Evidence for three respiratory terminals in "Trypanosoma cruzi" epimastigotes

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Evidence for three respiratory terminals in *Trypanosoma cruzi* epimastigotes

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Summary

The respiration of *T. cruzi* epimastigotes was studied using KCN (1 mM) and NaN_3 (5 mM) as inhibitors and glucose (33 mM) as substrate. NaN_3 inhibits 18% and KCN 58% of the oxygen uptake when *T. cruzi* cultures enter the stationary phase. The azide-inhibition pattern closely follows endogenous respiration pattern throughout the different growth phases. Titration of the oxygen uptake with cyanide and azide, with the results treated as Dixon plots, indicated the presence of: a) one respiratory terminal sensitive to both cyanide and azide; b) another cyanide-sensitive but azide-insensitive; c) a third insensitive to both inhibitors. Ethidium bromide (5 μg/ml) treatment caused the disappearance of the azide-sensitive terminal in the stationary phase.

Key words: *Trypanosoma cruzi;* respiration; ethidium bromide; cyanide; azide; respiratory terminals.

Introduction

The presence of cyanide-insensitive respiration in trypanosomes has been known for some time (von Brand and Tobie, 1947). In bloodstream forms of the *Trypanosoma brucei* group, oxygen consumption insensitive to cyanide is explained by the presence of an α-glycerophosphate system (Grant and Sargent, 1960; Grant et al., 1961). This terminal oxidase is inhibited by hydroxamates in *T. brucei* (Evans and Brown, 1973). In this species the transition of the bloodstream to culture form is followed by the appearance of a cyanide-insensitive oxidase and then by an oxidase which is cyanide-sensitive (Evans and Brown, 1971).

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In *T. mega*, cyanide-insensitive respiration has been associated with cytochrome *o*, which branches at the ubiquinone-cytochrome *b* region (Ray and Cross, 1972). Steady-state kinetics on the oxygen consumption led to the identification of three terminal oxidases in *T. mega* (Hill and Degn, 1977).

In *T. cruzi* it has been suggested that all three forms have the same degree of sensitivity to cyanide (Rogerson and Gutteridge, 1977). The oxidation of succinate in mitochondrial preparations from epimastigotes is partially sensitive to cyanide (Rogerson and Gutteridge, 1977). This inhibition varies with the age of the culture (Felix et al., 1978). The existence of cytochrome *o* has also been proposed to explain the cyanide-insensitive respiration in *T. cruzi* (Hill and Cross, 1973), although its photochemical action spectra has not been identified. Treatment of *T. cruzi* with the dyskinetoplastic agent ethidium bromide has led to the disappearance of the $a + a_3$ band, suggesting the presence of cytochrome *o* as an alternative oxidase terminal (Do Campo et al., 1978).

The present paper describes the effect of KCN and NaN_3 on the respiration of *T. cruzi* and the effect of ethidium bromide treatment on the activity of the two inhibitors.

Material and Methods

Growth of organism – T. cruzi. Y strain (Silva and Nussenzweig, 1953) was maintained in liver infusion-tryptose (LIT) liquid medium (Camargo, 1964) containing 50 U of penicillin G/ml of medium. For large-scale growth 10 ml of inoculum (5.0×10^7 cells/ml) were transferred to 500 ml Erlenmeyer flasks with 100 ml of LIT medium. The flasks were maintained under constant agitation (90 rpm) at 28°C in an incubator-shaker (New Brunswick Scientific Co., Inc., New York, USA). They were sampled for the respiration experiments after the third day of growth.

Ethidium bromide (EBr) treatment. For these experiments EBr (Sigma, St. Louis, Mo., USA) solution (0.5 mg/ml) was sterilized by Millipore filtration and added to the LIT medium to a final concentration of 5 μg/ml (Do Campo et al., 1978), immediately before inoculation. The cells were harvested at the stationary-phase of growth.

Preparation of T. cruzi cells. The cells were harvested by centrifugation at 2000 g for 15 min at 10–20°C in a refrigerated centrifuge (Sorvall RC-5). They were then washed $3 \times$ with 0.9% saline solution, and the final pellet was resuspended in a buffered-saline solution (Sylvester and Krassner, 1976) to give a final number of 10^8 cells/ml. The cells were counted in a Neubauer chamber.

Respiration experiments. Oxygen consumption was monitored polarographically in a Clark-type electrode (YSI oxygen monitor Model 53, Yellow Spring Inc. Co., Ohio, USA). All experiments were carried out at 28°C using 3.0 ml of cell suspension prepared as described previously. Inhibitors were added in a 50 μl sample to give the desired final concentration. The respiratory data were recorded in a 5-min period when there was a linear relationship between time and oxygen consumption. In a standard experiment KCN was added to a final concentration of 1.0 mM, and NaN_3 5.0 mM; at these concentrations both inhibitors showed their maximal inhibition of O_2-uptake (data not shown). Glucose was also added in a 50 μl sample to give a final concentration of 33 mM.
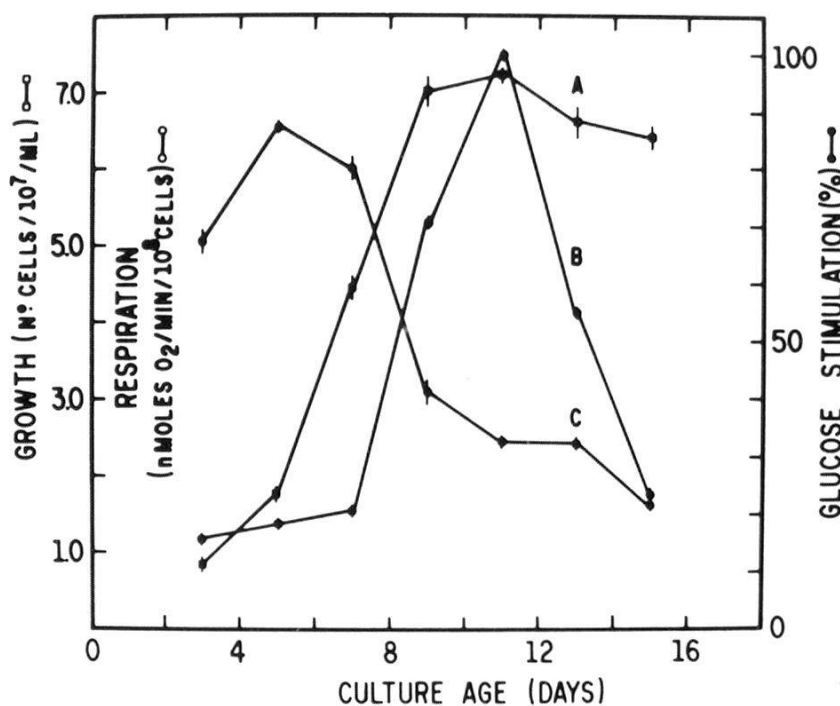
Fig. 1. Endogenous and glucose-stimulated respiration of *T. cruzi* cells as a function of the age of the culture. A typical growth curve for *T. cruzi* in LIT medium is shown (A). The glucose stimulus (B) of the respiration is expressed as the percentage of the endogenous. The endogenous respiration (C) is expressed in nmoles O_2/min/10^8 cells.

Results

Oxygen consumption during the growth curve and the effects of glucose, KCN and NaN_3

The endogenous respiration greatly decreases after the log phase and the respiratory stimulus caused by addition of glucose (33 mM) increases during the log phase of growth and decreases drastically during the stationary phase (Fig. 1). The inhibition pattern of KCN and NaN_3 as a function of the age of the culture is shown in Fig. 2. The azide inhibition (Fig. 2 C) decreases from the late log to the stationary phase at a much higher rate than KCN inhibition (Fig. 2 B). The azide inhibition pattern (Fig. 2 C) follows closely the endogenous respiration pattern shown in Fig. 1 C.

Titration of the oxygen uptake with KCN and NaN_3

Cyanide at saturating concentration inhibits 76% of the respiration at late log-phase (Fig. 3 A) and azide does not have any effect on the cyanide-resistent respiration (Fig. 3 B). Azide inhibits roughly 50% of the oxygen uptake (Fig. 3 C), and after the addition of cyanide a residual respiratory activity (23%), insensitive to both inhibitors, can be seen. These data led us to postulate the existence of three distinct respiratory sites.
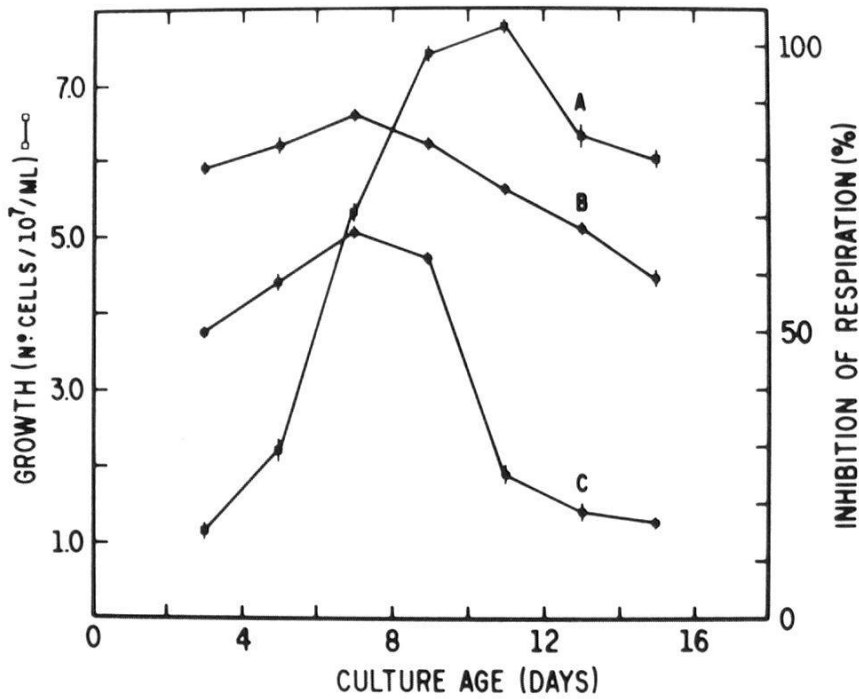
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Fig. 2. Effect of respiratory inhibitors on the glucose-stimulated respiration of *T. cruzi*. The inhibition of KCN (B) and NaN₃ (C) is expressed as percentage of the control-respiration in the absence of inhibitors, during the growth curve (A).
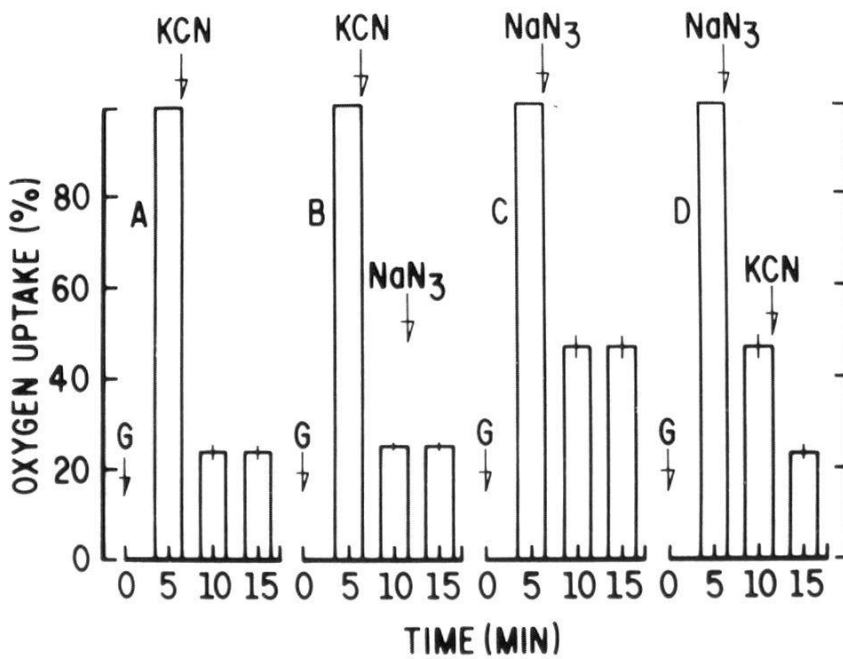

Fig. 3. Titration of oxygen uptake with respiratory inhibitors of late log-phase *T. cruzi* epimastigotes. The arrows indicate the addition of glucose (G), KCN or NaN₃. A. Control for KCN-inhibition. B. Effect of NaN₃ on the KCN-inhibition. C. Control for NaN₃-inhibition. D. Effect of KCN on the NaN₃-inhibition.
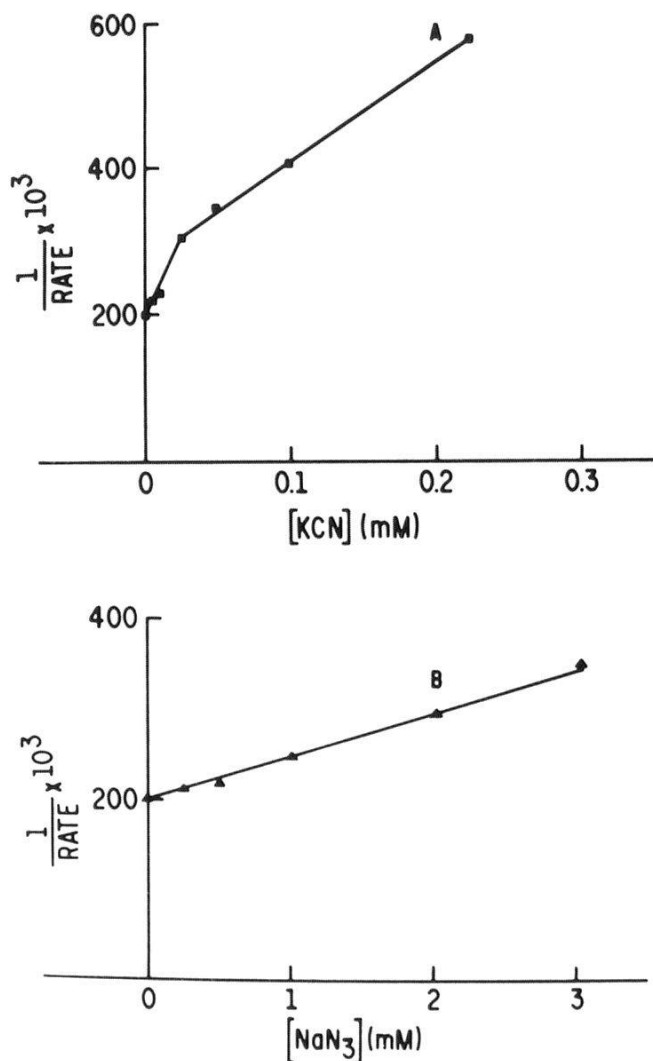
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Fig. 4. Dixon plot for the effect of inhibitors on the respiratory rate of late log-phase *T. cruzi* cells. Glucose was used as substrate and KCN (4A) or NaN_3 (4B) as inhibitors. The rate of oxygen uptake is expressed in nmoles O_2/min/10^8 cells.

To verify this postulate, an experiment with low KCN concentration was carried out, and the data were treated in a Dixon plot (Dixon, 1953). The results shown in Fig. 4 A indicated that *T. cruzi* might have two different inhibitory sites for KCN. When the same experiment was done for azide, the Dixon plot obtained indicated the existence of only one inhibitory site (Fig. 4 B).

Respiration experiments with EBr-treated T. cruzi cells

In the EBr-treated cells the growth was reduced to 60% and the endogenous respiration to 50% of the control (data not shown). The rate of oxygen uptake was the same in both EBr-treated and in the control cells when glucose was used as substrate.

EBr treatment led to the disappearance of the azide sensitive respiratory site (Fig. 5 C). Cyanide inhibited 50% of the respiration of *T. cruzi* (Fig. 5 A, D). Azide had no effect on the residual respiration of KCN (Fig. 5 B).
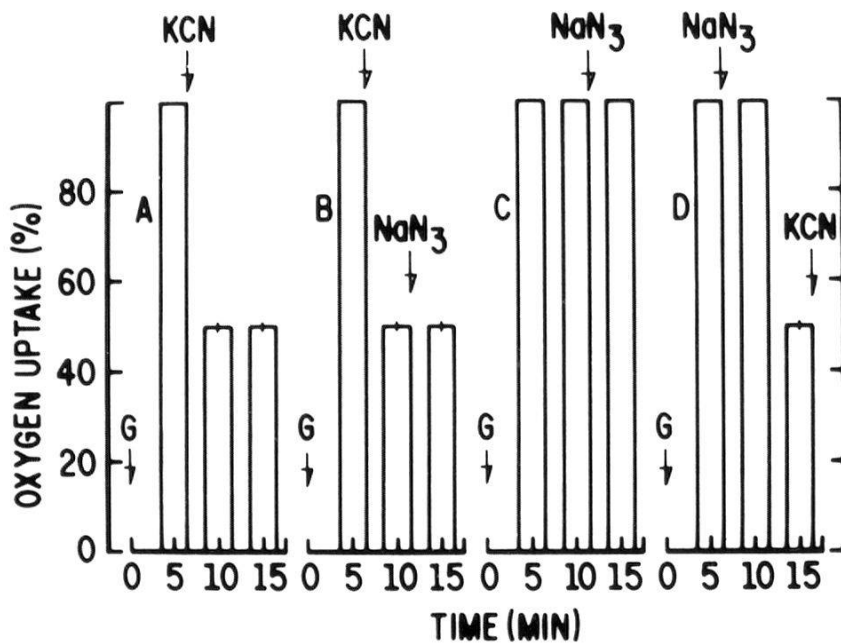
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Fig. 5. Titration of oxygen uptake with respiratory inhibitors of stationary-phase *T. cruzi* cells treated with EBr. The arrows indicate the addition of glucose (G), KCN or NaN_3. A. Control for KCN-inhibition. B. Effect of NaN_3 on the KCN-inhibition. C. Control for NaN_3-inhibition. D. Effect of KCN on the NaN_3-inhibition.

Discussion

The results shown in Fig. 1 and Fig. 2 indicate that the sensitivity to KCN and NaN_3 inhibition is somehow correlated with the age of *T. cruzi* cells. At the stage when the cells make less use of glucose for their respiration the main respiratory chain could be either reduced in concentration or activity. This conclusion is based on the extremely low respiratory stimulus caused by glucose after 15 days of growth (Fig. 1) and the low inhibition by NaN_3 at the same growth stage (Fig. 2). The difference in effectiveness of NaN_3 and KCN as inhibitors of the respiratory chain has already been reported in *Trypanosomatidae* (Hill, 1976). This difference varies with the age of the culture in *T. cruzi* epimastigotes as reported in the present paper. The changes observed in the inhibitory pattern for KCN and NaN_3 (Fig. 2) suggest the existence of different respiratory terminals. The titration of the oxygen uptake by *T. cruzi* cells at late log-phase (Fig. 3) and the Dixon plots at low concentrations of KCN and NaN_3 (Fig. 4) reveal the presence of at least three different respiratory terminals: a) one sensitive to both cyanide and azide; b) another cyanide-sensitive but azide-insensitive; c) a third insensitive to both inhibitors.

The presence of two oxidases in damaged cells of *T. mega* has been proposed based on spectral evidence and biphasic curves obtained in Dixon plots for inhibition of the respiration by KCN, NaN_3 and antimycin (Ray and Cross, 1972). Steady-state kinetics data have revealed the presence of three terminal oxidases in intact cells of *T. mega:* a) an azide-sensitive, which is sensitive to

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salicylhydroxamic acid (SHAM); b) an azide-sensitive, SHAM-insensitive, which is inhibited by CO; c) a third oxidase, insensitive to both azide and SHAM, but inhibited by CO, which was postulated to be cytochrome o (Hill and Degn, 1977).

Our data suggested the presence of only one oxidase sensitive to NaN_3 (Fig. 4) which disappeared in the EBr-treated cells (Fig. 5 B–C). This oxidase in *T. cruzi* has been described as the cytochrome $a+a_3$ system (Ryley, 1956). Furthermore, the treatment of *T. cruzi* epimastigotes with EBr has led to the disappearance of the spectral bands of the $a+a_3$ oxidase (Do Campo et al., 1978). Similar results have been obtained in dyskinetoplastic *C. fasciculata* (Hill and Anderson, 1969).

The oxidase more resistant to KCN inhibition (Fig. 4) is shown in our experiments to be insensitive to NaN_3 (Fig. 5 A–D). These data are in agreement with the presence of cytochrome o in *T. cruzi* (Hill and Cross, 1973). The presence of cytochrome o has been demonstrated in *T. mega*, *Blastocrithidia culicis* and *Leishmania tarentolae* based on action spectra data for the photo-chemical relief of CO-inhibition (Kronick and Hill, 1974). To our knowledge there is no similar evidence for *cyt o* in *T. cruzi*.

The third oxidase, which is insensitive to both NaN_3 and KCN, could be associated with the α-glycerophosphate (α-GP) system. In *T. brucei* this system is insensitive to KCN, NaN_3 and antimycin (Grant and Sargent, 1960; Grant et al., 1961) and it has two components, a dehydrogenase and an oxidase; the latter is of mitochondrial nature (Opperdoes and Borst, 1976). The dehydrogenase is inhibited by SHAM and the oxidase by suramin (Bowman and Fairlamb, 1976).

It has been shown that in *T. cruzi* epimastigotes, trypomastigotes and amastigotes, the α-GP oxidase activity is associated with the mitochondrial preparation, inhibited by suramin and insensitive to SHAM (Gutteridge and Rogerson, 1979). The dehydrogenase component was not detected in *T. cruzi*, but crude mitochondrial preparations presented a respiration sensitive to SHAM at the exponential phase of growth when succinate was used as substrate (Felix et al., 1978).

The existence of an α-GP oxidase directly associated with the main respiratory chain has been postulated, with evolutionary implications (Gutteridge and Rogerson, 1979). It is also possible that in *T. cruzi* there is a weak coupling of the two components of the α-GP system, which are maintained together during extraction depending on the procedure used. Nonetheless the question still remains as to the nature of the respiration of *T. cruzi* insensitive to KCN and NaN_3.

The physiological reason for the existence of different respiratory terminals in *T. cruzi* could be explained based on the need of the parasite to adapt to different environmental conditions (insect-gut, blood and animal tissues). In culture we observed a reduction in concentration or activity of the NaN_3-sensi-

tive terminal which is followed by the decrease in the utilization of glucose as substrate (Fig. 2). This might be followed by a shift of the oxygen consumption to a terminal more resistent to KCN inhibition (*cyt o*?). If that is the case, this shift could be related to the PO_2 of the medium and/or to the accumulation of organic acids. It has been shown, for instance, that succinic and malic acid are excreted in *T. cruzi* culture (Boiso and Stoppani, 1973). At low PO_2 it is assumed that a respiratory terminal with high affinity for O_2 should be more efficient. In *T. mega*, the *cyt o* has a $Km_{(O_2)} = 0.143$ M whereas the $Km_{(O_2)} = 0.350$ M for the $a + a_3$ oxidase (Hill, 1976). Our data suggest that the change in inhibition pattern of *T. cruzi* during the growth curve might have adaptive implications.

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