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Differential growth requirements of several *Leishmania* spp. in chemically defined culture media

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Summary

17 strains of *Leishmania* from 4 species: *brasiliensis, mexicana, donovani* and *garnhami* have been continually cultured at 26 °C, in the absence of proteins, in a medium containing salts, glucose, D-ribose, 2-deoxyribose, hemin, tricine, HEPES, 34 amino acids and intermediates of amino acid metabolism, 23 vitamins, 6 nucleotides and tetrahydrofolic acid. A wide variation in growth requirements was observed among leishmaniae which permitted the preparation of different minimum culture media for each *Leishmania* spp. Virulence of parasites was maintained after 30 passages in these chemically defined media. The requirements for differentiation to amastigotes also varied among the species as a function of the temperature of incubation and the protein content of the culture medium. Bovine serum albumin tryptic peptides substituted fetal bovine serum as growth factors at 30–34 °C.

Key words: *Leishmania;* protozoa, parasitic; hemoflagellate; nutritional requirements; temperature; promastigote; amastigote; chemically defined medium; minimum media; ultrastructure; metabolic diversity; growth promoting peptides.

Introduction

The majority of biochemical and immunological studies of different species and strains of *Leishmania* has been performed using culture media adapted from formulations used in the cultivation of mammalian cells in vitro. Most formulae contain amino acids, vitamins, heme and high concentrations of fetal bovine serum (Hendricks and Childs, 1979). New world *Leishmania* spp. have

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been cultured in insect cell culture media (Childs et al., 1978) and in peptone-yeast autolysate (Palomino, 1982), both containing high concentrations of fetal bovine serum; in medium 199 supplemented with high concentrations of water soluble vitamins, nucleotides and fetal bovine serum (Pan, 1984), and in monophasic high yielding liquid media (Al-Khateeb and Al-Azawi, 1981). Isolation of clones from *L. donovani* and *L. tropica* has been achieved in semi-solid agar containing commercially available culture media supplemented with fetal bovine serum or bovine serum albumin (Iovannisci and Ullman, 1982).

L. tarentolae has been cultured in a chemically defined medium; however, this medium can not support growth of other *Leishmania* (Trager, 1957). Some *L. donovani* isolates and *L. brasiliensis* have been cultured in defined media (Steiger and Steiger, 1976, 1977; Berens and Marr, 1978; Steiger and Black 1980); however, other *L. donovani* strains and other *Leishmania* species can not be cultured in these media. In order to get a high yield of antigenic material for the production of a vaccine, the cultivation of parasites in chemically defined media is important, since proteins from the culture medium have been found to be relevant contaminants associated with *Leishmania* membranes (O'Daly, 1979; Handman, 1982) as well as with cytoplasmic structures in other parasites (Bretaña and O'Daly, 1976) cultured in vitro.

In this paper we report the growth requirements of 5 subspecies of *Leishmania* and present minimum chemically defined media that support their continuous growth. Also differences in their potentiality for amastigote differentiation and degree of virulence for vertebrate hosts are presented.

Materials and Methods

Parasite strains: L. brasiliensis brasiliensis (Lbb: MHOM/VE/75/H-1, H-2 and H-4; MHOM/VE/75/H-12, H-14; MHOM/VE/80/H-24); *L. mexicana venezuelensis* (Lmv: MHOM/VE/80/H-16 and MHOM/VE/80/H-17); *L. mexicana amazonensis* (Lma: M1112, IFLA/BR/67/PH8, and MHOM/BR/73/M2269); and *L. donovani chagasi* (Ldch: MHOM/BR/74/PP75) were isolated from an endemic area in Barquisimeto, Lara State, Venezuela, by Dr. R. Bonfante-Garrido, who kindly gave them to us. The strains were also sent by Dr. Bonfante-Garrido to R. Lainson and J. J. Shaw of the "Wellcome Parasitology Unit" Belem, Para, Brazil and characterized by them, by isoenzymes, DNA hybridization and monoclonal antibodies as well as by other methods. The numbers in parentheses correspond to the International Reference Numbers Oswaldo Cruz Institute, International Reference Laboratory.

L. brasiliensis yaracuyensis (Lby), *L. mexicana pifanoi* (Lmp, Liverpool, N° LV96), *L. garnhami* (Lg, Scorza et al., 1979) and *L. donovani mediterranea* (Ldm, N° LUMP 1876), were donated by the "Instituto Nacional de Biomedicina", Caracas, Venezuela. Lmv, Lma, Lbb, Lby, Lmp, and Lg strains, were isolated from footpad nodules of infected hamsters, while Ldm and Ldch were obtained from the spleen of infected animals and cultured in enriched synthetic medium (ESM, O'Daly et al., 1987), containing 5% (v/v) fetal bovine serum (FBS, GIBCO). After the 3rd passage in this medium they were transferred to ESM without serum and maintained in this chemically defined medium (see below) by successive passages every week. The *L. mexicana mexicana* (Lmm, strain AZV donated by Dr. H. Pérez, IVIC) was transferred from blood agar medium to ESM containing 5% (v/v) FBS and afterwards to ESM free of protein supplements.

Preparation of culture media to study the growth requirements of Leishmania spp.

Using the enriched synthetic medium described for *Trypanosoma cruzi* growth (O'Daly et al., 1987), single component deletion experiments were undertaken. 64 culture media were prepared each based on a common salt solution (Table 2) and each containing all the substances presented in Table 1, except the one being considered as growth factor (Pirt, 1975). The complete medium was used as a control.

Each culture medium was sterilized through 0.22 μm Millipore filters, dispensed in 10 ml volumes into disposable flasks (Corning No. 25100) and incubated at 26°C with the parasites. The initial inoculum was 5×10^5 parasites/ml from the 10th passage in ESM culture medium. To minimize carry over of medium, parasites were washed 3 times in the basic salt solution (Table 2) by centrifugation at $900\times g$ for 15 min at 4°C. Samples (0.1 ml) were withdrawn every other day and parasites counted on a hemocytometer after diluting with phosphate buffered saline, PBS (0.01 M PO_4^-, 0.15 M NaCl, pH 7.2), containing 1% (v/v) formalin.

A component was considered not essential when growth in its absence was comparable to (i.e. at least 90% of) control values for more than 4 passages. All the experiments were repeated twice ($\simeq$2.700 growth curves) and population densities were determined until parasites reached the stationary phase of growth. All reagents were of the purest grade available from Sigma Chemicals. The maximum cell numbers obtained from the deletion experiment were expressed as percentages of the mean maximum number found in controls. Also, specific growth rates (u) during exponential growth phases were calculated from:

$$u = (\ln x_2 - \ln x_1) / (t_2 - t_1)$$

where x_1 and x_2 represent cell concentrations at times t_1 and t_2, respectively. Doubling times (td) were calculated from $td = \ln 2 / u$ (Pirt, 1975). Each value is the average of eight growth curves (2 experiments, 4 subpassages each). The standard deviations were less than 10% of the mean and are not shown for the sake of table simplicity.

Growth requirements of Lma and Ldm in chemically defined media were also compared with the requirements in semidefined protein containing media. Single deletion experiments were undertaken with the 64 culture media supplemented with 5% (v/v) FBS. Parasites were incubated at 26° and 30°C and processed as in the chemically defined media.

Preparation of minimum media for growth of Leishmania

Based on the results of Table 1, additional culture media were prepared for each *Leishmania* subspecies lacking all components considered non-essential (see: italicized substances, Table 1). (Minimum culture media which lacked substances, the individual absence of which decreased growth to 80% of control, could not sustain parasites.)

Preparation of culture media with serum substitutes for parasite growth

All *Leishmania* strains were cultured in ESM supplemented with: 1: *T. cruzi*-growth-promoting-peptide (O'Daly et al., 1987) at 50–100 μg/ml; 2: 2-mercaptoethanol (Taylor, 1974) at 4×10^{-3}–5×10^{-6} M; 3: bovine or human serum albumin at 100–1000 μg/ml; 4: bovine serum albumin tryptic peptides at 50–500 μg/ml.

To prepare the tryptic peptides, 500 mg of bovine serum albumin (BSA) (Cohn fraction V, Sigma) were dissolved in 0.1 M NH_4HCO_3, 0.1 mM $CaCl_2$ and digested with 1% (w/w) Diphenil carbamil chloride (DPCC) treated trypsin (Sigma Chemicals) previously dissolved in 0.1 mM HCl at 10 mg/ml. The mixture was incubated for 1 h at 37°C, keeping the pH at 8.2 with NH_4OH. The protein digest was then chromatographed in a G-50 Sephadex column (120×3 cm) equilibrated with 0.05 M NH_4HCO_3 at a rate of 40 ml/h. Three fractions were pooled, lyophilized and kept at –20°C until use. The growth promoting activity of each fraction was tested in ESM at a concentration of 100–500 μg/ml after calculating the protein content by the method of Lowry et al. (1951). All cultures were processed similarly to the chemically defined media, and incubated at 30° and 32°C. Neither protein-free ESM nor ESM containing *T. cruzi* growth-promoting-peptide supported parasite growth at 30°C. ESM supplemented with each one of 23 protein fractions isolated from FBS (see O'Daly,

1975), bovine, human serum albumin or 2-mercaptoethanol supported growth for 3–4 passages only, reaching densities of $6–7\times10^6$ cells/ml. The first pool of the BSA tryptic digest obtained after G-50 Sephadex, supported growth ($4–6\times10^7$ cells/ml) at 250 μg/ml of all strains at 30° and 32 °C for more than 10 passages (data not shown).

Test for virulence of culture parasites

Lby, Lmv and Lma parasites at the stationary phase of growth, cultured for more than 30 passages in ESM at 26 °C (promastigotes) and in ESM supplemented with 5% (v/v) FBS, at 30 °C or 32 °C (amastigotes) were washed 3 times in PBS. One $\times10^7$ parasites in 50 μl of PBS were injected into the footpad outbred male hamsters weighing 120–140 g. As control, the same amount of amastigotes from footpad nodules of infected animals were used. The increase in footpad diameter was measured with a Vernier every week and expressed as follows:

$$\frac{\text{infected footpad} - \text{normal footpad}}{\text{normal footpad}} \times 100$$

Each value is the average of 5 different animals. The standard deviation was below 10% of the mean in all cases.

Ultrastructural studies

Lmv, Lbb and Lby cultured in ESM at 26 °C and in ESM-FBS at 26° and 30 °C were centrifuged at $900\times g$ for 15 min at 4 °C, washed in ESM and resuspended in ESM containing 2.5% (v/v) glutaraldehyde for 2 h at 4 °C. Afterwards, they were washed 3 times in ESM at 4 °C by centrifuging at $900\times g$ for 15 min at 4 °C and post-fixed in 1% (v/v) osmium tetroxide in distilled water. Subsequently, they were washed twice in distilled water, embedded in 2% (w/v) agar dehydrated in graded ethanol (70%, 90%, 100%), then propylene oxide and finally embedded in Epon. Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome and double-stained in uranyl acetate 2% and lead citrate 2.6%.

To determine the percentages of different stages of *Leishmania* present in the course of cultivation, 0.1 ml aliquots were withdrawn from the falcon flasks at 2–3 day intervals. Smears prepared on slides were dried at room temperature and stained according to the May-Grunwald-Giemsa method.

Results

Fig. 1 shows the growth curves of the *Leishmania* spp. used. Parasite densities were equal or higher in ESM medium alone than in ESM-FBS at 26 °C, except for Lmm, Lg and Lmv. No growth was obtained above 26 °C without protein supplements. ESM containing 5% (v/v) FBS (or FBS-1, the excluded protein pool obtained after G-10 Sephadex chromatography, free of low molecular weight substances) (see: O'Daly et al., 1987), supported growth of all strains at 30 °C with parasites densities similar to the values observed at 26 °C. Hemin was essential in protein free ESM or ESM containing BSA tryptic peptides, but was unnecessary in ESM containing FBS or FBS-1 at any temperature, since all leishmaniae have been growing in its absence for more than 100 passages at densities similar to those in hemin containing medium.

Typical promastigotes and promastigotes with the kinetoplast located close to the nucleus (paramastigotes?) (Fig. 2) were observed in ESM, ESM-FBS and ESM-tryptic peptides in all strains at 26 °C and in Lmp and Lg at 30–34 °C.

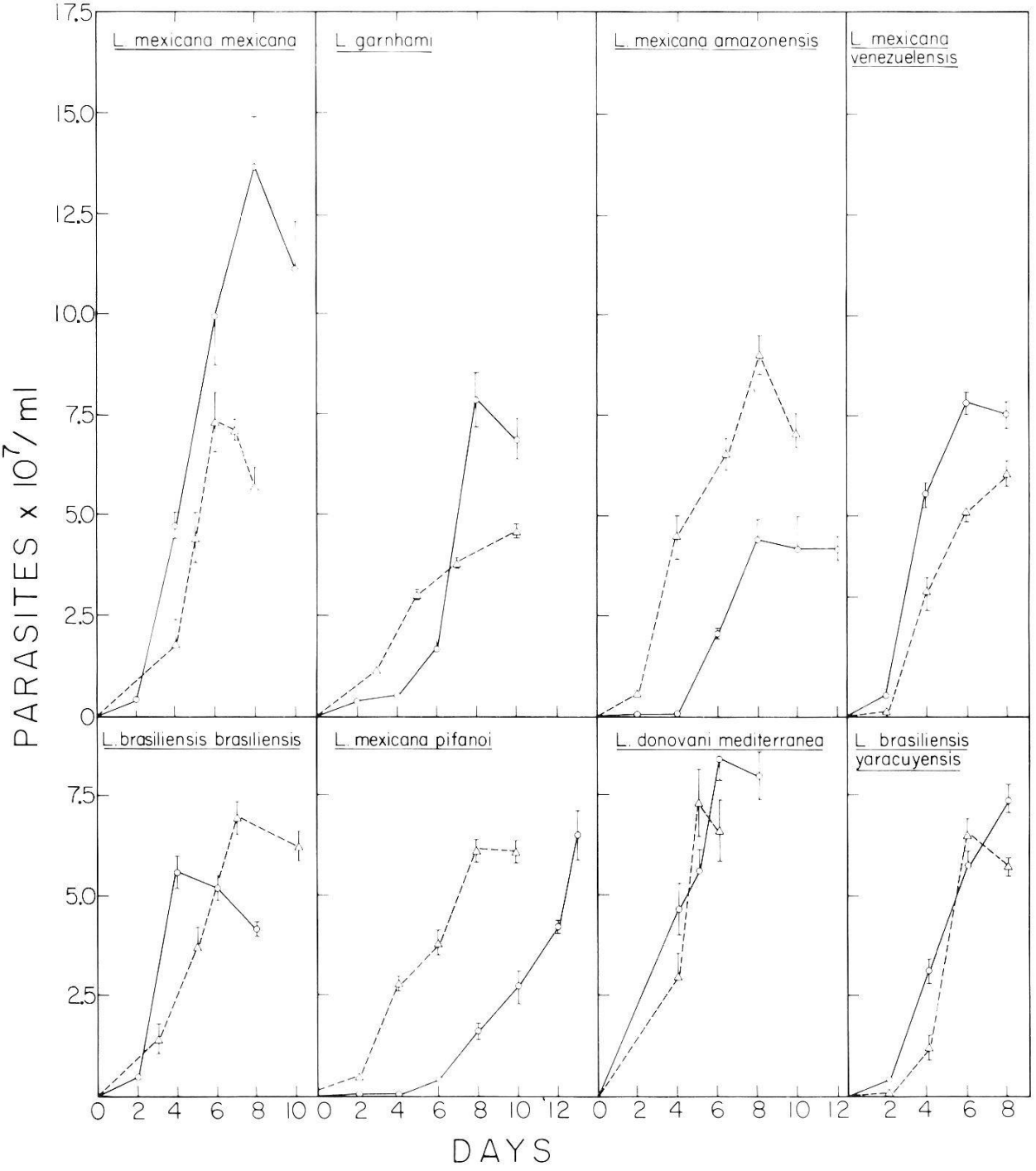

Fig. 1. Growth curves of several *Leishmania* species and subspecies in protein-free- ESM (△- - - -△) and in 5% (v/v) FBS-ESM (○——○) at 26 °C.

Lby, Lmv (H16 and H17) and Ldm transformed to 100% amastigotes in ESM-10% FBS at 30 °C and kept dividing as such in successive passages while Lbb (all strains), Ldch, Lmm and Lma (PH8, 1132) transformed at 32 °C. Lma (2269), Lmp, and Lg did not transform even at 34 °C the maximum temperature that permitted growth of these strains as promastigotes. Parasites failed to grow at temperatures above that necessary for promastigote to amastigote transformation. Transformation back to promastigotes was observed when amastigotes


ESM
26°
ESM-FBS
30°- 34°
Lmp
Lby
Lma
Lmv
Ldm
Lg

were cultured at temperatures lower than the temperature of amastigote transformation. BSA-tryptic peptides could substitute for FBS at 30–32°C and amastigotes kept dividing as such in successive passages. The strains that did not transform to amastigotes in ESM-FBS divided also as promastigotes in ESM-tryptic peptides.

All strains presented the same pattern of differentiation to amastigotes. After 24 h of incubation of Lbb, Lby or Lmv in ESM-FBS at 30°C, most parasites showed a densification of the membrane at the distal portion of the flagellar pocket, forming a constriction that clearly compressed the flagellum (Fig. 3a). The flagellar axoneme outside of the flagellar pocket is lost (Fig. 3b) and the flagellum within the pocket then convolutes or fragments (Figs. 3c, d and 4a). This process could be observed up to 72–96 h, after which many amastigotes in nuclear (Fig. 4b) and cytoplasmic division appeared (Fig. 4c) as well as amastigotes with flagella of more usual appearance in the flagellar pocket (Fig. 4d).

In chemically defined media all *Leishmania* strains required tryptophan, lysine, isoleucine, tyrosine, leucine, arginine, phenylalanine and valine as essential growth factors (0 growth in their absence), while taurine, β-alanine, sarcosine, phosphoethanolamine, proline, glutamic acid, glutamine, histidine, threonine and methionine were strictly required after a second passage. Riboflavin was an essential growth factor (0 growth) for all *Leishmania,* while E, K_3, niacinamide, ascorbic acid, myo-inositol, choline, panthotenic acid, tetrahydrofolic acid, 2′-deoxyadenosine-5′-monophosphate, 2′-deoxyguanosine-5′-monophosphate, and adenosine-5′-triphosphate were needed after a second passage. The remaining 15 vitamins and 16 amino acids, as well as the other nucleotides used showed variable results according to the species used for these studies. A gradual decrease in nutrient requirements was obtained from Lmp, and Lby going through Lma, up to Ldm and Lmv which are the strains that required the least amino acids, vitamins and nucleotides in the culture medium (Table 1).

Deletion of any of the compounds of Table 2 stopped growth in the first passage. The absence of Tricine and HEPES produced a marked drop in pH after 48 h of "in vitro" culturing at 26°C for all *Leishmania.*

Lma cultured in ESM containing FBS did not require cystine, sarcosine, glutamine, D_3, thymidine-5′-monophosphate and 2′-deoxyuridine-5′-monophosphate at 26°C and neither sarcosine, thioctic acid, U, D3, K1, and thymi-

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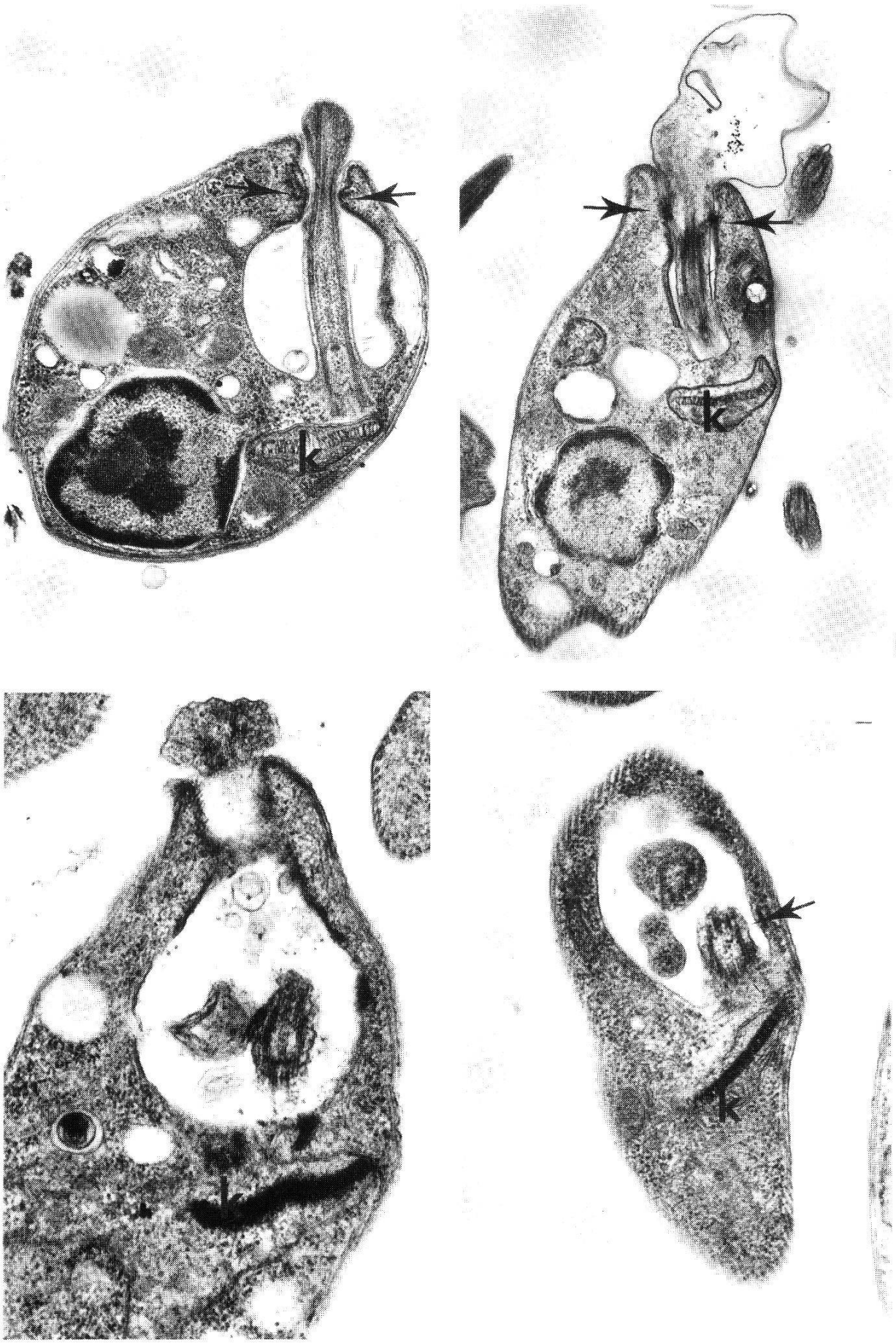
Fig. 2. Light microscopy of several *Leishmania* spp. in chemically synthetic medium (ESM) cultured at 26°C and in ESM containing fetal bovine serum (FBS) cultured: at 30°C for *L. brasiliensis yaracuyensis* (Lby), *L. mexicana venezuelensis* (Lmv) and *L. donovani mediterranea* (Ldm); at 32°C for *L. mexicana amazonensis* (Lma) (strain PH8), and at 34°C for *L. mexicana pifanoi* (Lmp) and *L. garnhami* (Lg). At 26°C in all strains and at 34°C in Lmp and Lg numerous promastigotes and promastigotes with kinetoplast close to the nucleus (paramastigotes?) are seen (arrows). Most strains transformed to amastigotes with the temperature rise except Lmp and Lg. ×1200.


k
k
k
k
A
B
C
D

dine-5′-monophosphate at 30°C. Ldm cultured in ESM containing FBS, could grow at 26°C without ornithine, citrulline, cysteine, glutamic acid, glutamine, thioctic acid, A, E, carnitine, K_1, and thymidine-5′-monophosphate; while at 30°C only A, U, E, and thymidine-5′-monophosphate could be taken out of the culture medium (data not shown).

Parasites cultured in ESM and ESM containing FBS were virulent after inoculation in hamster footpads. The nodular lesion appeared 12 weeks after infection in Lma and Lmv and ulcerated at the 20th week. Lby inoculated animals presented a slow lesion starting 16 weeks postinoculation which did not ulcerate up to the 30th week. The controls inoculated with amastigotes from infected hamsters showed a nodule at the 3rd week which ulcerated 5 weeks after infection. Abundant amastigotes were obtained from the footpad nodules of hamsters inoculated with cultured parasites.

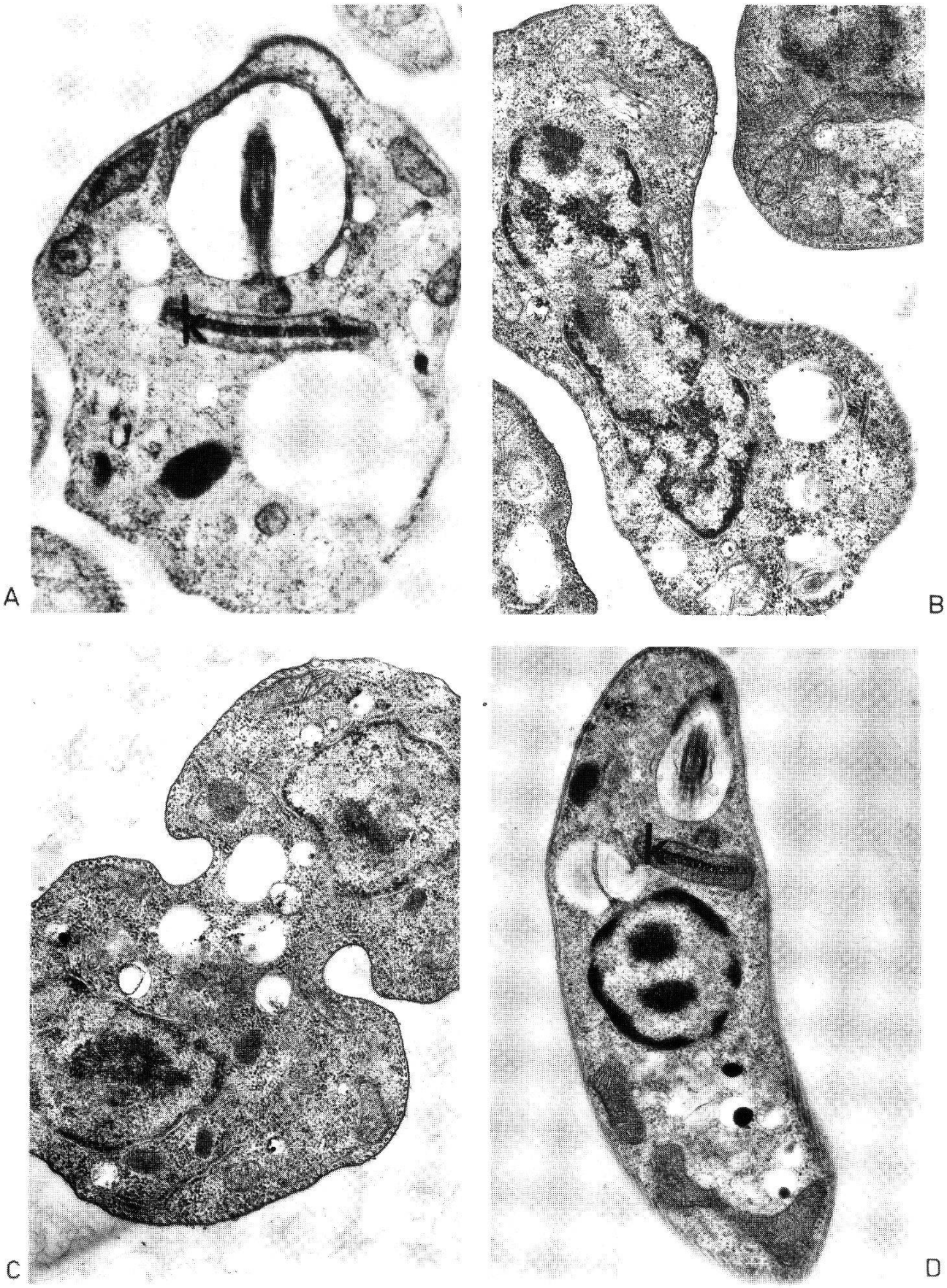
The parasite growth in minimum media confirmed the results obtained in Table 1, allowing the preparation of culture media for each subspecies which showed selectivity for proliferation of Leishmania. Lmp and Lby did not multiply in the less complex media made for the other species and Lma, Lmv, and Ldm either did not grow or proliferated for 1–3 passages only, in media different from their own. The growth of all *Leishmania* in Lmp medium is expected since it only lacks phosphoserine as compared with ESM, which could easily be substituted by serine. Each subspecies proliferated in its own minimum medium for more than 10 passages at $2–4\times10^7$ parasites/ml at 26°C. No growth was observed above this temperature.

Discussion

Continuous culturing of 17 strains of 7 subspecies: Lby, Lbb, Lmm, Lmv, Lmp, Ldm, and Ldch, of the 3 species: *brasiliensis, mexicana* and *donovani,* and also *L. garnhami* can be achieved in a chemically defined medium. In this medium all strains have been grown with passages every 7 days for the last 3 years at 26°C without any protein supplement.

Minimum culture media for each species and subspecies of *Leishmania* could be prepared with differences in nutrient composition. These varied from ESM in the absence of just 2 amino acids for the Lmp, up to 11 amino acids, 6 vitamins and 2 nucleotides for Lmv.

Fig. 3. Composite electronmicrograph of *L. mexicana venezuelensis.* A: Electron-dense material in the membrane of the distal portion of the flagellar pocket (arrows). The diameter of the flagellum decreases at the point of contact with the outer segment of the flagellar pocket. ×22,000. B: Electron-dense material (arrows) between flagellar membrane and membrane of outer segment of flagellar pocket. The tubular structure of the distal portion of flagellum is lost. ×12,450. C: The outer part of the flagellum is probably separated from the inner segment that seems to be fragmented inside the flagellar pocket. ×28,140. D: Absence of axoneme in flagellar fragments inside the flagellar pocket except the basal portion of the flagellum (arrow). ×19,140.


A
B
C
D

The classical studies of Rose et al. (1955), established that methionine, tryptophan, lysine, isoleucine, leucine, arginine, phenylalanine, valine, threonine, and histidine were essential amino acids for vertebrates. The carbon chains of arginine and threonine could be synthesized but at a very low rate to permit optimal growth. These amino acids were also found to be essential for mammalian cells cultured in vitro as also were cysteine, glutamine and tyrosine (Eagle, 1959). The former 10 amino acids were reported essential for a *L. brasiliensis*-like strain (Hopkins) and *L. donovani,* Sudan Strain 1S, by Steiger and Steiger (1977) as also were cystine, glutamine, proline and tyrosine (medium RE III) or these amino acids plus cysteine, glutamic acid and serine (medium RE I).

A surprising finding is the wide variation in growth requirements among leishmaniae. One group of 10 amino acids, belonging to several amino acid subgroups with common precursors and needed by all *Leishmania* after a first or second passage in defective media (threonine, methionine, tryptophan, lysine, isoleucine, tyrosine, leucine, arginine, phenylalanine and valine) are also essential for vertebrates and mammalian cells, which suggests that their biosynthetic pathways are blocked very early in eukaryotic evolution. The remaining 16 amino acids and amino acid precursors tested here change their role as nutritional requirements among the *Leishmania* populations. This suggests that different metabolic pathways are operational in the various species studied here and, therefore, a differential dependency exists on exogenous nutrients probably reflecting a high mutation rate in the *Leishmania* population.

In protein-free peptide containing media, *Trypanosoma cruzi* needs methionine, lysine, valine, isoleucine, tyrosine, tryptophan, arginine, serine, glycine, leucine and phenylalanine at all temperatures (O'Daly et al., 1987). These amino acids are also needed by leishmaniae in protein free ESM-medium except serine and glycine which are not required by some strains. In FBS containing media *T. cruzi* can grow without 18 amino acids at 26 °C and 7 amino acids at 37 °C (O'Daly et al., 1987), this was not the case for *L. mexicana* or *L. donovani,* grown in the same conditions. We have demonstrated that *T. cruzi* can incorporate proteins in its cytoplasm (Bretaña and O'Daly, 1976), which can act after degradation by proteinases, as a source of amino acids to the cytoplasmic free amino acid pool (O'Daly et al., 1983). Contrary to *T. cruzi, L. mexicana* or *L. donovani* do not show a major difference in growth requirements between protein free-ESM and ESM containing FBS, since only 3–4 of the amino acids are non-essential for parasites in FBS medium at 26 °C and all the amino acids become essential at 30 °C in both strains. We have never demonstrated gold-labelled fetal serum proteins in *Leishmania* cytoplasm

Fig. 4. Composite electronmicrograph of *L. mexicana venezuelensis.* A: Fragment of basal portion of flagellum inside flagellar pocket. ×27,000. B: Amastigote with abundant tubuli in nuclear matrix, evidence of nuclear division. ×17,000. C: Amastigote with abundant equatorial vesicles, evidence of cytoplasmic partition. ×17,000. D: Amastigote with basal portion of flagellum in flagellar pocket. ×14,300. K = kinetoplast.

Table 1a. Amino acid requirements of five *Leishmania* in chemically defined media at 26 °C

Growth without added amino acid, vitamin and nucleotide											
All amino acids except[a]		Lmp		Lby		Lma		Ldm		Lmv	
	mg/l	%[b]	td[c]	%	td	%	td	%	td	%	td
Anserine	17	*103*	20	*110*	19	*100*	20	*107*	21	*101*	25
Asparagine	165	16	25	71	20	77	16	*97*	16	*110*	27
Phosphoserine	23	*98*	28	76	22	*92*	20	*90*	21	*96*	36
Hydroxylysine	12	20	24	*91*	25	*100*	18	86	20	81	25
Alanine	512	57	30	*91*	19	75	19	*106*	17	88	22
Citrulline	50	55	45	79	21	*93*	20	*97*	17	77	28
Aspartic acid	120	44	48	79	23	81	18	*104*	22	*113*	23
α-Amino butyric acid	8	52	28	53	18	87	21	*93*	20	*93*	31
Hydroxyproline	263	84	25	73	25	86	19	*102*	22	*101*	20
Glycine	235	38	19	72	23	79	16	*95*	21	*93*	25
Ornithine	3	84	31	73	20	81	23	75	22	*97*	24
α-Amino adipic acid	3	67	21	47	24	85	18	*107*	17	*98*	21
Cystine	47	48	24	34	20	*109*	24	86	22	88	26
Carnosine	35	57	19	78	23	*91*	22	83	23	*94*	26
Serine	220	0	0	*92*	22	88	19	56	20	52	34
Cysteine	0.5	32	19	71	20	73	18	89	21	*90*	24
Taurine	6	27	34	74	22	76	23	86	20	78	36
β-Alanine	80	58	35	77	17	83	23	72	21	83	24
Sarcosine	57	37	22	74	19	61	22	75	22	77	23
Phosphoetanolamine	30	22	27	63	18	82	22	84	25	80	30

Table 1a (continued)

Growth without added amino acid, vitamin and nucleotide											
All amino acids except[a]		Lmp		Lby		Lma		Ldm		Lmv	
	mg/l	%[b]	td[c]	%	td	%	td	%	td	%	td
Proline	248	0	0	52	24	84	19	83	17	80	30
Glutamic acid	420	60	21	66	22	87	20	87	19	86	29
Glutamine	164	43	38	88	23	85	20	83	21	84	22
Histidine	6	0	0	73	19	71	22	77	20	82	40
Threonine	200	48	25	59	19	78	24	41	23	59	38
Methionine	140	17	45	0	0	41	22	37	33	58	44
Tryptophan	50	0	0	0	0	0	0	0	0	0	0
Lysine	337	0	0	0	0	0	0	0	0	0	0
Isoleucine	191	0	0	0	0	0	0	0	0	0	0
Tyrosine	210	0	0	0	0	0	0	0	0	0	0
Leucine	440	0	0	0	0	0	0	0	0	0	0
Arginine	413	0	0	0	0	0	0	0	0	0	0
Phenylalanine	240	0	0	0	0	0	0	0	0	0	0
Valine	266	0	0	0	0	0	0	0	0	0	0

The parasite densities and biomass doubling times in enriched synthetic medium (control) were: *L. mexicana pifanoi:* 66×10^6, 20 h; *L. brasiliensis yaracuyensis:* 60×10^6, 25 h; *L. mexicana amazonensis:* 90×10^6, 17 h; *L. mexicana venezuelensis:* 55×10^6, 25 h; *L. donovani mediterranea:* 57×10^6, 21 h. The minimal media lack the italicized substances.

[a] All the amino acids were added, except the one written out in the Table. [b] % with regard to control. [c] td = biomass doubling time in h.

Table 1b. Vitamin and nucleotide requirements of five *Leishmania* in chemically defined media at 26 °C

Growth without added amino acid, vitamin and nucleotide											
All vitamins and nucleotides except[a]		Lmp		Lby		Lma		Ldm		Lmv	
	mg/l	%	td	%	td	%	td	%	td	%	td
d-Biotin (H)	1	63	26	*92*	33	63	20	*102*	22	*99*	25
Ergocalciferol (D_2)	0.1	59	62	*91*	20	*97*	19	72	22	80	14
Pyridoxal · HCl	1	48	40	86	21	*98*	26	*108*	15	86	17
Folic acid	1	77	38	88	21	*93*	23	*100*	20	*94*	38
Cholecalciferol (D_3)	0.1	52	52	81	19	65	20	*107*	19	*104*	10
Thioctic acid	0.01	56	42	66	28	*97*	20	59	21	87	33
Retinol (A)	0.14	58	33	72	30	*93*	18	69	22	67	29
DL-Carnitine	0.05	47	33	64	21	*90*	22	*104*	24	81	31
Pyridoxamine · 2HCl	0.05	77	33	50	27	71	22	85	18	*97*	13
DL-methionine-5-methyl sulfonium chloride (U)	0.05	42	50	80	20	*93*	*18*	68	20	*91*	23
Thiamine (B_1)	1	46	26	66	19	70	26	95	28	89	37
p-Aminobenzoic acid	0.05	46	30	81	19	78	18	77	23	85	37
Pyridoxine (B_6)	0.025	22	29	88	16	70	20	73	22	*92*	20
3-phytylmenadione (K_1)	0.01	76	29	*99*	18	85	20	86	25	85	28
Cyanocobalamin (B_{12})	0.01	86	27	*108*	18	78	21	81	20	73	37
L-α-tocopherol (E)	0.01	75	23	67	26	87	23	62	28	77	29
Menadione (K_3)	0.01	79	52	75	20	88	23	70	22	87	27
Niacinamide	1	0	0	27	30	17	17	62	20	57	34

Table 1b (continued)

Growth without added amino acid, vitamin and nucleotide											
All vitamins and nucleotides except[a]		Lmp		Lby		Lma		Ldm		Lmv	
	mg/l	%	td	%	td	%	td	%	td	%	td
L-ascorbic acid (C)	0.05	42	34	81	18	74	22	84	21	73	32
Myo-inositol	2	57	40	80	24	81	17	74	19	79	27
Choline	1	80	35	38	22	80	25	82	19	89	13
Riboflavin (B_2)	0.1	0	0	0	0	0	0	0	0	0	0
D-Panthotenic acid	1	21	27	31	30	23	25	65	27	86	31
5-methyltetrahydrofolic · Mg (THF)	0.5	50	34	83	22	77	18	80	18	86	32
2′-deoxycitidine-5′-monophosphate (dCMP)	3.51	51	27	71	26	87	22	*92*	24	*103*	41
Thymidine-5′-monophosphate (TMP)	3.22	44	39	82	21	*92*	14	65	20	64	21
2′-deoxyuridine-5′-monophosphate (dUMP)	3.52	62	50	63	26	76	24	*91*	22	85	23
2′-deoxyadenosine-5′-monophosphate (dAMP)	3.31	47	26	63	22	75	21	81	21	77	31
2′-deoxyguanosine-5′-monophosphate (dGMP)	3.47	24	34	67	29	76	19	62	24	68	31
Adenosine-5′-triphosphate · Na_2 (ATP)	5.51	71	23	82	19	68	17	81	20	74	22

The parasite densities and biomass doubling times in enriched synthetic medium (control) were: *L. mexicana pifanoi:* 66×10^6, 20 h; *L. brasiliensis yaracuyensis:* 60×10^6, 25 h; *L. mexicana amazonensis:* 90×10^6, 17 h; *L. mexicana venezuelensis:* 55×10^6, 25 h; *L. donovani mediterranea:* 57×10^6, 21 h. The minimal media lack the italicized substances.

[a] All the vitamins and nucleotides were added, except the one written out in the Table.

Table 2. Salts and sugars present in enriched synthetic medium

	Conc. mg/l		Conc. mg/l
$CaCl_2$	200	Tricine	900
Fe (NO_3) $9H_2O$	0.72	Hemin*	1
KCl	400	HEPES	2,340
$MgSO_4$ $7H_2O$	200	Glucose	1,000
NaCl	6,800	D-ribose	10
$NaHCO_3$	2,200	2-deoxy-ribose	10
$NaH_2PO_4 \cdot H_2O$	140		

* Hemin was dissolved in 0.1 N NaOH, sterilized at 15 lb for 15 min and added aseptically.

(O'Daly, 1979) which probably explains the results obtained in ESM-FBS cultures and suggests that leishmaniae are unable to use culture medium proteins as a source of amino acids for the cytoplasmic free amino acid pool.

The need for protein molecules in *Leishmania* culture above 26 °C coincides with the appearance of amastigotes in most of the strains. The presence of low molecular weight substances in FBS that could be responsible for the induction of growth at 30 °C is discarded, since the FBS-1 pool, free of solutes (O'Daly et al., 1987) can induce growth and amastigote transformation as well as FBS. Also BSA tryptic peptides can substitute FBS and support growth of all strains as amastigotes or promastigotes, respectively. This suggests that fetal serum proteins or their peptides are playing a key role in the process of differentiation probably through membrane signals, and not as a source of nutrients for parasite growth.

The amastigote transformation is another variable among *Leishmania*, since Lby, Ldm and Lmv proliferated as amastigotes at 30 °C; Lmm, Lbb, Ldch and Lma (1132, PH8) at 32 °C while Lma (2269) Lmp and Lg did not transform at all. This suggests that vital information for the promastigote-amastigote transformation has been lost in some strains or that the requirements for transformation are different in the *Leishmania* spp. This variability is seen even in strains like PH8 and 1132 (amastigotes at 30 °C) and 2269 (promastigotes at 30–34 °C) belonging to the same subspecies (Lma) which also adds to the wide metabolic diversity within *Leishmania* found in this work.

The increase in growth requirements of Lma and Ldm in FBS containing ESM as the temperatures changed from 26 to 30 °C as well as the incapacity of some strains to grow above the temperature of amastigote transformation suggests the existence of a temperature sensitive enzyme as has been described in *T. brucei* (Cross and Manning, 1973) and *T. cruzi* (O'Daly et al., 1987).

The presence of proteins in the culture medium did not markedly change virulence of parasites as compared with the chemically defined medium although when compared with amastigotes from infected animals, the lesions in

both cases appeared later and the size increments of footpads were slower. Different strains presented variable degrees of virulence. The Lby, having more nutritional needs, presented the slower growing lesion which appeared 16 week after inoculation; while Lmv having less nutritional requirements presented the footpad lesion earlier, 7 weeks after infection.

It is noteworthy that immunization of hamsters with TLCK-killed amastigotes cultured with this procedures has induced protection against *Leishmania* infection (O'Daly and Cabrera, 1986).

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