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CITRIC-ACID CYCLE KEY ENZYME ACTIVITIES DURING IN VITRO GROWTH AND METACYCLOGENESIS OF *LEISHMANIA INFANTUM* PROMASTIGOTES

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ABSTRACT: The activities of 5 key regulatory enzymes in most energetic systems, namely citrate synthase (EC 4.1.3.7, CS), NADP-specific isocitrate dehydrogenase (EC 1.1.1.42, ICDH), succinate dehydrogenase (EC 1.3.99.1, SDH), L-malate dehydrogenase (EC 1.1.1.37, MDH), and decarboxylating malic enzyme (EC 1.1.1.40, ME), were measured during the growth and metacyclogenesis of a cutaneous (CL) and a visceral (VL) strain of *Leishmania infantum*. As occurs with other *Leishmania* species, infective promastigotes were present along all phases of growth, but their percentages were higher at the early stationary phase for VL and the end of the same phase for CL. High CS and SDH activities were detected in both strains, as compared with other trypanosomatids, bringing more evidence for an actively functional citric-acid cycle in *L. infantum*. Both strains showed higher levels of CS, ICDH, and MDH and lower SDH and ME activities when more metacyclic promastigotes were present, but in VL these changes paralleled an increase in glucose consumption, whereas in CL these changes coincided with an NH_3 hyperproduction. This suggests that the energy metabolism during *L. infantum* growth and metacyclogenesis is affected by regulated enzymes that probably respond to changes in the culture medium in the levels of glucose and amino acids.

Leishmania infantum is the only endemic agent of the 2 forms of human leishmaniasis, visceral and cutaneous, in most of the Mediterranean littoral (WHO, 1990). These 2 pathologies, however, tend to be caused by distinct zymodemes in the Old World (Gradoni and Gramiccia, 1994; Pogue et al., 1996; Noyes et al., 1997). Nevertheless, this clinical variability is not the unique feature of this peculiar species. In effect, there is abundant evidence indicating that *L. infantum* promastigotes depend mainly on respiration for their energy generation and have a poor capacity for anaerobic functioning (Van Hellemond et al., 1997). Certainly the energetic metabolism of most *Leishmania* spp. is characterized by the incomplete oxidation of glucose and amino acids to organic acids, in addition to carbon dioxide; but the excretion of succinate, pyruvate, acetate, L-alanine, L-malate, or glycerol shows species-dependent differences (Marr, 1980; Glew et al., 1988; Cazzulo, 1992; Blum, 1993; Van Hellemond et al., 1997).

On one hand, enzymes of the glycolytic pathway, citric-acid cycle, and pentose phosphate shunt have been described in both promastigotes and in amastigotes of *Leishmania* spp. (Martin et al., 1976; Hart and Coombs, 1982; Meade et al., 1984); and, on the other hand, sufficient evidence exists for the presence of typical trypanosomatid respiratory chain in promastigotes of *Leishmania tropica* (Martin and Mukkada, 1979) and *Leishmania donovani* (Santhamma and Bhaduri, 1995). Whereas the first 7 enzymes involved in the glucose conversion to 3-phosphoglycerate are located in a subcellular organelle called a glycosome (Hart and Opperdoes, 1984), most of the pyruvate kinase activity was found to be cytosolic (Mottram and Coombs, 1985b). This last enzyme may constitute the only regulatory enzyme of glucose metabolism in trypanosomatids (Van Schaftingen et al., 1985, 1987; Callens et al., 1991). Nevertheless, glucose is not the principal energy substrate for *Leishmania* spp. (Crowther et al., 1954; Mukkada et al., 1974; Marr, 1980). In fact, these species can grow in vitro without glucose, provided that amino acids such as proline and glutamate are present in the culture medium (Mukkada et al., 1974; Steiger and Meshnick, 1977). Once deaminated, amino acids can enter the *Leishmania* citric-acid cycle (Simon et al., 1983).

This paper refers to the energy metabolism of a cutaneous and a visceral strain of *L. infantum* during promastigote growth and differentiation into metacyclic promastigotes, determined by following glucose consumption, ammonia production, changes in pH of the medium, and the levels of 5 enzymes of the citric-acid cycle. Our data demonstrated that during the acquisition of promastigote infectivity, important physiological and biochemical transformations occurred. These changes might prepare the parasite to resist the multiple defense mechanisms of the host and to develop an infection that will evolve toward one or another clinical manifestation depending, partly, on strain-intrinsic factors (Sulahian et al., 1997).

MATERIALS AND METHODS

Cultures

Both visceral (MHOM/ES/85/DP153) and cutaneous (MHOM/ES/90/DP121) strains of *L. infantum* used in these studies were isolated from 2 human cases in southern Spain (Martín-Sánchez et al., 1996) and cultured in RPMI-1640 medium supplemented with 20% (v/v) heat-inactivated fetal calf serum (HIFCS), 1% (v/v) sodium pyruvate solution (100 mmol), 1% (v/v) BME vitamin concentrate solution (Sigma, St. Louis, Missouri), and 1% (v/v) of an antibiotic solution (5,000 U/ml of sodium G-penicillin and 5 mg/ml of streptomycin sulfate). The cultures were initiated by inoculation with mid-log-phase promastigotes into a volume of 25 ml within 75-cm^2 culture flasks and were incubated at 24 C. J774 mouse macrophages were cultured in RPMI-1640 medium supplemented with 10% (v/v) HIFCS and 1% (v/v) of an antibiotic solution (5,000 U/ml of sodium G-penicillin and 12 mg/ml of gentamycin sulfate) with a gas phase of 95% air and 5% CO_2 at 37 C.

Parasite lysis by rat serum

The lytic assay was carried out as described by Mallinson and Coombs (1989) with the exception that incubations were performed at 25 C (Bates and Tetley, 1993). The fresh blood was extracted directly from Wistar female rats (170–210 g) by cardiac puncture.

PNA agglutination

The assay of Sacks et al. (1985) is based on the fact that *Arachis hypogaea* lectin (PNA, 200 mg/ml) agglutinates nonmetacyclics (PNA^+) but not metacyclics (PNA^-). Once PNA^+ and PNA^- promastigotes were separated, their infection capacity was assayed within J774 macrophages.

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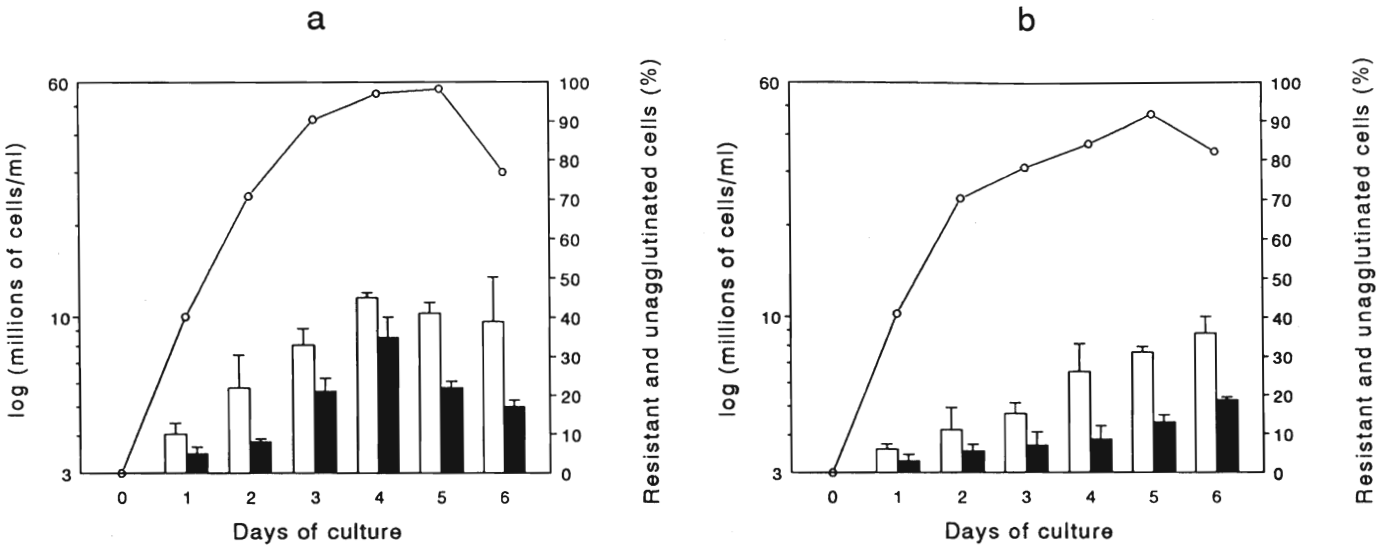

FIGURE 1. Promastigotes not agglutinated by *Arachis hypogaea* lectin (PNA^-, open bars) and parasites resistant to lysis by 50% fresh rat serum (closed bars) during the growth (○——○) of a visceral (a) and a cutaneous (b) strain of *Leishmania infantum*. The results are the means of 3 (VL) or 2 (CL) duplicated data ± SD.

Infectivity

The ability of promastigotes to invade and multiply within J774 macrophages in vitro was assessed as previously described (Sacks et al., 1985). Macrophages were washed twice in RPMI-1640 without serum and resuspended in the same medium with 10% (v/v) HIFCS and 1% (v/v) of an antibiotic solution (5,000 U/ml of sodium G-penicillin and 12 mg/ml of gentamycin sulfate). J774 cells in 0.5-ml aliquots were pipetted onto 8-mm round coverglasses that were previously placed in 24-well culture plates and were allowed to adhere overnight at 37 C in 5% CO_2. Nonadherent cells were removed by washing, and the remaining adherent macrophages were infected at a ratio of 10 promastigotes per macrophage. After 2 hr of interaction between parasites and host cells at 37 C in 5% CO_2, free promastigotes were removed by repeated washings with RPMI-1640 medium, and the cultures were additionally incubated in the same medium with 10% (v/v) HIFCS for 2 hr, 24 hr, 48 hr, and 72 hr postinfection (PI). The coverglasses, with parasitized macrophages, were fixed in absolute methanol for 2 min and were stained with 5% Giemsa for 30 min and examined by light microscopy at 1,000× to visualize intracellular parasites. At least 250 J774 cells were counted for each PI time to determine the number of amastigotes per parasitized macrophage. We used a 10:1 (parasites : macrophage) ratio because the population size of J774 cells remained almost constant throughout the PI time. At 96 hr PI, 37% of macrophages were lysed. For this reason, we stopped all the experiments at 72 hr PI when macrophage lysis percentage was 0. Under these conditions, the growth of amastigotes during PI time in comparison with the initial number of intracellular parasites could be described by the following equation $N_t/N_0 = \exp[(\alpha - \mu)t]$, where α and μ are the constants of replication (birth) and phagocytosis (death), respectively, per parasite per unit of time, and N_t and N_0 the numbers of amastigotes at time t and t = 0, respectively (Anderson, 1976).

Cell extract

For cell-extract preparation, parasites were removed by centrifugation at 3,000 *g* for 15 min at 4 C and washed 3 times with a large excess of 10 mmol phosphate-buffered saline (PBS) at pH 7.2. The leishmanias were resuspended in an adequate volume of tris-HCl, pH 7.4, with 5 mmol of EDTA to inhibit protease activities. The suspension of promastigotes was homogenized in a sonifier (4 μm, 4 × 30 sec, with 60-sec resting intervals in an ice bath). The homogenate was then centrifuged at 40,000 *g* for 30 min at 4 C. The supernatant fraction was used as a source of soluble enzymatic activity assays in the case of citrate synthase, NAD- and NADP-specific isocitrate dehydrogenase, L-malate dehydrogenase, and malic enzyme; the cell pellet was then homogenized in PBS in the presence of 0.1% triton X-100 to assess succinate dehydrogenase activity.

Enzymatic assays

Citrate synthase (EC 4.1.3.7, CS), NAD- (EC 1.1.1.41) and NADP- (EC 1.1.1.42) dependent isocitrate dehydrogenase (ICDH), and succinate dehydrogenase (EC 1.3.99.1, SDH) activities were measured as previously described (Adroher et al., 1988). L-Malate dehydrogenase (EC 1.1.1.37, L-malate : NAD oxidoreductase, MDH) activity was measured by Yoshida's (1965) assay, where malate oxidation was measured by optical-density increase at 340 nm caused by the reduction of NAD. The reaction mixture contained 100 mmol tris-HCl, pH 8.8; 3.33 mmol L-malic acid monosodium salt; 0.33 mmol NAD monosodium salt; and 0.03 mg of protein from the soluble fraction of the cell extract in 1 ml total volume. Malic enzyme (EC 1.1.1.40, L-malate : NADP oxidoreductase [decarboxylating], ME) was assayed by the method of Hsu and Lardy (1967). The final concentrations in the reaction mixture were 50 mmol tris-HCl, pH 7.4; 0.5 mmol L-malate monosodium salt; 0.23 mmol NADP monosodium salt; 4 mmol $MnCl_2$, and 0.03 mg of protein from the soluble fraction of the cell extract in 1 ml total volume. NADP formation was followed at 340 nm.

Protein concentration was determined according to Lowry et al. (1951), and all spectrophotometric determinations were carried out at 37 C. One unit of activity was defined as the amount of enzyme that catalyzes the oxidation or reduction of 1 μmol substrate per minute. Glucose and NH_3 concentrations were determined by procedures recommended by Sigma for their 510 and 170-UV kits, respectively. Results are expressed as mean ± SD. Statistical comparisons between data of different phases of *L. infantum* growth were done using *t*-tests.

Chemicals

Biochemicals were supplied by Boehringer Mannheim (Mannheim, Germany), Aldrich Chemicals (St. Louis, Missouri), or Sigma. RPMI-1640 medium was purchased from Bio-Whittaker (Walkersville, Maryland).

RESULTS

Lysis by normal rat serum and agglutination with *Arachis hypogaea* lectin (PNA) were used to determine and quantify the metacyclogenic process. Promastigotes resistant to complement-mediated lysis and PNA^- organisms were present in all phases of growth, but their percentages were highest at the

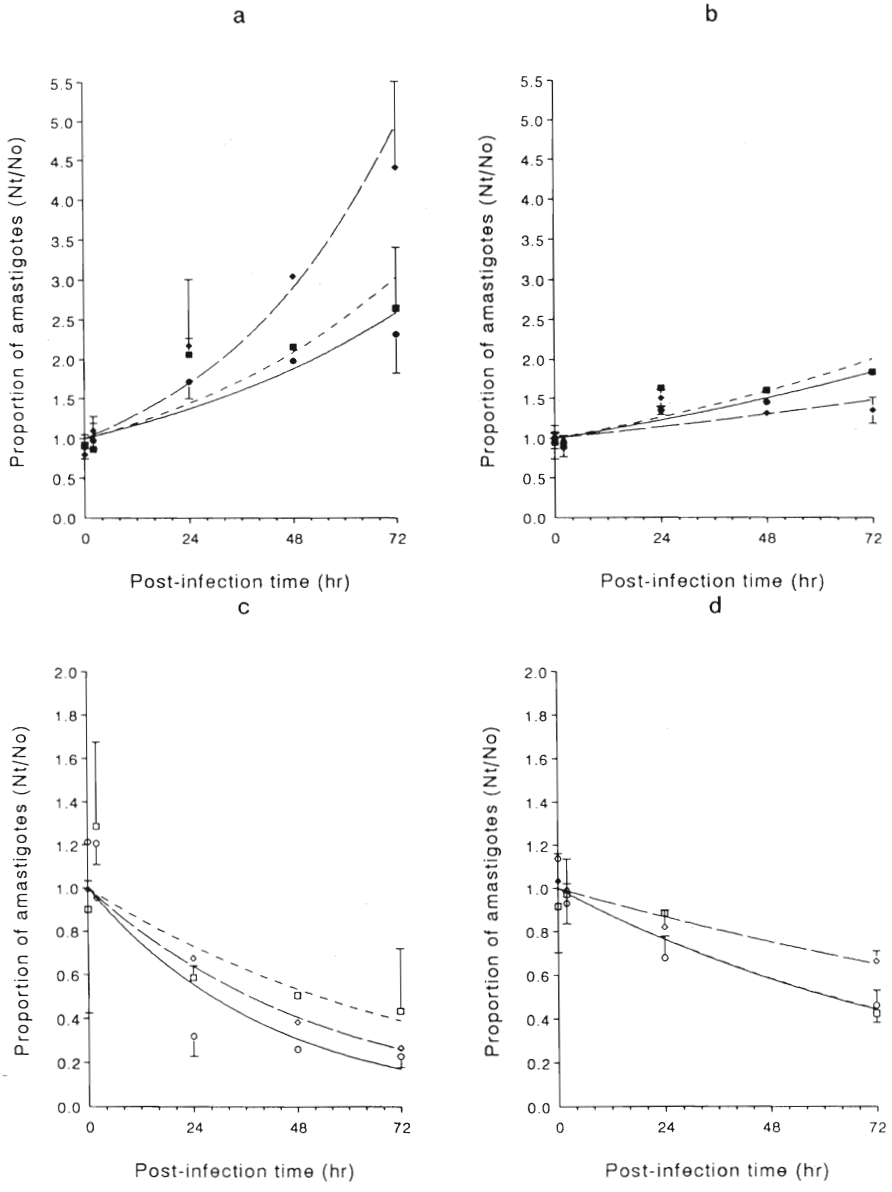

FIGURE 2. Dynamic behavior of PNA$^+$ (a and b) and PNA$^-$ (c and d) amastigotes within J774 macrophages. The proportion of amastigotes into J774 macrophages during PI time followed the equation $N_t/N_0 = \exp[(\alpha - \mu)t]$, where α and μ are replication and phagocytosis constants, respectively, per parasite per unit of time, and N_t and N_0 the numbers of amastigotes at time t and t = 0, respectively (Anderson, 1976). PNA$^+$ organisms originated from the second (●——●), the fourth (◆– – –◆), and the sixth (■·····■) days of VL (a) and CL (b) cultures. PNA$^-$ cells originated from the second (○——○), the fourth (◇– – –◇), and the sixth (□·····□) days of VL (c) and CL (d) cultures. Each point is the mean of duplicate determinations ± SD. The correlation coefficient r was ≥0.95 for all of the exponential plots except for PNA$^+$ parasites on the fourth day of growth (r = 0.84). The CL-PNA$^-$ on the second and the sixth days showed the same fitted curve (d). Notice that PNA$^+$ promastigotes treated with 10 mmol D-galactose, to liberate PNA-binding sites, behaved within J774 macrophages as PNA$^-$ promastigotes.

fourth day of visceral *Leishmania* (VL) culture (early stationary phase, Fig. 1a) and the sixth day of cutaneous *Leishmania* (CL) culture (late stationary phase, Fig. 1b). Notice the clear correlation between resistance of promastigotes to serum-mediated lysis and the prevalence of PNA$^-$ promastigotes (Fig. 1a, b).

To study the infective capacity of PNA$^-$ and PNA$^+$ and to measure the variation in the levels of glucose, NH_3, and pH in the culture medium, we chose 3 points of growth curves sufficiently separated in time that were characteristic of the mid-log phase (second day), early stationary phase (fourth day), and late stationary phase (sixth day). In the case of infectivity experiments, the 2 subpopulations separated by PNA followed the equation $N_t/N_0 = \exp[(\alpha - \mu)t]$, but in a different way. For PNA$^+$ parasites (Fig. 2a, b), this biological model was characterized by exponential growth ($\alpha - \mu > 0$) in contrast to PNA$^-$ organisms (Fig. 2c, d) that exhibited an exponential decline ($\alpha - \mu < 0$) with PI time. It is important to note that VL-PNA$^+$ amastigotes (Fig. 2a) showed a greater ability to multiply within the host cells than their CL homologues (Fig. 2b).

The variations of glucose and NH_3 concentrations and pH were similar in CL and VL (Fig. 3). The main difference between the 2 strains was the day of maximum glucose con-

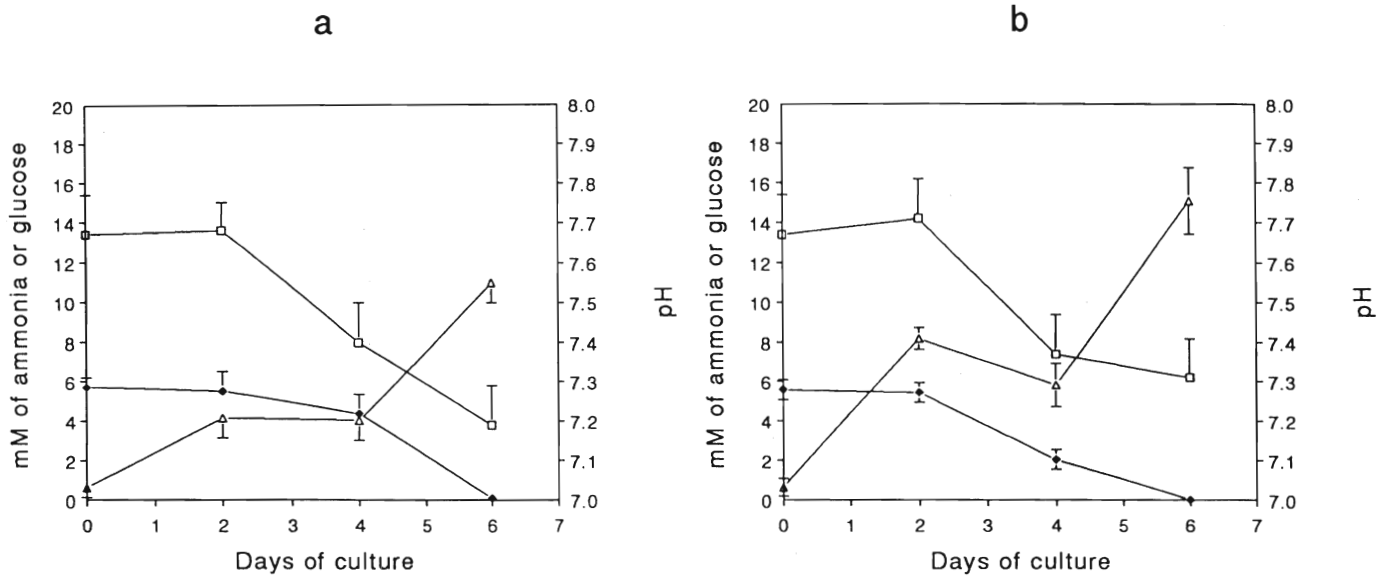

FIGURE 3. Changes in the levels of glucose (◆——◆), NH_3 (△——△), and pH (□——□) in the culture medium during growth and metacyclogenesis (see Fig. 1) of VL (a) and CL (b). The results are the means of triplicate determinations, and the bars represent their SD.

sumption—the sixth for VL (Fig. 3a) and the fourth for CL (Fig. 3b). The production of NH_3 during the second day of culture could have been related to amino acid catabolism that was required for the energetic needs of both *L. infantum* strains. In this way, *Leishmania mexicana mexicana* respires on amino acids excreting large quantities of urea and ammonia in the culture medium (Coombs and Sanderson, 1985). NH_3 production reached its maximum when the extracellular glucose was exhausted by the sixth day of CL and VL cultures (Fig. 3a, b), indicating that *L. infantum* growth and metacyclogenesis depend on glucose at the beginning of the stationary phase (fourth day) and on amino acid catabolism at the mid-log phase (second day) and the end of the stationary phase (sixth day). This finding agrees with previous reports on *L. tropica* (Mukkada et al., 1974), *L. donovani* (Marr and Berens, 1977), and *L. mexicana* (Cazzulo et al., 1985). It seems that amino acids are initially the preferred substrates (Crowther et al., 1954; Mukkada et al., 1974; Marr and Berens, 1977; Steiger and Meshnick, 1977). The progressive pH decline during *L. infantum* culture could have been related to secretion of acidic products by promastigotes in the medium as occurs with other *Leishmania* species (Hart and Coombs, 1982; Cazzulo et al., 1985).

Figure 4 shows the variation of CS, NADP-ICDH, SDH, MDH, and ME levels throughout the growth of both *L. infantum* strains. When the percentages of metacyclic promastigotes were higher, the behavior of the enzymes studied did not differ from 1 strain to another. In both strains there were significant increase in CS activity (Fig. 4c, d), maximal activities of NADP-ICDH (Fig. 4c, d) and MDH (Fig. 4e, f), and minimal activities of SDH (Fig. 4a, b) and ME (Fig. 4e, f) by the fourth day of VL growth and the sixth day of CL growth.

DISCUSSION

The presence of metacyclic promastigotes was detected by PNA agglutination and resistance to lysis with rat serum at all phases of *L. infantum* growth (Fig. 1) as occurs with other species of the genus (Sacks et al., 1985; Mallinson and Coombs, 1989; Bates and Tetley, 1993), but their numbers were higher at the early stationary phase (Fig. 1a) of VL culture (fourth day) and at the end of the same phase (Fig. 1b) for CL (sixth day).

It is well established that the metabolism of *Leishmania* species, growing in vitro, depends on the available energy sources, such as glucose, amino acids, and fatty acids in the growth media. Whereas the reason for preferential use of glucose or amino acids as substrates for growth of trypanosomatids remains unknown (Cazzulo, 1992), there is no doubt that *Leishmania* promastigotes do not oxidize free fatty acids very rapidly (Hart and Coombs, 1982). Promastigotes of *L. infantum* could have used amino acids as substrate throughout the logarithmic and late stationary phases, whereas they exhibited increasing glucose consumption as they entered the stationary phase of growth (Fig. 3). Notice that *Leishmania panamensis* oxidizes more glutamate than glucose (Keegan et al., 1987). Also, the presence of glucose in the growth medium increases the oxidation rate of certain amino acids (Mukkada and Simon, 1977; Darling et al., 1989).

Various authors (Martin et al., 1976; Coombs et al., 1982; Meade et al., 1984; Cazzulo et al., 1985; Mottram and Coombs, 1985a) have found very low (<3.1 mU/mg protein) or even undetectable activity of CS, α-ketoglutarate dehydrogenase, and SDH in different species of *Leishmania*. These findings suggest that the citric-acid cycle has little importance in the metabolism of these parasites (Glew et al., 1988). In contrast, SDH (Fig. 4a, b) and CS (Fig. 4c, d) were active in *L. infantum*. In fact, the activity of the first enzyme of the citric-acid cycle, CS, is low (0.04–0.29 mU/mg protein) in *Leishmania braziliensis, L. donovani, L. mexicana,* and *L. tropica* (Martin et al., 1976; Meade et al., 1984) or even undetectable in *L. mexicana* (Cazzulo et al., 1985). In the metabolic studies carried out in *Leishmania braziliensis panamensis* with labeled substrates, Darling et al. (1989) have suggested low CS activity. However, CS is

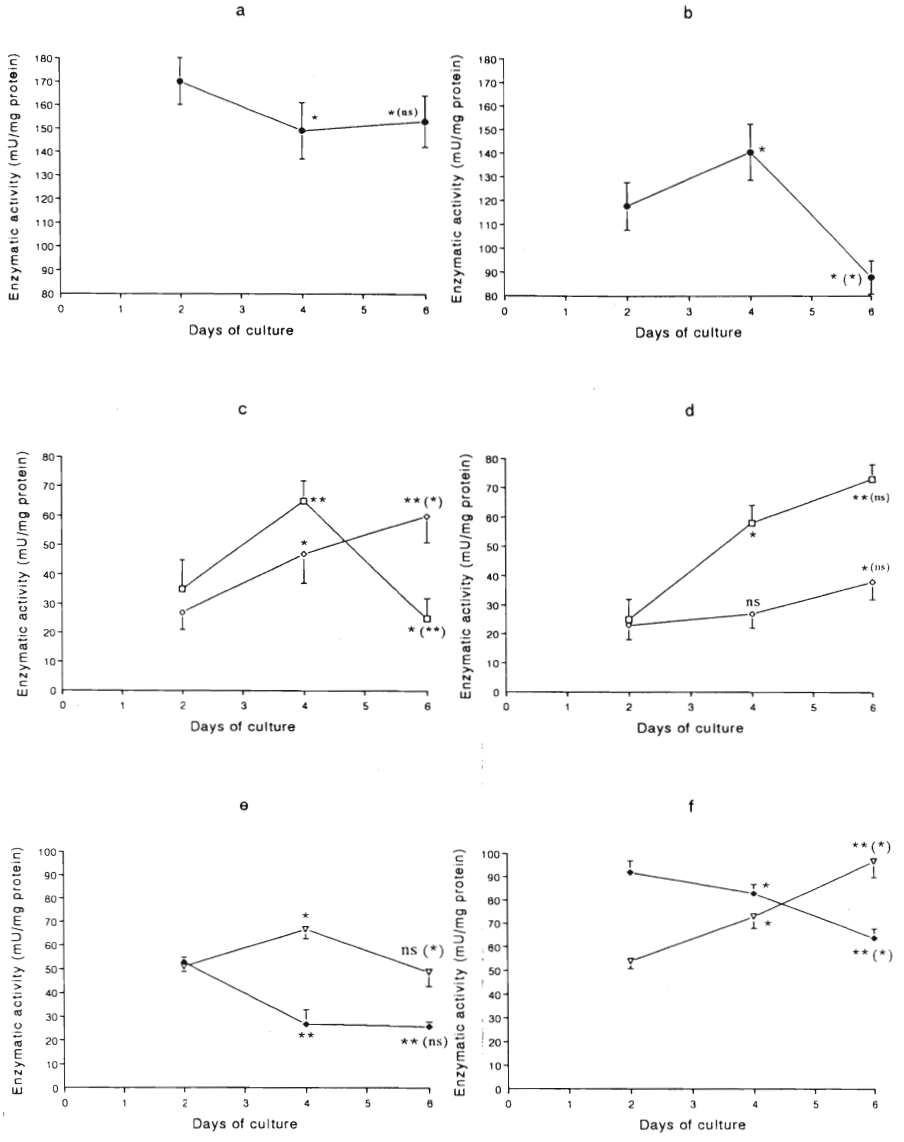

FIGURE 4. Citrate synthase (◊——◊), NADP-specific isocitrate dehydrogenase (□——□), succinate dehydrogenase (●——●), L-malate dehydrogenase (▽——▽), and malic enzyme (◆——◆) activity variations throughout the growth and metacyclogenesis (see Fig.1) of VL (a, c and e) and CL (b, d and f). The results are the means of triplicate determinations, and the bars represent their SD. Statistical comparisons were done between the second and fourth or sixth days and in brackets between the fourth and sixth days. $*P < 0.05$, $**P < 0.01$, and ns = not significant.

active in other trypanosomatids such as *Crithidia fasciculata, Trypanosoma cruzi* (Juan et al., 1976; Cazzulo et al., 1985; Adroher et al., 1988), and *L. infantum* (Fig. 4c, d). Furthermore, maximal SDH activities for CL (142 mU/mg protein) and for VL (170 mU/mg protein) were 28–55 times more than the maximal activity determined for other *Leishmania* species (Martin et al., 1976; Meade et al., 1984; Mottram and Coombs, 1985a), and it was even higher than other trypanosomatid SDH activities (Klein et al., 1975; Adroher et al., 1988).

We have not detected any NAD-ICDH activity in either strain of *L. infantum,* a finding consistent with the data of Martin et al. (1976), where none of the 4 studied *Leishmania* species shows activity for this enzyme. We demonstrated activity only when we used NADP as coenzyme for the same enzyme (Fig. 4c, d), as occurs with other trypanosomatids (Martin et al., 1976; Cazzulo et al., 1985; Adroher et al., 1988).

NAD-MDH activity can be found in mitochondria, the cytosol, and glycosomes in promastigotes of *L. m. mexicana* (Mottram and Coombs, 1985b). Mottram and Coombs (1985c) have purified glycosomal MDH in amastigotes of *L. m. mexicana,* suggesting that it reduces oxaloacetate, in contrast with mitochondrial MDH that oxidizes L-malate. Both *L. infantum* strains possess, at least, 2 isoenzymes with MDH activity as detected by the oxidation of L-malate (Martín-Sánchez et al., 1996). They may correspond to the mitochondrial and cytosolic MDHs described in promastigotes of *L. m. mexicana.* In the 4 *Leishmania* species studied by Martin et al. (1976), NAD-MDH is active and its activity varies with *L. m. mexicana* and *L. donovani* life cycles (Meade et al., 1984; Mottram and Coombs, 1985a; Mallinson and Coombs, 1986). Moreover, the metacyclic or stationary promastigotes present an activity 1.5–3 times higher than those of logarithmic organ-

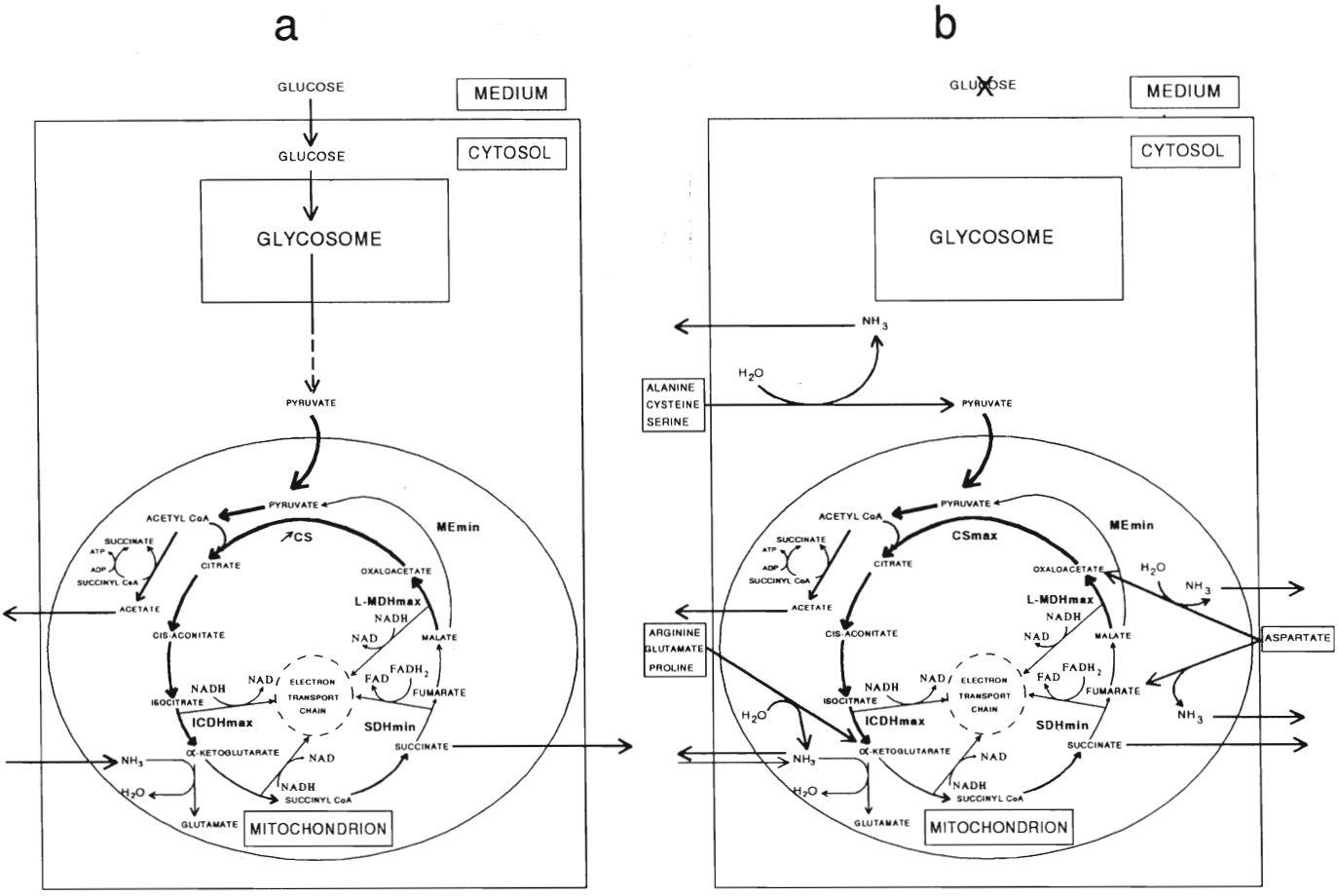

FIGURE 5. Proposed scheme for citric-acid cycle flows in *Leishmania infantum* and entry of some amino acids, after their deamination, into this cycle on the days when the percentages of metacyclic promastigotes were highest, the fourth for VL (a) and the sixth for CL (b). Heavier arrows indicate the principal flows. The activation of CS and ICDH can lead to the formation of α-ketoglutarate that can be converted to glutamate and detoxify or organically degrade NH_3 (Glew et al., 1988) accumulated in *L. infantum* culture medium (Fig. 3a, b). The α-ketoglutarate can be transformed through the action of citric-acid cycle enzymes to succinate, which is particularly released in *Leishmania* spp. (Marr, 1980; Cazzulo, 1992; Blum, 93), including *L. infantum* (Van Hellemond et al., 1997). Succinate can be converted to acetate, which is another major end product of *L. infantum* catabolism (Van Hellemond et al., 1997), via acetate : succinate Co-A transferase (Van Hellemond et al., 1998). It can also be oxidized to fumaric acid by SDH. However, in *L. infantum*, minimal levels of SDH (Fig. 4a, b) and ME (Fig. 4e, f) were seen on the days when the percentages of metacyclic promastigotes were highest. This later observation suggests that SDH could have been involved in the control of the complete oxidation of dicarboxylic acids, and ME could have been involved in the control of carbon flow toward aerobic fermentation (pyruvate and/or acetate release) or biosynthetic purposes (NADPH production) as has been suggested for *L. donovani* (Saadala and Rassam, 1988). Because of its highest activity, MDH might have used additional malate of glycosomal origin (Blum, 1993) for VL, or of aspartate origin (Blum, 1994) for CL after oxidative deamination of aspertate and its transformation into fumarate.

isms, at least in *Leishmania major* and *L. mexicana* (Mallinson and Coombs, 1986).

Based on the conclusions of Saadalla and Rassam (1988) and Mottram and Coombs (1985b) and the results of Martín-Sánchez et al. (1996), it is clear that at least 2 ME isoenzymes are present in *L. infantum,* with the mitochondrial form more active at pH 7.4 and the cytosolic more active at pH 9.5. In our work, we have measured ME activity at pH 7.4; therefore the major activity observed must have been mitochondrial.

To understand the energetic strategies adopted by both human strains throughout the metacyclogenic process, we have summarized in Figure 5 the main results determined on the days when percentages of metacyclic promastigotes were highest (the fourth for VL [Fig. 1a] and the sixth for CL [Fig. 1b]). In effect, both strains showed higher levels of CS, ICDH (Fig. 4c, d), and MDH (Fig. 4e, f) and lower SDH (Fig. 4a, b) and ME (Fig. 4e, f) activities when more metacyclic promastigotes were present. In VL these changes paralleled an increase in glucose consumption (Fig. 3a), whereas in CL these changes coincided with NH_3 hyperproduction (Fig. 3b). These results suggest that energy metabolism during *L. infantum* growth and metacyclogenesis is affected by regulated enzymes that probably respond to changes in the culture medium in the levels of glucose and amino acids.

On the other hand, the levels of ME and MDH (Fig. 4e, f) were higher on the sixth day of CL culture than on the fourth day of VL culture ($P < 0.01$). It seems that the complete oxidation of dicarboxylic acids was relatively more important during the metacyclogenesis of CL as compared with VL. This hypothesis is consistent with NH_3 hyperproduction observed on the sixth day of CL culture (Fig. 3b), reflecting active amino acid catabolism that can feed the citric-acid cycle with new

carbon skeletons after glucose depletion (Fig. 5b). Nevertheless, resistance to complement-mediated lysis, loss of PNA agglutination (Fig. 1), and infectivity within J774 cells (Fig. 2) were higher in VL at the fourth day than in CL at the sixth day. Thus, it is tempting to contradict other hypotheses that have related the high pathogenicity of trypanosomatids to their high catabolic activity (Mancilla and Naquira, 1964; Zeledon and Monge, 1967; Janovy, 1972) and to suggest that VL, which is able to evade the skin immune system of human hosts and disseminate into the reticuloendothelial tissue in vivo and produce more infective parasites in vitro, is catabolically less active than CL that is blocked at the cutaneous level and produces fewer metacyclic promastigotes during culture.

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