# CARBON MONOXIDE-REACTIVE HAEMOPROTEINS IN PARASITIC FLAGELLATE *CRITHIDIA ONCOPELTI*

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## 1. Introduction

*Crithidia* (*Strigomonas*) *oncopelti* is a trypanosomatic flagellate parasitic in the latex of the common milkweed and is transmitted from plant to plant by the bite of hemipterous insects. Respiration of the flagellate is thought to be mediated to a large extent by the cytochrome system [1, 2]; however, a clear understanding of cytochromes linked to mitochondrial terminal oxidase is lacking. Early spectroscopic studies of whole cell suspensions demonstrated the presence of a strong $\alpha$-band at 558 nm which was probably due to a modified *b* or *c* component, a weaker $\beta$-band at 528–530 nm, and an extremely weak band due to *a* at 605 [2]. In 1963, Baernstein [3] noted that the respiratory system of parasitic protozoa including *Crithidia* lacks cytochromes $aa_3$. Hill and Anderson [4] stated that all cyanide-sensitive insect trypanosomatids have a cytochrome system localized in mitochondria similar to that of *Crithidia fasciculata*, in consisting of cytochromes $a, a_3, b, C_{555}$, and cytochrome oxidase *o*. Particulate preparations of the human pathogenic flagellate *Trypanosoma rhodesiense* does not show absorption bands characteristic of cytochrome $aa_3$ [5]. In view of the uncertainty about the presence of cytochrome $aa_3$ and the fact that *C. oncopelti* differs from other members of the trypanosomatidae in having no requirement for haematin (a compound which could give a CO-binding spectrum similar to cytochrome *o*), it became necessary to reinvestigate the cytochrome system of the flagellate with more sensitive methods.

The present spectral study confirms the presence of two different CO-binding pigments, one an $a_3$ type and one an *o* type cytochrome. The results further indicate that cytochrome oxidase *o* may be functioning as the only terminal oxidase during the logarithmic growth phase and later cytochrome $a_3$ is synthesized as a second terminal oxidase in the stationary growth phase.

## 2. Materials and methods

The protozoa were cultivated in a sterile medium containing 2% bacteriological peptone (Oxoid Ltd.), 0.6% NaCl and 0.8% glucose, pH 7.2; incubation was carried out at 25°. The flagellates, either in their logarithmic growth phase or stationary phase, were isolated from culture medium by centrifugation at 1250 *g* for 10 min. The protozoa were washed twice in Krebs-saline and were immediately used for mitochondrial preparation.

The cells were sonicated in 0.3 M sucrose, 24 mM Tris, 1 mM EGTA (ethylene glycol-bis-($\beta$-aminoethyl ether)-tetracetic acid), pH 7.4 for 3 min at 3 A, at which time examination of the slurry by phase-contrast microscopy indicated 95% cell breakage. The sonicated mixture was centrifuged at 1250 *g* for 10 min. The mitochondrial fraction was collected from the supernatant fluid at 15,000 *g* 10 min. The resulting pellet was washed once with the buffer. All operations were performed at 0° to 4°. Standard spectrophotometric methods [6] with a SP 820 spectrophotometer fitted with a potentionetric recorder were used for the analysis of cytochrome systems in the mitochondrial fraction of the protozoa. The mitochondrial fraction was suspended in 100 mM phosphate buffer, pH 7.4.

## 3. Results

Fig. 1 illustrates the difference spectra of the mitochondrial fraction isolated from *C. oncopelti* in early logarithmic growth phase. Cytochromes *b* and *c* having a combined α-maximum at 556 nm with corresponding β and γ maxima at 528 and 430 respectively can be detected. It is worth noting that the presence of cytochromes *c* and *b* have been further clarified by detecting characteristic maxima for pyridine haemochrome *b* (557, 525 and 418.5) in the Acetone-HCl extract and for pyridine haemochrome *e* (551–55, 520 and 415) in the residual fraction of mitochondria or whole cells [7]. The repeated absence of the α-maximum at 604 nm and the γ maximum at 444 nm suggests that the protozoa in their early logarithmic growth phase lack cytochromes of the *a* type. However, a pigment with absorbance maxima at 570, 540 and 418 nm appeared after the reaction with CO. The spectral properties of this pigment resemble that of bacterial cytochrome oxidase *o* [8]. The CO-difference spectrum of the supernatant showed no CO-binding pigments. Also, as the growth medium contained no haemoglobin or methaemoglobin, the possibility of a protohaemoprotein complex capable of binding CO and so give a CO-difference spectrum similar to cytochrome *o* did not exist.

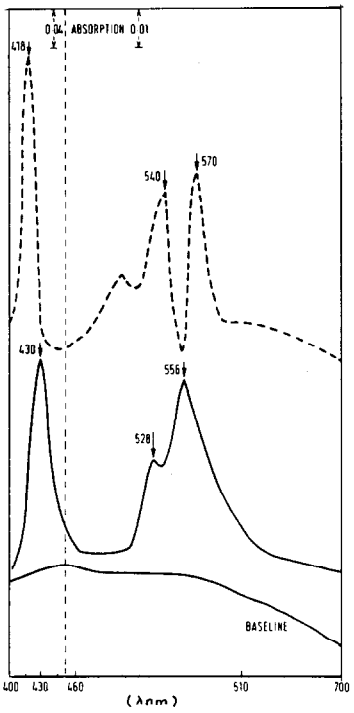


Fig. 1. Difference spectra of mitochondria of *Crithidia oncopelti* harvested in the early logarithmic growth phase. Straight line represents the difference spectra of mitochondria with pigments reduced in the presence of diothionite compared with mitochondria with the pigments oxidized by aeration. Dashed line represents the difference spectrum of mitochondria reduced in the presence of dithionite and saturated with carbon monoxide compared with mitochondria reduced in the presence of diothionite. The temperature was 25°, and the optical path length of the cuvettes was 10 mm. The mitochondria were suspended in buffer at a concentration of 2.0 mg of mitochondrial protein per ml.

The difference spectra of mitochondrial fraction isolated from the protozoa in their stationary growth phase is presented in fig. 2. Unlike fig. 1, a broad peak at 600 nm which could be due to an *a* type cytochrome can be seen in the dithionite difference spectrum. In the CO-difference spectrum, the following two CO-binding pigments are evident: cytochrome $a_3$ with α-peak at 595 nm and a Soret band at 431

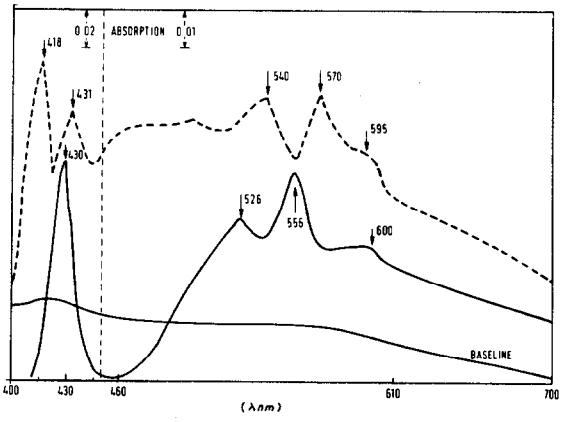


Fig. 2. Difference spectra of mitochondria of *Crithidia oncopelti* harvested in the stationary growth phase. Straight line represents the difference spectra of mitochondria with pigments reduced in the presence of diothionite compared with mitochondria with the pigments oxidized by aeration. Dashed line represents the difference spectrum of mitochondria reduced in the presence of diothionite and saturated with carbon monoxide compared with mitochondria reduced in the presence of diothionite. Experimental conditions as in fig. 1.

nm and cytochrome *o* with peaks at 570, 540 and 418 nm.

## 4. Discussion

The occurrence of cytochrome oxidase $a_3$ in *C. oncopelti* [2] and in another similar protozoa *C. fasciculata* [4] has been reported. Spectral evidence for the presence of cytochrome oxidase *o* in addition to cytochrome oxidase $a_3$ in *C. fasciculata* has also been presented [4]; however, as the growth medium contained haemin, the possibility of any artifact giving CO-maxima at 570, 540 and 418 nm [9] was not resolved. The results presented here clearly indicate that *C. oncopelti* like *C. fasciculata* possesses two CO-binding pigments, one an $a_3$ type and one an *o* type cytochrome. Since no cytochrome $a_3$-CO complex ($\alpha$-maximum at 590–595 nm and $\gamma$-maxima at 430–432 nm) was detected in the mitochondrial fraction prepared from the flagellate harvested during early logarithmic growth phase, it appears that a terminal oxidase linked to cytochrome *o* may be functioning exclusively in the logarithmic growth phase, and later cytochrome $a_3$ is synthesized as a second terminal oxidase in the stationary growth phase. An unusual feature of *C. oncopelti*, however, is the presence of one or two basophilic rod-shaped particles or 'bipolar bodies' which may be endosymbiotic bacteria in the cytoplasm of the flagellate [10]. If the bipolar body is indeed an andosymbiont with considerable synthesis ability then the cytochrome oxidase *o* as observed in both the stationary and logarithmic phases of growth could be attributed to these bacteria-like structures of the protozoa. Although the evidence concerning the possible bacterial nature of the bipolar bodies remains controversial [10, 11], the present spectral evidence for the occurrence of cytochrome oxidase *o* as the only terminal oxidase functioning during the logarithmic growth phase lends credence to the view that cytochrome *o* is indeed intrinsic with the protozoa, and not an artifact from the medium. It should be emphasized, however, that the combination of a cytochrome with CO is not considered definitive evidence that it is a terminal oxidase [12]. Since cytochrome $aa_3$ is missing in the logarithmic growth phase of the protozoa, it is likely that the sole oxidase here may be cytochrome *o*. Recent observation that cytochrome *o* reacts as rapidly with oxygen as does the mammalian cytochrome oxidase $aa_3$ [12] further supports this view. To finally prove that the CO-binding pigment (cytochrome *o*) is functional as a terminal oxidase depends on the determination of a photochemical action spectrum. Until such an action spectrum has been determined the function of cytochrome *o* as a terminal oxidase cannot be firmly established.

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