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# Respiratory Pigments of *Crithidia fasciculata*

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Mitochondria were isolated from the heme-requiring insect trypanosomatid, *Crithidia fasciculata*, which had respiratory activity, showed a P/O ratio with succinate of 0.5 to 1.0, and contained 40 to 50% of the heme *a* and heme *c* found in the intact cells. Cytochromes *b*, $c_{555}$, possibly $c_1$, cytochrome oxidase, a carbon monoxide-binding pigment, and flavoproteins were detectable in the spectra of both intact cells and mitochondria. Cytochrome $c_{555}$ is a basic protein that was extracted from cells and mitochondria with salt solutions. The molar ratio of heme *c* to heme *a* was approximately 2:1 in both cells and mitochondria. This organism could possibly serve as a model for studies of the respiratory activity of the pathogenic trypanosomes.

*Crithidia fasciculata* is a trypanosomatid that can be isolated from the intestine and rectum of the mosquito, *Anopheles quadrimaculatus*. This organism requires mitochondrial activity for growth but is apparently unable to synthesize protoporphyrin (17). The organism can be grown in vitro in the presence of either protoporphyrin or heme.

*C. fasciculata* has been reported to have unusual cytochromes (1). Growth and respiration are inhibited by cyanide, although cytochrome oxidase was reported to be absent (17). Cytochromes with absorption maxima at 555 and 530 mμ were detected with a hand spectroscope (17). Toner and Weber (23) were able to detect cytochrome oxidase and cytochrome *b* but not cytochrome *c*. These authors found a P/O ratio with succinate as substrate of 0.7 for a cell-free homogenate of *C. fasciculata*. In this study, evidence is presented which shows that a mitochondrial fraction that contains the cytochromes characteristic of the intact organism has been isolated from *C. fasciculata*.

Since the pathogenic trypanosomes are very difficult to obtain in amounts sufficient for biochemical studies, the related trypanosomatid *C. fasciculata* has been examined. It can be grown in vitro with reasonable yields. The mitochondria of *C. fasciculata* resemble those found in the stumpy form of the pathogenic trypanosomes, both in their morphology (12) and in their response to inhibitors (13).

## MATERIALS AND METHODS

*Organism and conditions of growth. C. fasciculata* (ATCC no. 11745) was obtained from S. Hutner of Haskins Laboratories, New York, N.Y. The protozoa were grown at 25 C in a 22-liter bottle that was inoculated with 200 ml of cells in the logarithmic phase of growth. The culture was stirred with a Teflon magnetic stirrer and gassed with air at a rate of 24 liters/min through a glass sparger. The medium contained 0.5% Trypticase (BBL), 0.5% yeast extract (Difco), 0.01% liver extract concentrate (Nutritional Biochemicals Corp., Cleveland, Ohio), and 0.05 M sucrose. The *p*H was adjusted to 8.0 with KOH. After the medium was autoclaved for 2 hr at 121 C, it was cooled, and protoheme was added to a final concentration of 3.0 μM. The stock solution of protoheme was dissolved in a small amount of pyridine and was diluted with 0.5 M $NH_4OH$ to a final concentration of 30 mM. This solution was kept in the dark at −16 C. The protoheme was added to the medium after autoclaving because experiments showed that during autoclaving 75% of the protoheme was destroyed. The α maximum of the pyridine hemochrome of the heme remaining after autoclaving was shifted from 556 to 553 mμ. Antifoam B (Dow Corning Corp., Midland, Mich.) was used to prevent excessive foaming. The doubling time was 5.0 hr, determined as the time necessary to double the absorbance at 750 mμ (measured in 13-mm test tubes) between absorbancies of 0.01 and 0.5. The protozoa were grown to the late-log phase. This usually required 3 to 4 days.

*Preparation of mitochondria.* Protozoa were harvested by centrifugation at 7,000 × *g* for 10 min and washed with a buffer containing 0.25 M sucrose, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.6. The protozoa were transferred to a cold mortar. Alumina (neutral alumina, AG-7, 100–200 mesh, Bio Rad Laboratories, New York, N.Y.) was added at a ratio of 2 g per g (wet weight) of protozoa. The paste was ground for 2 min, at which time examination of the slurry by phase-contrast microscopy indicated 98 to 100% cell breakage. The mixture was centrifuged at 1,000 × *g* for 10 min to remove the cell debris, whole cells, and alumina. The pellet was

washed twice with the buffer, and all the supernatant liquids were combined. The mitochondria were collected from the supernatant fluids by centrifugation at 16,000 × *g* for 10 min. The mitochondrial pellet was washed twice with the buffer. All the above procedures were performed at 0 to 4 C. The pellet consisted of mitochondria and associated kinetoplasts examined with the electron microscope (G. C. Hill, Ph.D. Thesis, New York Univ., New York, N.Y., 1968). No nuclei were present, but dense bodies which were observed in the intact cell were present.

*Difference spectra.* The difference in absorption between anaerobic and aerobic suspensions of intact cells or mitochondria was measured with the technique developed by Chance (3). A suspension of cells or mitochondria with their respiratory pigments reduced was compared to a portion of the same suspension with the respiratory pigments oxidized. Spectra were obtained with a Cary model 14 CM dual-beam recording spectrophotometer, as described (27). The intact protozoa had endogenous respiration which led to reduction of the respiratory pigments in the oxidized sample. The endogenous reduction of the respiratory pigments was decreased by aerating the suspension of cells for 15 min at 0 C before measurement. The mitochondria did not have significant endogenous respiration. The respiratory pigments in the mitochondria were reduced in the presence of 10 mM succinate. The reduction was complete in 60 min at 25 C. The interaction of carbon monoxide and the reduced respiratory pigments was measured by comparing a suspension of cells with pigments reduced and saturated with carbon monoxide to a part of the same suspension in which the pigments were reduced. Difference spectra were measured at 25 and −190 C. The low-temperature spectra were measured in an apparatus similar to that described by Estabrook (7) and constructed by T. Orr. Absolute spectra were obtained by comparing suspensions of protozoa to antifoam suspensions or ground glass of proper light-scattering properties, as described (19).

*Protein.* Protein was determined colorimetrically with the Folin-Ciocalteu phenol reagent (Harleco Corp, Philadelphia, Pa.) after alkaline hydrolysis (32) with bovine albumin as standard.

*Extraction of cytochrome* $c_{555}$. Cytochrome $c_{555}$ was extracted from mitochondria swollen with hypotonic KCl according to the method of Jacobs and Sanadi (14). The cytochrome solution was dialyzed overnight against 20 mM sodium phosphate buffer, *p*H 8.0, and applied to an Amberlite IRC-50 column previously equilibrated with the phosphate buffer. The cytochrome was eluted from the cation resin with 1.0 M NaCl in 0.02 M phosphate buffer.

Cytochrome $c_{555}$ was extracted from protozoa, essentially as described by Margoliash and Walesek (18). The cells were suspended (40 g, wet weight) in 40.0 ml of 1.5 μM aluminum sulfate, *p*H 4.5, and subjected to ultrasonic vibration for a total of 15 min. This treatment resulted in the rupture of nearly all the cells. The broken cell preparation was centrifuged at 16,000 × *g* for 5 min, and the supernatant liquid was filtered through fluted Whatman no. 588 paper. The temperature was maintained below 5 C during these operations. The *p*H was adjusted to 8.5 with NaOH, the filtration was repeated, and the solution was applied to an Amberlite IRC-50 column. The cytochrome $c_{555}$ was completely reduced as isolated from column chromatography.

*Extraction of the hemes.* Protoheme and heme *a* were extracted from the cells or mitochondria as follows. A mixture of acetone-water-concentrated HCl (50:10:2.5) was added to the protozoa or mitochondria at 0 to 4 C. The suspension was homogenized with a Teflon homogenizer and centrifuged at 1,000 × *g* for 10 min. The cold acid acetone extraction was repeated four times. The pellets were suspended in water, and the heme *c* was determined by preparing the pyridine hemochrome. The supernatant portion of the acid acetone extraction was then extracted with ether in the presence of HCl. The aqueous layer was back extracted with more ether. The combined ether layers containing protoheme and heme *a* were washed with two portions of 0.27 M HCl, and the ether was evaporated to dryness in vacuo. The pyridine hemochrome of the residue was then prepared as described by Falk (8). The concentration of the hemes was measured from the reduced minus oxidized difference spectra of the pyridine hemochromes by a procedure described by Falk (8). Heme *a* was measured as the absorbance increment between the α maximum at 587 mμ and the minimum at 610 mμ with $\epsilon = 21.7 \times 10^3$ (20); heme *c* was measured as the absorbance increment between the α maximum at 553 mμ and the minimum near 535 mμ between the α and β maxima with $\epsilon = 22.0 \times 10^3$ (15); protoheme was measured as the absorbance increment between the α maximum at 556 mμ and the minimum at 540 mμ between the α and β maxima with $\epsilon = 20.7 \times 10^3$ (30).

*Oxidative phosphorylation.* Oxidative phosphorylation was measured with conventional manometric techniques (24). In the determination of oxidative phosphorylation, each vessel contained 2.0 mM adenosine triphosphate (ATP), 40.0 mM glucose, 20.0 mM $HPO_4^=$, 6.67 mM Tris buffer, *p*H 7.4, 250 mM sucrose, 6.67 mM $MgCl_2$, 16.67 mM KF, 0.3 mM EDTA, and 0.5 mg of yeast hexokinase (Type II; Sigma Chemical Co., St. Louis, Mo.). The final volume was 3.0 ml. The flasks contained between 60 and 120 mg of mitochondrial protein. Succinate (10 mM) was tipped in after 15 min of equilibration. Phosphate disappearance in the presence of glucose and hexokinase was used as a measure of ATP formation. Inorganic phosphate was determined by the method of Bartlett (2), as adapted for the Technicon autoanalyzer by R. L. Lester.

*Oxygen utilization.* The initial oxygen uptake of mitochondria or cells was measured poloragraphically in the apparatus described by White (28).

*Materials.* The diethyl ether was washed with acidified ferrous sulfate solution to remove peroxides (8). Other materials were the best grade commercially available. The chicken heart cytochrome *c* was provided by S. K. Chan.

## RESULTS

*Spectra of the protozoa.* The spectrum of the protozoa with the respiratory pigments reduced

by the activity of endogenous metabolism is given in Fig. 1. Cytochrome *b* with an $\alpha$ maximum at 560 m$\mu$ can be detected. The addition of dithionite to the cell suspension produced an 11% increase in the absorbancy increment at 560 m$\mu$. Nearly all the protoheme that could be extracted from the protozoa with acid acetone was accounted for in the cytochrome *b*. Approximately 87% of the 3.8 m$\mu$moles of protoheme per 10 mg of protein could be reduced by the endogenous respiratory activity of the protozoa.

A difference spectrum in which the protozoa with pigments reduced by endogenous metabolism were compared to cells in which the pigments were oxidized by bubbling with air for 15 min at 0 C is illustrated in the lower part of Fig. 1. Approximately 26% of the total enzymatically reducible cytochrome *b* can be detected in the difference spectrum. Cytochrome *b* with maxima at 560, 527, and 430 m$\mu$ can be detected. The $\alpha$ maximum at 604 m$\mu$ and the $\gamma$ maximum at 444 m$\mu$ suggest the presence of cytochromes of the *a* type. When reduced cytochrome oxidase $a_3$ reacts with carbon monoxide, a shift in the $\alpha$ maximum to 590 m$\mu$ and a shift in the $\gamma$ maximum to 430 m$\mu$ occur (16). In the difference spectrum of *C. fasciculata* between cells with pigments reduced and cells with pigments reduced but saturated with carbon monoxide, there was a decrease in the absorbance at 443 m$\mu$ but no detectable increase in absorbance at 430 or 590 m$\mu$.

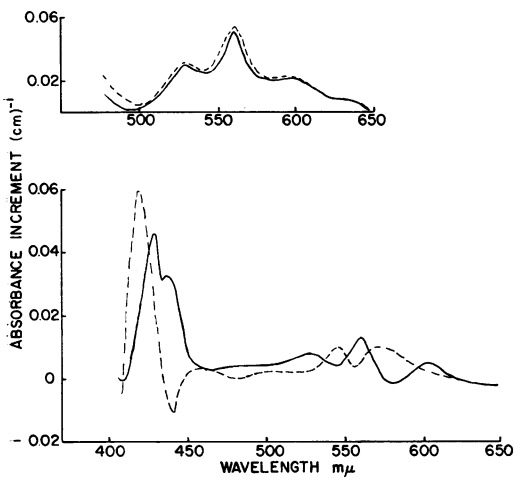


FIG. 1. *Spectra of Crithidia fasciculata. In the upper curve, the straight line represents the absorption of intact protozoa in which respiratory pigments were reduced by the endogenous metabolism of the cells compared to an aqueous suspension of antifoam B of suitable light-scattering properties (29); dashed line indicates the spectrum in which the respiratory pigments were reduced in the presence of dithionite. In the lower part of the figure, straight line represents the difference spectrum between a cell suspension in which respiratory pigments were reduced by endogenous metabolism compared to a portion of the same suspension in which the respiratory pigments were partially oxidized by vigorous aeration for 15 min at 0 C; dashed line represents the difference spectrum of the cell suspension with pigments reduced by endogenous metabolism and saturated with carbon monoxide compared to part of the same suspension in which the respiratory pigments were reduced. The cells were suspended in 0.25 M sucrose, 0.1 mM EDTA, and 0.1 M Tris buffer at pH 7.6 at a density of 10 mg of protein per ml. The temperature was 25 C, and the optical path length of the cuvettes was 10 mm.*

A pigment with absorbance maxima at 570, 540, and 419 m$\mu$ appeared after the reaction with carbon monoxide. This pigment has the spectral characteristics of bacterial cytochrome oxidase *o* (22). Complete reduction of the pigments by the addition of dithionite resulted in a fourfold increase in the pigment resembling cytochrome oxidase *o*. This increase may be due to cytochrome oxidase *o* which is not reduced completely with succinate. It is also possible that the CO-binding pigment is a protohemoprotein complex due to nonspecific binding of hemin in the growth medium.

*Difference spectra of the mitochondria.* Difference spectra of the mitochondria isolated from *C. fasciculata* are illustrated in Fig. 2. In these spectra, the respiratory pigments were allowed to reduce in the presence of 10 mM succinate for

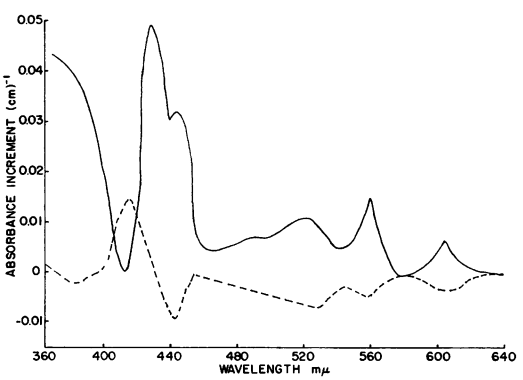


FIG. 2. *Difference spectra of mitochondria of Crithidia fasciculata. Straight line represents the difference spectra of mitochondria with pigments reduced in the presence of 10 mM succinate compared with mitochondria with the pigments oxidized by aeration. No additional reduction of pigments occurred after 60 min in the presence of succinate. Dashed line represents the difference spectrum of mitochondria reduced in the presence of succinate and saturated with carbon monoxide compared with mitochondria reduced in the presence of succinate. The mitochondria were suspended in buffer at a concentration of 1.5 mg of mitochondria-prote in per ml as in Fig. 1.*

60 min at 25 C. Incubation for longer than 60 min did not result in further reduction of the cytochromes. Other substrates such as reduced nicotinamide adenine dinucleotide (NADH), DL-$\alpha$-glycerol phosphate, and pyruvate + malate at 10 mM did not decrease the time required for reduction of the cytochromes or the amounts of the cytochromes that were reduced. Addition of dithionite to the mitochondria resulted in the reduction of 50% more cytochrome, indicating that in mitochondria prepared with alumina from cells grown in 3.0 $\mu$M hemin only half the cytochrome is enzymatically reducible.

There was a marked sharpening of the $\alpha$ absorbance maxima if the spectra were measured at $-190$ C. Figure 3 illustrates the difference spectrum of mitochondria reduced in the presence of succinate minus the absorbance of mitochondria whose pigments were oxidized. In addition to the $\alpha$ maximum of cytochrome *b* at 556 m$\mu$, there was a distinct shoulder near 554 m$\mu$. The shoulder was more prominent in a difference spectrum of mitochondria reduced in the presence of succinate minus mitochondria reduced in the presence of succinate and $10^{-4}$ M antimycin.

*Properties of extracted cytochrome* $c_{555}$. A cytochrome *c* with an $\alpha$ maximum at 555 m$\mu$ could be extracted from both mitochondria and ruptured cells with salt solutions. The cytochrome was adsorbed on Amberlite IRC-50 at *p*H 8.5 and eluted from this resin with 1.0 M NaCl in 0.02 M sodium phosphate buffer, *p*H 8.2. These are properties very similar to those of mammalian cytochrome *c*. The absolute spectrum of the isolated cytochrome $c_{555}$ is illustrated in Fig. 4. There were maxima at 555, 525, and 420 m$\mu$ at 25 C. When measured at $-190$ C, there were maxima at 555 and 551.5 m$\mu$, 531 and 523.5 m$\mu$, and 419 m$\mu$. The pigment showed maxima at 563, 526, and 413 m$\mu$ when oxidized in the presence of $10^{-3}$ M ferricyanide. The reduced pyridine hemochrome of the isolated cytochrome $c_{555}$ had an $\alpha$ maximum at 553 m$\mu$, whereas that of chicken heart cytochrome *c* was 550 m$\mu$. On the basis of the heme content calculated from the reduced pyridine hemochrome of the isolated cytochrome $c_{555}$, the extinction coefficient at 555 m$\mu$ for the reduced cytochrome was $\epsilon = 29.3 \times 10^3$. Assuming a molecular weight of 12,500 and one heme per protein molecule, the cytochrome $c_{555}$ represents 40% of the protein at this stage of purification.

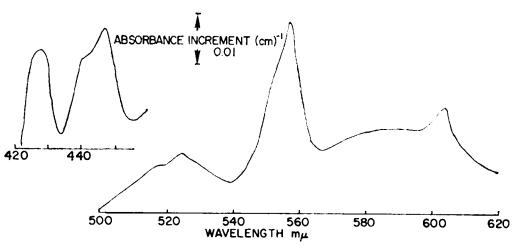


FIG. 3. *Difference spectra of the mitochondria of Crithidia fasciculata measured at $-190$ C. Difference spectra of the mitochondria reduced in the presence of succinate compared with mitochondria oxidized by aeration, whose absorbance is illustrated with the solid line in Fig. 2, were measured in leucite cuvettes with 1-mm optical path at $-190$ C. In Fig. 2 the optical path was 10 mm.*

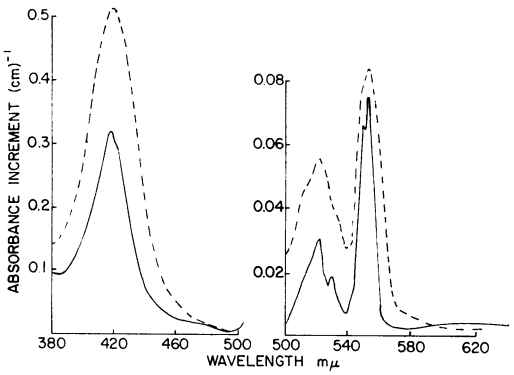


FIG. 4. *Spectra of reduced cytochrome* $c_{555}$ *from Crithidia fasciculata. Upper curve represents the absolute spectrum of the reduced cytochrome* $c_{555}$ *extracted from cells and concentrated by column chromatography as described in Materials and Methods and measured at 25 C. Lower curve represents the absolute spectrum of the same preparation measured at $-190$ C in leucite cuvettes of 1 mm-path length.*

The salt extraction removed approximately 1.2 $\mu$moles of cytochrome $c_{555}$ (measured as heme *c*) per kg (wet weight) of protozoa. About 19% of the total heme *c* of the cells was removed by the salt extraction. The residual cytochrome *c* could represent tightly bound cytochrome $c_{555}$, cytochrome $c_1$, or both.

*Estimation of heme a, heme c, and protoheme.* Hemes that are not covalently bound to the apoproteins can be removed by extraction with acid acetone (8). The protoheme and heme *a* can be extracted with acid acetone from intact cells or mitochondria. Treatment of the protozoa or mitochondria with four portions of acid acetone removed 99% of the total heme *a* and protoheme. The residue after acid acetone extraction contained heme *c* but not detectable heme *a* or protoheme as measured by the pyridine hemochrome. The heme of cytochrome *c* was bound covalently to the apocytochrome by thioester linkages and was not extractable with acid acetone.

The relative proportions of the hemes from three mitochondria preparations and the intact

protozoa are given in Table 1. The ratios of the hemes in both the intact protozoa and the mitochondria were essentially identical.

These protozoa were grown with a protoheme concentration of 3.0 mμmoles of protoheme per ml of medium. With this protoheme concentration there was no limitation of the growth rate of the organism. After a growth cycle during which approximately 0.5 mg of protozoal protein per ml was produced, 38% of the total protoheme in the medium at the start of the experiment was present at the end of the growth cycle. The protozoa incorporated 5.7% of the protoheme present at the start of the experiment of which 4.1% was found in protoheme, 1.0% was found as heme *c*, and 0.6% was found as heme *a*. Approximately 56% of the protoheme present at the start of the experiment could not be detected in the media or the protozoa as heme at the end of the experiment. The fate of the protoheme which has not been accounted for is unknown.

Comparison of the heme *c* and heme *a* concentrations of the intact protozoa and the mitochondria derived from these cells indicates that, after isolation, the mitochondria that were recovered contained 41% of the total heme *a* and 46% of the total heme *c* that was found in the intact cells. The isolated mitochondria contained 23% of the total protein of cells from which the mitochondria were isolated.

*Metabolic activities of the isolated mitochondria.* The intact protozoa have an initial endogenous respiratory rate of 29.4 mμmoles of oxygen per min per mg of protein. The isolated mitochondria have an initial respiratory rate of 10.3 mμmoles of oxygen per min per mg of protein with succinate as the electron donor. Succinate was used as substrate for the measurement of the oxidative phosphorylation activity of the isolated mitochondria. Five experiments were carried out with different mitochondrial preparations. The P/O ratio with succinate as substrate was found to vary from 0.5 to 1.0.

TABLE 1. *Heme a, heme c, and protoheme concentrations in mitochondria and intact cells of Crithidia fasciculata*

| Component | Mitochondria | | | | Intact cells | Ratio[a] | |
|---|---|---|---|---|---|---|---|
| | Expt 1 | Expt 2 | Expt 3 | Avg | | Mitochondria | Intact cells |
| Heme *a*[b] | 0.88 | 0.92 | 0.68 | 0.83 | 0.17 | 1.0 | 1.0 |
| Heme *c* | 1.99 | 2.37 | 1.45 | 1.94 | 0.38 | 2.3 | 1.6 |
| Protoheme | 5.83 | 8.17 | 3.93 | 5.98 | 1.14 | 6.9 | 6.9 |

[a] Ratio indicates the molar proportions of the hemes with heme *a* taken as 1.0.

[b] The concentration of heme *a* and protoheme was determined in the acid acetone extracts of mitochondria as the reduced pyridine hemochromes and expressed as mμmoles of heme/10 mg of protein. The reduced pyridine hemochrome of the residue after the extraction was used to measure heme *c*. The reduced pyridine hemochromes were prepared and measured as described in Materials and Methods.

## DISCUSSION

A significant portion of the electron transport system responsible for the respiratory activity of the heme-requiring trypanosomatid *C. fasciculata* is localized in a fraction which contains mitochondria when examined in the electron microscope (G. C. Hill, Ph.D. Thesis, New York Univ., New York, N. Y., 1968). Evidence for the mitochondrial localization of the respiratory system is as follows: (i) the intact cells and the isolated mitochondria contain the same cytochromes; (ii) these cytochromes are reversibly oxidized and reduced; and (iii) both the intact cells and the isolated mitochondria contain protoheme, heme *c*, and heme *a* in the same molar proportions. Evidence indicates that the present isolation procedures for the mitochondria may damage the electron transport system. The proportion of cytochrome *b* that is not enzymatically reducible increases from 11% in the intact cells to 50% in the mitochondria. When the cells are grown in lower amounts of hemin (e.g. 0.76 to 1.5 μM), the amount of the nonenzymatically reducible pigment at 559 mμ decreases. Although this may be due to a nonsubstrate reducible hemoprotein, it is possible that a portion of the electron transport system is not reduced by succinate or the other electron donors examined in the study. The respiratory rate per mg of protein decreases markedly after the isolation of the mitochondria, and the P/O ratio of the isolated mitochondria is 0.5 to 1.0 and not the expected 2.0 with succinate as the electron donor. About 40 to 50% of the heme *a* and heme *c* in the intact cells are recovered in the mitochondria isolated as described in this study.

Despite previous statements that the respiratory system of these organisms lacks cytochromes $a + a_3$ or cytochrome *c* (1, 23), the mitochondria of *C. fasciculata* have many features in common with the mitochondria of other systems. The level of cytochromes, the respiratory activity, the extractability of cytochrome $c_{555}$ with solutions of high ionic strength, and the ratio of heme *a* and heme *c* are all typical of other mitochondria (4–6, 9, 14, 18, 19, 22, 25). More extractable protoheme

is present in this mitochondrial fraction. A cytochrome *c* with spectra similar to cytochrome $c_{555}$ has been isolated from heterotrophically grown *Euglena gracilis* (31).

Changes in the respiratory apparatus are characteristic of the metamorphosis of the pathogenic trypanosomes (26). The vertebrate forms of the trypanosomes are very difficult to obtain in sufficient quantities for biochemical study. *C. fasciculata* can be grown in vitro in large quantities and forms mitochondria that are similar to the mitochondria of the stumpy form of the pathogenic trypanosomes. Similarities exist in the mitochondrial morphology by electron microscopy (12), in the behavior to inhibitors (13), and in the mitochondrial enzyme activities (26). *C. fasciculata* also forms low levels of the $\alpha$ glycerol phosphate dehydrogenase (C. J. Bacchi, Ph.D. Thesis, Fordham Univ., New York, N.Y., 1968) that is part of the cyanide-insensitive terminal oxidase in the slender form of the pathogenic trypanosomes (10, 11). *C. fasciculata* may serve as an excellent tool for the development of more effective chemotherapy that involves the transition from a cyanide-insensitive to a cyanide-sensitive respiratory system. This transition is essential for the development of infectivity for the vector in the infectious cycle of trypanosomes.

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