New Functions for Parts of the Krebs Cycle in Procyclic
Trypanosoma brucei, a Cycle Not Operating as a Cycle*

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We investigated whether substrate availability influences the type of energy metabolism in procyclic Trypanosoma brucei. We show that absence of glycolytic substrates (glucose and glycerol) does not induce a shift from a fermentative metabolism to complete oxidation of substrates. We also show that glucose (and even glycolysis) is not essential for normal functioning and proliferation of pleomorphic procyclic T. brucei cells. Furthermore, absence of glucose did not result in increased degradation of amino acids. Variations in availability of glucose and glycerol did result, however, in adaptations in metabolism in such a way that the glycosome was always in redox balance. We argue that it is likely that, in procyclic cells, phosphoglycerate kinase is located not only in the cytosol, but also inside glycosomes, as otherwise an ATP deficit would occur in this organelle. We demonstrate that procyclic T. brucei uses parts of the Krebs cycle for purposes other than complete degradation of mitochondrial substrates. We suggest that citrate synthase plus pyruvate dehydrogenase and malate dehydrogenase are used to transport acetyl-CoA units from the mitochondrion to the cytosol for the bio-synthesis of fatty acids, a process we show to occur in proliferating procyclic cells. The part of the Krebs cycle consisting of α-ketoglutarate dehydrogenase and succinyl-CoA synthetase was used for the degradation of proline and glutamate to succinate. We also demonstrate that the subsequent enzymes of the Krebs cycle, succinate dehydrogenase and fumarase, are most likely used for conversion of succinate into malate, which can then be used in gluconeogenesis.

Trypanosoma brucei is a unicellular eukaryote that causes sleeping sickness in humans and nagana in livestock. African trypanosomes undergo a complex life cycle through the bloodstream of their mammalian host and the blood-feeding insect vector, the tsetse fly (Glossina spp.) (1). During their life cycle, trypanosomes encounter many different environments and respond to these by dramatic morphological and metabolic changes, including adaptation of their glucose metabolism. The long slender bloodstream form has the simplest pathway for the degradation of glucose, viz. glycolysis, and excretes pyruvate as the sole end product (2–4). However, the glycosomal and mitochondrial metabolism of T. brucei changes significantly upon transformation from the long slender bloodstream form to the procyclic form. In procyclic forms, the end product of glycolysis (pyruvate) is not excreted, but is further degraded inside the mitochondrion. In addition to carbohydrates, procyclic T. brucei forms are also able to use amino acids such as proline and threonine for energy generation (5, 6).

In our previous investigation, we studied the contribution of the Krebs cycle to the energy metabolism of wild-type and aconitate knockout procyclic cells by metabolic incubations using labeled glucose and proline (7). We showed that, under standard in vitro culture conditions, proline and glucose are not degraded via the Krebs cycle to CO₂. Instead, proline is mainly degraded to succinate, and glucose is degraded to acetate, succinate, and alanine. It has been shown that succinate production from glucose occurs predominantly inside the glycosome (8). Furthermore, no difference in excreted end products was detected between wild-type and aconitate knockout procyclic cells, demonstrating that complete Krebs cycle activity is not involved in energy generation in procyclic T. brucei under the conditions studied (7).

Many microorganisms are able to adapt their metabolism for optimal utilization of the carbon sources available in the environment. One of the major mechanisms by which cells adapt is by regulation of gene expression. Analysis of genomic expression has revealed that, in many organisms, multiple genes are differentially transcribed in response to varying glucose levels, a process called glucose repression (9). In the yeast Saccharomyces cerevisiae, glucose repression affects the enzymes required for the metabolism of the sugars sucrose, maltose, and galactose and non-fermentable carbon sources such as ethanol and acetate, as well as the enzymes involved in the Krebs cycle, gluconeogenesis, and oxidative phosphorylation (10). When carbohydrates are abundantly available, S. cerevisiae exhibits a fermentative metabolism, even under aerobic culture conditions. Under glucose-limited conditions, however, chemostat cultivation of S. cerevisiae induces activation of the Krebs cycle (11). The question now arises whether also in procyclic T. brucei the (near) absence of carbohydrates leads to activation of the Krebs cycle for the complete oxidation of substrates.

To investigate whether the availability of substrates for glycolysis influences the type of energy metabolism in procyclic T. brucei cells, we performed radioactive incubations with cells cultured in the absence and presence of glucose and/or glycerol. Our investigations showed that the absence of car-
bohydrides did not induce a shift from a fermentative metabolism to the use of the Krebs cycle for complete oxidation of substrates. We therefore investigated whether the Krebs cycle enzymes, which have been shown to be present in procyclic T. brucei, might be involved in processes other than the degradation of acetyl-CoA. Our data led to the proposal that the Krebs cycle does not function as a cycle in procyclic T. brucei, but that parts of the Krebs cycle machinery were used in other processes such as partial degradation of amino acids, but also for biosynthetic purposes such as fatty acid biosynthesis and gluconeogenesis.

EXPERIMENTAL PROCEDURES

Trypanosome Culture Conditions—The pleomorphic procyclic T. brucei brucei TREU 927 strain (a gift from Jeremy Mottram, Wellcome Centre for Molecular Parasitology, Glasgow, Scotland, United Kingdom) was grown at 27 °C in the presence of 5% CO2 in SDM-79 medium with different glucose and/or glycerol concentrations. SDM-79 medium without glucose was prepared with glucose-free Medium 199 and minimum Eagle’s medium, and no additional glucose was added. This medium was supplemented with 10% dialyzed fetal bovine serum (In Vitrogen), resulting in a final glucose concentration of 0.02 mM. The three other culture media that we investigated were obtained by addition of glucose (10 mM final concentration) and/or glycerol (13 mM final concentration) to the above-described medium without glucose. Standard culturing was carried out in SDM-79 medium containing 10 mM glucose, and changes to other media were performed by dilution (1:5) in the new medium for at least 5 consecutive days before the metabolic studies were performed. This procedure allows possible adaptations to take place.

Metabolic Incubations—Incubations were performed using procyclic cells cultured in SDM-79 medium containing glucose and glycerol or procyclic cells that were adapted for 5 days to SDM-79 medium without glucose and/or glycerol. Metabolic experiments were started with 5 × 10⁸ cells/ml, and incubations were carried out for 17–24 h at 27 ℃ in sealed Erlenmeyer flasks containing 5 ml of incubation medium. Before sealing, the flasks were flushed for 1 min with a gas phase of 95% air and 5% CO₂. Incubations were started after addition of 5 μCi of [6-14C]glucose (2.07 GBq/mmol; Amersham Biosciences), 5 μCi of [U-14C]proline (9.47 GBq/mmol; Amersham Biosciences), 5 μCi of [U-14C]glycylglycinol (5.07 GBq/mmol; Amersham Biosciences), or 5 μCi of [U-14C]threonine (6.48 GBq/mmol; ICN Biomedica). Incubations were terminated by addition of 40 μl of 6 M HCl to lower the pH to 3.5. Analysis of the end products was performed as described (13). Glucose concentrations were determined enzymatically using hexokinase and glucose-6-phosphate dehydrogenase and by measuring the NADPH formed. Protein was determined by the Lowry method using defatted and dialyzed bovine serum albumin (Roche Applied Science) as the standard.

Fatty Acid Analysis—Procyclic T. brucei cells were incubated for 17 h in the presence of [6-14C]glucose, [U-14C]proline, or [U-14C]threonine as described above under “Metabolic Incubations.” Afterward, the trypanosomes (~5 × 10⁷ cells) were pelleted by centrifugation at 2000 × g for 15 min at 4 ℃ and washed with ice-cold buffer containing 10 mM Tris-HCl (pH 7.4) and 140 mM NaCl. Lipids were extracted according to the method of Bligh and Dyer (14). Organic extracts were dried under nitrogen, and lipids were subsequently hydrolyzed in 2.5 ml of 0.3 M NaOH in methanol for 1 h at 75 ℃. Non-saponified material was extracted three times with 1 volume of petroleum ether. After acidification of the methanolic phase with 150 μl of 6 M HCl, the fatty acids were extracted three times with 1 volume of petroleum ether (15). The isolated fatty acids were dried under nitrogen, converted into their phenacyl derivatives, dried under nitrogen, and redissolved in acetone/titration (16). The obtained fatty acid phenacyl esters were loaded onto a 250 × 4-mm LiChrospher 100 RP-18e column (5 μm; Merck, Darmstadt, Germany) and eluted isocratically with acetonitrile at a flow rate of 1 ml/min. Detection of the fatty acids was performed at 242 nm using PerkinElmer Life Sciences analytical software for data analysis. Fractions were collected every 30 s in scintillation vials, evaporated to dryness, and counted for radioactivity after addition of tritolum scintillation fluid (15). To investigate whether radioactive carbons were incorporated into saturated or unsaturated acyl chains, fatty acids obtained after petroleum ether extraction were either analyzed directly or first dissolved in methanol and hydrogenated by hydrogen gas in the presence of platinum(IV) oxide for 2 h before conversion into phenacyl derivatives.

RESULTS

Metabolic Incubations—To investigate whether the availability of substrates for glycolysis influences the type of energy metabolism in procyclic T. brucei, we performed radioactive incubations using four distinct labeled substrates. We used two substrates for glycolysis, [6-14C]glucose and [U-14C]glycerol, and two substrates for mitochondrial metabolism, [U-14C]proline and [U-14C]threonine. Glucose, proline, and threonine are considered to be main carbon sources for energy generation in procyclic T. brucei (5, 6). When present, glycerol was also a main carbon source for procyclic T. brucei, as was shown previously for cultured Trypanosoma rhodesiense cells (17). The catabolism of each of these four substrates was investigated separately under standard culture conditions in the presence of SDM-79 medium alone, with glycerol, without glucose, or without glucose but with glycerol (see Fig. 1) for an outline of all the radioactive incubations performed.

Metabolic Pathways in the Presence of Glucose (10 mM), Glycerol (15 mM), Proline (5 mM), and Threonine (3 mM)—The incubations performed with [U-14C]glucose and [U-14C]glycerol demonstrated that acetate and succinate were the main excreted end products of glucose metabolism (Fig. 1A), which is in agreement with previous reports (7, 8, 18). Only a very limited amount of glucose (~1%) was broken down to labeled CO₂. Because significant production of labeled CO₂ from [6-14C]glucose can occur only when pyruvate is degraded by the Krebs cycle, this result confirms that the activity of a complete Krebs cycle is negligible in procyelic cells (7). The incubations performed with [U-14C]-glycerol showed that, in the presence of both substrates, next to glucose, a considerable amount of glycerol was consumed, which was mainly metabolized into succinate (Fig. 1B). A minor part of the consumed glycerol was degraded to acetate, with the concomitant release of an equimolar amount of labeled CO₂, which is produced during the oxidative decarboxylation of pyruvate to acetyl-CoA. Analysis of the radioactive end products from [U-14C]proline revealed that the major end product was succinate, with the concomitant release of an equimolar amount of carbon dioxide (Fig. 1C), as was shown previously (7). Minor amounts of labeled acetate were detected upon [U-14C]proline degradation. The incubations performed with [U-14C]threonine demonstrated that considerable amounts of L-threonine were metabolized to acetate (Fig. 1D), which is in agreement with previous studies (6). However, at the same time, an equimolar amount of glycine has been reported to be produced (19), but this end product cannot be detected by the method we used because threonine (the labeled substrate) elutes close to glycine. Calculations of the amounts of various substrates consumed in the presence of all four substrates showed that the consumption ratio of the substrates was ~5: 13:1:4 for glucose, glycerol, proline, and threonine, respectively (Fig. 1). However, it should be realized that, during glycolysis, degradation of one molecule of glucose results in the formation of two molecules of triose phosphate and therefore results in twice the amount of end products compared with glycerol, proline, and threonine.

Metabolic Pathways in the Near Absence of Glucose (0.02 mM; with Glycerol, Proline, and Threonine Present at the Original Concentrations Indicated Above)—Procyclic cells grown in the presence of only a tracer amount of labeled glucose had a 50% increased doubling time (calculated from days 5 to 10) compared with procyclic cells grown in SDM-79 medium with both glucose and glycerol (Fig. 2). No change in the excreted end product pattern of proline, glycerol, and glucose metabolism was detected (Fig. 1, A–C). Also, no increase in glycerol or proline consumption was measured when the glucose consumption was negligible in the medium without glucose. Apparently,
Glucose metabolism in the absence of glycerol (with glucose, glycerol, proline, or threonine in the incubation medium. End product formation/mg of protein was calculated using the amount of protein present at the end of the incubations. The values represent the means ± S.D. of three or the average of two independent experiments.

Fig. 2. Growth of procyclic T. brucei cells. The growth of procyclic T. brucei cells was monitored in the presence and/or absence of glucose (glc) and glycerol (gly). Cell density was maintained between 3 × 10⁶ and 1 × 10⁷ cells/ml. Cumulative cell numbers reflect normal-ization for dilution during cultivation. All four cultures started with cells grown in the presence of glucose and glycerol. From day 1, the cells were diluted with SDM-79 medium containing glucose and/or glycerol or with SDM-79 medium without glucose and glycerol. It should be noted that only after four dilutions was the minimal glucose concentration of 0.02 mM reached (from day 4 onward). The cell numbers were counted every 24 h using a Burker counter. A representative example of three independent experiments is shown.

there is no change in the use of catabolic pathways and no up-regulation of the degradation of other substrates when glucose is absent.

Glucose Metabolism in the Absence of Glycerol (with Glucose, Proline, and Threonine Present at the Original Concentrations Indicated Above)—A small negative growth effect (25%) was detected in procyclic cells cultured in the absence of glycerol (Fig. 2). Metabolic incubations using labeled glucose showed that acetate and succinate were still the main end products of glucose catabolism, but the ratio in which these products were excreted changed significantly (Fig. 1A). Acetate production was increased from an average of 30 ± 8% to an average of 55 ± 1%, whereas succinate production dropped from 70 ± 8 to 45 ± 1%. In addition, glucose degradation was halved in the absence of glycerol.

Fig. 1. Radioactive end products of [6-14C]glucose, [U-14C]glycerol, [U-14C]proline, and [U-14C]threonine metabolism in procyclic T. brucei. Shown are the labeled end products from breakdown of [6-14C]glucose (A), [U-14C]glycerol (B), [U-14C]proline (C), and [U-14C]threonine (D). Upper panels, end products quantified as a percentage of the total radioactive end products produced; middle panels, amount of labeled substrate consumption; lower panels, presence or absence of glucose, glycerol, proline, or threonine in the incubation medium. End product formation/mg of protein was calculated using the amount of protein present at the end of the incubations. The values represent the means ± S.D. of three or the average of two independent experiments.

Amino Acid Metabolism in the Absence of Glucose and Glycerol—Although both substrates for glycolysis were absent, proliferation continued, albeit at a slower rate (35% decrease compared with procyclic cells grown in the presence of SDM-79 medium with both glucose and glycerol) (Fig. 2). The absence of glucose and glycerol induced a 2-fold increase in proline degradation (Fig. 1C), but no increase was detected in threonine degradation (Fig. 1D), and also no difference was seen in the end product pattern produced from threonine breakdown. Because labeled threonine is broken down to labeled acetate via labeled acetyl-CoA, an active Krebs cycle would result in significant excretion of labeled CO₂. Therefore, the absence of significant labeled CO₂ production upon threonine degradation showed that, even in the near absence of glycolytic substrates, the Krebs cycle was not induced, although the proliferating cells had 5 days to adapt their metabolism to the medium (see “Experimental Procedures”). The end product pattern produced from [U-14C]proline showed a dramatic increase in CO₂ production compared with the incubations containing carbohydrates (Fig. 1C), whereas excretion of succinate was not increased. This high production of labeled CO₂ has to imply that also more labeled succinate was produced. Apparently, this labeled succinate was not secreted, but was instead used for biosynthetic purposes such as the gluconeogenic pathway to produce carbohydrates (see “Discussion”).

Incorporation of Radioactive Carbons into Fatty Acyl Chains—Comparison of the isolated fatty acids present in the serum added to SDM-79 medium (Fig. 3A) with the fatty acids isolated from procyclic T. brucei cells (Fig. 3B) showed that the fatty acid composition in procyclic T. brucei cells differed from that in the medium. Therefore, procyclic cells either took up fatty acids selectively or synthesized fatty acids de novo or performed both activities. Fig. 3B demonstrates that fatty acids were synthesized de novo by procyclic cells, as fatty acid peaks marked with asterisks showed significant incorporation of labeled carbons derived from [6-14C]glucose or [U-14C]threonine (dpm up to 100 times the background levels). The only way to
incorporate $^{14}$C from these substrates into fatty acids involves a mitochondrial acetyl-CoA intermediate. No difference in fatty acid profile was observed in procyclic cells cultured in the presence or absence of glucose and glycerol (data not shown). All these experiments show that part of the mitochondrial acetyl-CoA pool is used for fatty acid biosynthesis in proliferating procyclic T. brucei cells. As could be expected from the observed low absolute flux of proline degradation in combination with the relative small amount of acetate that was produced from this substrate (Fig. 1), no significant radioactive carbon incorporation from [U-$^{14}$C]proline into fatty acids could be detected.

In contrast to the bloodstream form fatty-acid synthase system, which is specialized for myristate synthesis, procyclic forms synthesized mainly 16- and 18-carbon fatty acids, which is in agreement with previous studies in cell-free systems (20). Procyclic forms also synthesized myristate, which can be concluded from the detection of radioactive label in the C14:0 species after hydrogenation (Fig. 3C), but the incorporated amount of label was <10% of that present in 16- and 18-carbon fatty acids. No radioactive carbons could be detected in the C22.5 and C22.6 species; and therefore, the abundance of these fatty acids in procyclic cells compared with their presence in the medium can be explained only by selective uptake or modification of specific fatty acids from the medium.

**DISCUSSION**

**Influences of Substrate Availability**—The absence of glucose and/or glycerol in SDM-79 medium did result in decreased growth, but did not result in halted proliferation or cell death of procyclic cells of this pleomorphic T. brucei strain. This observation confirms previous studies on monomorphic strains (21, 22). Therefore, it can be concluded that glucose and glycerol (and hence glycolysis) are not essential for normal functioning and proliferation of this pleomorphic strain because procyclic T. brucei cells can adapt their metabolism accordingly. However, some morphological changes were observed upon changing substrate levels. Omission of glucose from the medium (with glycerol still present) resulted in an extension of the total length (body + flagellum) of the procyclic cells from 44 ± 5 to 63 ± 8 μm. Elongation of the flagellum in response to glucose limitation has been described previously in T. cruzi (23). Our various incubations also resulted in changes in the procyclin coat of the parasites, but these changes were not studied extensively. This aspect has been described in detail previously, and it is known that glucose and glycerol availability influences the expression of procyclins present on the surface coat of procyclic trypanosomes (22, 24, 25).

**Redox and ATP Balance in Glycosomes of Procyclic T. brucei**—Glucose metabolism in the absence of glycerol (with glucose, proline, and threonine present) resulted in an altered acetate/succinate ratio (1:1) compared with the acetate/succinate ratio (1:3) in incubations performed in the presence of glycerol (Fig. 1). Apparently, glucose metabolism was affected by the presence of glycerol in the medium, and the question is why? The pathways of glycerol and glucose degradation via glycolysis overlap, starting at the formation of dihydroxyacetone phosphate and glycerol 3-phosphate. However, in the formation of dihydroxyacetone phosphate/glycerol 3-phosphate, glycerol catabolism consumes one molecule of ATP/triose phosphate and produces one molecule of NADH/triose phosphate, whereas glucose catabolism also consumes one molecule of ATP/triose phosphate, but produces no NADH. Could this difference in NADH production possibly explain the altered acetate/succinate ratio?

It should be realized that, in procyclic T. brucei, glucose and glycerol catabolism and amino acid degradation result in a significant net production of NADH. This substantial amount of NADH is reoxidized by the respiratory chain because procyclic cells are known to consume significant amounts of oxygen by alternative oxidase and cytochrome c oxidase activities. Furthermore, this observed net NADH production is in agreement with the essential function of the respiratory chain in procyclic T. brucei cells, which are rapidly killed when both
branches of their respiratory chain are inhibited simultaneously (7).

Based on the current carbohydrate metabolism models of procyclic \textit{T. brucei}, the net production or consumption of cofactors in the various organelles can be calculated for the degradation of each carbohydrate substrate into the specific end products acetate and succinate. Using the current models that consider a cytosolic location for phosphoglycerate kinase (PGK), \textsuperscript{1} glucose degradation to acetate results (per molecule of acetate produced) in the formation of two molecules of NADH (one produced inside the glycosome) and in the net formation of two molecules of ATP (but in this process, one molecule of ATP is consumed inside the glycosome) (Table I). Glucose degradation to succinate results in the consumption of one molecule of NADH (inside the glycosome) and yields one molecule of ATP (no ATP formed inside the glycosome). Glycerol degradation to acetate yields three molecules of NADH (two inside the glycosome) and two molecules of ATP (one consumed inside the glycosome). Glycerol degradation to succinate is redox neutral (also in the glycosome) and yields no ATP inside the glycosome. In the case of a putative glycosomal localization of PGK, one extra molecule of ATP is produced inside the glycosome during the degradation of both glucose and glycerol to acetate and succinate at the expense of one molecule of ATP in the cytosol (Table I).

We used our metabolic data on glucose and glycerol consumption and the end product formation pattern (Fig. 1) to calculate the use and production of cofactors. In the presence of glucose only (Table II) the redox balance inside the glycosome appeared to be almost neutral (+38). Also in the presence of glucose plus glycerol (Table III), the total redox balance was nearly maintained (+26). The ratio of end products produced under these two conditions is strikingly different, however. In the absence of glycerol, glucose degradation was shifted from more succinate toward more acetate production (Fig. 1A). Apparently, the (combined) metabolism of glucose and glycerol is adapted in such a way that the net NADH consumption and production inside the glycosome are in redox balance under both conditions.

In the presence of only glycerol (Table IV), an excess of NADH was produced inside the glycosome, which means that, under these circumstances, the glycosome is not in redox balance. Therefore, alternative pathways, such as the dihydroxyacetone phosphate/glycerol 3-phosphate shuttle that is operative in bloodstream forms, should be involved in the oxidation of this surplus NADH. The fact that the growth of procyclic cells cultured in the absence of glucose but in the presence of glycerol was heavily affected (Fig. 2) might be the result of the unfavorable redox status inside the glycosome under these conditions. Apparently, this is a condition that the cells cannot handle adequately because any acetate produced will result in accumulation of NADH. This NADH produced cannot be balanced by another catabolic process, as it is the catabolism of only glucose to succinate, and not that of glycerol, that results in net NADH consumption inside the glycosomes (Table I).

It is remarkable that, using the current models for glycosomal metabolism in procyclic cells, all three experimental conditions that we tested (both glucose and glycerol present or only one of these two substrates at a time) resulted in a large ATP deficit inside the glycosome (Tables II–IV). In principle, two possibilities exist to solve this problem. (i) ATP can be imported from outside the glycosome, or (ii) another ATP-producing process exists in the glycosome. As there is no evidence for an ATP/ADP translocator in the glycosomal membrane, it is likely that another ATP-producing process should be identified, which can compensate for the deficit in glycosomal ATP balance observed during the metabolism of glucose and/or glycerol.

A possibility to balance the ATP inside the glycosome could be the use of a pyruvate-phosphate dikinase. This enzyme utilizes phosphoenolpyruvate + AMP + PP\textsubscript{i} to synthesize pyruvate + P\textsubscript{i} + ATP (26). The PP\textsubscript{i} required for this reaction is produced in various biosynthetic reactions that are also present in the glycosome, such as hypoxanthine-guanine phosphoribosyltransferase and orotate phosphoribosyltransferase. Because there is no pyrophosphatase present in glycosomes, the pyruvate-phosphate dikinase could link the glycosomal catabolic and anabolic functions. However, as the catabolic flux of glucose is much greater than the anabolic flux, it seems unlikely that the glycosomal ATP yield of the catabolic pathway is balanced by the anabolic pathway. Furthermore, the observation that procyclic \textit{T. brucei} is not affected by pyruvate-phosphate dikinase gene knockout does not indicate an important role for this enzyme in energy metabolism (27). Although pyrophosphate (PP\textsubscript{i}) is known to be involved in several enzymatic reactions in trypanosomes (28), the precise role of pyruvate-phosphate dikinase in the glucose metabolism of procyclic \textit{T. brucei} clearly needs further investigation.

To solve this obvious problem in glycosomal ATP balance, we would like to suggest that another ATP-consuming reaction occurs in the glycosome. We propose that to maintain ATP balance in the glycosome under all conditions, there are two PGKs, one active in the cytosol and one active in the glycosome. However, the latter is not necessarily the glycosomal PGK, which is expressed in \textit{T. brucei} bloodstream forms. In metabolic schemes of procyclic \textit{T. brucei}, PGK is usually depicted in the cytosol only, and not in the glycosome. It has been shown, however, that some 10\% of the PGK activity remains glycosomal (29, 30). Furthermore, in most trypanosomatids, including

\textsuperscript{1} The abbreviation used is: PGK, phosphoglycerate kinase.

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### Table I

| Substrate | End product$^a$ | NADH/mol product$^a$ | ATP yield/mol product$^a$ |
|-----------|-----------------|----------------------|--------------------------|
| Glucose   | Acetate         | +2                   | +2                       |
| Glucose   | Succinate       | −1                   | +1                       |
| Glycerol  | Acetate         | +3                   | +2                       |
| Glycerol  | Succinate       | 0                    | 0                        |

$^a$ All of the succinate referred to is glycosomal, whereas acetate is produced inside the mitochondrion.

$^b$ In the production of acetate and succinate from glucose and glycerol, NADH is produced in the reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase, glycerol-3-phosphate dehydrogenase, and pyruvate dehydrogenase, whereas NADH is oxidized in the reactions catalyzed by phosphoenolpyruvate carboxykinase and fumarate reductase (see Fig. 4).

$^c$ PGK, theoretical value with PGK active only in the cytosol; gPGK, theoretical value with PGK active only in glycosome.
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Table II
Calculated NADH and ATP balances of glucose metabolism in the absence of glycerol in procyclic T. brucei based on end product analysis (combination of data in Fig. 1 and Table I)

| Substrate | End product | NADH/mol product | ATP yield/mol productb |
|-----------|-------------|------------------|-----------------------|
|           |             | Total            | Inside the glycosome  |
| Glucose (195) | Acetate (107) | +428             | +214                  |
| Glucose (195) | Succinate (88) | −176             | −176                  |
| Net balance |             | +252             | +38                   |

a Substrate level phosphorylation.
b cPGK, theoretical value with PGK active only in the cytosol; gPGK, theoretical value with PGK active only in the glycosome.

Table III
Calculated NADH and ATP balances of glucose and glycerol metabolism based on end product analysis (combination of data in Fig. 1 and Table I)

| Substrate | End product | NADH/mol product | ATP yield/mol productb |
|-----------|-------------|------------------|-----------------------|
|           |             | Total            | Inside the glycosome  |
| Glucose (510) | Acetate (153) | +612             | +306                  |
| Glucose (510) | Succinate (357) | −714             | −714                  |
| Glycerol (1279) | Acetate (217) | +651             | +434                  |
| Glycerol (1279) | Succinate (844) | 0                | 0                     |
| Net balance |             | +549             | +26                  |

a Substrate level phosphorylation.
b cPGK, theoretical value with PGK active only in the cytosol; gPGK, theoretical value with PGK active only in the glycosome.

T. brucei, there exists a third PGK gene, PGKA, the gene product of which is also found in the glycosome (31, 32). It could be this activity that takes care of the PGK reaction in procyclic glycosomes. PGK activity is also reported to be present in the glycosome as well as in the cytosol in T. cruzi epimastigotes and Leishmania mexicana and Leishmania major promastigotes and amastigote-like forms (Ref. 33 and references therein).

Tables I—IV show that the suggested presence of PGK inside the glycosome would result in a surplus of ATP produced inside the glycosome instead of a deficit. Therefore, in case of a bi-location of PGK activity, the ATP balance inside the glycosome can be easily maintained (or can even be positive) by altering the ratio of the fluxes from 1,3-bisphosphoglycerate to 3-phosphoglycerate via the two locations.

Functions of the Krebs Cycle Enzymes in Procyclic T. brucei—We recently demonstrated that, in procyclic T. brucei cells grown under standard in vitro culture conditions, the Krebs cycle is not used for energy generation by complete oxidation of acetyl-CoA (7). However, in that study, the standard medium used for culturing procyclic forms of T. brucei (SDM-79) contained an unrealistically high glucose concentration (10 mM) compared with its natural environment, the midgut of the tsetse fly. In the midgut, the amount of glucose originally present in the blood meal of the fly will not be replenished after consumption by the host and parasite. Therefore, the glucose concentration in the midgut will drop rapidly in time. It could be argued that the presence of large amounts of fermentable substrates present in the standard medium prevents the Krebs cycle from being used or from being induced, similar to the glucose repression of the Krebs cycle reported for bacteria and several types of yeast (10, 11). However, our present results show that, in procyclic T. brucei, it was not the presence of a large supply of fermentable carbohydrates that prevented the oxidation of acetyl-CoA via the Krebs cycle. Apparently, a Crabtree effect does not occur in T. brucei, as the absence of carbohydrates does not induce a shift from a fermentative metabolism to the use of the Krebs cycle for the complete oxidation of substrates. As glucose was, physiologically speaking, absent in some of our incubations, which still contained the usual amounts of amino acids, it is rather unlikely that the Krebs cycle will be used for the complete oxidation of substrates in other stages of the life cycle in the tsetse fly or under other conditions because a situation more compelling to induce the activity of a complete Krebs cycle can hardly be imagined.

Genes for all eight enzymes of the Krebs cycle are present in T. brucei (see below), and the expression of all enzymes of the Krebs cycle is reported to be induced upon transformation of the bloodstream form to the procyclic form (12). The question then arises as to why the Krebs cycle does not function in its usual way, as a complete cycle. It is conceivable that the kinetic properties of the enzymes in combination with the cellular conditions (for instance redox status) prohibit the cycle from functioning. On the other hand, it is also possible that the activity of one or more enzymes of the cycle is just too low compared with the activities of those enzymes diverting metabolites from the cycle, such as acetate/succinate-CoA transferase, which pulls acetyl-CoA toward acetate production (34). In this respect, it is worth mentioning that the activity of the
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mitochondrial NADP-dependent isocitrate dehydrogenase in *T. brucei* is very low (5–7 nmol/min/mg of protein) (35) and is not induced upon transformation from the bloodstream form to the procyclic stage, suggesting that this enzyme is not involved in any function of the Krebs cycle that is induced upon transformation (see below). However, no matter what the true reason is, the fact remains that, at least under all conditions tested so far, the Krebs cycle does not function as a true cycle in procyclic *T. brucei*.

If the Krebs cycle is not used as such, then what can its function in procyclic *T. brucei* be? We propose that large parts of the machinery are used for purposes other than the complete degradation of acetyl-CoA. These tentative processes are catabolic as well as anabolic. It is obvious that the part of the cycle from α-ketoglutarate to succinate is used for the degradation of proline, a clear catabolic function. On the other hand, our observation that, in intact proliferating procyclic forms, glucose and threonine can be used for the biosynthesis of fatty acids implies that these substrates are first converted into acetyl-CoA, a process that occurs inside the mitochondrion. For the biosynthesis of fatty acids, this acetyl-CoA has to be transported from the mitochondrion to the cytosol. This transport has not been investigated in *T. brucei*; but in all other systems studied, this transport proceeds via citrate. Therefore, we propose that the first enzyme of the Krebs cycle (citrate synthase) is used in procyclic *T. brucei* mainly for anabolic purposes, the formation of citrate for the biosynthesis of fatty acids. For this reaction to occur, also the last enzyme of the cycle (malate dehydrogenase) has to participate in the formation of oxaloacetate, which is needed in the citrate synthase reaction.

Another anabolic function that we propose for part of the Krebs cycle in procyclic *T. brucei* is its participation in gluconeogenesis by the conversion of succinate into malate, which can then be transported to the cytosol for the synthesis of phosphoenolpyruvate. The anabolic function of this part of the Krebs cycle follows directly from our demonstration that succinate (and hence proline) can be converted into acetate or be used for gluconeogenesis in the absence of glucose and glycero1. Proline is degraded mainly to succinate, but also to significant amounts of acetate (Fig. 1C). Since no direct pathway to acetate exists in the degradation of proline, this indicates that a small but significant amount of succinate is further metabolized to acetate. The most plausible pathway would be the conversion of succinate via fumarate into malate by the usual Krebs cycle enzymes, followed by malate export to the cytosol, where either malate dehydrogenase and phosphoenolpyruvate carboxykinase will convert the malate into phosphoenolpyruvate, which can then be degraded via pyruvate to acetate via the usual pathway, also used for the degradation of glucose and glycero1 to acetate, or malic enzyme will convert the malate directly into pyruvate, and the NADPH so produced will be used for biosynthetic purposes. These pathways explain the observed production of acetate from proline. Alternatively, phosphoenolpyruvate could be used in the standard gluconeogenic pathway for the synthesis of carbohydrates, or ribose 5-phosphate could be used for the biosynthesis of pyrimidine ribonucleotides. Indications for this latter gluconeogenic pathway were found in our incubations with labeled proline (Fig. 1C). When performed in the presence of glucose and/or glycero1, these incubations resulted in the near equimolar production and subsequent excretion of succinate and carbon dioxide. As 1 mol of carbon dioxide was produced during the degradation of 1 mol of proline to succinate, this indicates that only a small portion of the succinate formed was further metabolized under these conditions. On the other hand, in the absence of glycolytic substrates, proline degradation resulted in a very high ratio of carbon dioxide to succinate. This demonstrates that, under these conditions, a larger proportion of succinate was further metabolized. Our observation that, under these conditions, acetate production was relatively low also indicates that, in the absence of glycolytic substrates, further metabolized succinate was used for gluconeogenesis instead of being converted into acetate.

If these reactions of the Krebs cycle are indeed used mainly for the indicated purposes, this means that six of eight enzymes of the cycle have found a new function in the mitochondrial metabolism of procyclic *T. brucei*. This also implies that, only for the mitochondrialaconitate and NADP-isocitrate dehydrogenase, no function in procyclic *T. brucei* has been found yet. This correlates with the absence of a specific phenotype in the energy metabolism of theaconitate knockout mutant of procyclic *T. brucei* (7) and with the reported low activity of isocitrate dehydrogenase (35).

**Anabolic and Catabolic Functions of Parts of the Krebs Cycle**—Based on all our results combined with previous studies, we suggest a new metabolic scheme for the energy metabolism of procyclic *T. brucei* (Fig. 4). All enzymes (except for two enzymes in fatty acid biosynthesis, which are supposed to be present, but are not yet identified) presented in this scheme are encoded in the genome of *T. brucei* and, as far as is known, are expressed in the procyclic forms, and their location in the various compartments (glycosomes, cytosol, or mitochondrion) is as shown, which is concluded from the presence or absence of specific target sequences or from actual enzyme localization studies (Table V).

The proposed reactions occurring in the glycosome during the degradation of glucose are not different from those proposed previously (3, 8). When present, glucose is the main substrate for glycolysis, but our studies showed that, if present, glycero1 is also an important glycolytic substrate. Glucose and glycero1 are predominantly degraded to acetate and succinate, and this succinate is mainly produced inside the glycosome by the soluble fumarate reductase shown to be present in glycosomes (8). As discussed above, based on enzyme activity measurements and considerations of energy balance inside the glycosome, we propose a dual location for PGK, being active in the cytosol as well as in the glycosome.

In the cytosol, 3-phosphoglycerate and 1,3-bisphosphoglycerate are degraded to pyruvate, which is then either imported into the mitochondrion for further degradation or, to a lesser extent, transaminated to alanine. A gene for lactate dehydrogenase is absent in *T. brucei*; and therefore, no lactate production from pyruvate is depicted.

Inside the mitochondrion, pyruvate is degraded via pyruvate dehydrogenase to acetyl-CoA, which is then converted into acetate via the acetyl-CoA transferase/succinyl-CoA synthetase cycle as depicted (34). However, we reported recently that this cycle is not the only acetate-producing pathway in procyclic *T. brucei* (36). Our present analysis of the genome of *T. brucei* revealed that there is one other candidate for acetate production in procyclic forms, acetyl-CoA synthetase (Table V). Alignment of the amino acid sequence of this *T. brucei* enzyme with other known acetyl-CoA synthetases indicated that the trypanosomal enzyme is very similar to AMP-forming acetyl-CoA synthetases (~50% identical). Analysis of the sequence did not reveal a clear mitochondrial target sequence, and it is possible that the reaction occurs in the cytosol (Table V). This trypanosomal enzyme is currently under further investigation.

Next to pyruvate, also proline and threonine are important.

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2 F. R. Opperdoes, unpublished data.
Fig. 4. Schematic representation of pathways involved in carbohydrate and amino acid metabolism in procyclic *T. brucei*. Substrates are shown in blue boxes, and end products in black boxes. Numbered reactions are the ones catalyzed by enzymes that we searched for in the trypanosome data base and that are identified by these same numbers in Table V. The colored thick arrows in the background of the Krebs Cycle Functions in *T. brucei*.
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TABLE V
Data base analysis of metabolic pathways present in T. brucei (see also Fig. 4)

| Pathway/process and protein | Identification | Location |
|-----------------------------|----------------|----------|
| **Krebs cycle**             |                |          |
| 1. Citrate synthase         | Tb10.05.0150   | mmm      |
| 2. Aconitase                | Tb10.61.2880   | AF127457 |
| 3a. Isocitrate dehydrogenase (NADP) | Tb08.10J17.710 | m mm |
| 3b. Isocitrate dehydrogenase (NADPH) | Tb11.03.0230 | mm |
| 4. a-Ketoglutarate dehydrogenase | Tb11.01.1740 | mm |
| 5. Succinyl-CoA synthetase   | Tb03.4508.710  | m 40     |
| 6. Succinate dehydrogenase  | Tb08.30K1.380  | m 35     |
| 7. Fumarase                 | Tb11.02.2700   | mmm 40   |
| 8. Malate dehydrogenase (mitochondrial) | Tb10.70.5120 | AF027739 |
| 9. Malate dehydrogenase (cytosolic) | Tb11.01.3040 | AA83037 |
| 10. Phosphoenolpyruvate carboxykinase | Tb9.27.2.4210 | AAQ15878 |
| 11. Fructose-1,6-bisphosphatase | Tb09.211.0540 | A301578 |
| **Glycogenogenesis**        |                |          |
| 12. Pyruvate degradation     |                |          |
| 13. Acetyl-CoA carboxylase   | Tb08.30K1.730  | mm       |
| 14. Acetyl-CoAACP transacylase | Tb08.10J17.20 | mm       |
| 15. Malonyl-CoAACP transacylase | Tb09.211.3020 | mm |
| 16. 3-Ketoyl-ACP synthase    | Tb09.27.3.910   | XP 340489 |
| 17. 2-Amino-3-ketobutyrate:CoA ligase | Tb08.11J15.760 | m |
| **Transporters**            |                |          |
| 18. Proline dehydrogenase    | Tb07.3P12.290   | mmm      |
| 19. L-Threonine 3-dehydrogenase | Tb07.22O1.470 | m m |
| 20. Aspartate aminotransferase | Tb09.160.4310 | AF095907 |
| 21. Proline dehydrogenase    | Tb09.27.3.910   | XP 340489 |
| 22. Aspartate aminotransferase | Tb07.11.2740 | mm |
| 23. L-Threonine 3-dehydrogenase | Tb06.5F5.290 | AF529241 |
| 24. Malic enzyme             | Tb11.02.3130   | mmm      |
| 25. Malic enzyme             | Tb11.02.3120   | mm c 41  |
| **Pyruvate degradation**     |                |          |
| 26. Pyruvate dehydrogenase   | Tb07.3P12.290   | mmm      |
| 27. Acetyl-CoA synthetase    | Tb07.11.2740   | mm |
| 28. Pyruvate dehydrogenase   | Tb07.3P12.290   | mmm      |
| 29. Pyruvate transporter     | Tb11.01.5950   | mm       |
| 30. Pyruvate transporter (tricarboxylate transporter) | Tb09.160.2910 | mmm |
| **Glutamate degradation**    |                |          |
| 31. Alanine aminotransferase | Tb09.160.4241 | AF095907 |
| 32. Pyruvate dehydrogenase   | Tb07.3P12.290   | mmm      |
| 33. Acetyl-CoA synthetase    | Tb07.11.2740   | mm |
| 34. Pyruvate dehydrogenase   | Tb07.3P12.290   | mmm      |
| 35. Lactate dehydrogenase    | Tb07.11.2740   | mm c 43  |
| 36. Pyruvate carboxylase      | Tb07.3P12.290   | mmm      |

* Temporary systematic identity in the T. brucei genome data base (GeneDB Version 3).
* Predicted location based on target signal using TargetP/Psort/Predator. m, mitochondrial; (m), mitochondrial prediction, but with low score; g, glycosomal; –, no target signal detected.
* Location experimentally shown. c, cytosolic.
* A. Aranda, D. Maugerib, A. Uttaro, F. Opperdoes, J. J. Cazzulo, and C. Nowicki, submitted for publication.

The dark green arrow indicates that part of the cycle used during glycogenogenesis (see “Discussion” for further explanations). Genes for lactate dehydrogenase and pyruvate carboxylase are absent in T. brucei; and therefore, no lactate or oxalacetate production from pyruvate is depicted. AOX, alternative oxidase; GPI, glycosylphosphatidylinositol.

substrates for mitochondrial degradation in T. brucei. We have show that these amino acids were degraded mainly to succinate and acetate (+glycine), respectively, which is in accordance with previous reports (6, 18).

Degradation of the mitochondrial substrates pyruvate, proline, and threonine results in the production of reduced equivalents, NADH and FADH$_{2}$ (Fig. 4). Via these reduced equivalents, the electrons originating from the substrates are ultimately transferred to oxygen by means of a branched electron transport chain consisting of a plant-like alternative oxidase and a classical chain with complexes I–IV (37, 38). As we demonstrated previously, procytic T. brucei cells are completely dependent on the use of oxygen as the terminal electron acceptor by this branched electron transport chain because they die rapidly when both branches are inhibited simultaneously (7). Electron transport via this respiratory chain results in proton translocation, which can be used for oxidative phosphorylation. This process is used by procyclic T. brucei cells under standard conditions and contributes significantly to energy production, but is probably not essential, as inhibition of the ATP synthase by oligomycin results in a decrease in growth rate, but not in death of the organisms (27). This adaptation to inhibition of oxidative phosphorylation again demonstrates the metabolic flexibility of procytic T. brucei.

As we demonstrated previously, the Krebs cycle is not operative as such in procytic T. brucei (7). Here, we have presented evidence for a new metabolic scheme in which parts of the Krebs cycle are used for purposes other than the complete cycle represent functions of those parts of the cycle that are discussed in this study and that are active in procytic forms. The pink arrow stands for the flux from pyruvate and oxalacetate to citrate in the transport of acetyl-CoA units from the mitochondrion to the cytosol. The light green arrow represents that part of the cycle that is used for the degradation of proline and glutamate to succinate. The dark green arrow indicates that part of the cycle used during glycogenogenesis (see “Discussion” for further explanations). Genes for lactate dehydrogenase and pyruvate carboxylase are absent in T. brucei; and therefore, no lactate or oxalacetate production from pyruvate is depicted. AOX, alternative oxidase; GPI, glycosylphosphatidylinositol.
degradation of mitochondrial substrates. We propose that the enzymes pyruvate dehydrogenase, malate dehydrogenase, and citrate synthase result in the formation of citrate from malate and pyruvate. This citrate is not further degraded in the Krebs cycle, but leaves the mitochondrion to enter the cytosol, where it is cleaved again into oxaloacetate and acetyl-CoA. This acetyl-CoA can then be used for fatty acid synthesis (Fig. 4). The mitochondrial inner membrane is impermeable to oxaloacetate, which therefore has to be converted by cytosolic malate dehydrogenase into malate. This malate can then be transported either directly back into the mitochondrion or after conversion via malic enzyme into pyruvate. All the enzymes necessary for this whole system to function are present in T. brucei and in fact act as a shuttle to transport acetyl-CoA from the mitochondrial to the cytosol using a small part of the Krebs cycle (Fig. 4). The section of the Krebs cycle consisting of α-ketoglutarate dehydrogenase and succinyl-CoA synthetase is used by T. brucei for the degradation of proline and glutamate to succinate (Fig. 4). Following the observations and reasoning described above, the subsequent reactions of the Krebs cycle (succinate dehydrogenase and fumarase) are most likely used for the conversion of succinate into malate, which can then be used in gluconeogenesis (Fig. 4).

This metabolic chart of the metabolism of procyclic T. brucei forms demonstrates not only that the Krebs cycle is used for purposes other than the complete oxidation of substrates, but also that these cells have in fact a much greater flexibility than was anticipated previously. It is also clear, however, that still several questions need further investigation.

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