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Activity of key enzymes in glucose catabolism during the growth and metacyclogenesis of *Leishmania infantum*

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Abstract This paper follows the development in the activity of the key enzymes of glycolysis and dehydrogenases of the pentose phosphate shunt throughout the in vitro growth and metacyclogenesis of two human strains of *Leishmania infantum* – one visceral (VL) and the other cutaneous (CL) – together with changes in the glucose, ammonium, and proton concentrations in the culture medium. In the first stage, ammonium was generated and no glucose was consumed. Later on, all the glucose was consumed and, finally, ammonium was generated again. The ammonium concentration increased 16- and 21-fold in cultures of VL and CL strains, respectively. The activities of the glycosomal enzymes hexokinase and phosphofructokinase differed in each strain, always being higher in CL than in VL and increasing throughout the culture period in CL while decreasing in VL. This probably indicates a different capability to adapt to the culture medium conditions. The activities of the pentose phosphate shunt enzymes examined indicate that 6-phosphogluconate dehydrogenase is possibly a rate-limiting enzyme for this pathway. Pyruvate kinase is a cytosolic control enzyme of glycolysis in trypanosomatids, and its activity decreased throughout the growth and differentiation of both strains of *L. infantum*, as occurs in other trypanosomatids. It was also observed that glucose catabolism was more active in the cutaneous strain than in the visceral one.

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Introduction

Leishmania infantum is an etiological agent of the two endemic forms of human leishmaniosis (visceral and cutaneous) throughout most of the Mediterranean region (WHO 1990). The consumption of glucose by *Leishmania* spp. is characterized by the variable excretion of reduced products such as succinate, pyruvate, acetate, L-alanine, L-malate, D-lactate, or glycerol, depending on the species and the incubation conditions (Marr 1980; Cazzulo 1992; Blum 1993; Van Hellemond et al. 1997). The universal regulatory enzymes of the glycolytic pathway, hexokinase (HK) and phosphofructokinase (PFK), are either not regulated or only slightly so in trypanosomatids, including *Leishmania* spp. Similarly, glucose 6-phosphate dehydrogenase (G6PDH), a key enzyme in the pentose phosphate pathway (PPP), does not appear to be controlled (Berens et al. 1980). However, the activity of pyruvate kinase (PK), the third key enzyme of glycolysis, is strongly regulated in this group of parasites (Cazzulo 1992).

Furthermore, it is known that promastigotes of *Leishmania* are capable of oxidizing [1-^{14}C]-glucose to $^{14}CO_2$ between 2 and 6 times faster than they can oxidize [6-^{14}C]-glucose, which indicates that this protozoan consumes an important quantity of glucose throughout the PPP (Berens et al. 1980; Keegan et al. 1987), in addition to that used by glycolysis. Despite the presence of all enzymes necessary for a functional PPP in some species of *Leishmania* (Ghosh and Datta 1971; Janovy 1972; Ghosh and Honigberg 1976; Martin et al. 1976; Berens et al. 1980; Coombs et al. 1982; Meade et al. 1984; Mottram and Coombs 1985a, b), this cycle seems operative only until the necessary ribose phosphates are produced for nucleotide synthesis, and the parasite apparently does not obtain energy in this way.

The existence of a metacyclic promastigote stage in *Leishmania* has been described by Sacks and Perkins (1984). Blum (1993) has commented that profound changes occur as the promastigotes enter the stationary

phase of growth (sta-P), becoming infective forms called metacyclics, but an understanding of intermediary metabolism as a function of the stage of differentiation has barely begun.

The aim of this study was to investigate the behavior of glycolysis and PPP activity throughout the in vitro metacyclogenesis process by consideration of the particular features of glycolytic enzyme compartmentation, the use of glucose via the PPP, and the biochemical and molecular changes that take place during metacyclogenesis. For this purpose, we carried out a comparative study of some metabolic changes during the growth and differentiation of two human strains of *L. infantum* – a visceral (VL) and a cutaneous (CL) strain – and also of the activity of the most important enzymes commonly involved in the regulation of the glycolytic and PPP pathways. Our results clearly indicate significant changes in the activities of the assayed enzymes throughout the metacyclogenesis process in both VL and CL strains of *L. infantum*.

Materials and methods

Both the VL (MHOM/ES/85/DP153; zymodeme MON-183) and CL (MHOM/ES/90/DP121; zymodeme MON-28) strains of *Leishmania infantum* used in these studies were isolated from human clinical cases from Granada province, southern Spain (Martín-Sánchez et al. 1996). Parasites and mouse macrophages J774 were cultured as described elsewhere (Louassini et al. 1998).

Promastigote agglutination by peanut agglutinin (PNA), complement-dependent lysis by serum, and in vitro infectivity for macrophages were assayed as described previously (Louassini et al. 1998). The metacyclic forms were not agglutinated by PNA (PNA−) and were resistant to lysis by serum (Louassini et al. 1998). For determination of the infectivity ratio (%I) at 72 h postinfection (p.i.), at least 250 macrophages were counted for each experiment. The infectivity ratio is calculated as *%I = 100 × the number of intracellular amastigotes per parasitized macrophage at 72 h p.i./the number of amastigotes per parasitized macrophage at 2 h p.i.* (Louassini et al. 1998).

Parasite extracts

For the preparation of parasite extracts, promastigotes were removed by centrifugation, washed, resuspended, and homogenized as previously described (Adroher et al. 1988a). The homogenate was then centrifuged at 40 000 *g* for 30 min at 4 °C. The cell pellet was discarded and the supernatant fraction was used as the source of soluble enzymatic activity in assays.

Enzyme assays

Glucose 6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGDH; E.C. 1.1.1.44) were assayed following the procedures of Corpas et al. (1995a, 1995b). Hexokinase (HK; E.C. 2.7.1.1), phosphofructokinase (PFK; E.C. 2.7.1.11), and pyruvate kinase (PK; E.C. 2.7.1.40) activities were assayed as described elsewhere (Adroher et al. 1990). All spectrophotometric determinations were carried out at 37 °C. The enzyme activities are expressed as enzyme units, whereby 1 unit of G6PDH, 6PGDH, and HK was defined as the amount required to catalyse the reduction of 1 μmol $NADP^+$ into NADPH in 1 min and 1 unit of PFK and PK was defined as the amount required to catalyse the oxidation of 2 and 1 μmol of NADH in 1 min, respectively.

Glucose, ammonium, and protein determinations

Glucose and ammonium concentrations were determined by a micromodification of procedures recommended by Sigma Chemical Co. for their 510 and 170-UV kits, respectively. The protein concentration was determined according to Lowry et al. (1951) using crystalline bovine serum albumin as the standard.

Analysis of data

Results are expressed as mean values ± SEM. Statistical comparisons were done using Student's *t*-distribution.

Results

Metacyclogenesis and variations of glucose and ammonium concentrations in the medium throughout *Leishmania infantum* development

Figure 1 shows the variations observed in glucose and ammonium concentrations throughout the growth and metacyclogenesis of both VL and CL strains of *L. infantum*. The growth of the organisms was clearly associated with an uptake of glucose and an excretion of ammonium. During the first 2 days of culture the glucose uptake was minimal; however, a noteworthy level of ammonium excretion was detected. From the 2nd day of culture onward the organisms started to consume glucose and did not excrete ammonium. At the same time, metacyclic differentiation started as indicated by PNA agglutination (Louassini et al. 1998). From the 4th day onward, ammonium excretion began again. Glucose uptake increased in the VL strain and decreased in the CL strain (Fig. 1a, b). Under these conditions the cellular differentiation process of *L. infantum* takes place in association with a continuous increase in metacyclic forms up to the 4th day for VL (50% PNA− forms) and up to the 6th day for CL (38% PNA− forms; Fig. 1c, d).

For confirmation of the results obtained by the PNA agglutination technique an assay of promastigote resistance against rat serum was carried out together with an infectivity assay using macrophages J774. In all cases the highest percentage of PNA− promastigotes matched, in time, both the highest percentage of promastigotes resistant to rat serum and the highest infectivity ratio in macrophages J774. This happened on the 4th day of culture for the VL strain and on the 6th day of culture for the CL strain of *L. infantum* (results not shown). Additionally, it is noteworthy that the highest glucose consumption in both strains coincided with the sharpest decreases in pH (Fig. 1a, b), probably due to the excretion of organic acids from glucose catabolism. The level of ammonium increased 16 and 21 times from the beginning to the end of the culture of the VL and CL strains, respectively, reducing the tendency for the decrease in pH. This ammonium could have originated in proteins and amino acids from the culture medium.

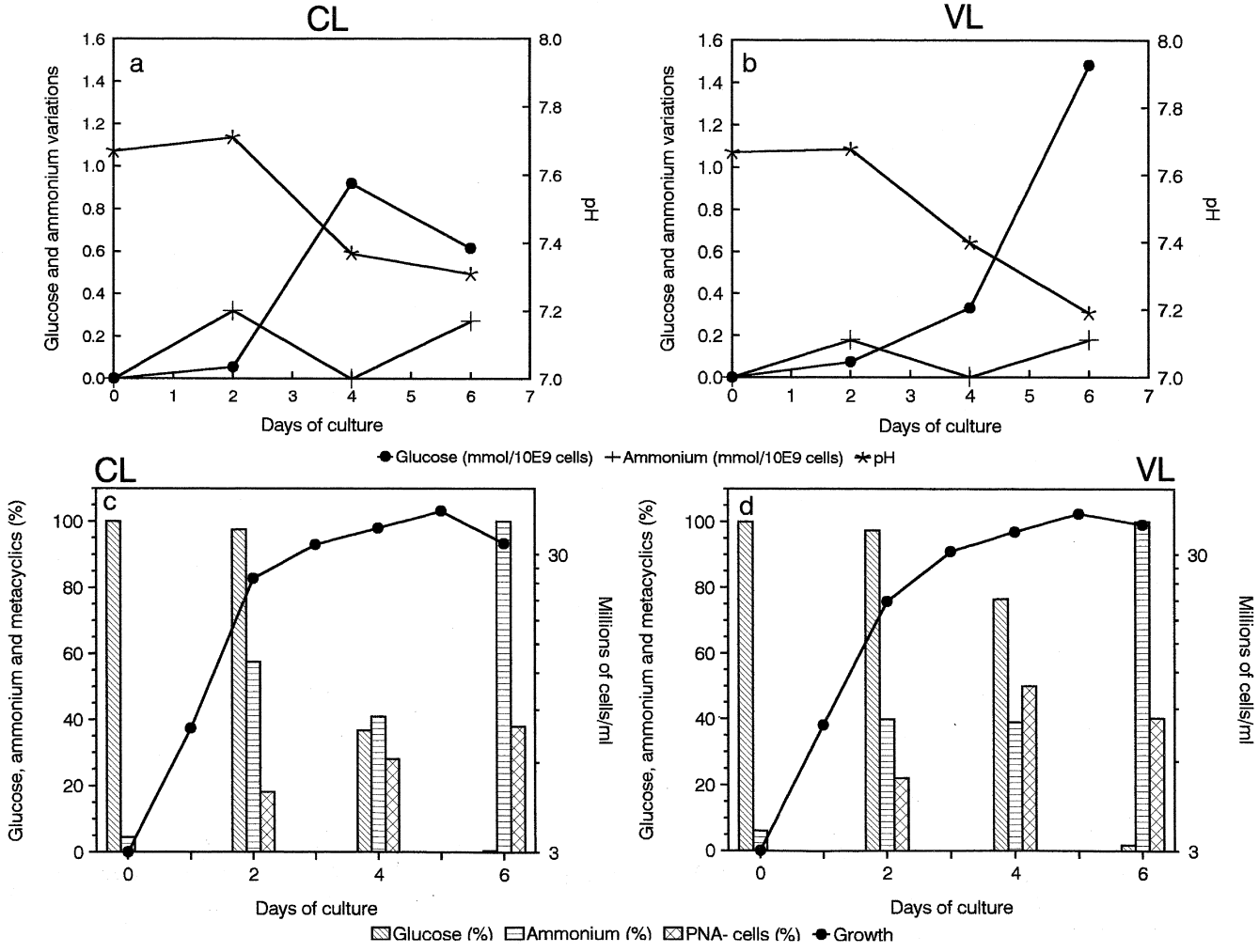

Fig. 1a–d Variations in the levels of glucose, ammonium, and protons in the culture medium throughout the growth and metacyclogenesis of cutaneous (*CL*) and visceral (*VL*) strains of *Leishmania infantum*. **a**, **b** For the calculation of each point, only the variation in the concentration of glucose (●) and ammonium (+) in relation to the previous point is determined, and this variation is then related to the number of cells in the medium at that particular time of culture and expressed as mmol/10^9 cells. The proton concentration (*) is plotted as pH. **c**, **d** Glucose and ammonium variations during the growth (●) and metacyclogenesis (*PNA- cells*) of *L. infantum* are expressed as a percentage of the initial and final values, respectively. The initial glucose concentrations were 5.6 m*M* for both CL and VL strains. The final ammonium concentrations were 15 m*M* for CL and 11 m*M* for VL

Enzyme activities

Glucose-6-phosphate dehydrogenase

The activity of G6PDH increased 1.35 times from the 2nd to the 6th day of growth ($P < 0.05$) of CL (Fig. 2a). It is noteworthy that the day of maximal activity of G6PDH, the 6th day of growth of CL, was the same as that with the maximal percentage of PNA– cells. This activity was similar to that observed on the 4th day of culture of VL (maximal PNA– cells; Table 1). No significant change in G6PDH activity was observed during the growth of VL (Fig. 2b).

6-Phosphogluconate dehydrogenase

6PGDH activity increased 3.1-fold during the culture of CL (Fig. 2a) from the 2nd to the 6th day ($P < 0.05$), whereas this activity decreased 78% in VL ($P < 0.05$). The activity noted on the 4th day of growth of VL (Fig. 2b) was lower than the maximum recorded for CL (Fig. 2a) on the 6th day ($P < 0.05$; Table 1).

Hexokinase

During the growth of *L. infantum* the specific activity of HK (Fig. 2c) increased about 1.4 times from the logarithmic phase of growth (log-P) to the sta-P ($P < 0.05$) in CL and was constant in VL. HK activity was higher ($P < 0.01$; Table 1) on the 6th day of growth of CL (Fig. 2c) than on the 4th day of culture of VL (Fig. 2d).

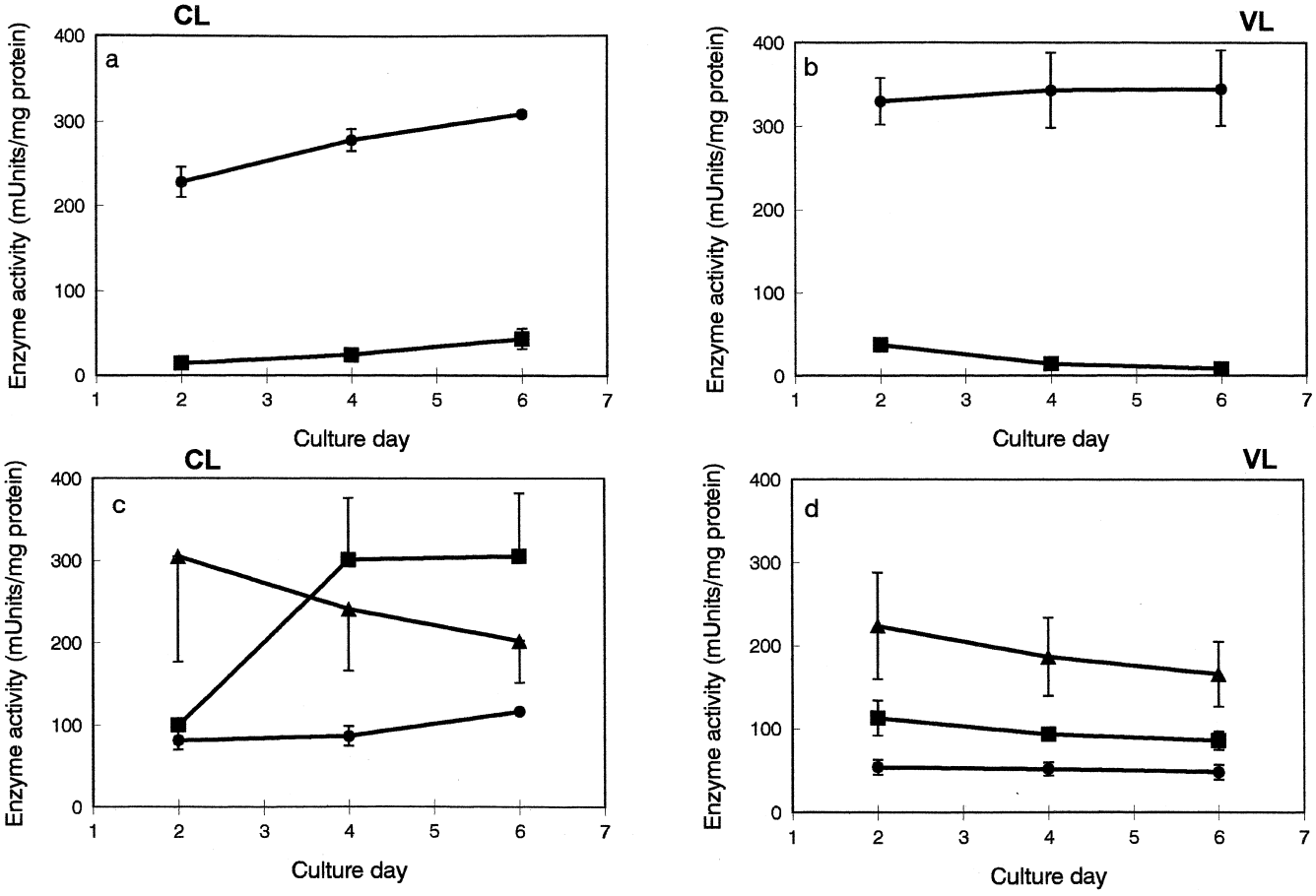

Fig. 2a–d Variations in enzyme activities throughout the growth and differentiation of **a**, **c** cutaneous (*CL*) and **b**,**d** visceral (*VL*) strains of *L. infantum*. The results represent mean values ± SEM for three or four experiments run in triplicate. **a**, **b** (●) G6PDH, (■) 6PGDH. **c**, **d** (●) HK, (■) PFK, (▲) PK

Phosphofructokinase

The activity of this enzyme was enhanced 3-fold during the period of culture of CL ($P < 0.05$; Fig. 2c), whereas the same enzyme in VL (Fig. 2d) decreased its activity by 24% during culture ($P > 0.05$). Moreover, the PFK activity observed on the 6th day of development of CL (Fig. 2c) was 3.2 times greater ($P < 0.05$; Table 1) than that seen the 4th day of growth of VL (Fig. 2d).

Pyruvate kinase

No statistically significant difference was found between the activities of PK throughout the culture of the two strains (Fig. 2c, d). PK activity decreased (by 34% for CL and 26% for VL) during growth and differentiation in both strains. Moreover, no significant differences was observed when PK activity was compared on the 4th day of culture of VL versus the 6th day of culture of CL (Table 1).

Discussion

Glucose catabolism in Trypanosomatidae is characterized by incomplete oxidation. During this "aerobic fermentation," excretion of organic acids into the growth medium is accompanied by a decrease in the pH of the culture medium in all Trypanosomatidae studied thus far (Cazzulo 1992), especially when the rate of glucose consumption is highest (Fig. 1 in the present paper; Cazzulo et al. 1985; Lupiáñez et al. 1987; Adroher et al. 1988b, 1990). This acidification is slow but progressive in *Leishmania mexicana* (Hart and Coombs 1982; Cazzulo et al. 1985) and in both human strains of *L. infantum* (Fig. 1). *Trypanosoma cruzi* epimastigotes consume glu-

Table 1 Ratio of the activities, of some key enzymes of glucose metabolism of *L. infantum* between the days on which the percentages of metacyclics were maximal, the 4th day for CL and the 6th day for VL (NS Not significant)

Enzymes	"6th day of CL/4th day of VL" activity ratio
G6PDH	0.89^{NS}
6PGDH	3.07**
HK	2.32***
PFK	3.24*
PK	1.07^{NS}

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Table 2 Variation of enzyme activity ratios during the growth and differentiation of *Leishmania infantum* human strains

Days of culture	Activity ratio		
		Cutaneous strain	Visceral strain
2	G6PDH/PFK	2.28	2.95
4		0.92	3.66
6		1.00	4.01
2	6PGDH/G6PDH	0.06	0.11
4		0.09	0.04
6		0.14	0.02
2	HK/PFK	0.81	0.47
4		0.29	0.55
6		0.38	0.56
2	PK/(2 × PFK)	1.52	1.02
4		0.40	0.99
6		0.33	0.94

cose preferentially when both glucose and amino acids are present (Cazzulo et al. 1985). This is not true of *Leishmania*. In our experiments, we believe that the slight reduction in protein concentration (results not shown) and the important ammonium production (Fig. 1) suggest an uptake of amino acids for the purpose of obtaining energy at a time when the glucose is not consumed, at the beginning of the culture, and/or when the glucose is exhausted, as occurs at the end of culture. This is based on previous papers reviewed by Blum (1994), who states that "In all species of *Leishmania* so far examined, however, amino acids from the medium are consumed in preference to glucose during the exponential growth, with glucose consumption increasing during stationary phase," and that "the source of amino acids groups is likely to be via protein degradation [in *L. tropica*] ... which [proteins] are degraded more rapidly when the nutrients in the medium are exhausted...," as occurs with the glucose. In addition, some amino acids (such as alanine) partially inhibit the rate of oxidation of [6-^{14}C]-glucose by *L. donovani* and *L. braziliensis* promastigotes (see Blum 1994). This leads us to suggest that the amino acids could be consumed by *L. infantum* before and after glucose consumption.

The glucose in the culture medium was exhausted faster in CL than in VL (Fig. 1). This could be explained by the observation that HK, PFK, G6PDH, and 6PGDH activities increased during the culture of CL (Fig. 2), resulting in more rapid consumption of glucose by CL than by VL (Fig. 1), in which these activity levels remained stable (HK, G6PDH) or even decreased (PFK, 6PGDH).

HK activity varies not only between species (Martin et al. 1976; Berens et al. 1980) but also between amastigote and promastigote forms of the same species, such as *L. mexicana* (Coombs et al. 1982; Meade et al. 1984), and even during the growth of promastigote forms (Cazzulo et al. 1985). In any event, the existing data show a greater degree of HK activity as compared with PFK activity in all the species studied, both in promastigotes and amastigotes (Martin et al. 1976; Coombs et al. 1982; Meade et al. 1984; Cazzulo et al. 1985; Mottram and Coombs 1985a). However, HK activity is always lower than PFK activity in both strains of *L. infantum* throughout the period of culture (Fig. 2, Table 2). Moreover, the HK activity in VL was not significantly modified during growth, whereas in CL a significant increase of 43% occurred at the end of culture ($P < 0.05$). This may be because less glucose remained in the medium, and an increase in HK activity would ensure that the necessary glucose could be obtained.

According to Berens and Marr (1977), PFK, which is the key regulatory enzyme in the glycolysis of many organisms, does not play an important role in *Leishmania*. PFK in *Leishmania*, and in all trypanosomatids studied, is very little affected by the typical effectors of this enzyme in most glycolytic systems (Cazzulo 1992). During the culture of VL it is not significantly modified, but in CL, PFK activity increases by up to 3 times when sta-P is beginning, probably because of the increased glycolytic flow with respect to the PPP activity, since G6PDH activity is 2.3-fold higher than PFK activity in the log-P. The activities of the two enzymes are very similar from the 4th day of culture onward (Fig. 2, Table 2), when the rate of glucose consumption is at its maximum (Fig. 1).

The activity of the first two enzymes of the PPP throughout the culture and differentiation of both strains of *L. infantum* was determined. However, the activity of this cycle with respect to glycolysis seems to be reduced when promastigotes pass from the log-P to the sta-P of growth, at least in *L. donovani* (Berens et al. 1980; Keegan et al. 1987). Various authors have proved the presence of the enzymes of this pathway in promastigotes as well as amastigotes of several species of *Leishmania* (Ghosh and Datta 1971; Janovy 1972; Ghosh and Honigberg 1976; Martin et al. 1976; Berens et al. 1980; Coombs et al. 1982; Meade et al. 1984; Mottram and Coombs 1985a, b), and it has been suggested that an appreciable fraction of glucose may be utilized via the PPP (Berens et al. 1980; Marr 1980; Keegan et al. 1987). In this regard, a high level of G6PDH activity was measured and, therefore, an important amount of glucose 6-phosphate generated by HK will be incorporated into the PPP, especially in the log-P. Subsequently, the behavior of the two strains differs. Whereas VL increases the G6PDH:PFK activity ratio from 2.9 to 4.0 throughout the period of culture, CL reduces this ratio from 2.3 to 1.0 (Table 2).

With regard to the meaning of this shunt in *Leishmania*, Berens et al. (1980) have suggested that the principal purpose of the PPP is the production of ribose for nucleotide synthesis. In *L. infantum*, however, the objective of the PPP could be the controlled production of important amounts of NADPH for biosynthetic or detoxification purposes, since less than 15% of the 6-phosphogluconate synthesized by the G6PDH activity in VL and CL (Table 2) could follow subsequent steps of the PPP to produce the pentose phosphates necessary for nucleic acid synthesis, due to the reduced activity of

6PGDH, as has occurred in other *Leishmania* species (Ghosh and Honigberg 1976; Martin et al. 1976).

PK is a cytosolic enzyme that plays an important role in the control of carbohydrate metabolism in Trypanosomatidae (Cazzulo 1992), and it seems to be regulated during the development of these parasites (Etges and Mukkada 1988). The maximal PK activity (Fig. 2) observed in the two strains of *L. infantum* was slightly lower than the activity determined for the same enzyme in *L. tropica* (= *L. major*; Martin et al. 1976) and higher than the activity found in *L. braziliensis*, *L. donovani*, and *L. mexicana* (Martin et al. 1976). A reduction in PK activity during growth and metacyclogenesis occurred in *L. infantum* (Fig. 2), as has also previously been reported in *L. m. mexicana* and *L. major* (Mallinson and Coombs 1986). This reduction in PK activity throughout culture and differentiation is the only common feature, apart from ammonium production (Fig. 1), observed in both strains of *L. infantum* studied.

In conclusion, our data demonstrate a significant variation in metabolism during the culture and differentiation of *L. infantum* as well as the existence of important differences between the two human strains studied. The glucose uptake and ammonium production by *L. infantum* promastigotes lead us to suggest that during culture, this parasite, like other species of the genus *Leishmania*, does not use a significant amount of glucose until it reaches sta-P, and it prefers amino acids both initially and after glucose depletion to satisfy its energy requirements (Blum 1994). The first two enzymes of the PPP were active, demonstrating the importance of at least the oxidative part of the PPP. Indeed, this alternative pathway is potentially capable of oxidizing an appreciable quantity of glucose 6-phosphate generated by HK to form pentose phosphates. However, the high activity of G6PDH with respect to 6PGDH leads us to hypothesize that the principal function of this pathway in both human strains is the production of NADPH for biosynthetic uses and/or for a detoxification process. The activity of the glycosomal enzymes HK and PFK evolved differently in the two strains, manifesting different adaptation capabilities to changes in the culture medium. The cytosolic PK, a key enzyme in the regulation of glycolysis in Trypanosomatidae, showed a high level of activity at log-P, which decreased during culture in both *L. infantum* strains. This also occurs in other species of the genus (Cazzulo et al. 1985; Mallinson and Coombs 1986) as well as in other trypanosomatids (Cazzulo et al. 1985; Adroher et al. 1990) and is probably a response to the exhaustion of glucose in the medium. This adaptation of PK activity to glucose concentration in the medium could be related to a control of pyruvate production and its posterior incorporation into the Krebs cycle, which seems to be fed by amino acid catabolism, as may be reflected by the production of ammonium by both strains.

In summary, glycolysis and the PPP were more active in CL than in VL, especially when the number of metacyclics was maximal (Table 1). This would appear to contradict the idea suggested by authors who relate the high pathogenicity of trypanosomatids to their high level of metabolic activity (Mancilla and Náquira 1964; Zeledón and Monge 1967; Janovy 1972), since the VL strain, which is capable of evading the skin immune system in the human host and of disseminating into the reticuloendothelial system, could be considered more pathogenic than the CL strain, which is blocked at the cutaneous level. Further studies are needed to clarify the regulation of intermediary metabolism during growth and metacyclogenesis in *Leishmania* species and the relationship of this metabolism to their tropism.

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