

Adaptation of Metabolic Enzyme Activities of *Trypanosoma brucei* Promastigotes to Growth Rate and Carbon Regimen

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The insect stage of *Trypanosoma brucei* adapted the activities of 16 metabolic enzymes to growth rate and carbon source. Cells were grown in chemostats with glucose, rate limiting or in excess, or high concentrations of proline as carbon and energy sources. At each steady state, samples were collected for measurements of substrate and end product concentrations, cellular parameters, and enzyme activities. Correlation coefficients were calculated for all parameters and used to analyze the data set. Rates of substrate consumption and end product formation increased with increasing growth rate. Acetate and succinate were the major nonvolatile end products, but measurable quantities of alanine were also produced. More acetate than succinate was formed during growth on glucose, but growth on proline yielded an equimolar ratio. Growth rate barely affected the relative amounts of end products formed. The end products accounted for the glucose consumed during glucose-limited growth and growth at high rates on excess glucose. A discrepancy, indicating production of CO₂, occurred during slow growth on excess glucose and, even more pronounced, in cells growing on proline. The activities of the metabolic enzymes varied by factors of 2 to 40. There was no single enzyme that correlated with consumption of substrate and/or end product formation in all cases. A group of enzymes whose activities rigorously covaried could also not be identified. These findings indicate that *T. brucei* adapted the activities of each of the metabolic enzymes studied separately. The results of this complex manner of adaptation were more or less constant ratios of the end products and a very efficient energy metabolism.

The carbohydrate metabolism of *Trypanosoma brucei*, causative agent of African sleeping sickness and of nagana in cattle, has been explored in great detail in order to identify targets for chemotherapy. In the mammalian host, it relies solely on glycolysis for ATP production, converting glucose to pyruvate (17). The procyclic form, corresponding to the insect stage, metabolizes glucose to CO₂, acetate, succinate, and alanine (7, 17). Seven enzymes of the glycolytic pathway are located in the glycosome, an organelle that is unique to the *Kinetoplastida* (17, 18). In the bloodstream form, the mitochondrion is involved only in maintaining the redox balance. The procyclic form has a more developed mitochondrion containing an active electron transport chain (16, 33). Despite this, the metabolism remains primarily fermentative even in the presence of oxygen.

The procyclic form grows even better on amino acids such as proline or glutamic acid than on glucose (8, 9). These carbon sources are abundantly available in the midgut of the tsetse fly. In most organisms, proline is metabolized in mitochondria, where it enters the tricarboxylic acid cycle as α -ketoglutarate after sequential conversion to pyrroline-5-carboxylate, glutamate γ -semialdehyde, and glutamate (24). This is assumed also to be the pathway in *T. brucei* (7); thus, CO₂ can be expected to be the major end product, but succinate and acetate are also produced.

In the bloodstream of the mammalian host, *T. brucei* benefits from constant conditions, including a steady supply of glucose. In the insect vector, the organism encounters variations in the availability of glucose and amino acids, depending on the feeding status of the fly. Shifts in metabolic patterns are needed to maintain functional homeostasis (11). Thus, the *T. brucei* cul-

ture form is suitable for the study of metabolic adaptations to changing environmental conditions. The extent of the metabolic adaptations will give important information on the physiological limits of the organism. Such investigations cannot be performed in vivo but are adequately simulated in chemostats, which enable the long-term growth of cultures under constant conditions that can be manipulated according to experimental design (32). The involvement of the mitochondrion in the energy metabolism can be appraised by measuring ratios of end products at different growth rates and under different carbon regimens. Specifically, it would seem logical to expect that when the glucose supply is low, oxidation would be more complete. Changes in the activities of metabolic enzymes forced upon the organism by alterations of the external conditions can reveal the internal organization of the overall energy metabolism.

Chemostat studies on *T. brucei* promastigotes (28, 29) were performed previously (i) to describe the organism's physiology in general terms and (ii) to study the mutual adaptation of glucose transport across the plasma membrane and the phosphorylation step trapping the substrate inside the cell. The most important conclusions from this research were that *T. brucei* has an opportunistic metabolic strategy aimed at maximizing yield at the cost of ecological flexibility and that the transport and subsequent metabolic steps are mutually adjusted to saturate the metabolic machinery without creating unnecessary overcapacity.

This study had three aims: (i) to determine the rates of substrate consumption and end product formation under various growth rates and carbon regimens, (ii) to investigate the adaptations of enzyme activities induced by changes in the external conditions, and (iii) to identify those enzymes whose cellular activities correlate best with the rates of overall metabolism. Taken together, these three types of information can be used to understand the organization of the metabolism of *T. brucei* and identify enzymes that could be promising targets for

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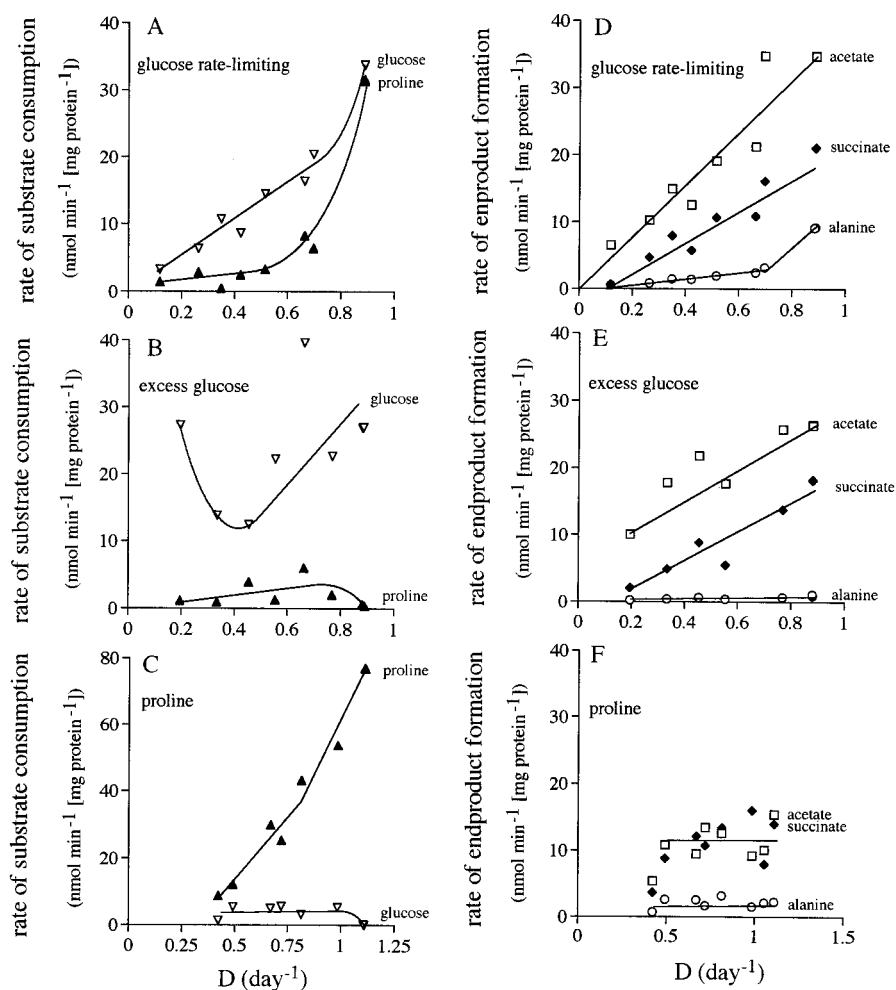


FIG. 2. Rates of glucose (A and B) and proline (C) consumption and end product formation (D to F) of *T. brucei* under the carbon regimens indicated. The units for rates are nanomoles consumed or produced per minute per milligram of total cellular protein. Note that the scales of the growth rate (D in doubling times per day) are different for glucose- and proline-grown cultures.

products acetate, succinate, and alanine and of the substrates glucose and proline were measured.

Correlation coefficients were calculated between all measured parameters and used for analysis of the data set. A high degree of correlation between enzymes would indicate that their activities are regulated by a single mechanism. Identification of enzymes appearing to exert most rate control over the entire pathway was attempted by using correlation between enzyme activities and rates of substrate consumption and end product formation. On the one hand, it cannot be concluded with certainty that the metabolic flux is controlled by these enzymes, because correlation does not prove causal relationship. On the other hand, lack of correlation makes a rate-controlling role unlikely.

The following topics were selected for presentation: (i) rates of substrate consumption and end product formation as a function of the dilution rate, (ii) cellular activities and extent of activity changes of metabolic enzymes, (iii) correlation between activities of individual enzymes and the rates of substrate consumption and end product formation, and (iv) the degree of covariation of different enzymes.

Substrate consumption. The specific rate of glucose consumption (Fig. 2A) increased linearly with increasing growth

rate when glucose was rate limiting. Proline was present in the glucose-based medium in a concentration of 0.5 mM. It accounted for a smaller part of the total carbon consumption at the low growth rates, but almost as much proline as glucose was used at the highest D values. Glucose consumption by cells growing on excess glucose far exceeded that of organisms grown under glucose limitation at low growth rates (Fig. 2B). In fast-growing cultures, the rates of glucose consumption were equal. The maximum growth rate of *T. brucei* on proline was about 20% higher than that on glucose (Fig. 2C). Surprisingly, attempts to grow cells on proline at less than 0.4 doubling time per day were not successful. The compositions of the proline and glucose media differed greatly, but fetal bovine serum was added to both in the same concentration. Glucose was present and was consumed in the amino acid-based (proline) medium but could not substitute as the sole carbon and energy source at the low dilution rates. At D values exceeding 1 per day, glucose was no longer consumed.

Rates of end product formation. The measured end products were acetate, succinate, and alanine. The concentrations of lactate, pyruvate, and glycerol were the same in the culture fluid and in the medium (less than 0.2 mM) and were thus neither consumed as substrate nor formed as end products.

TABLE 2. Carbon budgets for chemostat cultures of *T. brucei*^a

Culture	Dilution rate	Glucose consumed	Proline consumed	Acetate produced	Succinate produced	Alanine produced	CO ₂ (glucose only) calculated	CO ₂ (glucose + proline) calculated
Glucose rate limited								
	0.119	9.18	4.1	18.47	1.78	1.37	6.91	27.41
	0.264	9.20	4.1	14.76	6.75	1.19	-4.89	15.61
	0.350	9.01	0.4	12.54	6.64	1.24	-1.30	0.70
	0.423	6.61	1.9	9.57	4.37	1.08	-0.20	9.30
	0.516	9.17	2.1	12.11	6.75	1.24	0.08	10.58
	0.664	3.95	2.0	5.13	2.62	0.59	1.19	11.19
	0.698	4.44	1.4	7.56	3.51	0.69	-4.59	2.41
	0.889	2.14	2.0	2.20	1.33	0.58	1.38	11.38
Glucose non-rate limited								
	0.195	31.93	1.3	11.77	2.41	0.24	157.68	164.18
	0.334	16.49	1.1	21.19	5.85	0.47	31.75	37.25
	0.454	16.67	5.2	29.29	11.91	0.88	-8.84	17.16
	0.554	14.23	0.8	11.29	3.50	0.24	48.08	52.08
	0.662	18.55	2.8	5.33	1.61	0.0	94.20	108.20
	0.768	19.28	1.7	21.86	11.70	0.58	23.42	31.92
	0.881	18.24	0.4	17.91	12.33	0.61	22.47	24.47
Proline-based medium								
	0.422	1.22	7.5	4.52	3.09	0.61	-15.91	21.59
	0.492	3.18	7.1	6.33	5.14	1.50	-18.64	16.86
	0.670	2.02	11.9	3.76	4.82	1.01	-17.71	41.79
	0.720	2.22	10.0	5.31	4.23	0.66	-16.20	33.80
	0.814	1.11	15.0	4.37	4.66	1.09	-23.99	51.01
	0.984	1.53	15.3	2.62	4.55	0.43	-15.55	60.95
	1.109	0.0	16.8	3.37	3.05	0.49	-20.41	63.59
Avg for proline-based medium		1.61		4.33	4.22	0.83	-18.34	

^a The difference between concentrations of substrates (consumed) and end products (formed) in the medium and filtered culture fluid is expressed as millimolar. These differences are a measure of the metabolic activity of the culture, not corrected for biomass. The production of CO₂ is calculated from the difference between substrate(s) consumed and end products formed, each converted to C₁ units. The first value for CO₂ production takes only the consumption of glucose into consideration; the second is based on both glucose and proline. Since proline serves both as a carbon and energy source and as a source of amino acids, the actual production of CO₂ is probably between the two values.

Acetate was under all conditions formed as an end product (Fig. 2D to F; Table 2). When glucose was the primary carbon and energy source, rates of succinate production were very low at the lowest growth rates and increased with increasing growth rates. The rate of increase was similar to that of acetate production (Fig. 2D and E). Therefore, the ratio of the two end products changed gradually as a function of the growth rate (Table 2). Alanine was produced in small but measurable quantities. The formation of CO₂ was calculated from the balance of carbon consumed and produced in the form of nonvolatile end products (Table 2). Taking only glucose consumption into consideration, the substrate consumed was approximately accounted for by the end products formed in glucose-limited cultures and for cells growing on excess glucose at rates exceeding half the maximum growth rate. When proline is also calculated, there is a slight deficit in end products, but proline is incorporated in proteins in addition to being used as a carbon and energy source. This finding implies that little or no CO₂ is excreted as an end product. A larger deficiency, indicating significant production of CO₂, occurred in cultures growing at low rates on excess glucose and even more so during growth on proline. In the amino acid-based medium, proline was the main carbon and energy source, even though glucose was present in concentrations similar to those in the glucose-limited cultures. Acetate and succinate were produced in a 1:1 molar ratio, and surprisingly, their concentrations remained constant independent of the dilution rate (Fig. 2F; Table 2).

Enzyme activities. The activities per total cellular protein of the enzymes indicated in Fig. 1 are presented in Table 1. The

highest and lowest values and their ratios are given. Capacity, defined as activity divided by the flux of metabolites of the corresponding steady state, is presented in the same manner. The specific activities were very similar to those reported by Hart and coworkers (10), with the exception of that of glycerol-3-phosphate dehydrogenase (G-3-PDH). The ratios between the highest and lowest values differed between 2 and 42 if the very low values for ICDH are disregarded. The capacities indicated that the specific activities of most glycolytic enzymes could account for the flux of metabolites, allowing a factor of up to 5 for nonoptimization of the *in vitro* assays. *In vivo* rates may be higher due to compartmentalization in the glycosome. Exceptions were aldolase and pyruvate kinase (PK). RNA is a known inhibitor of aldolase and probably has caused a reduction of the aldolase activity in the cell lysates used for the measurement. PK had a higher activity in the presence of fructose-2,6-bisphosphate (31). The minimum capacity exceeded the flux significantly in only one case (TIM), and the maximum capacities always accounted for the measured fluxes. Large excess capacities were observed only for, in descending order, TIM, hexokinase (HK), malate dehydrogenase (MDH), and phosphoglucose isomerase (PGI).

Of all enzymes examined, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and acetate:succinate coenzyme A transferase (ASCT) (30) correlated best to substrate consumption and also to the formation of end products in *T. brucei* growing under glucose limitation (Fig. 3). Another enzyme, G-3-PDH, correlated best to end product formation in cells growing on excess glucose (Fig. 4). Unexpectedly, the final enzyme of

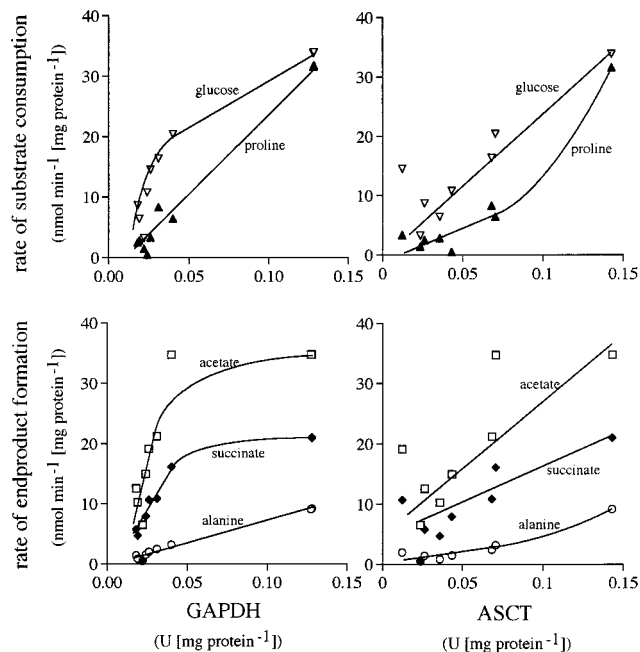


FIG. 3. Rates of glucose and proline consumption (upper panels) and formation of acetate, succinate, and alanine (lower panels) in glucose-limited-grown cells as a function of the specific activity of GAPDH (left panels) and ASCT (right panels), the only enzymes to correlate well to these rates.

acetate formation, ASCT, correlated positively to substrate consumption but negatively to acetate formation. The production of metabolic end products in organisms growing on proline was correlated not only to ASCT but also to enolase, an

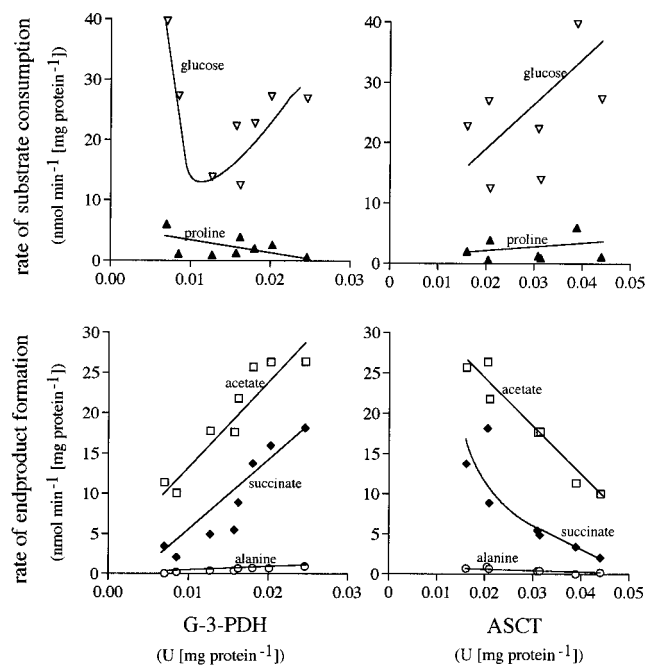


FIG. 4. Rates of substrate consumption and end product formation for excess-glucose-grown organisms. In this case, ASCT (right panels) correlated best with substrate consumption and G-3-PDH (left panels) was the only enzyme correlating well to rates of end product formation.

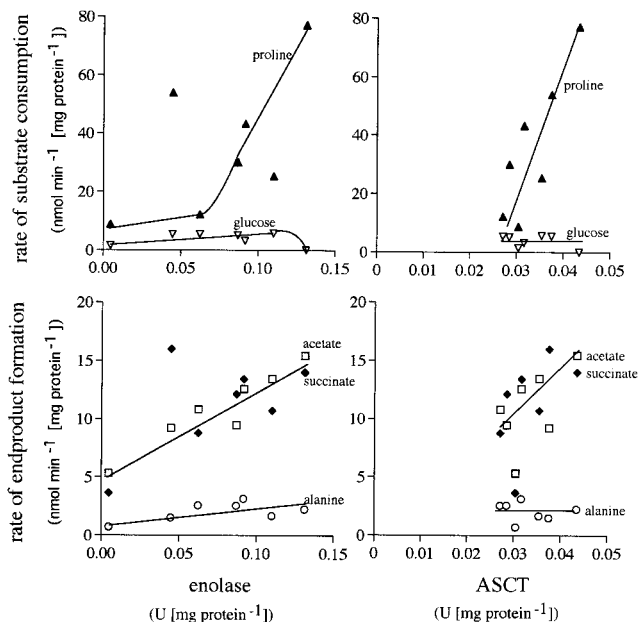


FIG. 5. Rates of substrate use and end product formation for cells growing on proline. Of the measured enzymes, enolase and ASCT correlated best to both processes.

enzyme that does not have a role in proline metabolism (Fig. 5).

By using correlation coefficients, groups of enzymes whose specific activities most covaried were identified (Fig. 6). Even though the activities of some of the enzymes varied by more than an order of magnitude, there was little correlation between the activities of the different enzymes in most cases. For cells growing on glucose, G-3-PDH was the enzyme having the highest total of correlation coefficients of a group consisting of MDH, enolase, fumarate reductase, and PK for glucose-limited cultures and of PFK, PGI, malic enzyme, and PK for excess-glucose-grown organisms. A similar group in *T. brucei* grown on proline consisted of HK, PGI, malic enzyme, G-3-PDH, and PFK. In this respect, proline- and excess-glucose-grown cells resembled each other more than glucose-limited and glucose-excess cultures.

DISCUSSION

Physiology of intact *T. brucei*. In this study, the maintenance energy of *T. brucei* growing on glucose could be estimated because the relationship between substrate consumption and growth rate was linear when glucose was rate limiting. The specific rate of substrate consumption can thus be extrapolated to the hypothetical state that the growth rate is zero and cells do not multiply. In that case, all energy consumed is used for maintenance or nongrowth purposes (32). No linear relationship was observed in an earlier study in which the pH was not regulated (28), possibly due to changes in the carbon balance caused by variations in the pH of the culture. The estimated maintenance energy was very low; 1 to 2% of the total carbon consumption was used for nongrowth purposes, in the same range as what is found in yeasts (23). By comparison, *Leishmania donovani* (15 to 27%) (28) and *T. vaginalis* (up to 50%) (25) are far less energy efficient.

Other interesting observations are that the maximal growth rate on proline is higher than that on glucose and that growth

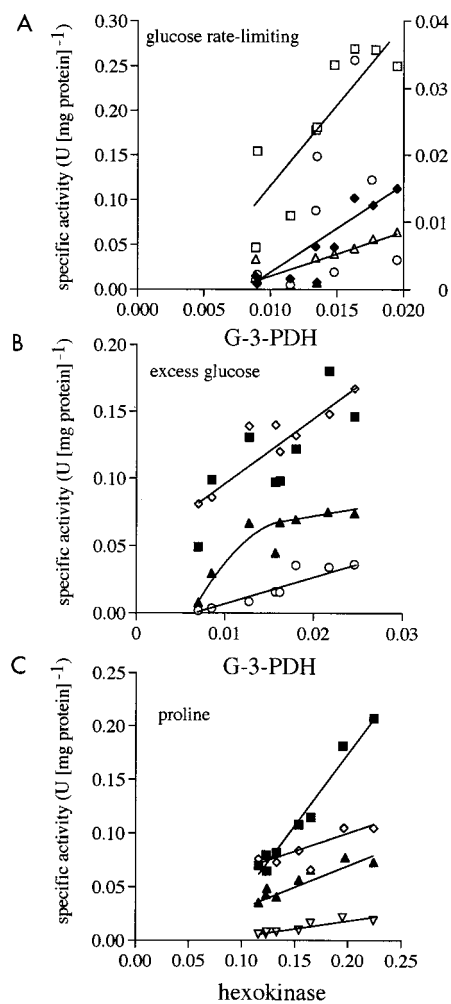


FIG. 6. Degree of covariation of the specific activities of metabolic enzymes. Groups of five enzymes were identified for each growth condition by means of correlation coefficients. Specific activities are plotted as a function of the specific activity of the enzymes having the highest combined correlation coefficient. (A [glucose-limited cells]) scale of 0 to 0.3, MDH (□) and enolase (◆); scale of 0 to 0.04, PK (○) and fumarate reductase (△). (B [excess-glucose-grown cells]) PFK (◇), PGI (■), PK (○), and malic enzyme (▲). (C [proline-grown cells]) PFK (◇), PGI (■), malic enzyme (▲), and G-3-PDH (▽).

on proline suppresses glucose consumption. This finding confirms the previously observed preference of procyclic *T. brucei* for proline as a carbon and energy source (8). In addition, glucose seems not to be consumed at growth rates exceeding the maximum growth rate on glucose. This observation indicates that the entire glycolytic pathway no longer functions at these high growth rates, not even to supplement the carbon and energy obtained from proline. Other components from the medium must then function as sources for the sugars needed in RNA, DNA, glycolipids, etc. *L. donovani* and *L. braziliensis* cultures growing on proline could use adenosine for this purpose (22). Another possible explanation is the occurrence of gluconeogenesis. In that case, parts of the glycolytic pathway would function in the reverse direction. This in turn could account for the low rates of glucose consumption during growth on proline.

The expected increase of the role of the respiratory chain under glucose starvation, which would lead to a higher yield, was not observed. The role of the mitochondrion in the overall

energy metabolism is to some extent counterintuitive. Least CO_2 is formed under glucose limitation, indicating less complete oxidation of the substrate when more would seem beneficial. The extra substrate consumed by cells growing on excess glucose at low rates is mostly converted to CO_2 but is not converted to additional cell mass. Judged by the calculated production of CO_2 , proline was oxidized more completely than glucose. Proline degradation occurs ultimately via the tricarboxylic acid cycle in the mitochondrion. Hence, this observation is not surprising, but it is unclear why glucose is not respired to a greater extent as well.

Metabolic adaptation. Four of the theoretically possible strategies for regulating and/or adapting metabolic fluxes in cells are (i) allowing the variations of metabolite concentrations to change the overall flux through the pathway while maintaining constant enzyme activities, (ii) changing all enzyme activities proportionally at higher substrate availability to increase the flux, (iii) sensing concentrations of intermediates and increasing the amount of enzyme converting those that accumulate, and (iv) using low-molecular-weight effectors to regulate activities of constant amounts of enzyme in response to changing concentrations of intermediates. All four could be operational in one cell at the same time. In *T. brucei*, enzyme concentrations varied considerably but not proportionally. This observation can be explained by the third strategy or by a combination of the first and second, but neither in an extreme way. The second strategy was found to be the primary mechanism for metabolic adaptation in *T. vaginalis* (26). The third was demonstrated to function in yeast (15), but there is no unambiguous evidence for it operating in *T. brucei*. Only in the case of PK has the fourth been confirmed (31), but the low capacities of, e.g., aldolase may be explained by a similar, until now undiscovered, type of regulation. A combination of the third and fourth strategies can be expected to lead to a complex system adapting enzymes individually. Such system can produce rather constant ratios of end products if it is aimed at maintaining metabolic homeostasis. Therefore, the present data set is best explained in terms of the latter two strategies. The manner of adaptation is clearly not influenced by the compartmentation of part of the glycolytic pathway, as there is no observable difference between glycosomal, cytosolic, or mitochondrial enzymes in this respect.

The far higher maintenance energy of *T. vaginalis* than of *T. brucei* suggests that the latter is more energy efficient. There may be three causes for this higher efficiency: (i) glycolysis is compartmentalized in the glycosome, (ii) the apparently optimal ratio of the end products is maintained within small margins, and (iii) the separate adaptation of each enzyme prevents excess protein synthesis. A study of overexpression of glycolytic enzymes in *Zymomonas mobilis* showed that excess protein synthesis severely decreased the growth rate (21). The earlier characterization of the metabolism of *T. brucei* as adapting its metabolism to achieve maximum energy efficiency (29) is confirmed by the present findings. In addition, the results of this study show that values reported for specific activities of metabolic enzymes are valid only for the growth conditions of the cells used for that particular measurement. Differences exceeding an order of magnitude are readily induced by changing environmental conditions. Smaller differences were observed by harvesting during different stages of the culture (6).

Regulation of the metabolic flux. It is difficult to discern which enzyme(s) exerts most flux control as defined in the sense of metabolic control analysis (12). Correlation between the activity of one enzyme and metabolic rates must be used with caution, because many enzymes changed simultaneously. Enzymes in the beginning of the pathway, like HK, are often

assumed to have a controlling role. The complete lack of correlation between activities of the early enzymes and overall rates contradicts that idea. Correlation coefficients suggest that the flux control for glucose metabolism of *T. brucei* resides largely at two points. Enzymes roughly halfway in the glycolytic pathway are likely to have a high control coefficient. For glucose-limited-grown cells, this enzyme is GAPDH; for excess-glucose-grown cells, it is G-3-PDH. The other point of control may be ASCT, which catalyzes the final step of acetate formation (30).

The reason that G-3-PDH varied with metabolic rates may be its role in maintaining the redox balance within the glycosome via the G-3-P/dihydroxy acetone phosphate shuttle. Maintaining this redox balance is thought to be essential for survival (7). In the bloodstream form of *T. brucei*, GAPDH and G-3-PDH had a high control coefficient when the V_{\max} of the plasma membrane glucose transporter was high (1). The rates of transport were not measured in the present study but were previously found to be higher in excess-glucose-grown than in glucose-limited cells (29). In one study on yeasts, G-3-PDH was found to exert considerable control, together with PK, over the glycolytic flux (4). However, overexpression of each of the glycolytic enzymes barely increased the flux (20).

For proper functioning of ASCT, only catalytic quantities of succinate are needed, as it is recycled from succinyl coenzyme A with the concomitant formation of ATP. This may explain the very low rates of succinate production at the lower growth rates. According to this line of reasoning, succinate is formed at the higher growth rates as a product of overflow metabolism. The fumarate reductase activity measured is the reverse of SDH. The low fumarate reductase activities measured correspond to low SDH, which at the higher metabolic rates leads to accumulation and excretion of succinate. Therefore, the overall rate of glucose metabolism would primarily be controlled by enzymes halfway the glycolytic pathway. The distribution of the end products is determined by the activities of the enzymes catalyzing the final steps of their respective pathways. The inverse relationship between ASCT and acetate production found in excess-glucose-grown cells is possibly due to a different pathway for acetate formation when energy is not limited. A similar distribution of control was also found in *T. vaginalis* (26), where the rate of glucose consumption primarily depended on PP₁-PFK. Rates of formation of two of three end products correlated to the activities of the final enzymes.

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