4-hydroxyquinoline-*N*-oxide; TMPD, *N,N,N'N'*,-tetramethyl-*p*-phenylene diamine.

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possess a respiratory chain which differs in many aspects from that of yeast and higher organisms and shows certain characteristics in common with the bacterial terminal oxidation pathways. First, it appears to have three *a* type cytochromes: a_1, a_2 [1] and $a+a_3$ [2, 3]. The most abundant is cytochrome a_2 which exhibits an absorption maximum at 616–618 nm and its spectrum overlaps that of cytochrome $a+a_3$. Kinetic studies [3] showed that both cytochrome a_2 and $a+a_3$ were oxidized rapidly enough to serve as terminal oxidases. Secondly, reduced mammalian cytochrome *c* is not oxidized by cell-free homogenates or mitochondria of *T. pyriformis* [4–7]. This is surprising in view of the report that cytochrome $a+a_3$ may be present, albeit in small quantities, in mitochondria of this organism. Third, the respiration of isolated mitochondria is relatively insensitive to rotenone and antimycin A and concentrations 1000-fold higher than those used in mammalian mitochondria are required to obtain the same level of inhibition [2]. Fourth, the ADP/O ratios were reported to be 0.8–1.5 for succinate and 1.3–2.3 for α-ketoglutarate [2] which would indicate that there are three phosphorylation sites between the NADH and oxygen. Since the data were calculated from the oxygen electrode traces on mitochondria with rather poor respiratory control a possibility existed that the results were an overestimation of the actual situation. Finally, two cytochromes *b* and two cytochromes *c* were described as the components of the respiratory chain on the basis of the low temperature difference spectra. One of the *b* cytochromes, *b*-560 was considered to be identical with cytochrome *o* [8, 9] and cytochromes *c*-553 and *c*-549 were suggested to be analogous to mammalian cytochromes c_1 and *c*. In spite of its elegance, the absorption spectroscopy may be a subject of certain errors due to the overlap of absorption bands of different cytochromes, the existence of multiple absorption bands for a single cytochrome, and interference from the oxidation or reduction reactions. It is therefore always desirable to establish the existence of a given cytochrome as a separate functional entity on the basis of yet another, independent ground.

The objectives of the present work were 2-fold: re-evaluation of the efficiency of oxidative phosphorylation in *T. pyriformis* mitochondria and characterization of the mitochondrial respiratory chain components with respect to their thermodynamic properties. The latter approach, used so successfully in mitochondria of higher organisms, gives us an independent tool in establishing the number of respiratory chain components and their relative positions in the chain.

MATERIALS AND METHODS

Growth of Tetrahymena. T. pyriformis strain ST (kindly provided by Dr. Y. Suyama, Department of Biology, University of Pennsylvania) was grown and maintained in a culture medium, which consisted of 2 % proteose peptone (Difco Laboratories, Detroit Michigan), 0.2 % yeast extract (Difco Laboratories), 1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.1 mM ferric citrate. Cultures were grown in 2-l Erlenmeyer flasks containing 300 ml of culture medium, without shaking at 28 °C ±1 for approx. 26 h.

Harvesting. Cells were harvested in the late logarithmic phase of growth by centrifugation at 2000 rev./min for 2 min (at 20 °C) in an IEC Model HN-S centrifuge equipped with a pear-shaped oil testing centrifuge head. The supernatant was removed by suction so as not to disturb the loosely packed pellet and cells were washed once in cold 0.25 M sucrose.

Isolation of mitochondria. The washed cellular pellet was resuspended in six times its final packed volume in 0.25 M sucrose, 10 mM KCl, 5 mM morpholinopropane sulfonate, and 0.2 mM EDTA (pH 7.2) medium and passed through a cold emulsion homogenizer (A. Thomas Co., Philadelphia) using moderate hand pressure. The cell homogenate was centrifuged at 2000 rev./min for 6 min in a Sorvall SS34 rotor. The supernatant was poured off and saved. The pellet was resuspended in half the previous volume, rehomogenized as before and centrifuged at 2000 rev./min for 6 min. The supernatant from each extraction was usually centrifuged separately at 6500 rev./min for 10 min and the sediment (mitochondrial fraction) was washed twice in 0.25 M sucrose, 10 mM KCl, 5 mM morpholinopropane sulfonate, 0.2 mM EDTA containing 0.2 % albumin (pH 7.2) by centrifugation at 8000 rev./min for 10 min. Care was taken to remove the colorless layer appearing on tops of the pellets and the black sediment which adhered firmly to the bottom of the centrifuge tube. It has been our experience that the second extraction yields more mitochondria and with higher P/O ratios.

Analytical methods

Protein determination. Proteins were assayed by the Biuret method [10] using crystalline albumin (Sigma Chemical Co) as a standard.

Respiratory control and oxidative phosphorylation. Respiratory control and ADP/O ratios were measured polarographically using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Cleveland, Ohio) at 24 °C. The mitochondria were suspended in 2 ml of 0.25 M sucrose, 10 mM morpholinopropane sulfonate, 5 mM phosphate, 2 mM $MgCl_2$, 0.7 mM EDTA, 20 mM glucose, and 10 mM NaF (pH 7.0). Succinate, 3-hydroxybutyrate, malate plus glutamate or α-ketogluturate were used as substrates. Respiratory control ratios were calculated according to the method of Chance and Williams [11].

Reactions for the assay of P/O ratios were carried out in the chamber used for respiration measurements in the medium described above (for ADP/O measurements) supplemented with 100 units of hexokinase (Type IV Sigma Chemical Co). Respiration was initiated by the addition of a respiratory substrate and 0.5–1 mM ADP. (The initial concentration of ADP ("zero time") was measured precisely in separate experiments in which ADP was added to the quenched sample in an amount equal to that used in subsequent measurements of P/O ratios). At various time intervals the samples were quenched with $HClO_4$ (4 % final concentration), neutralized with KOH and, after removal of the solid $KClO_4$ by centrifugation, aliquots of the supernatant were used for determination of glucose 6-phosphate and ADP. In calculations of P/O ratios corrections were made for ATP formed from ADP by the adenylate kinase reaction by subtracting the amount of ADP that was used up between the "zero time" and the moment of trapping.

Phosphorylation efficiency at sites 1 and 2 was measured according to the method of Lee et al. [12] using ferricyanide as the artificial electron acceptor. The incubation mixture contained: 0.2 M sucrose, 0.05 Tris/acetate (pH 7.4), 10 mM succinate (or 3-hydroxybutyrate), 1 mM KCN, 6 mM potassium phosphate (pH 7.4), 2 mM ATP, 50 mM glucose, 50 K.M. units yeast hexokinase, and 4–5 mg of mitochondrial protein. The reaction was started by the addition of 5 mM (final concentration) $K_3Fe(CN)_6$ and was carried out at 22 °C for 6–10 min.

Ubiquinone was measured by the extraction procedure as described by Kröger and Klingenberg [13].

Glucose 6-phosphate was determined enzymatically by the method of Lamprecht and Trautschold [14] and ADP by the procedure of Adam [15]. Inorganic phosphate was determined by the method of Martin and Doty [16].

Potentiometric titrations. Potentiometric titrations were carried out at 22 °C in an anaerobic chamber with an atmosphere of ultrapure argon gas. Simultaneous measurements of absorbance (Johnson Foundation dual wavelength spectrophotometer) and oxidation-reduction potentials were carried out as described by Dutton [17]. The mitochondria (approx. 10 mg protein/ml) were suspended in 0.2 M sucrose/50 mM morpholinopropane sulfonate (pH 7.2). Potassium ferricyanide was used as the oxidant and freshly prepared $Na_2S_2O_4$ was used as the reductant. The following redox mediators were used: phenazine methosulfate (Sigma Chemical Co); phenazine ethosulfate, pyocyanine perchlorate, 2-OH-1,4-naphthoquinone (K and K Laboratories); duroquinone and diaminodurene (Aldrich Chemical Co.). The results are presented graphically as the logarithm of the ratio of oxidized to reduced forms of the cytochromes (abscissa) as a function of the oxidation-reduction potential (ordinate). Separation of the titration curves into individual components was performed as described by Wilson and Dutton [18] and Dutton et al. [19].

Spectrophotometric measurements. Spectrophotometric measurements were performed using Johnson Foundation dual wavelength spectrophotometer or dual wavelength scanning spectrophotometer. The dual wavelength instrument was used to determine the extent of reduction of cytochromes as described by Chance [20], while spectra were obtained in the scanning spectrophotometer whose operation is described in refs. 21 and 22. The detailed experimental conditions are given in the figure legends.

Photochemical action spectrum. Simplified photochemical action spectra were obtained in the following way. Light from a 45 W tungsten-iodine lamp was directed through a shutter onto a 1.5×1.5 cm glass chamber equipped with a side arm which housed a Clark-type oxygen electrode connected to a derivative circuit. The oxygen electrode was covered with two membranes: on top of the standard membrane was placed a plastic ring approx. 1.5 mm thick which created a small "chamber" into which a suspension of submitochondrial particles, mitochondria, or cells was placed (3–4 mg protein/ml in 0.2 M sucrose/0.05 M morpholinopropane sulfonate buffer at pH 7.2 containing 0.04 M succinate). The "chamber" was closed with the same type of membrane that was covering the surface of the oxygen electrode and the membrane was secured onto the electrode with an O-ring. The cuvette was positioned so that the light fell directly onto the surface of the oxygen electrode. The main volume of the cuvette was filled with CO-saturated 0.2 M sucrose/50 mM morpholinopropane sulfonate buffer (pH 7.4) containing $1 \cdot 10^{-7}$ M catalase and stirred continuously with a small magnetic bar. The cells were allowed to respire the residual oxygen until the suspension became anaerobic and then a few microliters of 1 M H_2O_2 were introduced into the main chamber to raise the oxygen tension. Oxygen which diffused through the membrane sustained a slow but steady respiratory rate. When the shutter was opened and light illuminated the surface of the oxygen electrode, the respiratory rate increased 8–10-fold which was measured with high sensitivity with the oxygen electrode's derivative circuit. When the shutter was closed, the respiratory rate

returned to its previous CO-inhibited value and the O_2 returned to its original steady-state concentration. Light responses could be repeated many times without a decrease in the intensity of the response. Once the conditions were established, the experiment was carried out in the following way: after recording 2–3 dark-light cycles in response to white light at a less than 50 % saturation level, an appropriate filter (See Table III) was inserted and the dark-light cycles were repeated in its presence. This was followed by 2–3 dark-light cycles induced by white light. The procedure was repeated with the other filters and the responses obtained were calculated as percentages of change induced by the white light. Parallel experiments were carried out with suspensions of submitochondrial particles isolated from pigeon breast mitochondria.

RESULTS

Characteristics of respiratory control and oxidative phosphorylation

The respiratory control ratios of *T. pyriformis* mitochondria were generally low, 1.6–1.9 with the NAD-linked substrates and slightly above 2 in the presence of succinate (Table I). ADP : O ratios were always above 1 for succinate and about 2 for malate and glutamate oxidation. The P : O ratios for succinate oxidation were consistently above 1, both in the absence and presence of rotenone, with a mean value of 1.3 ± 0.25. 3-Hydroxybutyrate was oxidized with a P : O of almost 2 and in some experiments values as high as 2.2 were found. Malate plus glutamate were oxidized with a P : O ratio of 2.18 ± 0.11. The phosphorylation efficiency measured using ferricyanide as an artificial electron acceptor yielded $P/2e^-$ values for succinate oxidation of 0.83 ± 0.17 and 1.52 ± 0.17 for 3-hydroxybutyrate oxidation (Table I). Phosphorylation with succinate was completely inhibited by the addition of 100 μg of antimycin A/mg protein while that with 3-hydroxybutyrate was eliminated by the addition of 0.05 mM rotenone.

The respiratory rates were highest with succinate and lower with the NAD-linked substrates. Succinate oxidation was not stimulated further by the addition of any NAD-linked substrate. Since the concentration of cytochrome *c* was about 0.5 nmol/mg mitochondrial protein, the rate of 22.23 nmol O_2/mg protein per min observed with succinate as substrate corresponds to the value of approx. 45 nmol O_2/min per nmol of cytochrome *c*. The respiration of *T. pyriformis* mitochondria was inhibited more than 90 % by the addition of 1 mM cyanide, irrespective of the substrate used. Respiration of the NAD-linked substrates was sensitive to rotenone but at concentrations 1000-fold higher than those used in mammalian mitochondria. Similarly antimycin A and HQNO were much less effective in inhibiting electron transfer in *Tetrahymena* mitochondria (as compared with mitochondria of higher organisms) and concentrations as high as 1000 nmol/nmol cytochrome *c* had to be used to reach 90 % inhibition. These results are in excellent agreement with the findings of Turner et al. [2]. Salicyl hydroxamic acid used in a concentration which inhibits the alternate oxidation pathway in plant mitochondria [23] did not affect the respiratory activity of *Tetrahymena*.

Spectral studies in T. pyriformis mitochondria

(*a*) *Spectral studies in the α and β region.* The addition of succinate to mitochondria isolated from *T. pyriformis* induces the appearance of a small, rather broad

TABLE I

RESPIRATORY CONTROL, ADP : O AND P : O RATIOS AND RESPIRATORY RATES FOR MITOCHONDRIA ISOLATED FROM *T. PYRIFORMIS*

Respiratory control, ADP : O ratios and respiratory rates were calculated from polarographic traces as described by Chance and Williams [11]. P : O ratios were measured as described in Materials and Methods. Data are mean values ± S.E. The number of experiments is in parentheses.

Substrate	Respiratory control	ADP : O	P : O	Respiratory rate (nmol O_2/mg protein min)		P : $2e^-$
				State 4	State 3	
5 mM glutamate and 5 mM malate	1.96±0.53 (5)	1.91±0.36 (4)	2.18±0.11 (8)	7.28±0.78 (5)	14.13±3.22 (5)	–
10 mM 3-hydroxybutyrate	1.63±0.1 (2)	–	1.93±0.22 (11)	6.28±0.92 (2)	10.19±1.53	1.52±0.17 (3)
10 mM succinate	2.12±0.19 (6)	1.18±0.1 (5)	1.31±0.25	10.49±2.58 (6)	22.23±5.78 (6)	0.83±0.17 (3)
α-Ketoglutarate	1.89 (1)	–	–	5.67 (1)	10.70 (1)	–

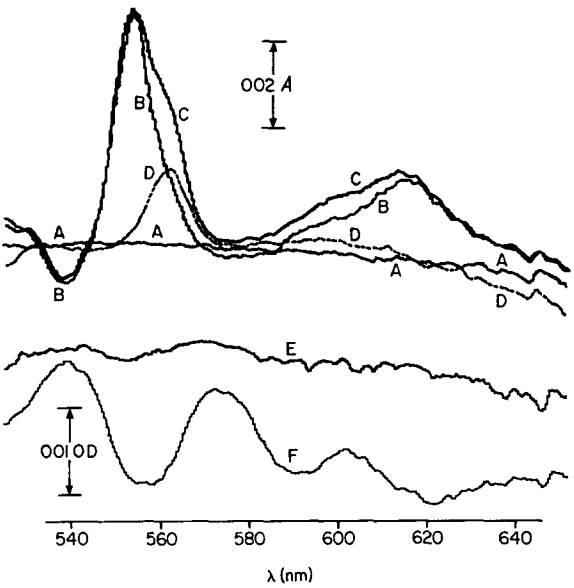

Fig. 1. Dual wavelength difference spectra of *Tetrahymena* mitochondria in the region from 510 to 640 nm. *Tetrahymena* mitochondria were suspended in 0.2 M sucrose/0.05 M morpholinopropane sulfonate (pH 7.2) buffer at a protein concentration of 3.0 mg/ml. The spectrum of the fully oxidized sample was measured and stored in the digital computer memory (note that any spectrum presented in this paper is the difference spectrum between that recorded and the one stored in the computer memory) and trace A which is the oxidized–oxidized spectrum appears therefore as a straight line. 10 mM succinate was then added and the sample was allowed to become anaerobic. Trace B is the anaerobic minus oxidized sample. Dithionite was then added and trace C was obtained (dithionite reduced minus oxidized). Trace D is the dithionite-reduced minus the anaerobic spectrum. The spectrum of the dithionite-reduced sample was stored in the computer memeory as in A (trace E) and the sample was saturated for 60 s with CO gas. Spectrum F is the difference between (dithionite-reduced+CO)−(dithionite reduced). The reference wavelength was at 540 nm.

absorbance change with a maximum at about 560 nm (not shown). As the sample becomes anaerobic, an absorption spectrum appears which is dominated by an asymmetric peak with a maximum at 552–553 nm (trace B of Fig. 1). The 552 nm peak is characteristic of cytochrome *c*, the 560 nm shoulder of a *b* type cytochrome. In addition there are absorbance changes in the 510–530 nm region and above 590 nm. The absorption change in the former region, i.e. at the short wavelength side of the cytochromes *b* and *c* peak consists of the *β* bands of *b* and *c* type cytochromes. A broad band in the region between 590 and 630 nm (with a maximum at 616 and a shoulder around 592 nm) is due to the overlapping absorbance bands of *a* type cytochromes. Dithionite causes an additional increase in absorption mostly at the long wavelength side of the cytochrome *c* peak (trace C of Fig. 1), and a small change around 592 nm. These changes are seen more clearly in the difference spectrum between the dithonite-reduced and anaerobic samples (trace F). A symmetric peak with a maximum at 560 nm is attributed to *b* type cytochromes.

The addition of ascorbate and TMPD to cyanide-inhibited mitochondria leads to the appearance of a symmetric absorbance change with a maximum at 552–553 nm (trace B, upper part of Fig. 2) and a small peak at around 605–620 nm. Under anaero-

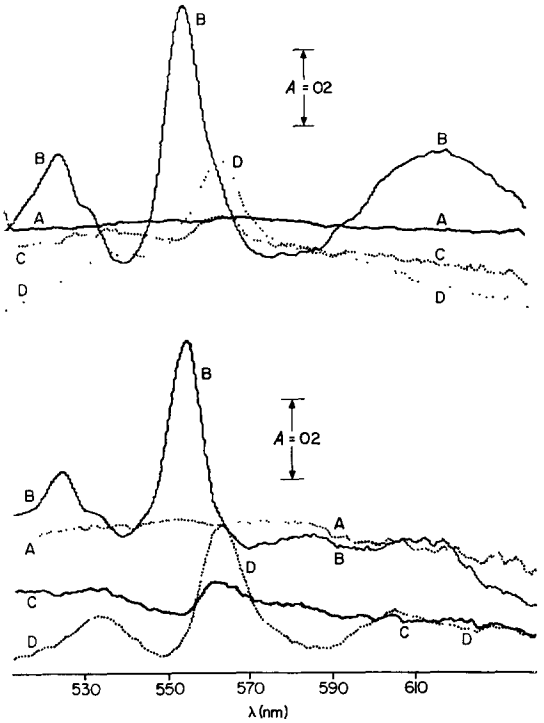

Fig. 2. The effect of ascorbate plus TMPD on the spectral changes in *Tetrahymena* mitochondria. The mitochondria were suspended as described in the legend of Fig. 1 and oxidized−oxidized spectrum was measured (A). 10 mM ascorbate and 10 μM TPMD were then added and the sample was either allowed to become anaerobic (upper part) or 1 mM KCN was added (lower part). Trace B is therefore either anaerobic−aerobic (upper part) or (KCN-reduced) − aerobic (lower part). Spectrum B was subsequently stored in the computer memory and 10 mM succinate was added. Trace C is the difference obtained in the presence and absence of succinate. Spectrum C was again stored in the computer memory and the sample reduced by the addition of dithionite. Spectrum D is the difference between the dithionite-reduced sample and either the anaerobic sample (with succinate, upper part) or the KCN-reduced (with succinate, lower part). The reference wavelength was at 540 nm.

bic conditions (trace B, lower part) a shoulder is observed at the long wavelength side of cytochrome *c* due to the reduction of cytochrome(s) *b* and a large increase in absorption with a maximum at 615 nm caused by the reduction of *a* type cytochromes. Further addition of succinate to either the anaerobic or cyanide-reduced sample results in a small additional reduction of *b* type cytochromes (trace C of Fig. 2). Substantial reduction of cytochrome(s) *b* is obtained upon further addition of dithionite (trace D) and it can be seen that only approx. 40–50 % of cytochrome(s) *b* is reduced by the substrates by anaerobiosis, the remaining fraction being reduced by dithionite.

The CO-difference spectrum (trace D, Fig. 1) shows absorption maxima at approx. 535 and 570 nm; there is little change in absorbance at around 589–590 nm. A trough at around 615–617 nm coincides with a small absorbance increase at 630–640 nm, too small to be characterized precisely.

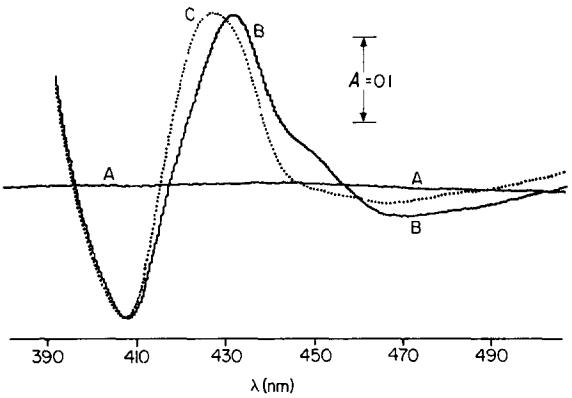

Fig. 3. Soret dual wavelength difference spectra of *Tetrahymena* mitochondria. Conditions are those of Fig. 1. A, oxidized−oxidized; B, (dithionite-reduced)−(oxidized); C, (dithionite-reduced+CO) −(oxidized). The reference wavelength was at 460 nm.

(*b*) *Spectral changes in the Soret region.* Fig. 3 compares the dithionite-reduced spectrum (dithionite reduced−oxidized) of the suspension of *Tetrahymena* mitochondria with the CO-induced change ((dithionite reduced+CO)−oxidized). The dithionite-reduced spectrum exhibits an absorbance maximum at 433 nm and a shoulder at 447 nm; the CO-induced change shows an absorption maximum at 426–427 nm. A more detailed account of the events is seen in Fig. 4. The addition of succinate and subsequent anaerobiosis lead to a difference spectrum depicted by B. A deep trough at 408 nm and an absorption maximum at 426 nm in the difference spectrum (anaerobic−oxidized) are characteristic of the mixture of cytochrome(s) *c* and *b*. Moreover, there is a pronounced shoulder at 448 nm, typical of *a* type cyto-

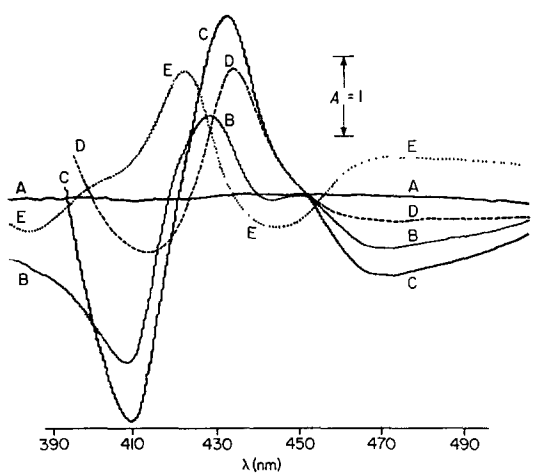

Fig. 4. Dual wavelength difference spectra of *Tetrahymena* mitochondria in the Soret region. Conditions are the same as in Fig. 1. A, oxidized−oxidized; B, anaerobic−oxidized; C, (dithionite-reduced)−oxidized; D, (dithionite-reduced)−anaerobic; E, (dithionite-reduced+CO)−(dithionite-reduced). The reference wavelength was at 460 nm.

chromes. The addition of dithionite to an anaerobic sample gives a spectral change shown in trace C which, as seen from the dithionite-reduced—anaerobic spectrum (trace D), arises from a component with the reduced absorption maximum at 434 nm. These absorption characteristics indicate that a *b* type cytochrome is reduced upon the addition of dithionite and confirm the results presented above carried out in the visible region of the spectrum.

The CO-difference spectrum of the dithionite-reduced sample is characterized by an absorption maximum at 422–424 nm and a trough at 442–444 nm (trace E). The difference spectrum of an anaerobic mitochondrial suspension ((anaerobic+CO)—anaerobic) shows an absorption maximum at 424 nm. Subsequent addition of dithionite leads to a much larger absorption change with a maximum at 426 nm and a shoulder at 434 nm and a trough at 448 nm.

Oxidation-reduction potentials of the cytochromes in mitochondria isolated from T. pyriformis

(*a*) *Oxidation-reduction potential measurements at 552–540 nm.* Fig. 6A shows that the plot of the ratio of the oxidized to reduced cytochrome against the oxidation-reduction potential (E_h) is a straight line with a slope of 1 (60 mV per 10-fold change in the ratio of the oxidized to reduced cytochrome). The half-reduction potential determined from this plot, at pH 7.2 and 22 °C, is 0.225 V. There were no further absorption changes at these measuring wavelengths either above an E_h of 0.29 V or below an E_h of 0.16 V. This indicates that if there is yet another type of cytochrome *c* in these mitochondria, it must have a half-reduction potential within 0.03 V of that measured in Fig. 6A.

(*b*) *Oxidation-reduction potential measurements at 615–575 nm.* Anaerobic potentiometric titration of the absorbance change at 615–575 nm gives a sigmoid curve characteristic of two components (Fig. 6B) with half-reduction potentials of 0.345 and 0.245 V (at pH 7.2 and at 22 °C) both components being one-electron donors/

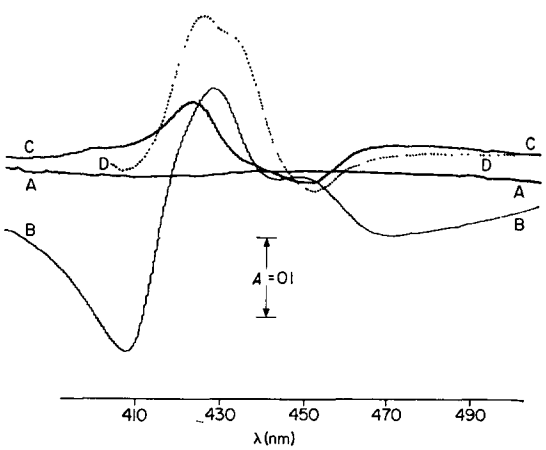

Fig. 5. CO-difference spectra of *Tetrahymena* mitochondria. The spectra were recorded as described in the legend of Fig. 1. A, oxidized—oxidized; B, anaerobic—oxidized; C, (anaerobic+CO)—anaerobic; D, (dithionite-reduced+CO)—(anaerobic). The reference wavelength was at 460 nm.

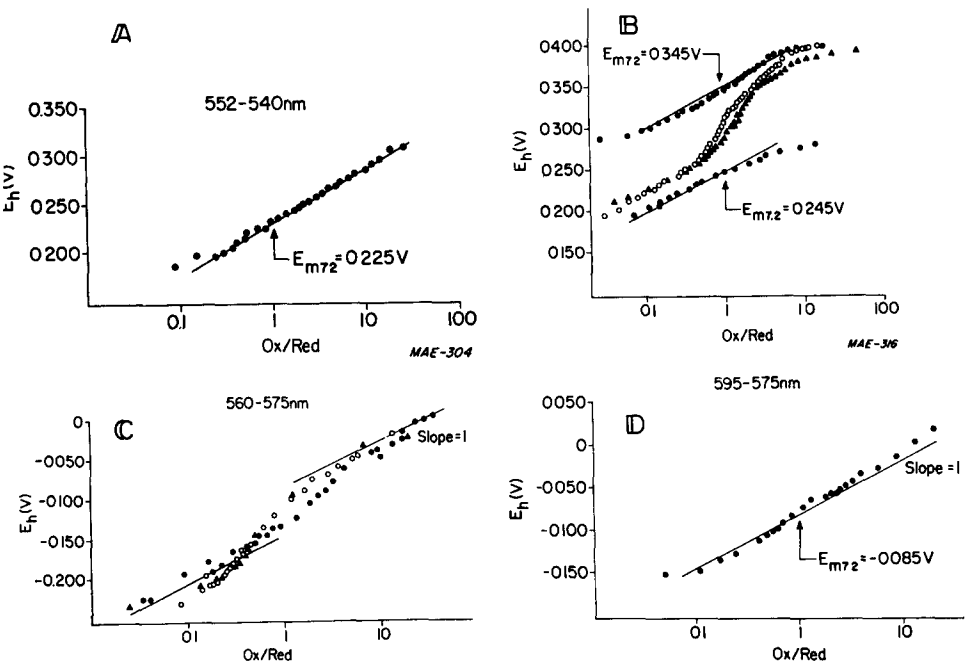

Fig. 6. Plot of the course of oxidation-reduction of various cytochromes in *Tetrahymena* mitochondria against the oxidation-reduction potential (E_h). A, absorbance changes at 552–540 nm; B, absorbance changes at 615–575 nm; C, absorbance changes at 560–575 nm; D, absorbance changes at 595–575 nm. Anaerobic potentiometric titrations were carried out in the dual wavelength spectrophotometer as described in Materials and Methods. Mitochondria was suspended in 0.2 M sucrose/0.05 M morpholinopropane sulfonate (pH 7.2) buffer at a protein concentration of 10 mg/ml. The redox mediators used were: 20 μM diaminodurol and 40 μM phenazine methosulfate (A and B). 40 μM phenazine ethosulfate, 40 μM duroquinone, 5 μM pyocyanine, 20 μM 2-OH-1,4-naphthoquinone, and 10 μM riboflavin (C and D). The same symbols are used for the oxidative (with ferricyanide) and for the reductive (with $Na_2S_2O_4$) titrations. Solid lines represent theoretical $n = 1$. Triangles in B represent the titration carried out at 605–575 nm. The different symbols in C represent oxidative and reductive titrations carried out on three different mitochondrial preparations.

acceptors. The upper potential component accounts for 60–70 % of the absorbance changes at this wavelength pair. The same sigmoid titration curve was obtained when the reference wavelength was at 640 nm and the values for the midpoint potentials were identical to those obtained in the titrations at 615–575 nm.

Titrations carried out at 605–575 nm also gave sigmoid curves with identical half-reduction potentials except that the total absorbance change was almost 50 % smaller than that at 615–575 nm. It was also noted in some titrations that the component with the lower half-reduction potential contributed more (up to 50 %) to the overall absorption change.

(*c*) *Anaerobic potentiometric titration of the absorbance change at 595–575 nm.* Anaerobic potentiometric titrations at pH 7.2 and 22 °C of the absorbance changes at 595 nm showed the presence of one component with a slope of 1 and a half-reduction potential of -0.085 ± 0.02 V (Fig. 6D). Owing to the difficulties in equilibration between the redox components and the oxidant or reductant in this potential region

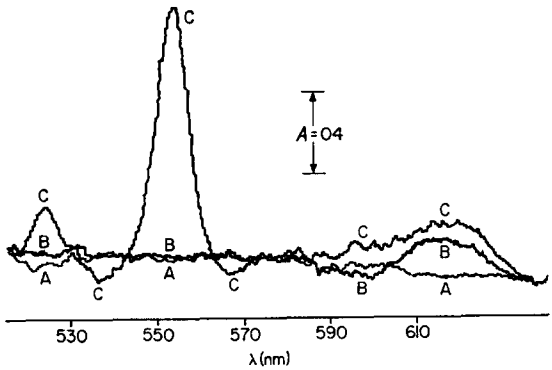

Fig. 7. Difference spectra of the cytochromes of *T. p. riformis* mitochondria obtained during anaerobic potentiometric titrations. The mitochondria were suspended at a protein concentration and under the same conditions as those in Fig. 6 and placed in an anaerobic titration vessel (see Materials and Methods) in a dual wavelength scanning spectrophotometer. The preparation was made anaerobic in the presence of 20 μM diaminodurene and 40 μM phenazine methosulfate and then reoxidized with ferricyanide to an E_h of 0.400 V. The anaerobic oxidized spectrum at this E_h value was measured and stored in the computer memory. The sample was then reduced with dithionite to an E_h of 0.295 V and spectrum B was recorded (i.e. it represents the absorbance change which occurs in the E_h span between 0.400 and 0.295 V). The sample was further reduced to an oxidation-reduction potential of 0.145 V. Spectrum C is therefore the absorbance change observed in the potential range between 0 and 0.145 V. The reference wavelength was at 575 nm.

(which were greater in *Tetrahymena* mitochondria than in other types of mitochondria [18, 19, 24]) there is certain degree of uncertainty in this half-reduction potential value.

(*d*) *Anaerobic potentiometric titrations of the absorbance changes at 560–575 nm.* Anaerobic potentiometric titrations of the absorbance changes at 560–575 nm (Fig. 6C) for three different mitochondrial preparations show sigmoid curves characteristic of two components. The half-reduction potentials for the two components are only approximate and equal to −0.065 V for the upper potential component and

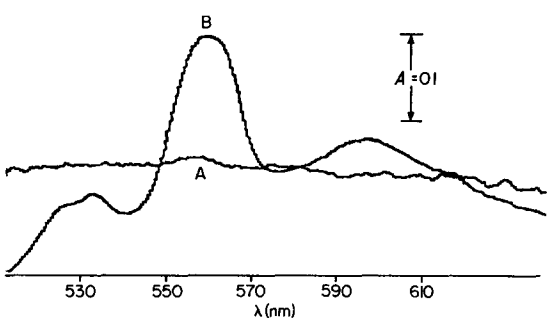

Fig. 8. Difference spectra of the cytochromes of *Tetrahymena* mitochondria below the E_h of 0.145 V. Conditions are the same as in Fig. 7. Spectrum C of Fig. 7 was stored in the computer memory to give trace A and the sample reduced by the addition of excess dithionite. Trace B is the difference spectrum between excess dithionite minus the absorbance change at the E_h of 0.145 V. The reference wavelength was at 575 nm.

−0.15 to −0.17 V for the lower potential one. Both appear to be one electron donors/acceptors. There was no change in absorbance above 0 V.

(*e*) *Difference spectra of the mitochondrial cytochromes obtained during the anaerobic potentiometric titrations.* In the potential span between an E_h of 0.39 and 0.29 V (trace B of Fig. 7), the absorption spectrum exhibits a maximum at 615 nm characteristic of an *a* type cytochrome. In the potential range between an E_h of 0.29 and 0.05 V a reduction of an *a* type cytochrome is indicated by an increase in absorbance at around 603–605 nm (and a small additional increase at 615 nm) and a reduction of cytochrome *c* shown by the appearance of a symmetric absorption peak with a maximum at 553 nm. Reduction with excess dithionite (potential range below 0.15 V, Fig. 8) yields absorption maxima at 560 nm, characteristic of *b* type cytochromes, and at 593–595 nm possibly due to cytochrome a_1. Because of the difficulties in equilibration the oxidation-reduction potential region where the two *b* cytochromes absorb no attempt has been made to resolve their spectra.

Steady-state reduction of cytochromes in mitochondria isolated from T. pyriformis

Table II shows firstly that almost all of the cytochrome *c* and *a* is reduced under anaerobic conditions and very little additional reduction is obtained with dithionite and secondly, that there is a large fraction of cytochrome *b* which is not reduced by substrates under anaerobiosis. The extent of reduction of various cytochromes under steady-state conditions is as expected from their location in the respiratory chain, i.e. on the basis of our knowledge of their half-reduction potentials. Cytochrome *c* is about 13 % reduced with succinate as substrate and 5 % reduced in the presence of 3-hydroxybutyrate. Cytochromes *b* (treated as a single component with maximum absorbance at 560–575 nm) are more highly reduced than is cytochrome *c*. Because the absorption spectra of the two species absorbing at 560 nm are the same, the steady-state measurements do allow us to determine the extent at which either one of them is reduced under the steady-state conditions.

Steady-state reduction level of ubiquinone in T. pyriformis mitochondria

T. pyriformis mitochondria, contained ubiquinone at the concentration of

TABLE II

STEADY-STATE REDUCTION LEVELS OF CYTOCHROMES IN MITOCHONDRIA ISOLATED FROM *T. PYRIFORMIS*

The steady-state reduction levels were measured in the dual wavelength spectrophotometer at the wavelength pairs indicated in the table. 10 mM succinate or 10 mM 3-hydroxybutyrate were added to an anaerobic mitochondrial suspension in 0.2 M sucrose/0.05 M morpholinopropane sulfonate (pH 7.2), buffer (approx. 2 mg prot/ml) and the redox level was recorded. The suspension was allowed to become anaerobic, and dithionite was then added to detect any cytochromes which were not fully reduced by anaerobiosis. The results are expressed as percentages in A taking anaerobic reduction levels as 100 % reduced and in B taking the dithionite-reduced levels as 100 %.

Substrate	cytochrome *b* (560–575 nm)		cytochrome *c* (552–540 nm)		cytochrome *a* (615–640 nm)	
	A	B	A	B	A	B
Succinate	42	26	13	12	3	2
3-Hydroxybutyrate	28	16	5	4	–	–

TABLE III

RESPONSES TO LIGHT OF CO-INHIBITED SUBMITOCHONDRIAL PARTICLES ISOLATED FROM PIGEON BREAST AND *T. PYRIFORMIS* MITOCHONDRIA

Experimental conditions are described in Materials and Methods. Values represent fractional responses of the effect caused by the white light ±S.E. for the number of experiments in parentheses.

Filter (Kodak Wratten)		Pigeon breast submitochondrial particles	*T. pyriformis* submitochondrial particles	*Tetrahymena*/ pigeon breast
No.	Transmission characteristics			
23A	570 nm and above (11.0 %)	0.79±0.03 (6)	0.66±0.03 (7)	0.84
26	590 nm and above (2.9 %)	0.54±0.03 (7)	0.45±0.03 (7)	0.86
29	610 nm and above (10.5 %)	0.33±0.06 (5)	0.29±0.04 (6)	0.88
55	460–600 nm (0.2 %) (0.4 %) Maximum at 520–530 (≈ 65 %)	0.72±0.05 (5)	0.34±0.03 (9)	0.47
73	560–610 nm (2.24 %) (0.1 %) Maximum at 570 nm (≈ 6 %)	0.30±0.03 (5)	0.11±0.03 (7)	0.37
98	400–490 nm (6.1 %) (0.7 %) Maximum at 430–440 nm (≈ 40 %)	0.73±0.03 (4)	0.30±0.04 (5)	0.41

3.8–4.2 nmol per mg protein, 80–85 % of which was reducible by succinate under anaerobic conditions. In the steady state, ubiquinone was 58–62 % reduced with succinate as substrate and 40–45 % reduced in the presence of 3-hydroxybutyrate. These values are close to the ones found in other types of mitochondria [13, 25] under the same experimental conditions.

Photochemical action spectrum in T. pyriformis submitochondrial fragments

Respiration of *T. pyriformis* cells, mitochondria, and their fragments was inhibited by CO and, therefore, the photochemical action spectrum can provide us with the nature of the CO-sensitive, terminal oxidase of this organism. Two points should be considered before analyzing the results (Table III); first, filters of appropriate characteristics with broad transmission bands were used instead of monochromatic light. Second, the polarizing light was not adjusted to equal intensity in various spectral region thus a true photochemical action spectrum cannot be obtained. Since, however, the light intensity was kept sufficiently low, the effect of light of various wavelength (obtained in the presence of different filters) can be evaluated as fractional response of that caused by white light. Moreover, when parallel evaluation is made *Tetrahymena* mitochondria and on pigeon breast mitochondria information can be gained on the nature of CO-sensitive pigment in *Tetrahymena* mitochondria owing to the fact that pigeon breast respiratory chain contains well-characterized cytochrome aa_3 as the terminal oxidase. Inspection of the data presented in Table III leads to the following conclusions: first, the light sensitivity of the terminal oxidase in *T. pyriformis* is smaller than that of pigeon breast oxidase. Second, the CO compound of the terminal oxidase in *Tetrahymena* has relatively lower absorbance in the Soret region and in the region between 500 and 580 nm than does the CO compound of

cytochrome a_3. Third, the alpha absorbance maxima of the CO compound of the terminal oxidase in *Tetrahymena* lies on the long wavelength side of the absorption maximum of the cytochrome $aa_3 \cdot CO$ complex. The nature of the oxidase with such characteristics will be discussed below.

DISCUSSION

The respiratory chain in mitochondria isolated from *T. pyriformis* bears a number of characteristics which are not commonly encountered in mitochondria of higher organisms. This atypical respiratory chain, however, carries out oxidative phosphorylation with the same efficiency as does the respiratory chain of mitochondria of higher organisms irrespective of the nature of its terminal oxidase.

By combining spectra studies with potentiometric titrations a new insight was gained into the nature of the respiratory chain components in *Tetrahymena* mitochondria. The discussion is divided into three parts: (1) the characteristics of cytochrome(s) *c*; (2) the characteristics of cytochrome(s) *b*, and (3) the nature of cytochrome(s) *a*.

Potentiometric titrations of the absorbance change at 552–540 nm reveal the presence of a single component with a half-reduction potential of 0.225 ± 0.01 V. The absorption spectrum of this cytochrome and its thermodynamic properties are characteristic of a large number of different cytochromes *c* so abundantly distributed in nature [26]. Because of the nature of potentiometric titrations we were unable to exclude the existence of more than one type of cytochrome *c* with similar spectral properties and half-reduction potential of not more than 0.03 V apart. This situation occurs in mitochondria from higher organisms in which cytochrome *c* and c_1 have similar spectral and thermodynamic properties. Experiments are currently underway to investigate the same possibility in the respiratory chain of *T. pyriformis*.

The overall content of cytochrome *c* in *Tetrahymena* mitochondria was calculated to be 0.5–0.6 nmol/mg protein, which is not very different from the values found in other types of mitochondria [26]. (An assumption was made that the millimolar extinction coefficient for cytochrome *c* at 552–540 nm (reduced-oxidized) is 19 cm^{-1}.)

The region of cytochrome(s) *b* in *Tetrahymena* is equally intriguing as in the mitochondria of other organisms. There are two components with different half-reduction potentials which contribute about equally to the overall absorbance change at 560 nm (and to the Soret absorbance with maximum at 428–430 nm); the numerical values of the half-reduction potentials for the two components are, however, different from those in other types of mitochondria. The higher potential component has a half-reduction potential similar to that of cytochrome *b*-565 [18]. There is an apparent lack of the *b* cytochrome with a half-reduction potential corresponding to that of *b*-561 [18]. This suggests that it is present in either a concentration of not more than 10 % of that of the other components which absorb at 560 nm or it has a half-reduction potential very close (within ± 0.030 V) to that of the other *b* cytochromes thus giving rise to a single component in the redox titrations. It is interesting to mention in this context that it is the cytochrome *b*-561 which undergoes a spectral shift upon the addition of antimycin A [27, 28] and *Tetrahymena* mitochondria, which appear to lack cytochrome *b*-561, are very insensitive to antimycin A inhibition.

The other component which absorbs at 560 nm has a half-reduction potential far too negative to make it an efficient member of the pool of redox carriers at site 2. The negativity of the half-reduction potential of this component can in part explain its slow reducibility in the presence of respiratory substrates. A question arises whether this low potential component is not perhaps identical with cytochrome *o*, the CO-absorbing pigment present in bacteria [29] and reported to be present in *Tetrahymena*. The CO-difference spectrum (Fig. 1) with maxima at 570 and 534 nm confirms the presence of a cytochrome with the characteristics of cytochrome *o*. There is no indication in the literature [30] as to the possible value of the half-reduction potential of cytochrome *o* in vivo. However, one of the cytochromes *o* isolated by Webster and Hackett [31] from blue-green algae had a half-reduction potential of −0.09 V and reacted with oxygen very rapidly. So we can tentatively accept that the other cytochrome which absorbs at 560 nm is the cytochrome *o*. It is also clear that in *Tetrahymena* which carries out oxidative phosphorylation with a P : O ratio of 3 for the NAD-linked substrates, cytochrome *o* with an $E_{m7.2}$ of −0.15 V cannot possibly serve as the terminal oxidase, in agreement with the slow rates of oxidation of this pigment observed by Lloyd and Chance [3]. Our results provide, however, no information as to the possible function of cytochrome *o* in the respiratory chain.

The most baffling question is the nature of the terminal oxidase in *T. pyriformis* respiratory chain. There are three possible candidates which were reported to be present in the mitochondria of this protozoon: cytochrome aa_3, cytochrome a_2 (615 nm component), and cytochrome a_1 (590 nm pigment). If we take 20 cm^{-1} as the reasonable value for the millimolar extinction coefficient of cytochrome a_2 at 615-640 nm, we can calculate its concentration in mitochondria to be 0.15–0.2 nmol/mg protein. Cytochrome a_2 appears to consist of two components with *n* values of 1 and the half-reduction potentials amazingly close to those of cytochromes *a* and a_3 (0.245 V vs. 0.205 V and 0.345 V vs. 0.375 V for cytochrome *a* and a_3, respectively. A similar value of 0.32 V was tentatively assigned to the E_m of cytochrome a_2 by Kauffman and Van Gelder [32]). These values of the half-reduction potentials and the rapid rate of oxidation [3] of cytochrome a_2 make it a suitable candidate for the terminal oxidase.

The two-component nature of cytochrome a_2 may be the intrinsic property of this enzyme but it may also arise from the fact that the absorption spectrum of cytochrome aa_3 which might be present in *Tetrahymena* mitochondria overlaps with that of cytochrome a_2. Thus the component with the midpoint potential of 0.245 V of the 615–575 nm absorbance change is de facto cytochrome *a* of the aa_3 oxidase while the component with an E_m of 0.345 V is the mixture of cytochromes a_2 and a_3 which cannot be resolved on the basis of potentiometric titrations due to the close values of their half-reduction potentials. If this were true we would expect to be able to measure the half-reduction potentials of cytochrome aa_3 at 445–460 nm as the extinction coefficients are 5–6 times higher in the Soret region. The overall absorbance changes at 445–460 nm in the potential region between 0.18 and 0.4 V were, however, extremely small, much smaller than expected on the basis of the measurements at 605–640 nm (unpublished data). In addition, it was noted that most of the component which absorbs at 445–460 nm was reduced at an oxidation-reduction potential value more negative than 0.15 V. We therefore conclude that the 445 nm shoulder in the absorption spectra shown in Figs. 3–5 arises from the cytochrome a_1 [33] and not aa_3 and if

there is any cytochrome oxidase of aa_3 in *Tetrahymena* mitochondria, it is present at extremely low concentration such that it does not play a major role as the terminal oxidase pathway.

The third candidate for the terminal oxidase is cytochrome a_1. The value of the half-reduction potential of -0.085 V makes it, however, highly unlikely that cytochrome a_1 like cytochrome *o* functions efficiently as the terminal oxidase in *T. pyriformis*. It should be mentioned here that Kauffman and Van Gelder [32] found that slow reducibility of cytochrome a_1 in *Azotobacter vinelandii* to be incompatible with its role as the terminal oxidase.

The simplified photochemical action spectrum described above provides additional information on the nature of the terminal oxidase in *T. pyriformis*. The CO compound of the oxidase has the following characteristics: (1) little absorption in the Soret region, (2) small sensitivity to light, and (3) its α-maximum lies toward longer wavelength of the absorption peak of the CO compound of cytochrome aa_3. These characteristics eliminate cytochromes a_1, *o*, and aa_3 as terminal oxidases and indicate that cytochrome a_2 is the functional oxidase in *T. pyriformis*. The characteristics of cytochrome a_2 mentioned above are in agreement with those observed by Chance [34] and Castor and Chance [35] in various bacteria. It can be concluded, therefore, that the terminal oxidase in *T. pyriformis* is cytochrome a_2 which in its intimate two-component structure resembles cytochrome aa_3.

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REFERENCES

1 Baker, E. G. S. and Baumberger, J. P. (1941) J. Cell. Comp. Physiol. 17, 285–292
2 Turner, G., Lloyd, D. and Chance, B. (1971) J. Gen. Microbiol. 65, 359–374
3 Lloyd, D. and Chance, B. (1972) Biochem. J. 128, 1171–1182
4 Ryley, J. F. (1952) Biochem. J. 52, 483–492
5 Keilin, D. and Ryley, J. F. (1953) Nature 172, 451
6 Eichel, H. J. (1954) J. Biol. Chem. 206, 159–169
7 Kobayashi, S. (1965) J. Biochem. Tokyo 58, 444–457
8 Yamanaka, T., Nagata, Y. and Okunuki, K. (1968) J. Biochem. Tokyo 63, 753–760
9 Perlish, J. S. and Eichel, H. J. (1971) Biochem. Biophys. Res. Commun. 44, 973–980
10 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) J. Biol. Chem. 177, 751–766
11 Chance, B. and Williams, G. R. (1956) Adv. Enzymol. 17, 65
12 Lee, C. P., Sottocasa, G. L. and Ernster, L. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds.), Vol. 10, pp. 33–35, Academic Press, New York
13 Kröger, A. and Klingenberg, M. (1966) Biochem. J. 344, 317–336
14 Lamprecht, W. and Trautschold, I. (1965) in Methods of Enzymatic Analysis (Bergmyer, H. U., ed.), pp. 543–551, Academic Press, New York
15 Adam, H. (1965) in Methods of Enzymatic Analysis (Bergmyer, H. U., ed.), pp. 573–577, Academic Press, New York
16 Martin, J. B. and Doty, D. M. (1949) Anal. Chem. 21, 965–967
17 Dutton, P. L. (1971) Biochim. Biophys. Acta 226, 63–80
18 Wilson, D. F. and Dutton, P. L. (1970) Biochem. Biophys. Res. Commun. 39, 59–64
19 Dutton, P. L., Wilson, D. F. and Lee, C. P. (1970) Biochemistry 9, 5077–5082

20 Chance, B. (1957) Methods Enzymol. 10, 474
21 Chance, B. and Graham, N. (1971) Rev. Sci. Instr. 42, 941–945
22 Erecińska, M., Oshino, R., Oshino, N. and Chance, B. (1973) Arch. Biochem. Biophys. 157, 431–445
23 Schanbaum, G. R., Bonner, W. D., Storey, B. T. and Bahr, J. T. (1971) Plant Physiol. 47, 124
24 Leigh, Jr., J. S. and Erecińska, M. (1975) Biochim. Biophys. Acta 387, 95–106
25 Wilson, D. F. and Erecińska, M. (1975) Arch. Biochem. Biophys. 167, 116–128
26 Vanderkooi, J. M. and Erecińska, M. (1976) in Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. I, pp. 43–86, Plenum Press, New York
27 Sato, N., Wilson, D. F. and Chance, B. (1971) FEBS Lett. 15, 209–212
28 Sato, N., Wilson, D. F. and Chance, B. (1971) Arch. Biochem. Biophys. 253, 88–97
29 Castor, L. N. and Chance, B. (1959) J. Biol. Chem. 234, 1587–1592
30 Kamen, M. D. and Horio, T. (1970) Annu. Rev. Biochem. 39, 673–700
31 Webster, D. A. and Hackett, D. P. (1966) J. Biol. Chem. 241, 3308–3315
32 Kauffman, H. F. and Van Gelder, B. F. (1973) Biochim. Biophys. Acta 305, 260–267
33 Erickson, R. H., Hooper, A. B. and Terry, K. R. (1972) Biochim. Biophys. Acta 283, 155–166
34 Chance, B. (1953) J. Biol. Chem. 202, 397–406
35 Castor, L. N. and Chance, B. (1959) J. Biol. Chem. 234, 1587–1592