then only in colchicine-treated animals. This is in contrast to the findings with the tripeptide TRH, which fills the cell cytoplasm, dendrites, and axons and is densely concentrated in the external zone of the median eminence (*14*) and the interomediolateral column of the spinal cord (*16*).

We further demonstrated the distinct compartmentalization of immunostaining within neurons in the PVN by sequential immunochemistry (*16*), using antiserum 342 and then antiserum to TRH (*14*) in colchicine-treated animals fixed with acrolein (*17*). Evidence for the presence of the two antigens was seen in parvicellular neurons of the PVN, with antiserum 342 reacting with cytoplasmic components encircling the nucleus and antiserum to TRH delineating the entire cell soma and processes (Fig. 2).

Immunoreactivity in control sections was abolished by preincubating antiserum 342 with $10^{-6}M$ proTRH-SH (Fig. 1D) but not with $10^{-5}M$ synthetic TRH (Bachem). We prevented TRH immunostaining by pretreating TRH antiserum with $10^{-5}M$ synthetic TRH.

These results show that an antiserum (342) raised against a synthetic sequence of the frog-skin TRH precursor (proTRH) immunostains neurons in the rat brain that also contain the tripeptide TRH. Because of the distinct compartmentalization of immunostaining with antiserum 342 in the cell somata, highly suggestive of an association with the Golgi apparatus (*18*), we propose that this antibody reacts specifically with the rat proTRH. Further, since immunoreactivity is confined to the cell body and is not present in axon terminals as is the tripeptide TRH, it appears that processing of the proTRH does not occur during axonal transport as described for propressophysin, the precursor to vasopressin (*19*). Rather, the data indicate that the proTRH is rapidly converted to TRH within the neuronal perikaryon, perhaps even before leaving the Golgi apparatus, in a manner that may be similar to the processing of the enkephalin precursor (*20*).

The findings suggest that a TRH precursor analogous to that reported in amphibian skin is present in the rat brain and that TRH biosynthesis in the mammalian central nervous system occurs by post-translational cleavage of a precursor protein rather than by a nonribosomal enzymic process. The availability of an antibody that specifically recognizes the rat hypothalamic TRH precursor has recently enabled our group to elucidate its cDNA sequence, which encodes a protein with a molecular size close to 30,000 daltons (*21*). These developments confirm the validity of the prohormone antiserum and shed light on the factors involved in the regulation of TRH biosynthesis in the mammalian brain.

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Enhanced Metabolism of *Leishmania donovani* Amastigotes at Acid *p*H: An Adaptation for Intracellular Growth

Abstract. *Amastigotes (tissue forms) of* Leishmania donovani *isolated from infected hamster spleens carried out several physiological activities (respiration, catabolism of energy substrates, and incorporation of precursors into macromolecules) optimally at* p*H 4.0 to 5.5. All metabolic activities that were examined decreased sharply above the optimal* p*H. Promastigotes (culture forms), on the other hand, carried out the same metabolic activities optimally at or near neutral* p*H. This adaptation to an acid environment may account in part for the unusual ability of amastigotes to survive and multiply within the acidic environment of the phagolysosomes in vivo.*

ANTONY J. MUKKADA
JOHN C. MEADE
THERESA A. GLASER
Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221
PETER F. BONVENTRE
Department of Microbiology and Molecular Genetics, University of Cincinnati, Cincinnati, Ohio 45267

Leishmania donovani, the agent of visceral leishmaniasis, is a dimorphic protozoan parasite. In tissues of infected hosts, amastigote forms of the organism establish residence within macrophages of reticuloendothelial organs by phagocytic engulfment. Macrophages of human and animal reservoirs serve as obligatory host cells that permit the survival and intracellular multiplication of leishmania amastigotes. *Leishmania donovani* and other leishmanial species do not inhibit phagosome-lysosome fusion after ingestion by phagocytic cells (*1–5*), as do several other intracellular pathogens (*6–8*). Thus leishmanias have mechanisms that permit survival, growth, and cell division in an environment that is inimical to most life forms. Unlike the rickettsiae (*9*) and *Trypanosoma cruzi* (*10*), both of which escape from phagocytic vesicles into the cell

cytoplasm before extensive fusion with lysosomes occurs, leishmanias appear to have evolved means for survival in the presence of hydrolytic enzymes and in the acid environment of the phagolysosome compartment.

The intraphagosomal environment of mammalian phagocytes becomes acidified after the ingestion of bacteria or other particles (*11–13*). In rat and human polymorphonuclear leukocytes, the *p*H within particle-containing vacuoles is between 4.0 and 5.0 (*14, 15*). Similarly, the *p*H of murine peritoneal macrophages is 4.5 (*16*).

The precise mechanisms that permit survival and growth of *L. donovani* in an acid environment are of medical as well as biological interest. One possible explanation is that amastigotes may have become adapted for life in an acid environment because their metabolic functions are activated at high H^+ concentrations. Only one other human (intracellular) pathogen, *Coxiella burnetii*, shows a metabolic dependence on an acid environment (*17, 18*). In this study, evidence is presented suggesting that the tissue form of *L. donovani* also has adapted to life in an acid environment. The data show that catabolic and synthetic reactions of the amastigote form of the parasite proceed optimally in an acid environment. These metabolic reactions are markedly reduced at neutral *p*H.

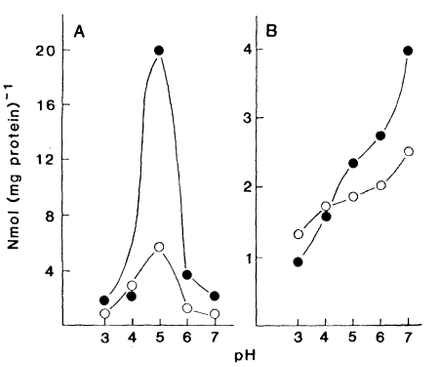

Fig. 1. Effect of *p*H on nucleoside incorporation in *L. donovani* amastigotes (A) and promastigotes (B): [^{3}H]thymidine (○--○) and [^{3}H]uridine (●--●). Cell suspensions in basal salts with 10 m*M* glucose as an energy source were dispensed into 25-ml Erlenmeyer flasks and incubated in a shaking water bath at 30°C for 10 minutes for temperature equilibration. [^{3}H]Thymidine or [^{3}H]uridine (New England Nuclear) were added at a final concentration of 0.4 m*M* and a specific radioactivity of 0.6 μCi/μmol. After incubation for 30 minutes, 1.0-ml samples were removed, filtered through glass-microfiber filters (1.2 μm porosity, Whatman GF/C), and immediately washed with 6 ml of cold 10 percent TCA. Filters with trapped TCA-insoluble material were rinsed with 2 ml of 95 percent ethanol, air-dried, and transferred to vials containing 10 ml of Biofluor scintillation fluid (New England Nuclear). The vials were agitated vigorously, and the amount of radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer (model 3003). The results are representative of four determinations.

Leishmania donovani amastigotes (Sudan strain 1S) were maintained by serial passage in female Syrian golden hamsters. Hamsters were killed 8 to 10 weeks after infection, and amastigotes from spleen homogenates were isolated (*19*). The isolation procedure recovers approximately 50 percent of the amastigotes originally present in the tissue homogenates. A typical preparation from two infected hamsters yielded approximately 2×10^9 to 5×10^9 amastigotes. These preparations were virtually free of host-cell contaminants, as judged by phase-contrast and electron microscopy (*19*). On the basis of acridine orange–ethidium bromide staining and their ability to transform to promastigotes, the amastigotes thus obtained were 85 to 95 percent viable. Promastigotes of *L. donovani* (Sudan strain 1S) were maintained at 26°C in medium 199 (Gibco) supplemented with 15 percent fetal bovine serum. The flagellated forms were harvested when cultures reached the middle of the exponential phase of growth (7×10^7 cells per milliliter) by centrifugation at 1100*g* for 10 minutes and washed twice in a basal salts solution composed of 5.2 g of NaCl, 0.5 g of KCl, and 10.3 g of Na_2HPO_4 dissolved in 1 liter of glass-distilled water and adjusted to *p*H 7.0 ± 0.1 (*20*). Both amastigotes and promastigotes were resuspended in the basal salts solution, which was adjusted to various *p*H's to a cell density corresponding to 0.35 mg of protein per milliliter for use in experiments. The *p*H of the reaction mixtures did not vary more than 0.1 to 0.15 *p*H unit during the course of the experiments. Both forms were fully viable after 2-hour incubations in the *p*H range used (*p*H 3 to 8); the amastigotes transformed to promastigotes when transferred to a suitable medium, and the promastigotes showed active motility and continued to divide and grow in the growth medium. Cell protein was determined by the method of Oyama and Eagle (*21*).

Incorporation of thymidine and uridine into trichloroacetic acid (TCA)–precipitable products in amastigotes occurred optimally at *p*H 5.0 (Fig. 1A). In contrast, nucleoside incorporation in promastigotes increased continuously from *p*H 3.0 to *p*H 7.0 (Fig. 1B). Amastigotes incorporated greater amounts of nucleosides, especially uridine, than promastigotes. This finding suggests that there is a higher rate of synthesis of nucleic acids, especially RNA, in amastigotes.

Catabolism of glucose and its incorporation into TCA-precipitable materials by amastigotes had a *p*H optimum at 4.0 to 5.0 with a sharp decline at higher and lower *p*H values (Fig. 2A). Incorporation of radioactivity from [^{14}C]glucose and CO_2 evolution from glucose in promastigotes followed the same pattern as in nucleoside incorporation, the levels being highest at *p*H 7.0 (Fig. 2B). The rate of glucose catabolism was considerably higher in promastigotes than in amastigotes. This is not surprising since the enzymes of the Embden-Meyerhof pathway were shown to be much more active in promastigotes than in amastigotes of *L. donovani* (*19*) and *Leishmania mexicana* (*22*). Proline metabolism in amastigotes, though more active than glucose

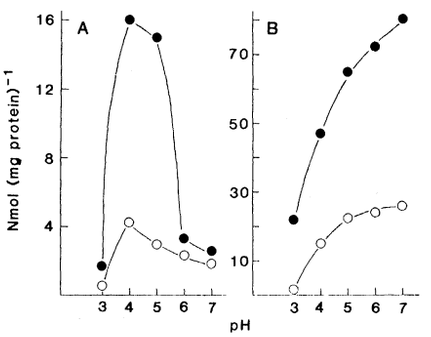

Fig. 2. Effect of *p*H on [^{14}C]glucose metabolism and incorporation in *L. donovani* amastigotes (A) and promastigotes (B). (●--●), CO_2 evolution; (○--○), incorporation into TCA-precipitable materials. Cells were prepared and equilibrated as described for nucleoside incorporation, except that the 10 m*M* glucose in the basal salts solutions was omitted. [U-^{14}C]D-glucose (New England Nuclear) was added at a final concentration of 0.4 m*M* and a specific radioactivity of 0.4 μCi/μmol. The flasks were immediately closed tightly with rubber stoppers, from which were suspended plastic cups containing fluted strips of filter paper (1 by 4 cm, Whatman 1) soaked with 0.2 ml of methylbenzethonium hydroxide (*35*) to trap the CO_2 evolved. Experiments were terminated after 2 hours by injection of 0.5 ml of cold 25 percent TCA through the rubber stoppers into the cell suspensions. The flasks were removed from the water bath and left undisturbed for 2 hours to allow absorption of all $^{14}CO_2$ into the filter papers. Cups containing trapped $^{14}CO_2$ were transferred directly to vials containing 10 ml of scintillation fluid and the radioactivity was determined. A 1-ml sample of TCA-extracted cell suspension was also removed from the flask, filtered through cellulose acetate filters (0.4 μm porosity, Millipore), and immediately washed with 5 ml of basal salts. Subsequent drying and radioactivity determination were identical to that for nucleoside incorporation. Residual radioactivity in the cells after extraction with cold TCA represents the extent of precursor incorporation into macromolecular material. The results are representative of four separate experiments.

metabolism, also showed a sharp peak at or near *p*H 5.0 (Fig. 3A). As expected, proline metabolism and incorporation in promastigotes were higher at or near neutrality (Fig. 3B). The rate of proline utilization in promastigotes was markedly higher than in amastigotes.

The effect of *p*H on respiration by amastigotes and promastigotes (Fig. 4) revealed a similar pattern. Oxygen uptake was highest at about *p*H 5.5 in amastigotes and at *p*H 7.0 in promastigotes. The values for respiration rates in *L. donovani* amastigotes and promastigotes were comparable to those reported for *L. mexicana* (*23*). In both species, the respiration rate of promastigotes was higher than that of amastigotes.

The extraordinary resistance of *L. donovani* to the killing mechanisms of macrophages is well documented (*24, 25*). How the amastigote form of this intracellular parasite manages to survive and to carry out its metabolic activities within macrophages successfully is unknown. Failure to evoke a significant oxidative burst upon ingestion by macrophages (*26*), possession of relatively high protease activity (*27*), a uniformly distributed acid phosphatase activity on its external membrane (*28*), and excretion of complex polysaccharides (*29*) have all been suggested as potential factors.

The data presented here demonstrate a potentially critical adaptation of *L. donovani* amastigotes, permitting growth in the phagolysosomal compartment of infected macrophages. Our results, based on the use of preparations of amastigotes purified from infected hamster spleens, suggest that this parasite has adapted to life in an acid environment. The data show that amastigotes carry out several important metabolic activities optimally between *p*H 4.0 and 5.5. Promastigotes, which are considerably less resistant to intracellular killing than amastigotes (*30, 31*), carry out these metabolic reactions best at or near neutral *p*H. Amastigotes use glucose and proline most efficiently at *p*H levels comparable to that within the phagolysosome. The same is true for the uptake and incorporation of nucleic acid precursors. The fact that these metabolic processes of amastigotes are markedly diminished in a neutral environment suggests that they are activated by H^+. Since several amastigote enzymes in cell-free extracts show *p*H optima at or near neutrality (*32*), the key to the *p*H dependence of intact amastigotes reported here probably resides at the level of membrane function.

Although it is generally accepted that the environment within phagolysosomes is acidic (*11–13*), the possibility exists that viable microbes may prevent acidification by production of basic substances within the membrane-bound compartment. Viable *Legionella pneumophila* prevent acidification of the phagosome (*13*) and inhibit fusion with lysosomes (*6*). In *Leishmania* sp., however, there is no evidence that amastigotes alter the acidity of their phagolysosomal environment. The principal excretory products of metabolism in the leishmanias are dicarboxylic acids such as succinate and oxaloacetate (*33*), which would only contribute further to the acidic microenvironment of the organisms in vivo. Furthermore, in a preliminary study Rivas and Chang (*34*) found that *L. mexicana* amastigotes do not measurably alter the low *p*H of the phagolysosomal compartment.

We found some variation in absolute values between different experiments

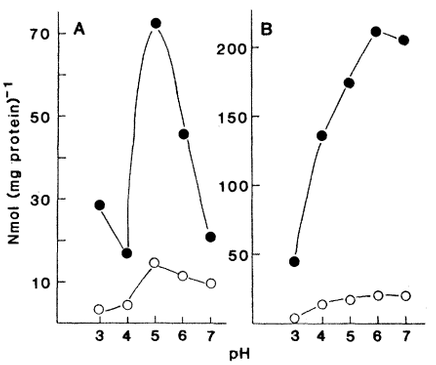

Fig. 3. Effect of *p*H on [^{14}C]proline metabolism and incorporation in *L. donovani* amastigotes (A) and promastigotes (B); (●--●), CO_2 evolution; (○--○), incorporation into TCA-insoluble materials. The experimental protocols were the same as for Fig. 2. [U-^{14}C]-proline was obtained from New England Nuclear. The results are representative of four separate determinations.

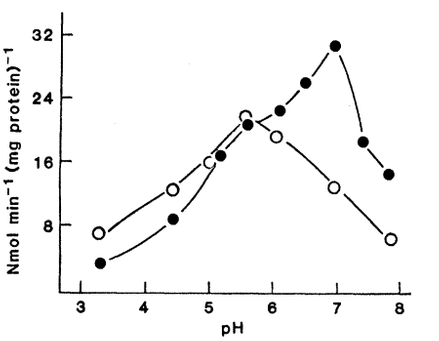

Fig. 4. Effect of *p*H on respiration in *L. donovani* amastigotes (○--○) and promastigotes (●--●). We measured oxygen uptake at 30°C in the presence of 10 m*M* succinate, using a polarographic technique with a Clark type oxygen electrode (Yellow Springs biological oxygen monitor, model 53) (*36*). The results are representative of three separate experiments.

with amastigotes. This may be due to the inherent difficulties associated with standardizing the physiological state of the amastigote preparations as a result of the diverse pathological states of the infected hamsters at the time they were killed and differences in parasite burdens. However, in multiple experiments of each type, all metabolic functions examined showed a peak in amastigotes at or around *p*H 4.0 to 5.5. It is noteworthy that intracellular organisms as phylogenetically diverse as the prokaryotic *C. burnetii* (*17, 18*) and the eukaryotic *L. donovani* have evolved similar mechanisms to ensure survival and growth in the mammalian cells they parasitize.

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