Terminal Oxidases and Carbon Monoxide-reacting Haemoproteins in the Trypanosomatid, *Crithidia fasciculata*

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SUMMARY

Respiration of *Crithidia fasciculata* was more than 90 % inhibited by 0.5 mM-KCN or by a 19:1, CO:O₂ gas mixture. Low-temperature difference spectra indicated the presence of *a*-, *b*- and *c*-type cytochromes and several CO-reacting haemoproteins. Photochemical action spectra revealed that cytochrome *a*₃ and another component (with an α -band in the CO-liganded state at 570 nm) act as functional terminal oxidases; no evidence was obtained for the presence of a functional cytochrome *o*. Extracts of the organism contain a cyanide-sensitive and CO-insensitive cytochrome *c* peroxidase. The possible identity of the haemoprotein(s) reacting with CO to give a Soret absorption maximum at 418 nm and previously referred to as cytochrome *o* is discussed.

INTRODUCTION

The respiratory system of the trypanosomatid *Crithidia fasciculata*, which is readily cultured and requires haemin, has previously been studied by Kusel & Weber (1968), and by Hill and his co-workers (Hill & White, 1968*a, b*; Hill, Brown & Clark, 1968; Hill & Anderson, 1970). These investigations revealed the presence of *a*-, *b*- and *c*-type cytochromes in this organism together with a CO-binding pigment which was designated cytochrome *o*. Cytochrome *c*₅₅₅ has been purified and characterized from *C. fasciculata* (Kusel, Suriano & Weber, 1969; Hill, Chan & Smith, 1971; Hill, Gutteridge & Mathewson, 1971). Similar cytochromes are also present in *C. oncopelti* (Srivastava, 1971). It has been suggested that, as in some bacteria (Castor & Chance, 1959), the cytochrome *o* of *C. fasciculata* may function as an alternative oxidase to that of the main phosphorylating electron transport chain (Hill, 1972), and this suggestion is of considerable interest in view of the claim that cytochrome *o* may participate as an alternative oxidase in the cyanide-insensitive respiration of *Trypanosoma mega* (Ray & Cross, 1972); alternative oxidases are also present in cyanide-insensitive bloodstream and culture forms of *T. brucei* (Evans & Brown, 1971).

We now report that the respiration of *Crithidia fasciculata* is almost completely sensitive to inhibition by cyanide and CO; that of the CO-binding pigments present, only cytochrome *a*₃ and possibly an unidentified component which is not cytochrome *o* act as functional oxidases; and that a cyanide-sensitive cytochrome *c* peroxidase may also mediate terminal electron transport.

METHODS

Growth and harvesting of the organism. Cultures of *Crithidia fasciculata* were kindly supplied by Dr J. Leaver, Department of Biochemistry, University of Edinburgh. The growth medium contained 2 % (w/v) proteose peptone, 0.1 % liver digest, 1 % (w/v) glycerol, 0.5 % Tween 80 and 0.6 % triethanolamine. Half the triethanolamine and all the other

components were dissolved in a sixth of the final volume of distilled water, heated to 100 °C, and centrifuged at 2000 g for 15 min to remove suspended solids. The supernatant was decanted and 25 mg haemin/l dissolved in the rest of the triethanolamine (made up as a 50 %, v/v, solution) were added. Folic acid dissolved in the minimum volume of 1.0 M-KOH was added to give 2.5 mg/l. The medium was adjusted to pH 8.0 to 8.2 with 2.0 M-HCl, made up to the final volume with distilled water and autoclaved (103 kNm⁻² for 20 min) in 200 ml amounts in 1 l conical flasks or in 6 l amounts in a 14 l Microferm Laboratory Fermentor (New Brunswick Scientific Co., New Jersey, U.S.A.). The organism was maintained in tubes containing 10 ml of this medium or on slopes of medium containing 2 % agar. Growth of organisms was measured by counting in a haemocytometer slide (Thoma, Type B, Hawksley, London). Cultures were inoculated with organisms from the late exponential phase of growth; the initial population was 2×10^6 organisms/ml. Cultures were grown at 29 °C in a rotary orbital shaker (Gallenkamp & Co., London E.C.2) for 48 h at 150 rev./min, or in the Fermentor with a stirring rate of 90 rev./min under forced aeration at 1 l air/l medium/min. Sterile silicone MS antifoam RD (0.05 %) was added to prevent foaming. The population at the stationary phase of growth was 2 to 2.5×10^8 organisms/ml; the doubling time in the exponential phase of growth was 5 to 5.5 h. Except where otherwise stated, cultures were harvested in the late exponential phase of growth when the population was 10^8 organisms/ml. Harvesting was by centrifugation at 4 °C for 10 min at 1500 g ($r_{av.} = 11.6$ cm) in the 6×250 ml rotor of an MSE centrifuge or at 1500 g ($r_{av.} = 24$ cm) in the 6×1 l rotor of an MSE Mistral centrifuge.

Organisms were washed in 20 mM-potassium phosphate buffer (pH 8.0) and finally resuspended to a known density in this buffer.

Preparation of cell-free extracts. Washed organisms were resuspended in a buffer containing 0.25 M-sucrose, 10 mM-tris HCl and 2 mM-MgCl₂ (pH 8.0) and treated with digitonin as described by Kusel & Storey (1972). Disruption was by repeated passages of a 40 % (w/v) suspension of organisms through a Chaikoff Press (Misco, Berkeley, California, U.S.A.; Emanuel & Chaikoff, 1957) until at least 50 % of the organisms were broken. Intact organisms and nuclei were removed by centrifuging at 680 g ($r_{av.} = 7.6$ cm) for 10 min in the 8×50 ml rotor of an MSE 18 centrifuge. The supernatant was termed whole homogenate. All procedures were conducted at 4 °C.

Analytical methods. Polarographic measurements of oxygen uptake by whole-cell suspensions were made with an oxygen electrode (Lloyd & Brookman, 1967). Manometric measurements of respiration were made in a conventional Warburg apparatus (Umbreit, Burris & Stauffer, 1957); flasks were shaken at 120 cycles/min and at 2.5 cm amplitude. Photochemical action spectra were also determined manometrically. The light source was a focused 300 W projector lamp at 25 cm from the Warburg flask; Filtraflex-B-40 interference filters (Balzers Ltd, Berkhamsted, Hertfordshire) were used to obtain light of different wavelengths. Difference spectra of samples at the temperature of liquid N₂ were obtained using a Unicam SP 1800 u.v. spectrophotometer fitted with special cuvettes. These were constructed from aluminium strips and had Perspex windows (path length 2 mm). They were positioned 3 cm from the photomultiplier tube and immersed up to the windows in liquid N₂ contained in a specially constructed Dewar vessel. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin as a standard.

Assays of cytochrome c peroxidase activity. Assays for cytochrome c peroxidase activity were based on the procedure described by Yonetani & Ray (1965). Cytochrome c (30 µM in 50 mM-potassium phosphate buffer, pH 7.0) was reduced by addition of a grain of sodium borohydride. Control experiments showed that extracts of *Crithidia fasciculata* were not

Fig. 1. Inhibition of respiration of *Crithidia fasciculata* by cyanide. Measurements of oxygen uptake were made polarographically on washed suspensions (10^9 organisms/ml) in the absence (a) or presence (b) of glycerol (9 mM). Cultures were harvested at the following concentrations: (▲), 3.2×10^7 organisms/ml (early exponential phase); (△), 1.03×10^8 (late exponential phase); and (●), 1.8×10^8 organisms/ml (stationary phase). Temperature of incubation 30°C .

able to catalyse reoxidation of this cytochrome *c* in the absence of H_2O_2 or ethyl hydrogen peroxide. Ethyl hydrogen peroxide (80 mM) used as substrate gave a non-enzymatic reoxidation of cytochrome *c* and the rates of this reaction ($2.5 \text{ nmol/min/2 ml}$ reaction volume) were subtracted from those obtained in the presence of the extracts.

Chemicals. Proteose peptone was obtained from Difco Laboratories, Detroit, Michigan, U.S.A., liver digest from Oxoid Ltd., London S.E.1, Tween 80 from Koch-Light Laboratories, Colnbrook, Buckinghamshire, and silicone MS antifoam RD from Hopkin & Williams, Chadwell Heath, Essex. Ethyl hydrogen peroxide was from Ferrosan, Malmo, Sweden; haemin (type III equine), cytochrome *c* (type III, horse heart) and digitonin were all from Sigma Chemical Co., Kingston-upon-Thames, Surrey.

RESULTS

Inhibition of respiration by cyanide and CO

Both the endogenous and glycerol-supported respiration of washed, whole organisms of *Crithidia fasciculata* were more than 90 % inhibited by 0.5 mM-KCN (Fig. 1). Respiration in the presence or absence of glycerol was decreased by 50 % when organisms were incubated in manometer flasks containing a 19:1, $\text{N}_2:\text{O}_2$ gas phase (Fig. 2). A 19:1, $\text{CO}:\text{O}_2$ gas mixture gave almost complete inhibition of respiration; partial release of this inhibition was produced by illumination with white light. The extent of inhibition by CO and CN^- under these conditions was similar for organisms harvested at early or late exponential phases of growth, or at stationary phase. Differences were evident in the sensitivities to inhibition by CO in the dark of organisms harvested at these three growth stages (Fig. 3). The extent to which illumination reverses this inhibition by CO depends upon both the relative concentrations of CO and O_2 , and the time of harvesting of cells.

Fig. 2. Inhibition of respiration of *Crithidia fasciculata* by CO. Each manometer flask contained 0.7 mmol KOH in the centre well, 20 mM-potassium phosphate buffer (pH 8.0) and 1.5×10^9 washed organisms from the late exponential phase of growth in the main compartment, and, unless indicated, glycerol (60 mM). Total liquid volume was 3.0 ml, and temperature of incubation 22 °C. Flasks 1 and 2 contained air, flasks 3 and 4 contained a 19:1, N₂:O₂ gas mixture and flasks 5 and 6 contained a 19:1, CO:O₂ gas mixture. Flasks 1, 3 and 6 contained glycerol; flasks 2, 4 and 5 were endogenous controls. Flasks 5 and 6 were illuminated with white light at the point indicated by an arrow.

Photochemical action spectra for the relief of CO inhibition

Respiration rates of organisms under a 19:1, CO:O₂ gas phase were measured during exposure to visible light of different wavelengths (Fig. 4). In the Soret region a single maximum of respiration occurs in the photochemical action spectrum at 426 to 432 nm. The α -band region has a major contribution at 592 nm and a second maximum is evident at around 570 nm. Similar action spectra were given by organisms harvested at different stages of growth.

Cytochromes in low-temperature spectra

Difference spectra of whole cell suspensions were recorded at the temperature of liquid N₂ in the split-beam spectrophotometer. The contents of the reference cuvette were maintained in the oxidized state by the addition of H₂O₂, and the sample cuvette contained a suspension of organisms allowed to become anaerobic in the presence of glycerol. The

Fig. 3. Inhibition of respiration of *Crithidia fasciculata* by $800\ \mu\text{M}$ -CO at different O_2 concentrations in light and dark. Respiration was measured polarographically in the presence of glycerol (9 mM) at 22°C in 20 mM-potassium phosphate buffer (pH 8.0) in the presence and in the absence of CO. Cultures were harvested at the following concentrations: (a) 3.45×10^7 organisms/ml (early exponential phase), (b) 9.4×10^7 organisms/ml (late exponential phase), (c) 2.03×10^8 organisms/ml (stationary phase). (A), Inhibition in the dark; (B), inhibition in white light.

Fig. 4. Photochemical action spectrum for the release of CO-inhibition of respiration of *Crithidia fasciculata*. Oxygen uptake of organisms harvested at late exponential phase of growth (1.08×10^8 organisms/ml) was measured manometrically under light of different wavelengths as described in Methods, in the presence (—○—) and absence (—□—) of 60 mM-glycerol. No correction was made for variation of energy content through the spectrum.

presence of cytochromes $a+a_3$ was indicated by maxima at 602 and 445 nm (Fig. 5). Absorption maxima at 565, 529 and 426 nm are due to b -type cytochrome(s), and those at 555, 552, 519 to 522, and 420 to 422 nm indicate the presence of c -type cytochrome(s). Other maxima are evident at 409, 438 and 513 nm.

Fig. 5. Low-temperature difference spectrum of *Crithidia fasciculata* harvested from the stationary phase of growth. The cuvettes contained 3×10^8 organisms/ml. Reduction was achieved in the presence of 9 mM-glycerol; the oxidized sample contained 100 mM- H_2O_2 . The samples were cooled to 77 °K in cuvettes (path length 2 mm) and the spectral bandwidth was 1 nm.

CO-reacting haemoproteins

A suspension of intact organisms was allowed to become anaerobic in the presence of glycerol in both reference and sample cuvettes, and the contents of the sample cuvette were then sparged with CO for 2 min. A difference spectrum indicated the presence of several CO-reacting haemoproteins (Fig. 6). Reaction of cytochrome a_3 with CO resulted in the formation of absorption minima at 600 and 445 nm; the reduced cytochrome a_3 -CO complex showed absorption maxima at 590 and at 435 nm. Absorption minima at 555 nm (shoulders at 560 and 550 nm) and at 426 to 429 nm indicate the presence of other CO-reacting haemoproteins. Shoulders at 568 to 570 nm, 453 nm and 418 nm result from the absorption

Fig. 6. Low-temperature CO-difference spectrum of *Crithidia fasciculata* harvested from the stationary phase of growth. Both cuvettes initially contained 3×10^9 organisms/ml allowed to become anaerobic in the presence of 9 mM-glycerol; the reference cuvette was bubbled with CO for 2 min, and both cuvettes were immersed in liquid N_2 before recording the absorption spectrum. Spectral bandwidth was 1 nm and the path length 2 mm.

Fig. 7. Inhibition of cytochrome *c* peroxidase in whole homogenate of *Crithidia fasciculata* by CN^- . The assay contained 30 μM -mammalian ferrocytochrome *c* in 50 mM-potassium phosphate buffer (pH 7.0), 1 mM-EDTA, and 80 mM-ethyl hydrogen peroxide together with a range of concentrations of KCN. The reaction was started by the addition of whole homogenate (0.675 mg of protein) and the reaction rate was measured at 550 nm in a total volume of 2.0 ml.

Table 1. Comparison of the CO-difference spectra of *Crithidia fasciculata* with those of haemin-containing growth medium, cytochrome *c* peroxidase, and cytochrome *a*₃

Preparation	Characteristics of the CO-difference spectra (λ_{max} , nm)											
<i>Crithidia fasciculata</i> (glycerol reduced + CO; Minima glycerol reduced, 77 °K)	Maxima	418		435		453	540				570	590
	Minima		426-429		445			550	555	560		600
Haemin-containing growth medium	Maxima	420					540				570	
	Minima								555			
Yeast cytochrome <i>c</i> peroxidase (Yonetani & Ray, 1965)	Maxima		433				542				570	
	Minima			438						560		
Cytochrome <i>a</i> ₃ (Chance, 1953)	Maxima		430									590
	Minima				444							600

of the CO-complexes formed. CO difference spectra of cells harvested at different stages of growth showed similar characteristics.

The presence of cytochrome c peroxidase

Fulton & Spooner (1959) found that extracts of *Crithidia oncopelti* do not catalyse the oxidation of reduced mammalian cytochrome *c* at a measurable rate. Extracts of *C. fasciculata* also show no activity in this conventional assay for cytochrome *c* oxidase activity, but a reaction does occur when ethyl hydrogen peroxide is present. The specific activity of cytochrome *c* peroxidase in whole homogenates was 160 nmol cytochrome *c* oxidized/min/mg protein. The rate of the reaction was not decreased when assays were performed in the presence of CO-saturated buffer, but 0.5 mM-KCN gave 90% inhibition of the enzyme (Fig. 7).

DISCUSSION

The respiration of *Crithidia fasciculata* is almost entirely mediated by cyanide-sensitive oxidases. Organisms incubated under a 19:1 mixture of N₂:O₂ respire at one-half the rate of those in air, and this result indicates the presence of an oxidase, alternative to cytochrome *a*₃, with a low affinity for O₂. Variation in sensitivity to inhibition by CO at different times of harvesting, and differences in the light sensitivity of the CO-inhibited oxidases, suggest that changes in the pathways of terminal oxidation may occur through the phases of growth in a batch culture. However, no significant alterations in CO-binding pigments or in photochemical action spectra of release from CO inhibition were detectable. These action spectra clearly indicate the presence of cytochrome *a*₃ as a functional oxidase, but do not give any support to the view that the haemoprotein showing an absorption minimum at 426 to 429 nm (λ_{max} at 418 nm for the CO-liganded form) plays any role in terminal oxidation. There is evidence, however, for another CO-binding component which contributes both to action spectra and CO difference spectra at approx. 570 nm. If this pigment has a Soret band in action spectra, then we have been unable to resolve it from that of the CO-complex of cytochrome *a*₃. This pigment is clearly different from 'cytochrome *o*' (Hill & Anderson, 1970; Hill, 1972) which shows absorption maxima at 570, 540 and 410 to 420 nm in the CO-liganded state. The spectral characteristics of this so-called cytochrome *o* are evident in the CO difference spectra presented here, but it is suggested that these absorption maxima may have contributions from one or more of the following haemoproteins containing protohaem in a high spin state: (i) it may have a contribution from haemin taken up by the

organisms from the growth medium; (ii) it may arise as a degradation product of cytochrome P-450 (Omura & Sato, 1964); (iii) it may represent a Crithidial haemoglobin; (iv) it might be due to a cytochrome precursor such as protohaem or the product of cytochrome *b* degradation, or (v) it might represent absorption due to cytochrome *c* peroxidase (Yonetani & Ray, 1965). The positions of absorption maxima and minima in CO difference spectra of *C. fasciculata* are compared in Table 1 with those of haemin-containing growth medium, cytochrome *a*₃ (Chance, 1953), and purified yeast cytochrome *c* peroxidase (Yonetani & Ray, 1965). This latter enzyme occurs in extracts of *C. fasciculata*, and accounts for the absorption maximum at 438 nm in reduced-oxidized difference spectra. This haemoprotein can by-pass cytochrome *a*₃ during electron transport in yeast mitochondria (Erecińska, Oshino, Loh & Brocklehurst, 1973). Further work is necessary to establish such a function for cytochrome *c* peroxidase in *C. fasciculata*.

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REFERENCES

- CASTOR, L. N. & CHANCE, B. (1959). Photochemical determinations of the oxidases of bacteria. *Journal of Biological Chemistry* **234**, 1587-1592.
- CHANCE, B. (1953). The carbon monoxide compounds of the cytochrome oxidases. I. Difference spectra. *Journal of Biological Chemistry* **202**, 383-396.
- EMANUEL, C. F. & CHAIKOFF, I. L. (1957). A hydraulic homogenizer for the controlled release of cellular components from various tissues. *Biochimica et biophysica acta* **24**, 254-260.
- ERECIŃSKA, M., OSHINO, N., LOH, P. & BROCKLEHURST, E. (1973). *In vitro* studies on yeast cytochrome *c* peroxidase and its possible functions in the electron transfer and energy-coupling reactions. *Biochimica et biophysica acta* **292**, 1-12.
- EVANS, D. A. & BROWN, R. C. (1971). Cyanide-insensitive culture form of *T. brucei*. *Nature, London* **230**, 251-252.
- FULTON, J. D. & SPOONER, D. F. (1959). Terminal respiration in certain mammalian trypanosomes. *Experimental Parasitology* **8**, 137-162.
- HILL, G. C. (1972). Identification of functional cytochromes in Trypanosomatids. *Journal of Protozoology* **19**, 5A133.
- HILL, G. C. & ANDERSON, W. A. (1970). Electron transport systems and mitochondrial DNA in Trypanosomatidae: a review. *Experimental Parasitology* **28**, 356-380.
- HILL, G. C., BROWN, C. A. & CLARK, M. V. (1968). Structure and function of mitochondria in *Crithidia fasciculata*. *Journal of Protozoology* **15**, 102-109.
- HILL, G. C., CHAN, S. K. & SMITH, L. (1971). Purification and properties of cytochrome *c*₅₅₅ from a protozoon, *Crithidia fasciculata*. *Biochimica et biophysica acta* **253**, 78-87.
- HILL, G. C., GUTTERIDGE, W. E. & MATHEWSON, N. W. (1971). Purification and properties of cytochromes *c* from Trypanosomatids. *Biochimica et biophysica acta* **243**, 225-229.
- HILL, G. C. & WHITE, D. C. (1968*a*). Characterization of the cytochrome system in *Crithidia fasciculata*. *Bacteriological Proceedings* **68**, 138-139.
- HILL, S. C. & WHITE, D. C. (1968*b*). Respiratory pigments of *Crithidia fasciculata*. *Journal of Bacteriology* **95**, 2151-2157.
- KUSEL, J. P. & STOREY, B. T. (1972). Evidence for the presence of two phosphorylation sites in mitochondria isolated from the trypanosomatid hemoflagellate, *Crithidia fasciculata*. *Biochemical and Biophysical Research Communications* **46**, 501-506.
- KUSEL, J. P., SURIANO, J. R. & WEBER, M. M. (1969). Isolation, purification and characterization of *Crithidia fasciculata* cytochrome *c*₅₅₅. *Archives of Biochemistry and Biophysics* **133**, 293-304.
- KUSEL, J. P. & WEBER, M. M. (1968). Enzymatic activities and cytochrome components of the terminal transport pathways of *Crithidia fasciculata*. *Bacteriological Proceedings* **68**, 139.
- LLOYD, D. & BROOKMAN, J. S. G. (1967). An oxygen electrode vessel. *Biotechnology and Bioengineering* **9**, 271-272.

- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- OMURA, T. & SATO, R. (1964). The carbon monoxide-binding pigment of liver microsomes. *Journal of Biological Chemistry* **239**, 2370–2379.
- RAY, S. K. & CROSS, G. A. M. (1972). Branched electron transport chain in *Trypanosoma mega*. *Nature, London* **237**, 174–175.
- SRIVASTAVA, H. K. (1971). Carbon monoxide reactive haemoproteins in parasitic flagellate *Crithidia oncopelti*. *FEBS Letters* **16**, 189–191.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). In *Manometric Techniques*, p. 1. Minneapolis: Burgess.
- YONETANI, T. & RAY, G. S. (1965). Studies on cytochrome *c* peroxidase. I. Purification and some properties. *Journal of Biological Chemistry* **240**, 4503–4508.