Biochimica et Biophysica Acta, 502 (1978) 466—476

BBA 47498

TRICARBOXYLIC ACID CYCLE OPERATION AT THE KINETOPLAST-MITOCHONDRION COMPLEX OF *TRYPANOSOMA CRUZI*

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(Received November 3rd, 1977)

Summary

Culture of *Trypanosoma cruzi* (Tulahuen strain) in the presence of ethidium bromide (1—20 μg/ml) resulted in dyskinetoplasty and inhibition of growth, to an extent depending on the dye concentration and the medium composition. The ethidium bromide-induced dyskinetoplasty caused a decrease of (a) the cytochrome content of epimastigotes (a,a_3 and b species); (b) the rate of respiration (endogenous or supported by D-glucose); and (c) the rate of production of $^{14}CO_2$ from [2-^{14}C]acetate and [1-^{14}C]glucose. [2-^{14}C]Acetate oxidation to $^{14}CO_2$ was affected by dyskinetoplasty more than [1-^{14}C]glucose oxidation, particularly at the exponential growth phase. With dyskinetoplastic epimastigotes, diminution of $^{14}CO_2$ production from [2-^{14}C]acetate largely exceeded that of oxygen uptake, while with [1-^{14}C]glucose, $^{14}CO_2$production and respiration were affected to about the same extent. Dyskinetoplasty also decreased the incorporation of [2-^{14}C]acetate carbon into intermediates of the tricarboxylic acid cycle and related amino acids, and modified the distribution pattern of ^{14}C in accordance with the decrease of respiration. Reduction of cytochrome content of epimastigotes by restriction of heme compounds during growth decreased $^{14}CO_2$ production from [2-^{14}C]acetate, like the ethidium-induced dyskinetoplasty. The same occurred after inhibition of electron transfer by antimycin and cyanide, though to a much more significant extent, thus confirming the functional association of electron transport at the mitochondrial cytochrome system of *T. cruzi* and the enzymatic reactions of the tricarboxylic acid cycle.

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Introduction

The epimastigote or culture forms of *Trypanosoma cruzi*, the agent of Chagas disease, oxidize [2-^{14}C]acetate and [3-^{14}C]pyruvate to $^{14}CO_2$ without a significant increase in overall respiration [1—3]. The oxidation involves, however, the tricarboxylic acid cycle as shown by (a) the incorporation of substrate ^{14}C into the cycle intermediates; (b) the earlier liberation of acetate carboxyl carbon as CO_2; and (c) the characteristic distribution of pyruvate and acetate carbon atoms in aspartic and glutamic acids [3]. In all plant and animal cells so far examined, the mitochondrion is the site of the tricarboxylic acid cycle operation, a process which depends on electron transfer at the mitochondrial cytochrome system. *T. cruzi* epimastigotes possess a unitary mitochondrion with a differentiated DNA-containing region, which is termed the kinetoplast [4—6]. Cytochemical and spectroscopic investigations of *T. cruzi* and other kinetiplastida have shown the presence of mitochondrial cytochromes [7—13] but the kinetoplast function in substrate oxidation reactions has not been established, in all probability because isolation of intact kinetoplast-mitochondrion complexes for metabolic studies is of great practical difficulty [5]. These circumstances prompted us to examine the influence of cytochrome deficiency on the oxidation of [2-^{14}C]acetate to $^{14}CO_2$ in culture forms of *T. cruzi*. This oxidation depends strictly on the tricarboxylic acid cylcle operation [3]. Epimastigotes were made deficient in cytochromes either by culturing in the presence of 3,8-diaminophenyl-5-ethylphenantridinium bromide (ethidium bromide) or by restriction of heme compounds during growth. Ethidium bromide is known to be able to affect mitochondrial (kinetoplast) DNA, producing biochemical and structural alterations (the dyskinetoplastic state) which lead to the loss of the ability to synthesize mitochondrial proteins [14—18]. The results here described confirm that in *T. cruzi* the biosynthesis of cytochromes a,a_3 and b is to a significant extent specified by the mitochondrial (kinetoplast) genome, as in yeast and higher eukaryotes [19,20].

Materials and Methods

Growth of organism. T. cruzi epimastigotes (Tulahuen strain) were grown in Roux bottles on a diphasic medium at 28°C. The solid phase consisted of: agar, 19 g (Bacto); brain-heart infusion, 38 g (Difco); calf-blood, 40 ml; and water, 1 l. The overlay contained: liver extract, 10 g; brain-heart infusion, 28 g; glucose, 10 g; and water, 1 l. 4 days after inoculation, the cells were collected and inoculated in (a) the same diphasic medium supplemented (or not) with blood, or (b) a liquid medium made of NaCl, 9 g; Na_2HPO_4, 7.5 g, KCl, 0.4 g; glucose, 4.0 g; tryptose, 15 g; liver extract, 5 g; yeast extract, 5 g; inactivated calf serum, 50 ml and water, 1 l, supplemented (or not) with hemin (20 mg (Sigma), dissolved in 8 ml 0.1 M NaOH containing 3.7 g triethanolamine hydrochloride). The liquid medium was maintained in a water bath at 30°C with constant shaking. Unless otherwise stated ethidium bromide was added to the medium at the beginning of growth, at the concentration stated in each case. Epimastigotes were harvested after 60 or 140 h culture (late exponential or stationary phase of growth, respectively) and washed

with 0.154 M NaCl as described in ref. 21. The concentration of epimastigote suspensions was estimated by weighing the washed cells after drying at 100—105°C for 24 h. 1 mg (dry weight) contained 4.5 ± 0.81 (S.D.) × 10^7 cells. When the effect of ethidium bromide was assayed in liquid media, cell concentration was determined by measuring absorbance in a photoelectric colorimeter fitted with a blue filter and precalibrated with epimastigote suspensions of known concentration. Cultures were performed in Erlenmeyer flasks provided with a tube directly adaptable to the photocolorimeter. A linear relationship was found between absorbance and cell concentration values [22].

Epimastigote fractionation. Epimastigotes were disrupted with a Polytron blendor and the homogenate was fractionated as described by Kusel and Storey [23]. The $12\,000 \times g - 480 \times g$ fraction is henceforth termed the "kinetoplast fraction".

Chemicals. Ethidium bromide was obtained from Sigma Chemical Co. Radioactive compounds were purchased from the Radiochemical Centre, Amersham, Bucks, England, and unless otherwise stated were diluted with carrier to obtain a specific activity (in cpm/mmol) as follows: [1-^{14}C]glucose, $2.5 \cdot 10^7$ and [2-^{14}C]acetate, $1.2 \cdot 10^7$. Other reagents were as described in refs. 3, 12, 21, 22 and 24.

Assays. Oxygen uptake measurements were made in duplicate. The cells (10—15 mg) were suspended in 3.0 ml of "standard saline solution" made up as follows: 1 vol. 0.22 M Tris · HCl buffer, pH 7.2, in 0.154 M NaCl; 0.2—0.4 vol. 0.08 M phosphate buffer (KH_2PO_4/Na_2HPO_4), pH 7.2; 1 vol. 0.154 M NaCl containing epimastigotes, and 0.154 M KCl to complete the reaction mixture (6 vols.); the final cation concentration was 0.103 M K^+; 0.035 M Tris; 0.051 M Na^+; pH 7.2. All incubations were at 30°C. 0.1 ml of 10 M NaOH (carbonate-free) was placed in the centre well of the Warburg flask (without filter paper) to collect respiratory $^{14}CO_2$, which was precipitated as $Ba^{14}CO_3$. In order to decrease the rate of endogenous respiration the epimastigotes were starved by incubation for 1—2 h before the addition of substrate. Other experimental conditions were as described in ref. 3.

Epimastigotes which had been incubated with the ^{14}C-labeled substrates were centrifuged at 2—4°C and $3000 \times g$ for 10 min and washed twice in the centrifuge with 0.5 ml of 0.154 M KCl. The pellets were thoroughly mixed with 5 ml of an 80% (v/v) methanol/H_2O mixture; 200 µl of methanolic cell suspension were plated in aluminum cups and counted to measure total ^{14}C incorporated by epimastigotes. After centrifuging the cell suspensions for 10 min at $3000 \times g$, 200 µl of the soluble extract was plated and counted. The activity represented the ^{14}C incorporated into the methanol/water-soluble substances. The supernatant fluids (containing the soluble cell fraction) were evaporated to a volume of 0.4—0.8 ml. The residues were chromatographed on paper, the chromatograms radioautographed and radioactive compounds were identified and counted for ^{14}C. The experimental conditions were as described in ref. 3.

Spectrophotometry. The cytochrome spectra of epimastigotes were obtained in a Johnson Research Foundation split-beam spectrophotometer.

Results

Effect of ethidium bromide on growth and cytochrome content of T. cruzi

The sensitivity of hemoflagellates to dyskinetoplasty-inducing drugs varies from strain to strain of a given species [25,26] and it therefore seemed convenient to determine the response of the Tulahuen strain employed to ethidium bromide. Fig. 1 shows typical growth curves in the liquid medium supplemented with hemin, as indicated. As expected, addition of ethidium bromide determined a slower rate of growth and a decrease in the cell density at the stationary phase. Table I summarizes the percentage inhibition of growth as a function of dye concentration, the inhibition values being calculated on the basis of epimastigote concentration after 4 days of culture (near the end of the exponential phase). Generally speaking, variation of medium composition did not affect the response of *T. cruzi* to ethidium bromide, except with the diphasic-blood medium where the drug was less effective. Hemin counteracted in some extent the inhibition of growth by 1.0 μg/ml ethidium bromide but that action could not be noted with 5 and 20 μg/ml. Comparison of the Tulahuen strain sensitivity with those of the Pasteur Institute [14], the Y and Costa Rica strains [26] reveals that the former was relatively less affected by ethidium bromide. The decrease of ethidium bromide effect in the diphasic-blood medium probably depends on dye binding by blood proteins. Epimastigotes cultured in the presence of 5 μg/ml ethidium (stationary growth phase) showed under the fluorescence microscope selective fixation of dye at the kinetoplast [15]; furthermore, electron microscopy of the same material revealed typical dyskinetoplastic lesions [16,27].

Spectral analysis of normal *T. cruzi* epimastigotes in the 650—500 nm range revealed the presence of absorption bands with broad maxima at about 603 nm

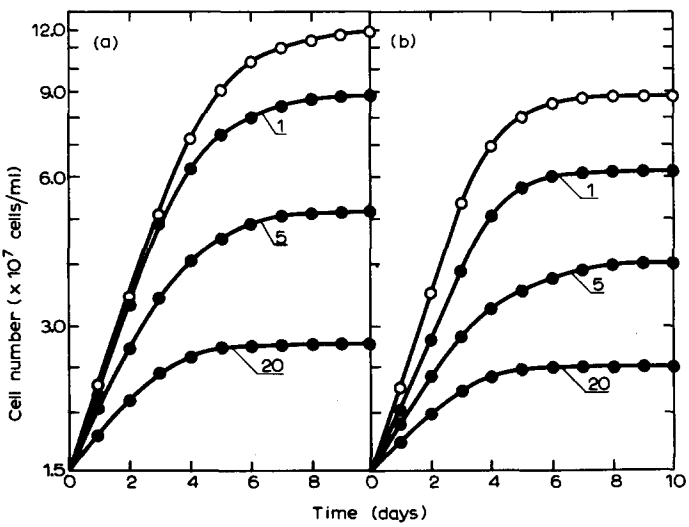

Fig. 1. Effect of ethidium bromide on growth of *T. cruzi*. Liquid medium supplemented (a) or not (b) with hemin. Time of culture was as indicated in the abscissa. The figures near the lines indicate ethidium bromide concentration in μg/ml. Other conditions were as described under Materials and Methods.

TABLE I

EFFECT OF ETHIDIUM BROMIDE ON GROWTH OF *T. CRUZI* (TULAHUEN STRAIN)

Experimental conditions as described in Fig. 1 and under Materials and Methods. The values represent percentage inhibition of growth after 4 days of culture and were calculated from the equation $(n_4 - n_0) - (n_{4EB} - n_0)/(n_4 - n_0)(X100)$ where n_4 and n_{4EB} are the number of cells after 4 days of culture, in absence and presence of ethidium bromide (at the concentration stated below), respectively, and n_0 is the initial number of cells (1.5 ± 0.26) (S.D.) X 10^7/ml).

Ethidium bromide (μg/ml)	Medium			
	Diphasic	Diphasic-blood	Liquid	Liquid-hemin
1	33	18	36	16
5	59	43	66	56
20	77	53	77	77

(henceforth the "a" band), 555 nm (henceforth the "b" band) and 525 nm (Fig. 2). Kinetoplast hemoproteins were partly responsible for the epimastigote absorption, as may be inferred from spectra in Fig. 2 (electron microscopy of the kinetoplast fraction revealed that it was constituted by mitochondrial membranes, flagella and kinetoplast DNA) (micrographs omitted). The epimastigote "a" band was relatively small, but it was always to be observed. It is assumed that the "a" band represented the terminal oxidase (cytochrome a,a_3) while the "b" band in all probability involved cytochrome *b* species [12,13], cytochrome *c*-558 [28], and microsomal hemoproteins [13,29]. Lack of resolution prevented determining the specific contribution of each cytochrome to the "b" band. In this connection it is worth recalling that a similar difficulty was met with by Agosin et al. [13] using more sophisticated

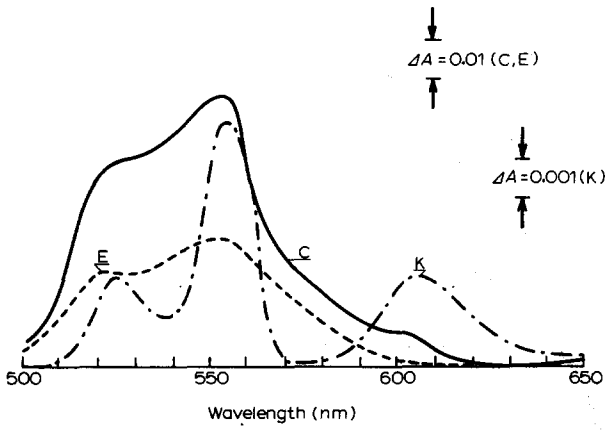

Fig. 2. Dithionite reduced-oxidized spectra of normal and dyskinetoplastic *T. cruzi*. Epimastigotes suspended in glycerol/standard saline solution (30 : 70, v/v) (20 mg cells/ml). C, control epimastigotes cultured in the liquid-hemin medium for 6 days; E, same, cultered in the presence of ethidium bromide (5 μg/ml); K, kinetoplast fraction of normal epimastigotes (6.0 mg protein/ml). The oxidized sample was saturated with oxygen. 5 mm optical path. Other experimental conditions were as described under Materials and Methods.

instrumentation. Nevertheless, the different values of the $\Delta A_{555nm}/\Delta A_{603nm}$ ratio for intact epimastigotes (>9) on the one hand and the kinetoplast fraction on the other (2.5) allows one to infer that the contribution of the extramitochondrial hemoproteins to "b" absorbance was certainly important. This assumption is in good agreement with Agosin et al. [13] observations. Dyskinetoplasty determined significant changes in the epimastigote spectrum, since the "a" band practically disappeared while the "b" band, measured at 555 nm, decreased by about 42%.

Substrate oxidation by dyskinetoplastic and cytochrome-deficient epimastigotes

Epimastigotes cultured in the presence of ethidium bromide were incubated with [2-^{14}C]acetate and examined for (a) oxygen uptake; (b) $^{14}CO_2$ production; (c) total ^{14}C incorporation; and (d) ^{14}C distribution in the methanol/water-soluble extract. In this experiment ethidium bromide was used at a concentration of 5.0 μg/ml, which is well below the concentrations required to inhibit the respiratory chain and uncouple oxidative phosphorylation [30,31]. The results presented in Table II show that dyskinetoplastic epimastigotes, in particular those collected at the stationary phase of growth, oxidized [2-^{14}C]acetate to $^{14}CO_2$ at a slower velocity than the normal ones. Dyskinetoplasty affected $^{14}CO_2$ production more than oxygen uptake, which in good agreement with previous observations [3], was not very different from the endogenous value. Similar results were obtained when [1-^{14}C]glucose was used as substrate, except

TABLE II

OXIDATION AND ASSIMILATION OF ^{14}C-LABELED SUBSTRATES BY NORMAL AND DYSKINETICPLASTIC *T. CRUZI*

Epimastigotes cultured in the liquid-hemin medium for 2.5 days (exponential phase) or 6 days (stationary phase) were suspended in 3.0 ml of standard saline solution and incubated in Warburg manometers for 1 h before substrate addition and for 2 h after substrate addition. Dyskinetoplastic epomastigotes were obtained by culturing in the presence of 5 μg/ml ethidium bromide. Other experimental conditions were as described under Materials and Methods and in ref. 3.

Epimastigotes (growth phase; condition; amount)	Substrate (mM)	Q_{O_2}	^{14}C in respiratory CO_2 (as $BaCO_3$) $\left(\frac{\text{total cpm}}{\text{mg cells}}\right)$
Exponential; normal; 8.3 mg	[2-^{14}C]Acetate (20)	14.0	750
	[1-^{14}C]Glucose (10)	19.5	2320
	None	13.0	—
Exponential; dyskinetoplastic; 10 mg	[2-^{14}C]Acetate (20)	9.4 (33) *	300 (60) *
	[1-^{14}C]Glucose (10)	10.3 (47)	1460 (37)
	None	7.9 (39)	—
Stationary; normal; 15 mg	[2-^{14}C]Acetate (20)	8.5	680
	[1-^{14}C]Glucose (10)	18.0	2770
	None	8.5	—
Stationary; dyskinetoplastic; 11 mg	[2-^{14}C]Acetate (20)	5.4 (36)	140 (79)
	[1-^{14}C]Glucose (10)	6.5 (64)	890 (68)
	None	4.4 (48)	—

* Percentage diminution, as compared with the corresponding control.

TABLE III

DISTRIBUTION OF ^{14}C AFTER OXIDATION OF [2-^{14}C]ACETATE BY NORMAL AND DYSKINETOPLASTIC *T. CRUZI*

Experimental conditions were as described in Table II. After incubation with ^{14}C-labeled substrates, the epimastigotes were taken, washed and analyzed for ^{14}C incorporation and distribution as described under Materials and Methods and in ref. 3.

Epimastigotes (growth phase; condition)	Total ^{14}C incorporated (cpm/mg cells)	^{14}C in the methanol/water-soluble fraction (cpm/mg cells)	Percent distribution of ^{14}C in the methanol/water-soluble substances (% of total)			
			Glutamic acid	Alanine	Succinate acid	Tricarboxylic acids
Exponential						
normal	445	400	52	28	5	2
dyskinetoplastic	240 (46) *	240 (40)	50	27	7	10
Stationary						
normal	300	300	87	11	0	2
dyskinetoplastic	159 (47)	115 (62)	63	7	17	7

* Percentage diminution, as compared with the corresponding control.

that: (a) respiration was increased well above the endogenous level, and consequently its inhibition was more significant than with [2-^{14}C]acetate and (b) with epimastigotes at the exponential growth phase, production of $^{14}CO_2$ from [1-^{14}C]glucose was less affected by dyskinetoplasty than that from [2-^{14}C]acetate, but at the stationary phase, dyskinetoplasty decreased the oxidation of both substrates to about the same extent.

Table III shows the effect of dyskinetoplasty on total incorporation and distribution of [2-^{14}C]acetate. It may be seen that the epimastigotes exposed to ethidium bromide incorporated less ^{14}C than the control ones. With the normal epimastigotes, ^{14}C distribution in the methanol/water-soluble extract was in accordance with that previously described by Boiso and Stoppani [3], and, therefore, presentation of data is now limited to the more strongly labeled compounds, namely glutamic, succinic and the tricarboxylic acids, and also alanine. It should be noted that (a) the combined radioactivity of the two amino acids amounted to more than 50% of the total in the soluble extract; (b) radioactivity in glutamic acid proved the labeling of 2-oxoglutaric acid, while (c) radioactivity in alanine involved the labeling and decarboxylation of L-malic acid (the "malic" enzyme reaction [32]). With epimastigotes at the exponential growth phase, the pattern of ^{14}C distribution was not significantly affected by dyskinetoplasty (except the tricarboxylic acids) but at the stationary phase, dyskinetoplasty decreased the incorporation of ^{14}C into glutamic acid and increased that into succinic and tricarboxylic acids, with little modification in the labeling alanine.

Since dyskinetoplasty produced decrease in the cytochrome content of epimastigotes, it seemed of interest to establish whether limitation of electron transfer was responsible for the reduced rate of substrate oxidation by dyskinetoplastic epimastigotes. In order to reduce the cytochrome content, the heme supplement to the culture media (blood or hemin) was omitted.

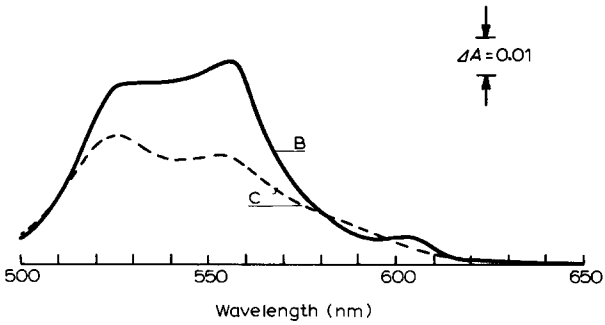

Fig. 3. Dithionite reduced-oxidized spectra of normal and heme-dificient *T. cruzi*. B, epimastigotes cultured for 6 days in the diphasic-blood medium; C, control epimastigotes cultured in the diphasic medium, less blood. Other conditions were as described in Fig. 2 and under Materials and Methods.

With the diphasic medium, exclusion of blood decreased the rate of growth by 25%, while the final epimastigote concentrations decreased to about the same extent (experimental data omitted). The modification of the epimastigote spectrum is illustrated in Fig. 3. It is to be seen that omission of blood determined the almost complete disappearance of the "a" band and a significant reduction in the absorbance of the "b" band. As expected, the cytochrome-deficient epimastigotes oxidized [2-^{14}C]acetate to $^{14}CO_2$ at a slower rate than the normal ones, the diminution of $^{14}CO_2$ production being nearly the same as that of the "b" band absorbance. The decrease of respiration (both endogenous and glucose-stimulated) was, however, relatively smaller (Table IV). Similar results were obtained with epimastigotes cultered in the liquid medium supplemented (or not) with hemin (data not shown).

The observations described above strongly support the functional association of electron transfer at the kinetoplast-mitochondrion cytochromes and the enzymatic reactions of the tricarboxylic acid cycle. This assumption is fully supported by the effect of antimycin and cyanide, two inhibitors of *T. cruzi*

TABLE IV

REDUCED RATE SUBSTRATE OXIDATION BY CYTOCHROME-DEFICIENT *T. CRUZI*

Epimastigotes (12 mg) cultured for 2.5 day in the diphasic medium supplemented (or not) with blood. Incubation for 1 h before substrate addition and 2 h after substrate addition. Other experimental conditions were as described in Table II and under Materials and Methods.

Addition to the culture medium	Substrate (mM)	Q_{O_2}	^{14}C in respiratory $^{14}CO_2$ (as $BaCO_3$) ($\frac{\text{total cpm}}{\text{mg cells}}$)
Blood	[2-^{14}C]Acetate (20)	8.8	480
	D-Glucose (10)	16.8	—
None	[2-^{14}C]Acetate (20)	5.6 (37) *	260 (46)
	D-Glucose (10)	12.0 (28)	—

* Percentage diminution, as compared with the corresponding control.

TABLE V

EFFECT OF ANTIMYCIN AND CYANIDE ON THE OXIDATION OF [2-^{14}C]ACETATE BY NORMAL AND DYSKINETOPLASTIC *T. CRUZI*

Experimental conditions were as described in Table II. Epimastigotes (11.4 mg) cultured for 6 days in the liquid-hemin medium (stationary growth phase); antimycin, 0.3 μg/ml; cyanide, 2 mM.

Epimastigotes	Inhibitor	^{14}C in respiratory $^{14}CO_2$ (as $BaCO_3$) ($\frac{cpm}{mg\ cells}$)
Normal	None	220 —
	Antimycin	6 (97) *
	Cyanide	4 (98)
Dyskinetoplastic	None	130 —
	Antimycin	5 (96)
	Cyanide	6 (95)

* Percentage inhibition, as compared with the corresponding control.

respiration [12], which decreased the oxidation of [2-^{14}C]acetate by more than 95%, either with normal or dyskinetoplastic epimastigotes (Table V).

Discussion

In yeast and higher eukaryotes cytochrome a,a_3 subunits are in part intramitochondrial coded proteins the synthesis of which is inhibited by ethidium bromide [19]. Absence of "a" band in the spectrum of dyskinetoplastic *T. cruzi* (Fig. 2) is, therefore, an important evidence for the identification of the corresponding pigment with an a,a_3-like hemoprotein. Assuming that the ΔE_{mM} value of *T. cruzi* oxidase is about that of the heart muscle cytochrome a,a_3 ($\Delta E_{605-630(mM)} = 20$; ref. 33), the spectrum of the kinetoplast fraction in Fig. 2 allows one to calculate a content of 0.05 nmol oxidase per mg of protein which is a small value, as compared with those of actively respiring systems, for example, pigeon heart [34] and ox-neck muscle mitochondrial membranes [35]. Consequently, in *T. cruzi* epimastigotes, electron transport resulting from the tricarboxylic acid cycle operation could not exceed the limit set by the concentration of hemoprotein carriers, and in order to be oxidized, exogenous substrates must compete with the endogenous ones. The reduction in the content of terminal oxidase, as determined by exposure to ethidium bromide or by heme restriction during growth, would cause a further reduction in the rate of the tricarboxylic acid cycle operation. Under these conditions, the escape of electrons through a branch of the respiratory chain involving cytochrome *o* [11,28] must be very small, since the antimycin/cyanide-insensitive oxidation of [2-^{14}C]acetate to $^{14}CO_2$ was less than 5% of the control value (Table V), in good agreement with the inhibition of oxygen uptake [3,12].

The results in Table II indicate that production of $^{14}CO_2$ from [2-^{14}C]-acetate, whose oxidation depends strictly on the tricarboxylic acid cycle, is a more reliable parameter than oxygen uptake for the detection of alterations in *T. cruzi* mitochondrial respiratory chain. On the other hand, reactions

of the phosphogluconate pathway play an important role for glucose oxidation in the Tulahuen strain of *T. cruzi* [36], and consequently, it is not surprising that oxidation of [1-^{14}C]glucose to $^{14}CO_2$ should be less affected by dyskinetoplasty than [2-^{14}C]acetate oxidation, since the reactions of the phosphogluconate pathway take place in the cytosol. The relatively high rate of $^{14}CO_2$ production from [1-^{14}C]glucose with proliferating, dyskinetoplastic epimastigotes (as compared with the resting ones) (Table II) is easily understood considering the high demand of pentose 5-phosphate for the synthesis of nucleotides during growth. Reoxidation of electron carriers pertaining to the phosphogluconate pathway may contribute to the overall parasite respiration, and to peroxide production as well [37].

The decrease of the "b" band in dyskinetoplastic epimastigotes indicates the presence of cytochrome *b* species in *T. cruzi* respiratory chain, since these cytochrome subunits, unlike cytochromes *c* and c_1 [38], are specified by the mitochondrial genome [20]. It is possible that, in the above-described observations, the reduction of *b* level in the respiratory chain contributed to limit the rate of electron transfer and substrate oxidation reactions.

Manaia and Roitman [39] recently reported observations with *Crithidia fasciculata*, which are consistent with those described in this paper.

Acknowledgments

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (C.O.N.I.C.E.T.), the National Program for the Study of Chagas Disease and the Scientific Office of the American States Organization. J.F.B. and A.O.M.S. are Career Investigators of C.O.N.I.C.E.T. and R.D. is Research Fellow of C.O.N.I.C.E.T.

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