The importance of a functional Krebs cycle for energy generation in the procyclic stage of *Trypanosoma brucei* was investigated under physiological conditions during logarithmic phase growth of a pleomorphic parasite strain. Wild type procyclic cells and mutants with targeted deletion of the gene coding for aconitase were derived by synchronous *in vitro* differentiation from wild type and mutant (*Δaco*):NEO/Δaco::HYG) bloodstream stage parasites, respectively, where aconitase is not expressed and is dispensable. No differences in intracellular levels of glycolytic and Krebs cycle intermediates were found in procyclic wild type and mutant cells, except for citrate that accumulated up to 90-fold in the mutants, confirming the absence of aconitase activity. Surprisingly, deletion of aconitase did not change differentiation nor the growth rate or the intracellular ATP/ADP ratio in those cells. Metabolic studies using radioactively labeled substrates and NMR analysis demonstrated that glucose and proline were not degraded via the Krebs cycle to CO₂. Instead, glucose was degraded to succinate. Importantly, there was absolutely no difference in the metabolic products released by wild type and aconitase knockout parasites, and both were for survival strictly dependent on respiration via the mitochondrial electron transport chain. Hence, although the Krebs cycle enzymes are present, procyclic *T. brucei* do not use Krebs cycle activity for energy generation, but the mitochondrial respiratory chain is essential for survival and growth. We therefore propose a revised model of the energy metabolism of procyclic *T. brucei*.

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bloodstream form T. brucei, procyclic form cells can also use amino acids, most notably proline, for the generation of energy (9).

To investigate the contribution of the Krebs cycle to carbon fluxes in the energy metabolism of procyclic trypanosomes, we studied the degradation of glucose and proline by a combined genetic and biochemical approach that was stimulated by our analysis of a targeted deletion of the single aconitase gene of T. brucei (19, 20). We noticed that procyclic trypanosomes devoid of any aconitate activity were viable and able to proliferate. To assess the Krebs cycle contribution to energy production in the normal procyclic form trypanosome, ideally a complete but conditional repression of a key enzyme would be most informative. Conditional repression of genes by RNA interference has become extremely popular but suffers from a variable degree of repression and often leakiness, complicating metabolic studies. Targeted gene deletion of a key enzyme, on the other side, may lead to selection of compensatory epigenetic changes. Here we have targeted the aconitate gene in the bloodstream stage of the parasite where the encoded enzyme is only marginally expressed and is dispensable for growth (19, 20). To be able to induce rapid and synchronous differentiation to the procyclic stage, we knocked out aconitate in a pleomorphic T. brucei strain for which we recently developed a gene transfer and selection procedure (21). Thus, we have constructed isogenic strains of procyclic form trypanosomes with and without expression of aconitate activity.

Our investigations showed that deletion of the aconitate gene had no effect on differentiation and growth in culture of procyclic T. brucei. Further analysis of the metabolism of glucose and proline by procyclic trypanosomes led us to conclude that under the conditions studied, these substrates were not degraded by Krebs cycle activity. In addition, no metabolic or growth differences could be observed between wild type and knockout strains, which confirmed that Krebs cycle activity is not used for the generation of energy in procyclic T. brucei cells.

EXPERIMENTAL PROCEDURES

Culture and Differentiation of Trypanosomes—The pleomorphic strain AnTat1.1 of T. brucei brucei was used in this study (for references see Ref. 21). Bloodstream form parasites were grown either on agarose plates (21, 22) or in HMI9 medium supplemented with 15% heat-inactivated fetal calf serum and 1.1% methylecellulose (MethcelMC 3000–5000 mPa.s. Fluka) (23). The cells were counted with a Neubauer hemocytometer (depth, 0.01 mm). In vitro differentiation to the procyclic stage (transformation) was initiated as described before (21). Procyclic cultures were maintained in SDM-79 supplemented with 2.0% (v/v) heat-inactivated fetal bovine serum (2). Growth was monitored in vitro, and the transformation rate at 37 °C was determined using a spectrophotometer thermostated at 25 °C according to the UV method (27). The reaction buffer 50 mM NaHPO4/Na2HPO4 (pH 7.4) was supplemented with 30 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) or trypanosome dilution buffer (5 mM KCl, 80 mM NaCl, 1 mM MgSO4, 20 mM NaHPO4, 2 mM Na2HPO4, 20 mM glucose, pH 7.7), resuspended in 0.5 ml of 50 mM Tris/HCl, pH 7.4, 10 mM N-isocitrate, and cracked by three cycles of freeze-thaw lysis. The lysate was homogenized by several passages through a 0.6-mm syringe and then diluted 1:2 with stock solutions of cysteine and sucrose (final concentrations, 10 and 250 mM, respectively). The extract was centrifuged (20,000 × g for 10 min at 4 °C), and the supernatant containing 1–5 mg/ml protein (26) was used for the determination of enzymatic activity. Part of the extract was treated 10 min at 4 °C with Fe3+ by adding Fe3+-loading buffer (0.5 mM FeCl3, 10 mM diethylthiocarbamate, 50 mM FeCl2, 50 mM FeCl3, pH 7.4), and the reaction was stopped by the addition of saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) was supplemented with 30 mM N-isocitrate as substrate. The reaction was started with protein extract, and the increase in absorbance at 240 nm was followed for 6 min. The specific activity was defined as the formation of nmol product min−1 mg−1 protein using an absorption coefficient of 3.4 mM−1 cm−1 (27). α-Ketoglutarate dehydrogenase activity was determined by monitoring the initial rate of NAD+ reduction at 340 nm. The reaction mixture contained 150 mM Tris/HCl, pH 7.4, 3 mM cysteine, 0.2 mM coenzyme A, 10 mM α-ketoglutarate, 4 mM NAD+, and protein extract. The reaction was started by adding NAD+ simultaneously. Isocitrate dehydrogenase activity was determined by monitoring the rate of NADP+ reduction at 340 nm. The reaction mixture contained 50 mM Tris/HCl, pH 7.4, 1.4 mM MnSO4, 5 mM N-isocitrate, 0.5 mM NADP+, and protein extract. The reaction was started by adding protein extract and NADP+ simultaneously. Glutamate dehydrogenase activity was measured by monitoring the initial rate of NADH oxidation at 340 nm. The reaction mixture contained 50 mM Tris/HCl, pH 7.4, 50 mM NaCl, 10 mM α-ketoglutarate, 0.2 mM NADH, and protein extract. The reaction was started by adding the protein extract and NADH simultaneously. Non-specific NADH oxidase activity was not detected in the same assay and isozyme A was tested without NaCl and α-ketoglutarate, and hence correction was not required.

Microscale Determination of Metabolite Concentrations by HPLC—Established procyclic form cells in mid-log growth phase were harvested and washed twice in trypanosome dilution buffer. The pellets were frozen in liquid nitrogen and stored at −80 °C. Metabolite concentrations were determined using the HPLC method with exchange columns (Dionex AS-11 and AG-11) and dual (conductivity and UV) detection (28). Briefly, the trypanosomes were homogenized in 0.5 ml of 60% acetonitril (v/v) using a Braun Dismembrator (B. Braun, Melsungen, Germany) cooled with liquid nitrogen. After thawing, the homogenate was centrifuged twice for 10 min at 10,000 × g in a cooled benchtop centrifuge at 0 °C. 5 ml of the supernatant was afterward diluted 1:100 using a vacuum centrifuge and resuspended in 300 ml of ice-cold water with a specific resistance of 18 mΩ or greater. After filtration through 0.22-μm syringe filters (Gelman Acrodisc), the analyte was injected into the HPLC system after appropriate dilution. The metab-
olite contents were normalized to total cellular protein content that was assessed from the redissolved pellet (in 90 °C NaOH) according to standard methods (26).

Metabolic Incubations—Incubations (5 × 10⁶ cells/ml) were carried out for 17 h at 27 °C in sealed 25-ml Erlenmeyer flasks, containing 5 ml of SDM-79 incubation medium. The incubations were performed after the addition of either 5 μCi of 2-[U-¹³C]glucose (20.7 GBq/mmol) or 5 μCi of L-¹³C-glucose (9.47 GBq/mmol) (both Amersham Biosciences). This implies that all of the incubations were performed in complete SDM-79 medium containing both glucose and proline with only one of these two radioactively labeled at a time. Incubations were terminated by addition of 40 μl of 6 M HCL to lower the pH to 3.5. Preceding acidification, 0.1 ml of 1 N NaHCO₃ was added through the rubber stopper, and the flasks were placed on ice. Immediately after acidification, the incubation flasks were flushed with nitrogen for 90 min at 0 °C. In this way all carbon dioxide is removed, whereas acetate remains in the incubation medium. The carbon dioxide was trapped in a series of four scintillation vials, each filled with 1 ml of 0.3 m NaOH and 15 ml of scintillation fluid (29). The radioactivity in this fraction was counted in Tritosil modified according to the method described by Pande (30). After removal of carbon dioxide, the acidified supernatant was separated from the cells by centrifugation (4 °C for 10 min at 500 × g) and neutralized by the addition of 40 μl of 6 N NaOH. Analysis of the labeled end products occurred by anion-exchange chromatography on a Dowex 1X8, 100–200 mesh column (Serva) (60 × 1.1 cm) in chloride form (31). The column was eluted successively with 200 ml of 5 mM HCl, 130 ml of 0.2 mM NaCl, and 130 ml of 0.5 mM NaCl. The fractions were collected and counted with 2 ml of Lumac LCS in a scintillation counter. All of the values were corrected for blank incubations. Labeled end products were identified by their Rₚ values. The identity of the major end products produced in the [¹³C]glucose incubations was checked by [¹³C] NMR spectroscopy. For that purpose both wild type and aco::NEO::aco::HYG procyclic trypanosomes were incubated in the same medium as described above, except now all of the glucose normally present in SDM-79 (10 mM) was replaced by 2-[U-¹³C]glucose (10 mM) (Sigma). [¹³C] NMR spectra of the incubation media were measured using a GARP sequence (globally optimized alternating phase rectangular pulses) for ¹H decoupling and a 2-s repetition time (32). A total of 30,000 scans was acquired using a spectral window of 31250 Hz and 32,000 data points after a 90° pulse of 10 μs. Chemical shifts were measured with respect to C-1 of β-glucose at 96.6 ppm relative to tetramethylsilane at 0 ppm. [¹³C] NMR spectra of succinate were also simulated, using the computer program NMRsim of the Aurelia software package of Bruker. Glucose was assayed enzymatically using a standard procedure (33), and the protein level was determined with the Lowry method, using bovine serum albumin obtained from Roche Molecular Biochemicals, defatted with active carbon, and dialyzed before use, as standard (34).

RESULTS

Genetic Ablation of Krebs Cycle Activity in Procyclic T. brucei—Our strategy to investigate the metabolic role of the Krebs
cycle in insect stage T. brucei included targeted deletion of the single gene encoding aconitase (20), which had been shown to be nonessential in the bloodstream stage of the parasite (19). Induced differentiation of the aconitase-deficient lines into the procyclic stage indicated that, in contrast to our expectation, aconitase was dispensable for growth of procyclic trypanosomes (not shown). However, inefficient differentiation of the monomorphic parental line introduced a selection step and did not provide us with mutant procyclic long term cultures for biochemical analysis of this phenotype. Therefore, homozygous deletion of \( ACO \) was performed in bloodstream form \( T. brucei \) of the pleomorphic strain AnTat1.1 that can be triggered to differentiate to the procyclic form in vitro with high efficiency and fast kinetics. Locus-specific targeting vector integration and deletion of both alleles of \( ACO \) in three independent double drug-resistant lines are documented in Fig. 1 (A and B). Cultures of \( aco::NEO:aco::HYG \) clones and wild type AnTat1.1 were then subjected to a standard in vitro differentiation protocol (21). Surprisingly, deletion of \( ACO \) did not alter the change in the availability of Krebs cycle intermediates, which confirms the kinetics of differentiation, providing a strong argument against selection, and did not change the growth rate of the resulting procyclic populations (Fig. 2). Absence of aconitase in these procyclic cultures was confirmed by Western blotting (Fig. 1C) and activity measurements (Table I). Total aconitase activity was reproducibly below the sensitivity of the spectrophotometric assay in whole cell extracts of several independent \( aco::NEO:aco::HYG \) clones (Table I and data not shown). In contrast, enzymatic assays for activities of isocitrate dehydrogenase, glutamate dehydrogenase, and \( \alpha \)-ketoglutarate dehydrogenase did not reveal any significant difference between the constitutive transgenics of \( ACO \) and two independent \( aco::NEO:aco::HYG \) clones (Table I).

**Metabolite Concentrations in Wild Type and \( aco::NEO/aco::HYG \) Procyclic T. brucei**—A microscale HPLC separation of the metabolome of wild type AnTat1.1 and two independent \( aco::NEO/aco::HYG \) clones was used to simultaneously quantitate the intracellular levels of Krebs cycle intermediates (citrate, isocitrate, \( \alpha \)-ketoglutarate, succinate, fumarate, and malate), of glycolytic intermediates (glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-biphosphate, fructose 2,6-bisphosphate, glyceraldehyde 3-phosphate, 2,3 bisphosphoglycerate, 2,3-phosphoglycerate, and phosphoenolpyruvate), and of ATP, ADP, AMP, and \( \text{P}_i \) (Table II). The citrate concentration was greatly increased (60–90-fold) in the aconitase knockout clones (Table II), confirming a complete block of the aconitase reaction. In contrast, the concentrations of all other Krebs cycle metabolites and glycolytic intermediates were not significantly different in wild type procyclic cells and \( aco::NEO/aco::HYG \) clones.

**End Products of Glucose Breakdown**—To investigate the role of the Krebs cycle in the glucose metabolism of procyclic \( T. brucei \), we performed radioactive incubations using \( [6-^{14}\text{C}]\text{glucose} \). Table III shows the end product pattern of radioactive glucose breakdown. The result shows an almost equimolar production of acetate and succinate for both the wild type and the \( aco::NEO/aco::HYG \) clone. Only a very limited amount of the glucose (−1%) was broken down to labeled \( \text{CO}_2 \). Because we used \( [6-^{14}\text{C}]\text{glucose} \), which only results in labeled \( \text{CO}_2 \) production when pyruvate is further degraded via the full Krebs cycle but not during the formation of acetate, this indicates that Krebs cycle activity is negligible. Furthermore, the very limited amount of labeled carbon dioxide detected upon \( [6-^{14}\text{C}]\text{glucose} \) incubations with wild type cells was equal to the amount detected in the incubations with the aconitase knockout cells, which cannot exhibit regular Krebs cycle activity because of the lack of aconitase. This clearly demonstrates that even this limited amount of carbon dioxide was not produced by complete Krebs cycle activity but, for instance, by a cyclic succinate production or by the complete degradation of glucose via a cycling pentose phosphate pathway (see “Discussion”).

Although only three labeled end products are mentioned in Table III, it should be noted that some minor amounts of other metabolites were also detected by anion-exchange chromatography. The total radioactivity of these (unidentified) metabolites never exceeded 10% of the total of acetate and succinate. Therefore, they have been omitted from the table.

We used \( ^{13}\text{C} \) NMR spectroscopy experiments to confirm the identity of acetate and succinate as major end products of glucose degradation in these incubations (Fig. 3). These studies using \( [U-^{13}\text{C}]\text{glucose} \) showed that apart from acetate and succinate as major end products, alanine was also produced as a major end product, because from the \( ^{13}\text{C} \) NMR spectra it could be estimated that the amount of alanine produced was comparable with that of succinate (Fig. 3). The production of alanine
Energy Metabolism of Procyclic T. brucei

Specific activities are given in nmol/min/mg of protein. The values represent the means ± standard deviations of three independent extract preparations and measurements. wt, wild type; ND, not detectable; —, not determined. NAD−-dependent isocitrate dehydrogenase activity was not detectable in the same extracts.

| Enzyme activities in procyclic T. brucei |
|----------------------------------------|
| wt Antat1.1 | Δaco clone 121 | Δaco clone 311 |
| Aconitase EC 4.2.1.3 | 81 ± 8 | ND | ND |
| Isocitrate-dehydrogenase (NAD+) | 57 ± 5 | 58 ± 9 | — |
| Glutamate dehydrogenase | 127 ± 27 | 127 ± 11 | — |
| α-Ketoglutarate dehydrogenase | 223 ± 54 | 148 ± 46 | 168 ± 92 |

Intracellular metabolite concentrations in procyclic T. brucei

TABLE II

| Metabolite | wt AnTat1.1 | Δaco clone 121 | Δaco clone 311 |
|------------|-------------|----------------|----------------|
| Citrate | 0.15 ± 0.02 | 9.2 ± 2.1 | 13.5 ± 3.6 |
| Isocitrate | 2.3 ± 0.5 | 2.5 ± 0.4 | 2.6 ± 0.9 |
| α-Ketoglutarate | 3.6 ± 0.3 | 6.0 ± 3.4 | 4.0 ± 1.5 |
| Succinate | 5.2 ± 1.8 | 5.5 ± 1.3 | 5.7 ± 1.3 |
| Fumarate | 0.30 ± 0.10 | 0.25 ± 0.07 | 0.29 ± 0.11 |
| Malate | 19.7 ± 5.3 | 24.2 ± 5.7 | 23.2 ± 5.4 |
| ATP | 19.3 ± 5.9 | 15.2 ± 1.0 | 14.8 ± 6.6 |
| ADP | 6.9 ± 2.9 | 5.1 ± 0.9 | 4.0 ± 0.9 |
| AMP | 1.3 ± 0.3 | 0.86 ± 0.15 | 0.84 ± 0.16 |
| F6P | 18.0 ± 4.6 | 24.1 ± 5.0 | 22.8 ± 8.8 |
| G1P | 18.0 ± 4.4 | 24.2 ± 5.0 | 25.7 ± 7.6 |
| G6P | 16.7 ± 4.9 | 14.5 ± 4.3 | 21.6 ± 9.2 |
| F16P | 1.1 ± 0.3 | 1.4 ± 0.7 | 2.4 ± 0.7 |
| F2,6P | 0.65 ± 0.33 | 0.97 ± 0.22 | 0.64 ± 0.17 |
| GAP | 0.22 ± 0.15 | 0.69 ± 0.71 | 0.41 ± 0.26 |
| 2,3BPG | 0.31 ± 0.34 | 1.0 ± 0.44 | 0.87 ± 0.32 |
| 2/3PG | 0.41 ± 0.13 | 0.32 ± 0.07 | 0.31 ± 0.22 |
| PEP | 11.2 ± 3.2 | 14.9 ± 3.6 | 12.5 ± 4.8 |

could not be quantified in our studies with radioactively labeled [1-13C]glucose, because alanine and glucose are not separated in the anion-exchange chromatography analysis necessary to resolve acetate and succinate. Production of alanine from glucose by procyclic T. brucei is in agreement with earlier studies (6). The complex resonance signals around 35 ppm stem from the labeled CH₂ groups of succinate (Fig. 3). The observed pattern of these peaks indicated that a large portion of the succinate was labeled at carbon atoms 1, 2, and 3, with also a significant amount of succinate containing 13C only in positions 2 and 3 (the single central peak at 34.7 ppm). However, the actual pattern of peaks can only be explained by a pathway where succinate is formed (Fig. 4). The presence of [1,2,3,4-13C]succinate can only be explained by incorporation of 13C-labeled carbon dioxide in PEP, which is then converted into succinate by the above described pathway (Fig. 4).

It should be noted that the observed label pattern of succinate (mainly 1,2,3- and 2,3-labeled succinate) cannot be explained via formation of succinate by Krebs cycle activity, neither via incorporation of labeled acetyl-CoA stemming from the labeled glucose nor via the use of labeled oxaloacetate as starting compound for a round of Krebs cycle ending in succinate. Furthermore, the observation that the labeling pattern of succinate formed in the incubations of wild type and aconitase knockout procyclic T. brucei was identical excludes the involvement of a functional Krebs cycle because the latter strain lacks aconitase.

Radioactive End Products of [U-14C]Proline Breakdown—Because it has been reported that proline T. brucei use proline as a major energy source (9), the breakdown of proline was also investigated and compared with that of glucose. Incubations were performed in the same complete SDM-79 medium used for the [14C]glucose incubations, except that a tracer amount of 13C-labeled proline was added instead of a tracer amount of labeled glucose. Analysis of the radioactive end products in the presence of labeled proline revealed that the major end product of proline degradation was succinate, with the concomitant release of an equimolar amount of carbon dioxide (Table IV). No differences in end products could be detected between the wild type and aconitase knockout strain.

Role of Electron Transport Chain—To investigate the relative importance of the two branches of the electron transport chain, we incubated both strains in the presence of KCN (inhibitor of the mammalian type respiratory chain) or SHAM (inhibitor of the plant-like alternative oxidase). The addition of SHAM or KCN individually resulted in an increase of the doubling time of both strains (Fig. 5). Inhibition of the mammalian type respiratory chain apparently resulted in a stronger growth reduction than inhibition of the alternative oxidase. Again no differences could be detected between the wild type and aconitase knockout procyclic cells (Fig. 5).

The simultaneous presence of KCN and SHAM resulted within 1 h in cell death of both the wild type and aconitase knockout procyclic trypanosomes (not shown). In addition, the complete removal of oxygen from the incubation (using ascorbate and ascorbate oxidase as was described previously (31)), also resulted in rapid death of the organisms (not shown). Apparently, procyclic T. brucei cells are completely dependent on the use of oxygen by the respiratory chain for their energy generation and survival.

DISCUSSION

In this paper we investigated the importance of the Krebs cycle for energy generation in the procyclic stage of T. brucei.
For these studies we compared a wild type pleomorphic strain and several independently derived mutant clones in which the single copy aconitase gene was deleted by gene targeting. No aconitase activity could be detected in the single copy aconitase gene was deleted by gene targeting. No and several independently derived mutant clones in which the wild type and aconitase knockout trypanosomes. In contrast to our previous study on iron uptake regulation (19), here we constructed the aconitase deletion in a fully differentiation competent, tsetse fly passagable strain background (AnTat 1.1) constructed the aconitase deletion in a fully differentiation competent, tsetse fly passagable strain background (AnTat 1.1) constructed the aconitase deletion in a fully differentiation competent, tsetse fly passagable strain background (AnTat 1.1)

Table III

| Incubation | Radiative glucose breakdowna | Labeled end products | Glucose degraded |
|------------|-----------------------------|----------------------|-----------------|
|            |                            | CO₂ | Acetate | Succinate | CO₂ | Acetate | Succinate |
| WT | 435 ± 143 | 7 ± 6 | 412 ± 94 | 354 ± 185 | 1 ± 2 | 55 ± 9 | 44 ± 11 |
| KO | 568 ± 401 | 10 ± 8 | 781 ± 488 | 556 ± 467 | 1 ± 1 | 60 ± 4 | 39 ± 5 |

a Radiative glucose breakdown was calculated from the total amount of radioactive end products.

b End product formation per mg of protein was calculated using the amount of trypanosomal protein at the end of the incubation.

Fig. 3. 13C NMR spectra. A. 13C NMR spectrum of excreted end products of [U-13C]glucose metabolism by procyclic T. brucei. Wild type and aconitase knockout organisms were incubated for 17 or 72 h, after which the incubation medium was analyzed by 13C NMR. All of the incubations resulted in identical patterns of the spectra. The spectrum of the 72-h incubation of the aco::NEO/aco::HYG clone 121 is shown. The asterisks indicate peaks that are also present in the blank incubations. B, close up of A (34 – 36 ppm). C, calculated spectrum of succinate (mixture of 60% [1,2,3-13C]succinate, 30% [2,3-13C]succinate, and 10% [1,2,3,4-13C]succinate).

In an independent biochemical approach, the unimportance of the Krebs cycle for energy generation in procyclic T. brucei was confirmed by our studies using labeled substrates. To investigate whether the absence of aconitase activity results in altered pathways of energy generation, we performed radioactive incubations using [6-14C]glucose. If the degradation of acetyl-CoA inside the mitochondrion occurred via multiple pathways, i.e. via acetate formation caused by ASCT activity, as well as via oxidation in the Krebs cycle, one would expect the acetate production to increase when Krebs cycle activity was blocked by the absence of aconitase. However, the knockout cells did not show an increased production of acetate compared with the wild type control. Both wild type and knockout procyclic T. brucei metabolize [6-14C]glucose to acetate and succinate as major end products. No significant amount of 14C-labeled CO₂ could be detected, indicating that a normally functioning Krebs cycle is not involved in energy production in wild type as well as in knockout cells. These results thus demonstrated the absence of oxidation of glucose via a complete Krebs cycle in procyclic T. brucei. NMR studies with [U-13C]glucose were performed to confirm the identities of acetate and succinate produced during glucose breakdown.

The pathway of succinate production by procyclic T. brucei is not yet completely resolved. Earlier reports suggested a mitochondrial origin of this succinate production involving the activity of a soluble fumarate reductase (35). Recently, however,
Besteiro et al. (18) showed that succinate production occurs mainly inside the glycosomes by a soluble glycosomal NADH: fumarate reductase, and our analysis of the position of the $^{13}$C labels in succinate can provide information on the route by which this succinate was formed. The NMR spectra demonstrated that PEP carboxykinase was involved in the production of succinate; PEP is carboxylated to oxaloacetate, which is subsequently reduced via malate to succinate. Close inspection of the resonance pattern combined with computer simulations showed that this succinate was produced by three variations on this pathway (Fig. 4); directly ($[1,2,3-^{13}$C$]$succinate), via cycling of the formed fumarate ($[2,3-^{13}$C$]$succinate), or including the incorporation of $^{13}$C-labeled carbon dioxide ($[1,2,3,4-^{13}$C$]$succinate). This last variation seemed enigmatic, because the source of the $^{13}$C-labeled carbon dioxide was unknown, whereas unlabeled carbon dioxide is abundantly present in the incubation medium. It is known, however, that a significant part of the glucose degradation inside the glycosomes occurs via the pentose phosphate pathway, which results in the formation of (labeled) carbon dioxide (36), and it was also shown recently that the major part of succinate production from glucose occurs inside the glycosome (18). The subcellular co-compartmentalization of these two pathways could explain the observed incorporation of labeled carbon dioxide in a fraction (10%) of the succinate formed. It should be noted that these variations of succinate production exclude the involvement of Krebs cycle activity in the forward direction but are in other aspects in full agreement with the pathway of succinate production suggested by Besteiro et al. (18).

Earlier studies that have characterized the amino acid uptake from the environment by the insect stage of T. brucei show that L-proline is a main source of energy and carbon (9, 37). In our incubations performed to study $[14$C$]$proline breakdown, labeled succinate was found to be a major end product. This was accompanied by the expected production of 1 mol of labeled CO$_2$/mol of labeled succinate formed during the conversion of $\alpha$-ketoglutarate to succinyl-CoA. Again, no differences could be detected between the wild type and knockout cells. This result also demonstrated that proline is only degraded to succinate and not further degraded via the Krebs cycle because this would have resulted in a higher CO$_2$/succinate ratio. The incubations in which we studied the glucose and proline degradation were performed in both cases in the same complete medium (SDM-79), the only difference being the tracer amount of

![Fig. 4: Pathways of labeled succinate formation from [U-^{13}$C$]glucose by procyclic T. brucei. The asterisks indicate $^{13}$C atoms.](image)

![Table IV: Radioactive end products of [U-^{14}$C$]proline breakdown by procyclic T. brucei.](table)
**Energy Metabolism of Procyclic T. brucei**

14C label added. This means that we studied for both substrates their degradation in the presence of the other substrate and that the observed rates of degradation and patterns of end products of glucose and proline occurred at the same time. Comparison of the total amount of end products produced from each substrate in complete medium (SDM-79) showed that in the simultaneous presence of glucose and proline, glucose is the preferred substrate. Under these conditions, the rate of glucose degradation was around three times as high as that of proline. It should be realized, however, that proline could well be the preferred substrate in vivo inside the insect gut where glucose concentrations are probably much lower.

We showed that procyclic T. brucei, either wild type or knockout mutants without aconitase activity, are equally unable to survive under anaerobic conditions. Furthermore, simultaneous inhibition of both the mammalian type respiratory chain and the plant-like alternative oxidase resulted in cell death within 1 h. On the other hand, survival and even growth continued when only one of the two terminal oxidases was inhibited. A stronger effect on growth (likely to be due to inhibition of energy production) was found upon KCN inhibition of the mammalian type respiratory chain as compared with SHAM inhibition of the plant-like alternative oxidase.

Again, no differences could be detected between the wild type and knockout procyclic T. brucei. These results confirm that both branches of the respiratory chain simultaneously play a role in the normal functioning of procyclic cells (38), but apparently, the activity of only one of the two branches of the respiratory chain is sufficient for survival and reduced growth.

The role of aconitase is still unresolved, but no matter what role aconitase plays in procyclic trypanosomes, it is clearly not in the generation of energy via the Krebs cycle. The end products of the incubations of the wild type strain with labeled glucose and proline demonstrated already that Krebs cycle activity does not play a significant role in the degradation of these substrates. The fact that no metabolic differences could be detected between procyclic wild type and procyclic aconitase knockout clones is solid confirmation of the unimportance of Krebs cycle activity for energy generation in the procyclic life cycle stage. On the other hand, the metabolism of prokaryotic T. brucei is not purely fermentative, because oxygen is required as a final electron acceptor for survival and growth. The earlier described absence of a growth phenotype upon RNA interference-mediated ablation of succinate dehydrogenase or α-ketoglutarate dehydrogenase in procyclic T. brucei (13) is in agreement with our observation that the Krebs cycle is not involved in energy generation in this developmental stage. The observed differences in the kinetics of induction of Krebs cycle enzymes during bloodstream to procyclic differentiation (Ref. 10 and references there in) are also compatible with a nonessential role of the Krebs cycle in the procyclic stage.

Mitochondria of African trypanosomes are unique in many aspects including the structure of the mitochondrial genome and the extensive RNA editing during mitochondrial gene expression, the fact that each cell contains a single mitochondrion whose division is closely linked to the cell cycle, and the developmental regulation of their morphological structure and function in the life cycle. From a bioenergetical point of view, it is surprising that mitochondria of procyclic form T. brucei ferment substrates to mainly acetate and succinate, whereas they contain the entire set of Krebs cycle enzymes and a functional respiratory chain including an alternative oxidase, which is otherwise only present in plants and fungi. The presence of a complete aerobic machinery together with the incomplete oxidation of substrates places these mitochondria in a special category in between classical aerobic mitochondria and the fully anaerobically functioning mitochondria of, for instance, adult parasitic helminths (39). This is also reflected by the presence of ASCT, an enzyme absent in classical type aerobic mitochondria.

Although our results demonstrate that apparently an intact Krebs cycle is not used for complete oxidation of glucose or proline, it should be realized that part of the Krebs cycle is used for the degradation of proline to succinate. The enzyme succinyl-CoA synthetase of this part of the Krebs cycle is also functioning in the ASCT cycle (16) and has been shown to be essential for substrate phosphorylation in isolated mitochondrial vesicles and for growth of procyclic trypanosomes (13). It should also be noted that the Krebs cycle (or at least the first two reactions) is probably active in the wild type strain, because citrate levels were dramatically increased in the aconitase knockout strain. At the moment, the possible function of aconitase and other Krebs cycle enzymes in procyclic trypanosomes is unknown, but no matter what the role is, it is clearly not related to the generation of energy in the procyclic stage. The most appealing interpretation is that the Krebs cycle plays a role in energy generation in other stages of the life cycle of the parasite in the tsetse fly (e.g. in the salivary gland) or under other environmental conditions, for instance when substrate availability is limited.

Even though clearly not all of the details are known yet, a combination of older and recently published studies (13, 18) with our experiments, in which we studied for the first time the glucose and proline metabolism of intact procyclic T. brucei of a pleomorphic strain under growing conditions, results in a model of the main pathways of glucose degradation as shown in Fig. 6. This metabolic scheme shows the fermentation pathways coupled to oxidative phosphorylation in these unique...
FIG. 6. The energy metabolism of procyclic *T. brucei*. Shown are the main pathways used by procyclic *T. brucei* for the degradation of glucose to the main end products, acetate, succinate, and alanine, for the degradation of proline to succinate via part of the Krebs cycle, and the essential role of the branched respiratory chain in the oxidation of NADH formed in these fermentation pathways. Degradation of glucose by the pentose phosphate pathway is not included in this scheme. Furthermore, it should be realized that, next to the transaminase reaction shown, the conversion of glutamate into α-ketoglutarate can also be catalyzed by glutamate dehydrogenase. AA, amino acid; AcCoA, acetyl-CoA; AO, alternative oxidase; 1,3BPGA, 1,3-bisphosphoglycerate; c, cytochrome c; Citr, citrate; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; Fum, fumarate; GAP, glyceraldehyde 3-phosphate; Glu, glutamate; G-3-P, glycerol 3-phosphate; αKG, α-ketoglutarate; Mal, malate; OA, 2-oxoacid; Oxac, oxaloacetate; 3-PGA, 3-phosphoglycerate; Pyr, pyruvate; Succ, succinate; Succ-CoA, succinyl-CoA; UQ, ubiquinone.
mitochondria of procyclic T. brucei and accounts for the degradation of glucose to acetate, succinate, and alanine, the degradation of proline to succinate via part of the Krebs cycle, and the essential role of the branched respiratory chain in the oxidation of NADH formed in these fermentation pathways.

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Procyclic *Trypanosoma brucei* Do Not Use Krebs Cycle Activity for Energy Generation

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