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Comparative Physiology of Two Protozoan Parasites, *Leishmania donovani* and *Trypanosoma brucei*, Grown in Chemostats

BENNO H. TER KUILE† AND FREDERIK R. OPPERDOES*

Research Unit for Tropical Diseases, International Institute for Cellular and Molecular Pathology, Avenue Hippocrate 74.39, B-1200 Brussels, Belgium

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Cultures of the insect stage of the protozoan parasites *Leishmania donovani* and *Trypanosoma brucei* were grown in chemostats with glucose as the growth rate-limiting substrate. *L. donovani* has a maximum specific growth rate (μ_{max}) of 1.96 day^{-1} and a K_s for glucose of 0.1 mM; the μ_{max} of *T. brucei* is 1.06 day^{-1} and the K_s is 0.06 mM. At each steady state (specific growth rate, μ, equals D, the dilution rate), the following parameters were measured: external glucose concentration (Glc_{out}), cell density, dry weight, protein, internal glucose concentration (Glc_{in}), cellular ATP level, and hexokinase activity. *L. donovani* shows a relationship between μ and yield that allows an estimation of the maintenance requirement (m_s) and the yield per mole of ATP (Y_{ATP}). Both the m_s and the Y_{ATP} are on the higher margin of the range found for prokaryotes grown on glucose in a complex medium. *L. donovani* maintains the Glc_{in} at a constant level of about 50 mM as long as it is not energy depleted. *T. brucei* has a decreasing yield with increasing μ, suggesting that it oxidizes its substrate to a lesser extent at higher growth rates. Glucose is not concentrated internally but is taken up by facilitated diffusion, while phosphorylation by hexokinase is probably the rate-limiting step for glucose metabolism. The K_s is constant as long as glucose is the rate-limiting substrate. The results of this study demonstrate that *L. donovani* and *T. brucei* have widely different metabolic strategies for dealing with varying external conditions, which reflect the conditions they are likely to encounter in their respective insect hosts.

Leishmania and *Trypanosoma* spp. are two closely related members of the family of the *Trypanosomatidae*, protozoan hemoflagellates, parasitic to most animals. These protozoa undergo complex life cycles which involve both a vertebrate and an insect vector. The sandfly (*Phlebotomus* spp.) serves as the vector for *Leishmania donovani*, which is the etiological agent of visceral leishmaniasis, or kala azar, in humans, while the tsetse fly (*Glossina* spp.) transmits trypanosomes of the *Trypanosoma brucei* complex, which cause sleeping sickness in humans and nagana in cattle (16). While the conditions to which the parasites are exposed are fairly constant inside the vertebrate host, the parasite must be able to adapt rapidly to changes in the alimentary tract of the insect vector. In particular, the concentration of the energy source (glucose and/or amino acids) is likely to be subject to wide variations. Insect stages of both *L. donovani* and *T. brucei* can be cultured in complex semidefined media at 25 to 28°C.

L. donovani accumulates glucose from the medium by a factor of approximately 80 by means of a pump driven by proton motive force (31, 32). It would not be advantageous for this organism if the proton motive force changed with the external pH. Indeed, *L. donovani* maintains its cytoplasmic pH and proton motive force constant over an extracellular pH range of 5.5 to 7.4 (33). Moreover, *Leishmania major* shows only a slight reduction in energy-dependent glucose uptake when going from the logarithmic to the stationary growth phase (8). These data suggest that *Leishmania* spp. have a tightly controlled physiology aimed at internal homeostasis, even when external conditions vary dramatically.

Contrary to the situation in *Leishmania* spp., the *T. brucei* bloodstream form does not accumulate glucose in an energy-dependent manner but relies on facilitated diffusion as an uptake mechanism (7, 10, 11, 25). These authors suggest that the uptake step is limiting the rate of the overall glucose metabolism. The K_m for glucose was estimated to be approximately 2 mM, sufficient in the blood of a mammalian host, but a K_m of this order of magnitude might severely limit the growth of the insect stage if uptake is indeed the overall rate-limiting step.

The insect stage of *T. brucei* contains cytochromes that are induced upon transition from the bloodstream to the insect form (13, 17, 28). Thus, the procyclic trypomastigotes are capable of at least partial oxidation of substrates, while the bloodstream form performs glycolysis only. This is advantageous when low glucose and high amino acid levels are encountered in the insect gut.

At present, few data are available on how both types of organism cope with environmental changes.

The most appropriate system to study physiological adaptations to varying substrate concentrations to date is the chemostat (continuous culture; see reference 29 for review). It allows the organism to be grown under constant conditions, at fixed rates, and with a growth rate-limiting factor chosen by the investigator. In particular, physiologically relevant parameters, such as K_s for the rate-limiting substrate, maximum growth rate, maintenance energy, and yield, can only be measured properly in a chemostat (23, 24, 29). In this article, we present physiological data obtained for *L. donovani* promastigotes and *T. brucei* procyclic trypomastigotes grown in such chemostats. The results clearly indicate that these two closely related organisms have entirely different physiological strategies. Furthermore, we show that *L. donovani* keeps its internal concentration of glucose constant as long as the organism is not energy depleted.

* Corresponding author.
† Present address: The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399.

MATERIALS AND METHODS

L. donovani infantum promastigotes and *T. brucei* strain 427 procyclic trypomastigotes were grown in chemostats with glucose as the growth-limiting substrate. The chemostats were of the single-stage flow-controlled type, as described by Veldkamp (29), with a working volume of 100 ml. The actual design has been described before (26). The glucose limitation was verified by observing the increase in cell density upon injection of a concentrated glucose solution and by the linear relationship between the glucose concentration in the medium reservoir (S_r) and the density of the culture at the concentrations used. When S_r exceeded 15 mM, glucose was no longer growth limiting. The medium used was filter-sterilized SDM 79 (2) supplemented with hemin and 10% (vol/vol) fetal calf serum having a lowered glucose concentration (S_r) was 4.553 and 10.842 mM in the case of *L. donovani* and *T. brucei*, respectively). In one series of steady-state cultures with *L. donovani*, glucose was not rate limiting (S_r = 14.91 mM). The medium reservoir was kept refrigerated in the dark, and all tubing and the culture vessel were shielded from the light by aluminum foil. The chemostats were driven by water-saturated air, and sampling was done by means of sterile syringes. The temperature was controlled at 27.5 ± 0.1°C by means of a waterbath. The oxygen concentration was measured on freshly drawn samples with an oxygen electrode. The oxygen level never went below 75% air saturation. The pH was subject to slight variations in the case of *T. brucei*, but values below 6.7 were never observed.

The density of the culture was monitored daily by counting cells in a Petroff-Hausser counting chamber. The density in the overflow was equal to the density in the culture vessel, indicating that at steady state, μ indeed equals D. When the culture had been growing under constant conditions for at least five doubling times and the density of the culture had not changed more than 1% for at least 24 h, the occurrence of a steady state was assumed. At every steady state, samples were taken for the following measurements: glucose concentration in the culture vessel (S), intracellular glucose concentration (Glc_{in}), cell density (as cell counts), protein concentration, ATP concentration, total dry weight, and hexokinase activity.

At this stage, metabolic products were not measured. In a continuation of this study, we intend to measure all products formed in order to describe carbon budgets.

The samples for measurement of the glucose concentration in the culture vessel were made by rapidly drawing a fresh 5-ml sample and filtering it into two test tubes. These were stored at −20°C, and the glucose level was determined enzymatically in duplicate by the method of Bergmeyer (1). Taking into consideration the specific consumption of glucose and the duration of the sampling procedure (11 to 13 s), our calculations suggest that during the sampling procedure, no more than 3% of the glucose was consumed. The standard deviation of the four determinations exceeded 1% of the measured value only in the case of samples containing less than 50 μM glucose.

The glucose concentration inside the organisms was determined by the silicone oil centrifugation technique (8). Ten samples of 250 μl were added to 400-μl microfuge tubes containing 25 μl of 1.7 M perchloric acid with 50 μl of 1-bromododecane on top of it, and these were centrifuged immediately. The samples so obtained were stored at −20°C and used for the measurements of Glc_{in} and ATP. Glc_{in} was measured enzymatically in duplicate for the combined pellets of four tubes, after the perchloric acid was neutralized, in an Aminco-Chance double-wavelength DW 2a spectrophotometer at the wavelength couple 340 and 400 nm. The volume of the interstitial water in the pellet was measured by using [^{3}H]inulin. The difference between the duplicates never exceeded 5% of the measured value.

The protein content of three 1-ml samples for each steady state was determined by the fluorescamine method (22) with bovine serum albumin as the standard. The standard error of this method is 6.9%.

ATP was measured for four replicate samples, obtained as for the internal glucose measurement, by the luciferin-luciferase method (Boehringer ATP bioluminescence CLS kit) after the perchloric acid was neutralized. The standard error of this method was at most 4.2% of the measured value.

The total dry weight was measured in duplicate as the additional weight of a preweighed Nucleopore filter (pore diameter, 0.4 μm) on which a pellet had been filtered. The filters were dried overnight in a hood. The average difference between duplicates was 7.6%.

The hexokinase activity was determined for duplicate samples that were resuspended in 0.1% Triton X-100 as described before (1). The suspension was added to a buffer containing all reactants required for glucose determinations as above, with 10 mM glucose added. The hexokinase activity was calculated from the rate of the reaction. It is reported in units (micromoles of product formed per minute) per milligram of total sample protein.

RESULTS

L. donovani. All measurements were carried out under steady-state conditions. Under these conditions, the specific growth rate (μ) equals the dilution rate (D). The μ_{max} and K_m were estimated by using the direct linear plot (6), which is based on straight lines connecting the measured substrate concentration (S) plotted on the negative x axis, with the corresponding μ on the y coordinate. The coordinates of the intersection point of the extrapolated lines give statistically more reliable estimates of μ_{max} and K_m than the classical $1/\mu$ versus $1/S$ plot, because all points are equally weighted (6). In the case of *L. donovani*, the four highest dilution rates gave a single intersection point at K_s = 0.1 mM and μ_{max} = 1.96 day^{-1}, corresponding to an average time between divisions of slightly more than 12 h (Fig. 1A). The lowest two dilution rates crossed at increased x values, suggesting slightly higher K_s values (Fig. 1A).

The plots of Glc_{out} (S), cell density, dry weight, protein content, and ATP content for *L. donovani* grown under glucose limitation against the dilution rate (D) shown in Fig. 2A resembled the typical curves obtained for prokaryotes grown in chemostats (see reference 29 for review) in most respects. However, the increase in the yield of cells per mole of glucose consumed with increasing D occurred in two stages: a slow increase between D = 0.314 day^{-1} and D = 1.090 day^{-1} and a more rapid increase between D = 1.090 day^{-1} and D = 1.860 day^{-1} (Fig. 2B). In this latter range, the internal glucose concentration (Glc_{in}) remained constant at about 53 mM (Fig. 2B). Even when glucose was not growth rate limiting, a similar value was found independently from the μ (Fig. 2D). At D below 1.090 day^{-1}, when glucose was limiting, Glc_{in} was reduced (Fig. 2B). The decrease in Glc_{in} coincided with a sharp decrease in cellular ATP content, from approximately 12 to 1 nmol/mg of protein (Fig. 2A and B), indicating a strong depletion of energy at low D in these

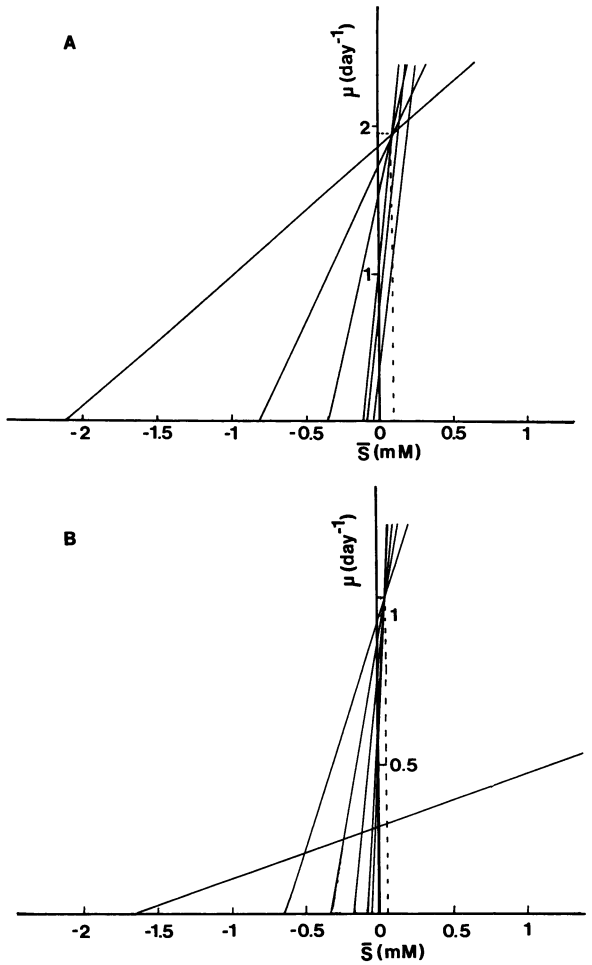

FIG. 1. Direct linear plot (6) of the relation between glucose concentration and specific growth rate (μ) for (A) *L. donovani* and (B) *T. brucei* grown in continuous culture. The substrate concentrations (millimolar) measured at each steady state are plotted on the negative x axis and are connected to the corresponding μ (day^{-1}) plotted on the y coordinate. The coordinates of the intersection point obtained by extrapolation indicate μ_{max} and K_s. Note that at the two lowest substrate concentrations, a higher value for K_s in the case of *L. donovani* is indicated. For *T. brucei* at the lowest dilution rate, glucose may not have been rate limiting.

organisms. At growth rates exceeding $D = 1\ day^{-1}$, the yield increased while the specific substrate consumption per hour (q) did not increase correspondingly (Fig. 2B). This suggests that a metabolic switch towards energy saving had occurred at increased dilution rates. The relative hexokinase activity was constant within the precision of the measurement (Fig. 2B), indicating that this energy saving is not caused by changes in the metabolic activity.

When *L. donovani* was not grown under glucose limitation (Fig. 2C and D), the relation between the external glucose concentration and the dilution rate was more or less linear (Fig. 2C). The cell mass, measured as cell number, dry weight, or protein, showed a slight decrease at the highest dilution rates (Fig. 2C). The intracellular ATP concentration remained constant (Fig. 2C), as did the internal glucose concentration (Fig. 2D). The yield and the specific substrate consumption (q) increased linearly with the dilution rate, as did the relative activity of hexokinase, in contrast to what was found in glucose-limited cultures (Fig. 2D). The yield per mole of substrate was much lower than under glucose limitation, while the q and the hexokinase activity were much higher (compare Fig. 2B and D). This indicates that from an energy point of view, the metabolism under conditions in which glucose is nonlimiting is rather wasteful, as has been described for other organisms.

T. brucei. The direct linear plot of the data set for *T. brucei*, with the exception of $D = 0.298\ day^{-1}$, had one intersection point only, indicating that μ_{max} and K_s were constant for the range above $D = 0.298\ day^{-1}$ (Fig. 1B). The glucose concentration in the culture vessel (S) at $D = 0.298\ day^{-1}$ was more than 30 times higher than at $D = 0.460\ day^{-1}$. At this low growth rate, lysis was not observed and the steady state remained stable, suggesting that in this case glucose was not rate limiting (Fig. 3A). Attempts to reach a steady state at even lower dilution rates were unsuccessful; the cells lysed and a stable steady state could not be reached. The μ_{max} of *T. brucei* was $1.06\ day^{-1}$, which corresponded to an average time between divisions of almost 23 h. A similar doubling time was found in batch cultures of *T. brucei* in the same medium. The K_s for glucose was 0.06 mM.

The results obtained for *T. brucei* were strikingly different from those for *L. donovani*. The yield was highest at the lower dilution rates and decreased strongly with increasing D (Fig. 3B), while the cellular ATP content increased slowly (Fig. 3A). At the two lowest D values, the yield equaled approximately the yield found for *L. donovani*. The specific substrate consumption (q) increased exponentially with D (Fig. 3B) rather than linearly, as is the case with other organisms, including *L. donovani*. At all dilution rates, the Glc_{in} was lower than or equal to the external glucose concentration (Fig. 3B), which indicates that active accumulation of glucose inside *T. brucei* did not occur. The relative levels of hexokinase did not change (Fig. 3B).

DISCUSSION

Overall energy metabolism of *L. donovani*. It is difficult to estimate the metabolic efficiency of *L. donovani*. The exact amount of ATP derived from each mole of glucose depends on the relative contribution of fermentation and mitochondrial oxidation and on the ATP yield of respiration. At present, it is estimated that both contribute equally (3–5). If a P/O ratio of about 1.5, as is found in yeasts (30), is assumed, 1 mol of glucose should give approximately 13 mol of ATP. The data of Keegan et al. (15) suggest that the partitioning of glucose metabolism over the pentose-phosphate pathway and the Embden-Meyerhof pathway changes little with culture age during log-phase growth. This indicates that, as long as glucose metabolism is the limiting factor of growth, the yield of ATP per mole of glucose does not change. Changes in the oxygen concentration, on the other hand, may affect glucose metabolism (5, 14), but this is unlikely to have occurred in the chemostat, as the air above the culture was continuously replaced and the culture itself was well stirred. The yield in milligrams (dry weight) per millimole of ATP, corrected for the maintenance requirement, increases slightly with D from 29 at $D = 0.314\ day^{-1}$ to 33 at $D = 1.704\ day^{-1}$. These values are approximately the theoretical maximum value (28.8 to 31.9 mg [dry weight] per mmol of ATP [23]). The data obtained for cultures in which glucose was not limiting (Fig. 2C and D) suggest higher values for m_s than in the case of glucose-limited cultures and possible waste of ATP.

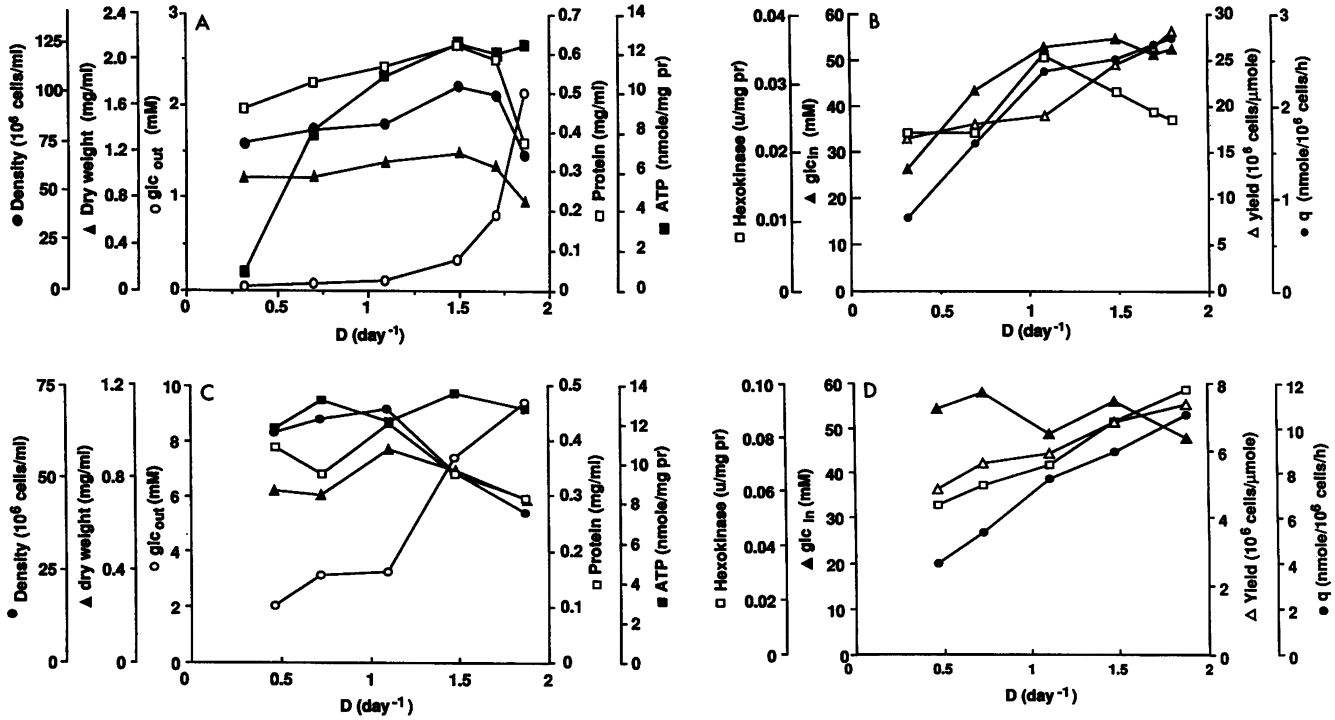

FIG. 2. *L. donovani* grown under glucose limitation (A and B) and non-glucose limitation (C and D). (A and C) Plot of glucose concentration in the culture vessel, dry weight, cell density, protein content, and internal ATP level (per milligram of protein [pr]) against D under steady-state conditions ($\mu = D$). (B and D) Plot of internal glucose concentration, yield, specific substrate consumption q, and relative hexokinase activity.

L. donovani is known to precisely regulate its internal pH and its proton motive force over the plasma membrane (33) and its internal calcium levels (19). Our results show that, as long as the organism is not depleted of energy, the internal glucose concentration (Glc_{in}) is strictly regulated around a value of approximately 50 mM and that this value is independent of the external glucose concentration. As a result, the ratio Glc_{in}/Glc_{out} may vary from 6 to 600 (Fig. 2B and D), depending on growth conditions. The transport of glucose is driven by proton motive force (32) and hence is energy dependent.

The energy needed for maintaining constant internal conditions is part of the maintenance energy. The term maintenance energy is applied to the ATP needed for all purposes other than growth, while the maintenance requirement (m_s) is the amount of substrate used for these purposes. Generally, m_s is a constant amount per hour, independent of the specific growth rate μ. The m_s is equal to the specific substrate consumption (q) at zero growth rate, which can be estimated by extrapolation of q to $D = 0$ (24). The value of m_s derived this way is 0.036 μmol/mg of protein/h. The maintenance requirement accounts for a rather large portion of the substrate consumed (15 to 27%) compared with the requirement in yeasts (less than 10%) (23, 30). While making these comparisons, it should be noted for each species that the portion of substrate used for maintenance decreases with increasing growth rate. Therefore, fast-growing organisms like yeasts use a much smaller portion of their energy source for nongrowth purposes than slower-growing organisms.

At values of D exceeding 1, *L. donovani* switches to an "energy-saving" metabolism, characterized by increasing yield, not accompanied by a corresponding increase in q.

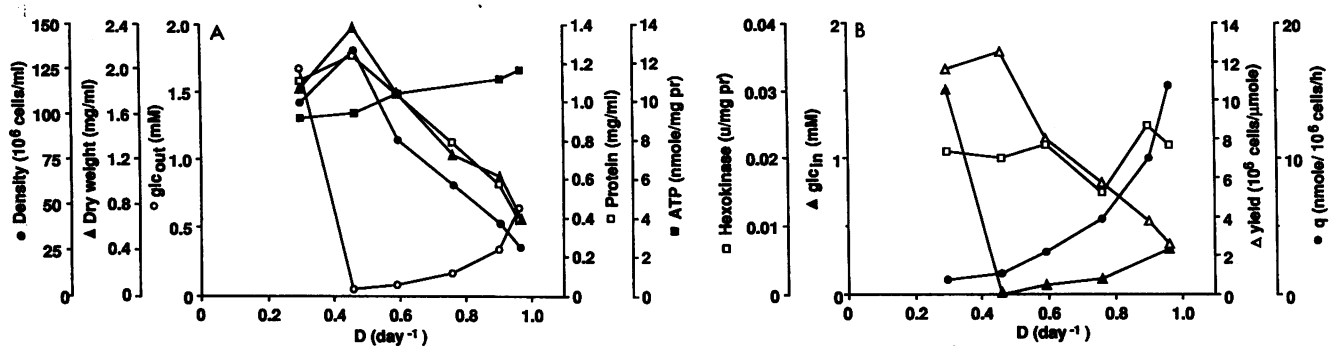

FIG. 3. *T. brucei* grown under glucose limitation. See Fig. 2 legend for details.

This switch occurred around the K_s of *L. donovani* for glucose and may be caused by a more efficient use of the substrate or a reduction in ATP use, e.g., for concentrating glucose. The physiology at the low growth rates differed from those at $D > 1$ in the following ways. (i) The intracellular concentration of free (unphosphorylated) glucose decreased. It was constant under all other conditions examined. (ii) The intracellular ATP concentration decreased, while it was constant at higher Glc_{out}, also in slowly growing non-glucose-limited cells. (iii) The K_s for glucose increased, but the ratio Glc_{in}/Glc_{out} continued to increase as well.

We have measured the internal volume of *L. donovani* at both $D = 0.5$ and 1.5 day^{-1} under glucose limitation and nonlimitation. Internal volume remained constant at approximately 2.1 μl/mg of protein under all conditions. Volume changes are therefore unlikely to be part of the energy-conserving mechanism, nor can they have interfered with our measurements.

Opportunistic metabolism of *T. brucei*. The metabolic strategy of the insect stage of *T. brucei* differs from that of *L. donovani*. Glucose is not concentrated inside the organism and is probably taken up by facilitated diffusion, as is the case for the bloodstream form of *T. brucei* (7, 10, 11, 25, 27). The observed K_s of 0.06 mM for glucose in the chemostat is in marked contrast to the affinity of the glucose carrier for glucose, as was determined both in short-term experiments on the bloodstream form (approximately 2 mM [7, 10, 11, 25]) and in short-term uptake experiments on the insect stage obtained from the chemostat (approximately 2 mM [27]). This suggests that the uptake of glucose by the insect stage is not the overall rate-limiting step in the glycolytic pathway. With a low-affinity facilitated diffusion carrier present in high abundance, the uptake rate under equilibrium conditions may be sufficient to saturate a subsequent metabolic step, which then becomes rate limiting. This is most likely the phosphorylation of glucose by hexokinase, an assumption that is based on three observations. (i) The uptake capacity of *T. brucei* for glucose is linearly correlated to its hexokinase activity (27). (ii) The K_m of the hexokinase of *T. brucei* for glucose is about 0.1 mM (unpublished observation), not too dissimilar from a K_s of 0.06 mM for glucose measured in the chemostat. (iii) The insect stage has a poor glycolytic capacity. This is mainly due to the low amount of the enzyme hexokinase (12), which drops in activity by 14-fold (from 0.76 to 0.053 U mg^{-1}) upon transformation from bloodstream to insect stage.

It is impossible to estimate the maintenance requirement of *T. brucei* as has been done for *L. donovani* because the yield decreases rather than increases with increasing μ. It can only be concluded that at low dilution rates ($D = 0.3$ to 0.5 day^{-1}), the yield is in the same range as that for *L. donovani* (100 to 120 mg of protein [mmol of glucose]$^{-1}$), assuming that only glucose is used as a carbon and energy source. Such a decreasing yield can be explained in two ways. (i) In a rich medium, at low μ, *T. brucei* oxidizes not only glucose but other components of the medium as well (9, 21). However, extrapolation of the density as a function of the glucose concentration in the storage vessel at $D = 0.5$ day^{-1} suggests that the cell mass derived from components other than glucose does not exceed 10% of the observed cell mass. (ii) At low μ, the substrate is more completely oxidized than at high μ. This is most likely the case with *T. brucei* insect forms, in which cytochromes are easily induced and repressed in response to changing culture conditions (13, 18, 28). Such a regulatory mechanism may fulfill an important role in the adaptation of the parasite to the changing physiological conditions in the midgut of the tsetse fly.

Metabolic strategies. In conclusion, it can be said that *L. donovani* and *T. brucei* have widely differing physiological strategies for dealing with changing environmental conditions. *L. donovani* aims at strictly maintaining internal conditions. *T. brucei*, on the other hand, has a yield that differs widely according to substrate availability. The range over which the dilution rate can be varied is much larger for *L. donovani* than for *T. brucei*. Below $0.28 \times \mu_{max}$, *T. brucei* lysed, while *L. donovani* still continued to grow down to $0.16 \times \mu_{max}$. These observations suggest that *L. donovani* has a greater ecological flexibility than *T. brucei*. These physiological differences may well reflect the different diets of their respective insect hosts. The sandfly, host and vector of *L. donovani*, feeds on plant juices as well as on blood, while the tsetse fly, host and vector of *T. brucei*, restricts itself to blood meals only.

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