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Leishmania donovani: In vitro culture and [^{1}H] NMR characterization of amastigote-like forms

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Abstract

When *Leishmania donovani* promastigote forms, were cultured in TC-199 medium at 28°C and subsequently incubated at 38°C, they turned into aflagellate (amastigote-like) forms. A return of the incubation-culture temperature to 28°C these amastigote-like forms to revert to promastigotes. The amastigotes obtained by heat-shock, were viable and retained antigenic capacity being recognized by the sera of naturally infected patients. These forms, remained also capable of multiplying inside the J-774A.1 macrophages. When the amastigote-like forms are kept in culture at 38°C retained their rounded appearance and their biological characteristics for more than 3 months subculturing every 6 days. These amastigote-like forms, when used for subcultures at 28°C, transformed into promastigotes capable of multiplying as flagellate forms. The amastigote-like forms obtained *in vitro* can be used in biochemical studies related to chemotherapy and immunology studies, as part of an effort to combat this parasite. The end-products of of glycolysis were studied in both the amastigote-like and promastigote forms of *L. donovani*, by proton magnetic resonance analysis of the culture media. Alanine, succinate, and acetate, were predominant, and to a lesser extent pyruvate, glycine and D-lactate. Our results suggest that both forms of *Leishmania* use different biochemical strategies to obtain their energy. (Mol Cell Biochem **142:** 89–97, 1995)

Key words: amastigote culture, nuclear magnetic resonance, *Leishmania donovani*

Introduction

Leishmania genus includes a wide group of species, among which *L. donovani* causes visceral leishmaniasis in humans. In the biological cycle of this parasite some species of haematophagous Diptera of the genus *Phlebotomus* act as intermediary hosts in which the ingested amastigote forms transform into promastigote forms. These promastigotes, inoculated by the insect into a new vertebrate host, are ingested by phagocytic cells, where they transform into the phagosome in intracellular amastigotes [1, 2].

Many attempts have been made to obtain axenic amastigote cultures of different *Leishmania* species [3–7]. Some authors have described *in vitro* transformation when the promastigotes are subjected to a sharp changes in temperature, leading to amastigote forms that showed antigenic and morphological similarities with the intracellular amastigotes [8–11]. Doyle *et al.*, 1991 [12], obtained continuous axenic cultures of the aflagellate forms, in *L. donovani* using 100% foetal bovine serum at 37°C, as culture medium. When the temperature was lowered from 37–28°C the aflagellate forms change to flagellates. Nevertheless, the transference to a semi-defined medium kills the protozoa.

In this work we have not only studied the culture of aflagellate (amastigote-like) forms in a semi-defined medium, but we have also investigated using [^{1}H] NMR the catabolites produced by both forms of the parasite and its ultrastructural characteristics.

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Material and methods

Parasite strain and maintenance

The strain of *Leishmania donovani* LRC-L133 (Leishmania Reference Center, Jerusalem) was isolated in 1967 from a clinical case in Begemder (Ethiopia). The parasites were maintained in our laboratory by culture in Nicole Novi and MacNeal (NNN) medium supplemented with Minimal Essential Medium (MEM) plus 20% Inactivated Foetal Calf Serum (Md-NNN).

Mass culture of promastigote forms

Promastigote (1×10^6 parasites/ml), from Md-NNN medium, were transferred after centrifugation to Roux flasks of 75 cm^2 with 25 ml TC-199 medium (GIBCO, Grand Island, N.Y., USA) supplemented with 30% (v/v) Inactivated Foetal Calf Serum (IFC), and then cultured at 28°C in a 95% air and 5% CO_2 atmosphere and maintained for 7 days in a humid atmosphere.

Transformation from promastigote to amastigote-like forms

The promastigote forms in the exponential growth phase cultured at 28°C in TC199 medium, with a density of 3×10^6 cell/ml were transferred to 38°C to obtain amastigote-like forms, following the method of Darling and Blum [6].

Aliquots were taken every 24 h throughout the experiment. Cell-free supernatants of these aliquots (15 min centrifugation at 1500 g) were frozen with liquid N_2 for [^{1}H] NMR determination of the metabolites excreted by the parasite. Other aliquots were used to measure the pH of the medium.

Viability and multiplication capacity of amastigote-like forms

The amastigote forms, obtained after 5 days of culture at 38°C, following heat shock treatment, were separated from the flagellate forms according to Gamarro *et al.* [13]. More than 98% were amastigote-like forms, which were transferred to TC-199 and cultured at 38°C for 5 days. During this period, cell growth was recorded every 24 h.

The viability was determined by two procedures. First, by fluorescence microscopy with vital stain acridine orange (EGA-CHEMIE, Steinheim/Albuch. Germany) at a concentration of 15 µg/ml dissolved in TC-199 medium, after incubation (15 min 38°C) and centrifugation (1500 g for 15 min). The second procedure used [^{3}H]Thymidine labelling for 1 h at 37°C ([^{3}H]Thymidine specific activity 26 Ci/mM. Radio Chemical Centre, Ltd. Denmark), after which the cells were kept for 1 h at 37°C. After labelling, the cells were washed 3 times in cold medium with a concentration of unlabelled Thymidine 100 folds higher than the labelled one. The pellet containing the parasites was treated with Trichloroacetic Acid (TCA) (Merck) at 20%, and passed through a GFC filter (Whatman); TCA was eliminated with methanol, and the precipitable radioactive material was dried and solubilized in 0.1% SDS and 0.1% NaOH. The radioactivity was measured in a β-particle counter (BECKMAN, Palo Alto, CA, USA).

Macrophage culture and infection with amastigote-like forms

The macrophage cell line J-774A.1 (ATCC TIB 67) was used to study the cell-parasite interaction, they were cultured in RPMI 1640 (GIBCO) medium supplemented with 10% IFCS in Roux flasks of 25 cm^2 in a moist atmosphere with 5% CO_2. The amastigote-like forms were added to the macrophages at a 3:1 ratio. As control we used promastigote forms. After 24, 48 and 72 h the cultures were fixed with methanol and stained with Giemsa. The number of parasitized macrophages and the number of parasites per cell was counted to evaluate the multiplication of these forms.

Fluorography of the proteins synthesized by the different forms of culture

Cultures of 1×10^6 cell/ml of promastigotes kept at 28°C; amastigote like forms cultured at 38°C and treated during 2 h at 42°C; promastigotes obtained at 28°C from amastigote-like forms; amastigote-like forms cultured at 38°C during 30 days; and heat shock (42°C, 2 h) of cultured amastigote like forms of 30 days old, were labelled for 2 h with 20 µCi/ml of Trans ^{35}S label (ICN Radiochemicals) with a specific activity of 1132 Ci/mmol [≥ 70% L-Methionine ^{35}S, ≤ 15% L. Cysteine ^{35}S, ⟨ 7% L-Methionine sulfone ^{35}S, ≤ 3% L-Cysteic acid ^{35}S, ≤ 5% other ^{35}S-Compounds, and various non-labeled amino acids]. Next, the parasites were centrifuged 3 times and the radioactive medium was washed with non-radioactive medium. Afterwards, the pellet containing the parasites was resuspended in 50 µl of 2.5% SDS, 5.0% B-mercaptoethanol, 0.01% bromphenol blue, and immersed in boiling water during 5 min. Electrophoresis was carried out in (12.5%) SDS PAGE (Hames, 1981) [14] during 30 min at 250 V; 10 mA; 70 Vh in a PhastSystem equipment (Pharmacia, Upsala, Sweden), after which the gel was

dried, treated 20 min with EN³HANCE™ (DU PONT) and incubated with X-Ortonat film (Kodak) for 72 h at –70°C. The film was processed 3 min with developer for X-rays (Kodak), and fixed with (VALCA fixer, Bilbao, Spain). The gels were digitalized on a ScanMaker 1850-S scanner (Microtek) and processed with the Millipore programme BioImage Software.

Antigenic study of amastigote-like forms

The amastigote-like forms obtained *in vitro* were fixed in acetone at –20°C, in order to study the antigenicity of these forms against a immuniserum obtained against promastigote forms from a human case of Kala-azar from the Spanish Mediterranean region.

Transformation of amastigotes-like forms into promastigotes

The amastigote-like forms obtained *in vitro* by heat shock were transferred to Roux flasks (25 cm^2) with TC-199 medium, centrifugated at 1500 g for 15 min, and then cultured for 5 days at 28°C.

Transmission-electron microscopy

The promastigote and amastigote-like forms growing in TC-199 medium at 28 and 38°C, were collected during the exponential growth phase by centrifugation at 1500 g for 15 min, then fixed in 2.5% (v/v) buffered glutaraldehyde, postfixed in 1% (v/v) buffered OsO_4 for 30 min, and then dehydrated and embedded in Spurr resin. Ultrathin sections were stained with 8% uranyl acetate followed by 0.2% lead citrate, and were examined under a Zeiss ECM 10 SEM.

[¹H] NMR spectroscopy

The spectra of protonic [¹H] NMR were obtained with a Bruker AM-300 apparatus operating at 300.13 MHz in protons. The temperature of the probe was maintained at 27°C. The pulse technique and Fourier transformation was used with 90° pulses and a spectrum of 3287.5 Hz. To eliminate watermarks, the method of presaturation was used, selectively irradiating at the frequency of water for 2.5 sec with an interval between pulses of 7.5 sec, accumulating 160 FIDs, the total of which was multiplied exponentially with a line-broadening of 0.2 before Fourier transformation. The chemical shifts are expressed as parts per million (ppm) downfield from TSP.

Identification and quantification of metabolites

The resonances of a number of metabolites in *Leishmania donovani* medium were assigned by the addition of pure compounds, whereupon the chemical shifts were measured. By recording the spectra having different recycle times, we established that a recycle time of 8 sec did not cause any significant reduction in intensity, due to partial saturation, for the peaks of interest.

Standard curves were constructed by the addition of pure compounds dissolved in H_2O to fresh medium, and were linear over the range 0–20 mM. All correlation coefficients were greater than 0.98. Quantification of metabolites was assayed biochemically according to Bergmeyer [15].

Results

Figure 1 represents the *in vitro* transformation from promastigote to amastigote-like forms of *L. donovani,* and also their respective growth curves at 28 and 38°C. At 28°C the promastigote forms showed a normal exponential growth, whereas moments after raising the culture temperature to 38°C a drastic reduction was detected in the flagella lengths. After 3 days of culture at 38°C, 80% of the protozoa had taken on a rounded shape without flagella. Their viability, determined by acridine-orange staining, was never less than 90%. These aflagellates forms appear in Fig. 3b and 3c. The stability of the amastigote-like forms remains after 3 months of culture, with a exponential growth *in vitro* (Fig. 4).

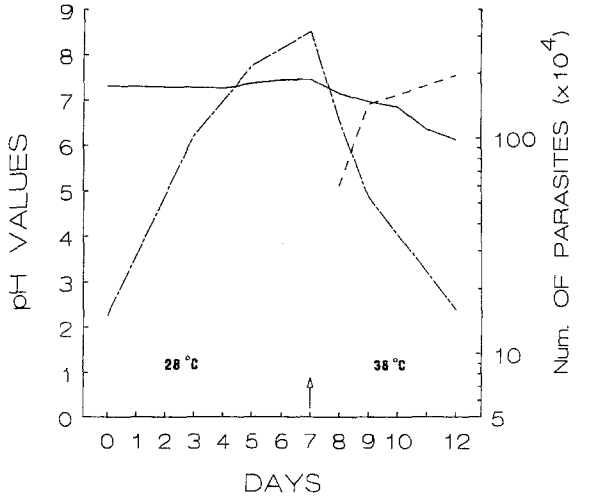

Fig. 1. Growth of *Leishmania donovani* in culture medium at different temperatures. (—• —• —) Promastigote forms. (---------) amastigote-like forms. (————) pH of the culture values during the culture, the arrow (↑) indicate the moment of temperature change. The results are the mean of 5 experiments.

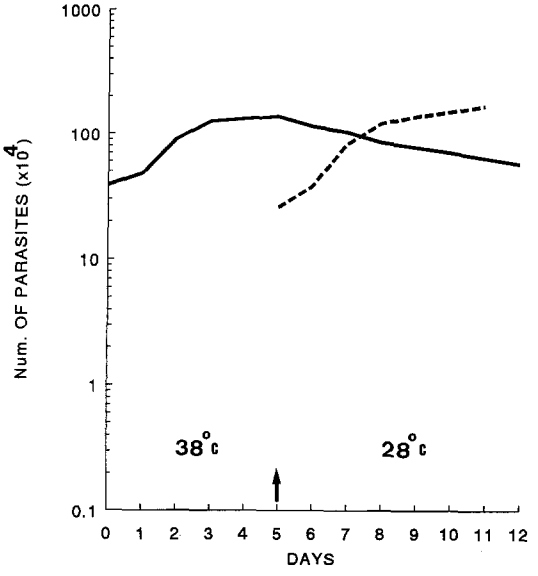

Fig. 2. In vitro transformation from amastigote-like (————) to promastigote forms (--------), the arrow (↑) indicate the moment of temperature change. The results are the mean of 5 experiments.

Transferring these protozoa to a new TC-199 culture at 28°C caused the parasites to return to their flagellate form (Fig. 2); after 48 h a great proportion of them began to develop a flagellum once more and after 5 days virtually 80% were flagellate. The growth curve of these new promastigote forms was similar to that of promastigotes cultivated *in vitro*.

The proteins expressed by the different forms after the labelling with ^{35}S aminoacids are shown in Fig. 5. The protein pattern of the amastigote-like forms and the promastigote forms (obtained from amastigote-like forms) are different. Also when the amastigote-like forms were treated at 42°C during 2 h, the pattern is different as the amastigote-like forms subjected at normal temperature of culture at 38°C (Fig. 5 and Fig. 6).

The ultrastructure of the promastigote forms of *L. donovani* cultured at 28°C, as is shown in Fig. 3a, is similar to that described by Molyneux and Killick-Kendrick [16]. One form has a wide flagellar sac (Fp), in which the flagellar root (Fr) is clearly visible (Fig. 3a); a very wide kinetoplast sack (K) can also be seen. The mitochondria (M) have a double membrane with several crests and are distributed throughout the cytoplasm. In transverse cuts of the flagellum there are traces of the paraxial cord near the axoneme. Besides this, there are a striking number intravacuole membranes surrounding empty vacuoles.

The amastigote-like forms obtained *in vitro* (Fig. 3b and 3c) are either round or oval, with a nucleus (N) in which the chromatine is uniformly distributed near the nuclear membrane. Mitochondrial cisterns are present with the crypts being practically absent. The quantity of lipid vacuoles (LV)

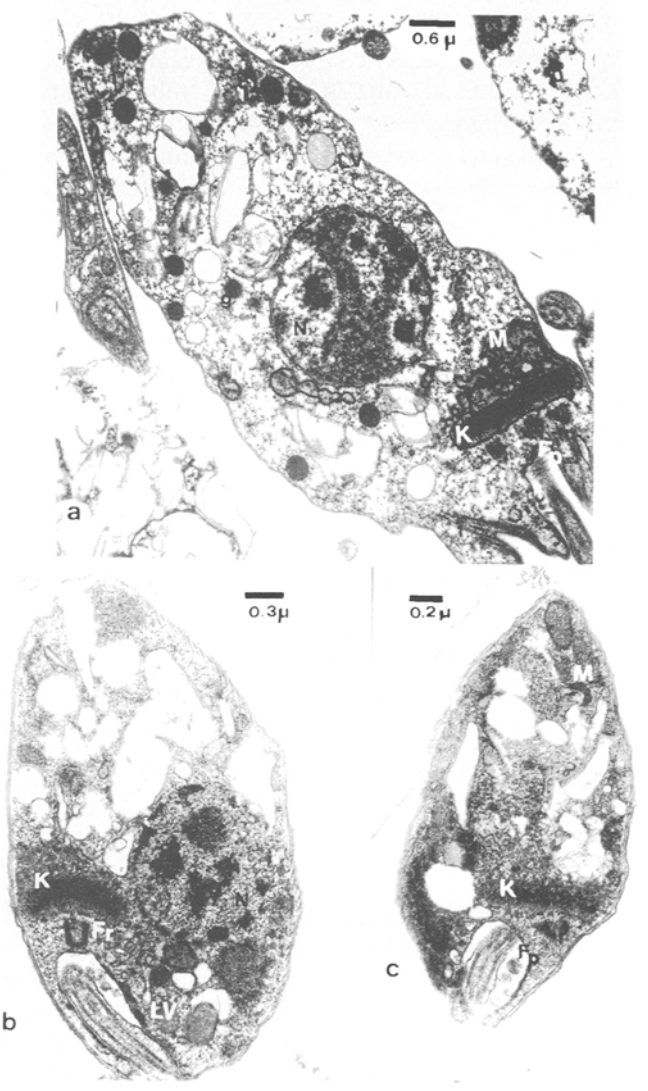

Fig. 3. Transmission electron micrographs of differents *Leishmania donovani* forms isolated *in vitro*. a: promastigote form (magnification × 16000). b and c: amastigote forms (magnification × 12000). M: mitochondria. g: glycosomes. N: nucleus. LV: lipid vacuoles. Fp: flagellar sac. Fr: flagellar root. K: kinetoplast.

is higher, whilst the number of axonemes and flagella is almost nil. A series of vacuoles is also present.

The results with [^{3}H]Thymidine (Fig. 7), indicate that after 48 h the incorporation of the analogue in cpm into the subculture of the amastigote-like forms was about double that in the same volume of the sample culture medium. This demonstrates the viability and multiplication capability of the amastigote like forms after 3 months of culture.

We obtained a titer of 1/1024 by immunofluorescence, using the serum from a kalaazar patient against the cultured amastigote-like forms. The same titer was obtained against promastigote forms of *L. donovani*.

The Survival and evolution of the amastigote-like forms

Table 1. Infectivity of amastigote-like forms in J774A.1 macrophage cell line. The parasite:cell ratio was 3:1 [%P: percentage of macrophages parasitized after different times (shown the infectivity of the different forms of the parasite). Ii: number of amastigotes per infected cell, (shown the multiplication capability of different forms)]. The results are the mean of 5 experiments ± SEM. Student's test, $p < 0.001$.

	24 h		48 h		72 h	
	%P	Ii	%P	Ii	%P	Ii
amastigotes-like	7.23 ± 0.80	1.25 ± 0.06	5.17 ± 0.81	3.32 ± 0.09	4.08 ± 0.57	7.15 ± 0.32
promastigotes	18.43 ± 1.34	1.42 ± 0.07	15.25 ± 0.77	5.16 ± 0.33	12.13 ± 0.98	10.27 ± 0.59

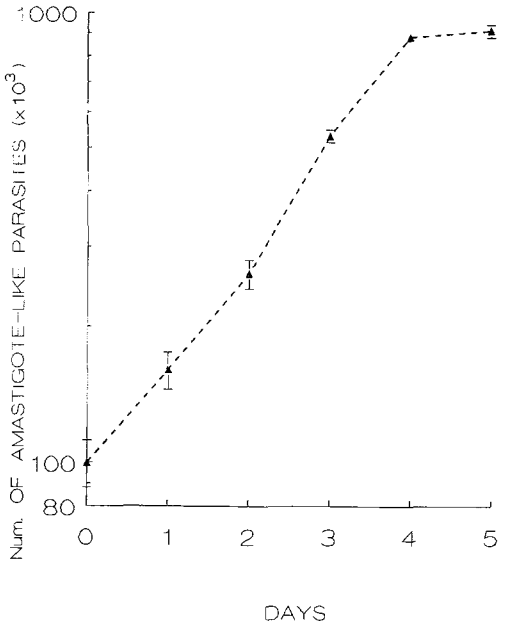

Fig. 4. Multiplication capability of the amastigote-like forms in culture medium TC-199, at 38°C. The results are the mean of 5 experiments (± SE).

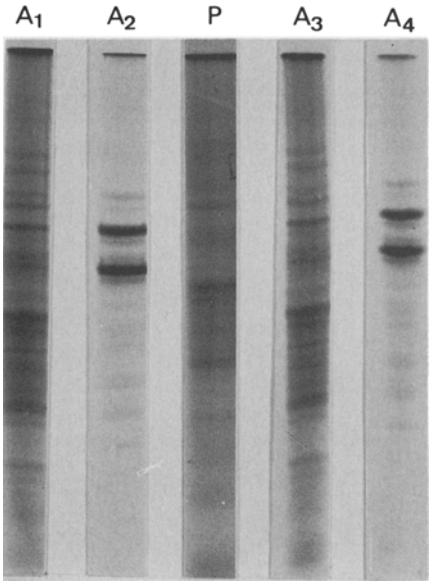

Fig. 5. Proteins expressed by the differents forms after the labelling with 35S aminoacids. A_1: (amastigote-like forms after 4 days of culture at 38°C). A_2: (amastigote-like forms after 4 days of culture at 38°C and treated at 42°C during 2 h). P_1: (promastigotes produced from amastigotes at 28°C). A_3: (amastigote-like forms after 1 month of culture at 38°C). A_4: (amastigote-like forms after 1 month of culture at 38°C and treated at 42°C during 2 h).

inside J-774 macrophages are shown in Table 1.

Examples of our [^{1}H] NMR spectra appear in Fig. 8. The first spectrum (Fig. 8.1) represents the fresh medium free of protozoa; the second (Fig. 8.2) reflects the medium after inoculating the promastigote forms and culturing them at 28°C for 7 days; and the third (Fig. 8.3) represents the medium of amastigote-like forms after culture during 5 days at 38°C. Some additional peaks, corresponding to metabolites produced and excreted during growth, can be made out in these spectra.

The metabolites excreted by the promastigotes of *L. donovani* were mainly succinate (S), alanine (Al) and acetate (A), and minor quantities of pyruvate (P), glycine (Gl) and D-lactate (L). The metabolites excreted by the amastigote-like forms obtained *in vitro* by heat shock were alanine (Al), acetate (A) and glycine (Gl), and to a lesser extent, succinate (S), pyruvate (P) and D-lactate (L).

The quantities of alanine and glycine excreted by the amastigote-like forms were significantly greater than those excreted by the promastigote forms. The quantities of succinate and pyruvate, on the other hand, were clearly smaller. The amount of acetate and D-lactate excreted by both forms were practically the same.

Discussion

Current chemical methods of culturing *in vitro* promastigotes of *Leishmania* have produced a great deal of morphological and metabolic information. Nevertheless, few articles describe a chemically defined medium capable of sustaining *in vitro* growth of the amastigote forms [17], and thus these parasite forms are not well understood. Present knowledge on this subject is based on forms isolated from previously infected macrophages but, unfortunately, the number of cells obtained in this way is inadequate for detailed metabolic studies.

Pan (1984) [3] described a stock of *Leishmania (mexicana) pifanoi,* which was capable of growing continuously in an axenic culture in the amastigote form. From this work

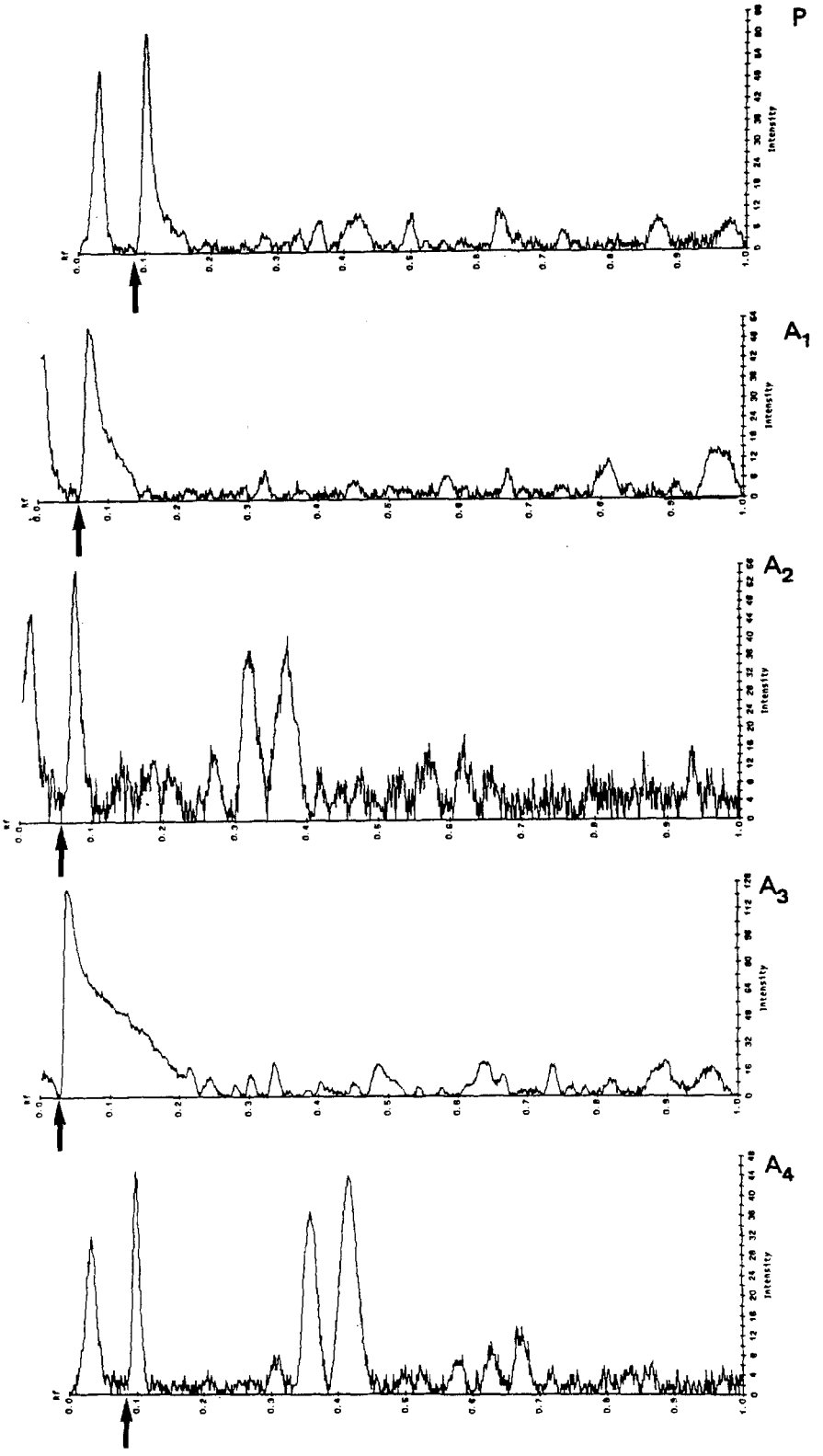

Fig. 6. Pattern of new proteins syntetized by the two forms of the parasite cultures *in vitro* after labelling with S^{35} aminoacids. The arrows shown the end of the stacking gel. A_1: (amastigote-like forms after four days of culture at 38°C). A_2: (amastigote-like forms after four days of culture at 38°C and treated at 42°C during 2 h). P_1: (promastigotes produced from amastigotes at 28°C). A_3: (amastigote-like forms after one month of culture at 38°C). A_4: (amastigote-like forms after one month of culture at 38°C and treated at 42°C during 2 h).

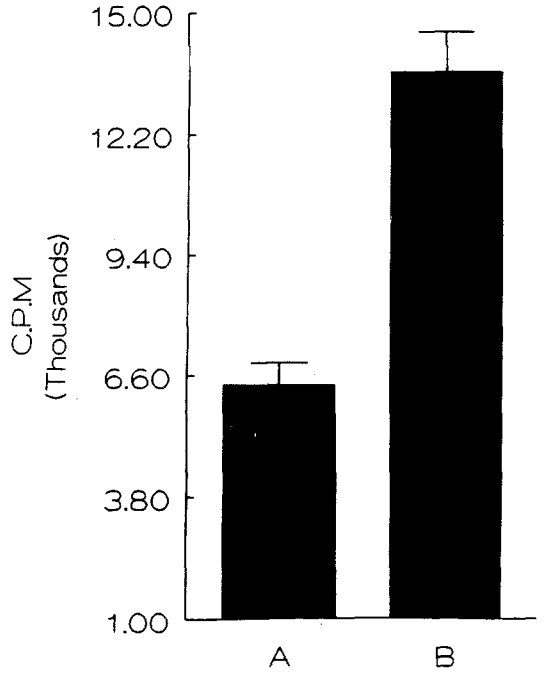

Fig. 7. Incorporation of [^{3}H] Thymidine by the purified amastigote-like forms. A: forms obtained from 1 ml of growth medium after 24 h of culture. B: forms obtained under similar conditions after 48 h of culture. The medium was removed by centrifugation and the organisms labelled as described in Materials and methods. The results are the mean of 5 experiments.

and subsequent studies [4–7] it is known that amastigotes growing *in vitro* are very similar in morphology, antigenicity and infectivity to the amastigote forms isolated either from infected animals or from infected macrophages.

The transformation of promastigotes to amastigotes occurs in *Leishmania* during phagocytosis by the host phagocytic cells, when exposed to an acidic environment [18]. Nevertheless, the factors involved in this transformation are as yet unknown [17]. Some researchers [19], believe that pH plays an important role in the mechanisms which triggers the transformation process, and note that a slight fall in pH is sufficient to induce the transformation of promastigotes into amastigotes. Other authors [6] have reported that a rise in the temperature of axenic cultures from 26–34°C causes cell changes, reflected in their morphology and metabolism.

From our results we conclude that temperature plays an important part in the mechanism that triggers the transformation from promastigote to amastigote forms. The heat shock applied must be drastic in order to achieve an adequate transformation rate; the promastigotes of *Leishmania major* underwent little change in cell morphology [19, 20] when subjected to a smooth change, whereas higher increments of heat shock resulted in significant morphological and biochemical changes [7]. Furthermore, a drastic reduction in the temperature of the culture medium causes amastigotes to revert into promastigotes.

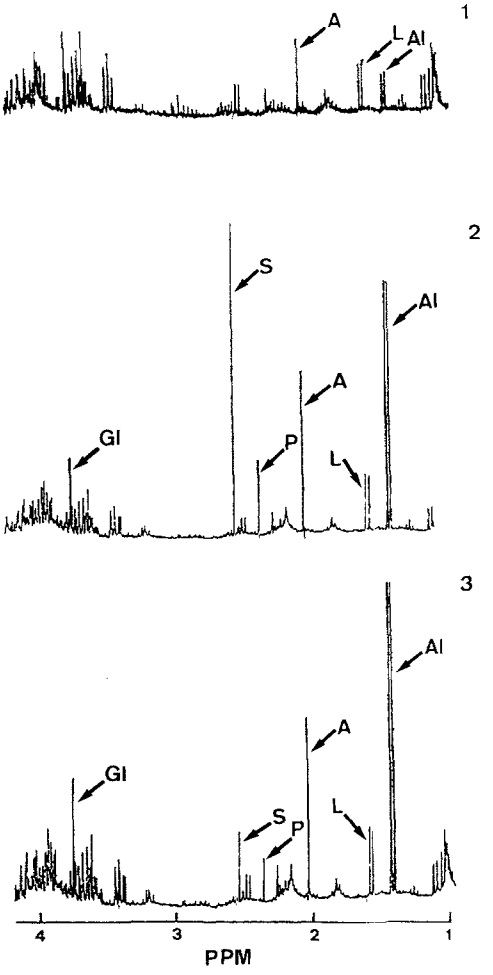

Fig. 8. [^{1}H] NMR spectra of *Leishmania donovani* cultures. Trace (1), fresh culture medium before inoculation of cells. Trace (2), medium from the promastigote culture after 7 days at 28°C. Trace (3) medium from the amastigote cultures after 5 days at 38°C. Chemical shifts are expressed as PPM. (S) succinate (s); (A) acetate (s); (Al) Alanine (d); (P) pyruvate (s) and (L) D-Lactate (d) and Gl glycine (s). (s): singlet; (d): doublet. The results are the mean of 5 experiments.

The fact that the transformation can be caused by acidification resulting from metabolites produced and excreted by the parasites suggests that acidity must play a part in the transformation process, but this relationship requires further investigation.

An examination of the proteins expressed by the different parasite forms indicates that the promastigote protein pattern differs from that of amastigote-like forms; when the amastigote are subjected to 42°C , there appears a series of major proteins which do not appear in cultures kept at 38°C and which could proteins typical of heat shock [21]. This indicates that the proteins appearing under stress disappear as proteins of major synthesis when the amastigote-like forms are cultured at a constant 38°C.

Examination by transmission electron microscopy reveals that the ultrastructural pattern of the amastigote-like forms

obtained *in vitro* by heat shock is similar to that described for amastigotes isolated from infected macrophages [16]. In the amastigote-like forms the occurrence of lipid vacuoles might suggest a lack of mitochondrial activity. The photograph of the transverse section of the flagellate promastigote form (Fig. 3a), shows the number of free axonemes present compared to a similar section of the amstigote form.

The viability and infectivity of the amastigote-like form is demonstrated in our results, showing the parasite to be capable of surviving and multiply in true amastigote form, inside cells with phagocytic capacity.

The higher percentage of parasitism shown by macrophages infected by promastigote forms may be due to the greater mobility of these forms, favouring the contact of the parasite with the cells; this situation contrasts with that of the amastigote like forms, in which the entry is only by passive phagocytosis.

[^{1}H] NMR spectroscopy is a recently established technique for studying metabolic routes in biological systems, most studies having so far used [^{13}C] NMR. Currently, [^{1}H] NMR is being extensively applied to metabolic studies of parasites, given that the majority of biological compounds have detectable protons [22].

Although glucose metabolism has been thoroughly studied in the promastigotes of different species of *Leishmania*, little is known about its metabolism in the amastigote forms, for the reasons already mentioned. By means of nuclear magnetic resonance we have studied the metabolic differences in the two parasite forms by detecting the metabolites produced and excreted into the medium.

Our *L. donovani* promastigotes cultured in TC-199 medium under aerobic conditions excrete the following final metabolites: principally succinate, alanine and acetate, and lesser amounts of pyruvate, glycine and D-lactate. Other studies of different *Leishmania* species, such as *L. pifanoi* [23] and *L. braziliensis* [6], indicate that besides CO_2 all three species produce succinate, acetate and alanine as major metabolites, while minor metabolites depend on the species in question. Rainey and Mackenzie (1991) [23] found that *L. pifanoi* excreted malate and citrate. Other authors [24, 25] did not detect this excretion, however, but did find pyruvate and, in cases such as *L. mexicana* promastigotes grown on a complex medium [26], considerable quantities of L-alanine and glycine. *L. major* also excretes alanine, succinate and acetate but instead of glycine small quantities of lactate are excreted [24]. Darling, Balber and Blum (1988) [27] found that the principal *Leishmania* species excreted D-lactate under anaerobic but not in aerobic conditions. As they themselves believe, it is possible that all the lactate formed is D-lactate, although they always tested for the production of L-lactate.

Like all these authors, we have found that succinate is the main metabolite excreted into the medium by the promastigote forms of *L. donovani.* This succinate may come from pyruvate via oxalacetate-malate-fumarate, from pyruvate via the tricarboxilic-acid cycle, or, as demonstrated for several species of *Leishmania,* via glyoxilate [28–30].

The second metabolite in quantity excreted by promastigote forms of *L. donovani* is alanine and this must come from pyruvate since it needs a source of nitrogen for its conversion. This confirms the viability of aminoacid catabolism in *Leishmania.*

Very little is known about the excretion of final metabolites of the amastigote forms of *Leishmania* [31]. Several authors think that in certain *Leishmania* species glycolysis is more important as far as energy is concerned in the promastigotes than in the amastigotes [26, 30]. This opinion is supported by the fact that under aerobic conditions promastigotes produce considerably more CO_2, and that the number of glycosomes per cell in promastigotes exceeds that in the amastigotes.

According to Darling and Blum (1987) [6] the amastigote forms appear to use less glucose and more fatty acids as energy substrates. The amastigote forms of *L. mexicana* excrete succinate, acetate and alanine [26] as final metabolites. Rainey and Mackenzie (1991) [23], have detected malate in addition to these metabolites. In our work we have found alanine, acetate, glycine, and in lesser quantities, succinate, pyruvate and D-lactate. Certain quantities of succinate and pyruvate appearing in the culture media of amastigote-like forms may be due to catabolites being produced during the transition between the promastigote and amastigote-like forms, rather than being produced by the actual amastigote-like forms. This suggests that the succinate-conversion metabolic path of the aflagellate form (via malate and fumarate) is less functional than in promastigote forms.

In conclusion, a sharp change in temperature of the *in vitro* cultures of *L. donovani* is one of the factors, through perhaps not the only one, involved in the transformation mechanisms of the promastigote forms and vice versa. The TC-199 medium supplemented with 30% (v/v) IFCS is an optimum medium to cultivate *in vitro* amastigote forms of *L. donovani.* These parasite forms obtained by heat shock are viable and are similar in appearance and antigenicity to amastigotes isolated from infected animals or obtained from the infection of macrophages. The biochemical strategies used by the parasite to obtain energy depend on the forms of its life cycle.

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