Studies on the promastigote morphotypes of *Leishmania mexicana* and *L. panamensis*

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Abstract

The purpose of this study was to investigate the occurrence of different developmental forms of leishmania promastigotes during growth *in vitro* and attempt to correlate these with forms that occur in different localities in the gut of infected sandflies. One aim was to identify and characterise biochemical differences between the promastigotes and so provide insights into the adaptations of the promastigotes to the different environments encountered in the sandfly. The study was initiated with *L. panamensis* to enable investigation of the hindgut forms, stages which occur in only a few *Leishmania* species. A comparative approach was adopted later, however, and involved *L. mexicana*, a species which does not undergo hindgut development. Several promastigote forms were distinguished on morphological grounds and were investigated biochemically in order to ascertain stage-specific features. Since amastigotes of *L. mexicana* were readily available pure and in reasonable quantities, these were also included in my studies in an attempt to identify biochemical differences between the sandfly and mammalian stages of the parasite.

Three morphologically distinct promastigote forms were identified in *in vitro* cultures of *L. panamensis* and *L. mexicana*. These forms morphologically resembled promastigotes that occur at different localities in and at different times after infection of, the sandfly host. Morphotype 1 promastigotes, which are similar in appearance to those found in the blood meal, occur primarily in early-log phase cultures *in vitro*. Morphotype 2 promastigotes have longer and more slender cell bodies than the rounded morphotype 1 forms and occur in large numbers in mid- to late-log phase *in vitro* cultures. They appear to resemble multiplicative mid-gut forms of *L. mexicana*. *L.*

panamensis promastigotes undergo a different developmental route in the sandfly and multiply as spatulate-shaped cells in the hindgut rather than the midgut. The morphotype 2 forms of this species may be equivalent to these developmental forms. Stationary phase cultures are less homogeneous, in terms of cell morphology, and contain a mixture of morphotype 2 and morphotype 3 promastigotes. The latter appear to be the same as the infective metacyclic forms that are thought to be responsible for transmission of infection from the sandfly to a mammalian host.

The three promastigote morphotypes were found to differ metabolically. Morphotype 1 promastigotes, of both species, consumed larger quantities of amino acids during short term incubations in simple media than morphotypes 2 and 3. *L. panamensis* morphotype 2 stages consumed smaller quantities of some of the amino acids than stationary phase cultures containing morphotype 3 promastigotes. The opposite pattern was observed with *L. mexicana* promastigotes. All three promastigote morphotypes of *L. panamensis* released alanine during the incubation while morphotype 1 promastigotes of *L mexicana* consumed large quantities of this amino acid. In contrast morphotypes 2 and 3 of *L. mexicana* consumed and released negligable amounts of alanine. When each form was incubated with amino acids and glucose, amino acid utilisation differed only slightly from when amino acids were the sole exogenous substrates. The main difference with promastigotes of *L. panamensis* was that alanine release was greater when glucose was available. Morphotypes 2 and 3 produced alanine under both conditions but in larger quantities when glucose was present. *L. mexicana* promastigotes behaved differently. In the absence of glucose, alanine was consumed by morphotype 1 promastigotes but not by morphotypes 2 and 3, the concentration not changing during the incubations. When glucose was introduced alanine was still consumed by morphotype 1s, although to a much smaller extent. Morphotypes 2 and 3

switched to production. Changing the gas phase from air to 95% air/5% CO_2, N_2 or argon appeared not to affect the consumption and production profiles for promastigotes of either species. Amino acid consumption by purified lesion amastigotes of *L. mexicana* differed considerably from that by each promastigote morphotype and by axenically cultivated amastigotes. Furthermore, consumption and production profiles by axenic amastigotes of *L. mexicana* varied considerably in the presence and absence of glucose and with the pH of the incubation medium.

A number of enzymes central to energy metabolism were assayed in each promastigote morphotype of *L. mexicana*. The activities of several exhibited stage specificity, in that they were enhanced or reduced in the different promastigote forms. This suggests that there are significant differences in the energy metabolism of the various forms, reflecting adaptations for the conditions prevailing in the different parts of the sandfly gut and at different times after blood meals.

The stage specificity of protein synthesis in *L. panamensis* and *L. mexicana* promastigotes was analysed by labelling with ^{35}S-methionine. This revealed proteins that exhibited differential synthesis in the three forms of both species, some proteins being synthesised in greater amounts in one form than in others.

Western blot analyses using a variety of different antibodies identified differences in protein expression by the three morphotypes of *L. mexicana*. Antibodies raised against Type 1 cysteine proteinases (encoded by the cpb gene array) of *L. mexicana* recognised two proteins in morphotype 3 promastigotes and in lesion amastigotes which were not recognised in either of the other two promastigote morphotypes. In contrast, antibodies raised against the Type II cysteine proteinases of *L. mexicana* recognised two proteins in all three promastigote forms and in lesion amastigotes of *L. mexicana*, although quantitative differences were detected.

Amastigotes contained higher quantities of both proteins than any of the promastigote forms. Moreover, both proteins were most abundant in morphotype 3 promastigotes and least abundant in morphotype 1 promastigotes. These antibodies failed to detect proteins of equivalent molecular mass in any of the promastigote morphotypes of *L. panamensis* although a protein of slightly lower molecular mass was recognised in all three forms by the antiserum raised against the Type II cysteine proteinases of *L. mexicana*.

Polyclonal antisera raised in rabbits against each promastigote morphotype of *L. mexicana* recognised a similar but distinct array of molecules in all three morphotypes. The most prominant differences were in molecules of 45-66 kDa which were recognised by all three antisera and appeared to be differentially expressed in the different promastigote morphotypes. A larger number of bands in this size range, and in greater quantity, were detected in morphotype 3 promastigotes than in either of the other two forms. An intermediate quantity were present in morphotype 2 promastigotes. Molecules of the same size were also detected in morphotype 3 forms, but not in the others, when lysates were probed with partially stage-specific antisera which were generated from the anti-morphotype 3 antiserum.

Developmental differences in proteinase expressions were discovered. The promastigotes of *L. panamensis* were shown to contain multiple proteinases. These were analysed using gelatin SDS-PAGE. These enzymes were most active in glycine buffer pH 3·5 or borate buffer pH 8·5 and showed no stimulation with DTT. Interestingly one additional proteinase for which the pH optimum was not investigated was stimulated by DTT. The activities of the *L. panamensis* proteinases changed little during growth in neutral medium, however when promastigotes were grown in acidic Schneiders' medium, enzyme activity in late-log phase and stationary phase cells was

higher than cells in the equivalent growth phase but grown in neutral medium. These findings suggest that the proteinases are most active in metacyclic promastigotes. Partial characterisation of proteinases was attempted by use of specific proteinase inhibitors. All activities were inhibited by o-phenanthroline, a metallo-proteinase inhibitor. Serine proteinase and cysteine proteinase inhibitors had no apparent effect.

Approximately 13 enzyme activities were detected in promastigotes of *L. mexicana* by gelatin SDS-PAGE and there were some clear changes in activity of some of these enzymes during promastigote growth *in vitro*. The most obvious changes were in activities of enzymes between 20-24 kDa in size. All of these were least active in morphotype 1 promastigotes, and most active in morphotype 3s. Morphotype 2 proteinases exhibited intermediate activity.

Proteinase profiles of *L. mexicana* amastigotes differed from those of promastigotes. The proteinase of lowest molecular mass (approximately 21 kDa) was not detected in amastigotes. Almost all others, however, appeared more active than any of the promastigote forms. Infection of J774 macrophage-like cells allowed production of *L. panamensis* amastigotes. Analysis of these parasites using gelatin SDS-PAGE revealed only one proteinase band which was also detected in promastigotes.

Three nuclease activities were detected in promastigotes of *L. panamensis*. Only one of these appeared to undergo stage-regulation, being more active in morphotype 1 promastigotes than either of the other two forms. Investigation of *L. mexicana* promastigotes for stage-specific nucleases proved positive. The nuclease profiles differed in all three promastigote forms. Amastigotes and stationary phase promastigotes (mainly morphotype 3) of *L. mexicana* exhibited similar nuclease profiles, although the nucleases were at higher activity in amastigotes.

Infectivity of each promastigote form of *L. panamensis* and *L. mexicana* to peritoneal exudate cells was assayed. The numbers of infected macrophages and the average number of amastigotes per macrophage were compared for each promastigote morphotype. All three promastigote morphotypes of *L. panamensis* behaved similarly for both features analysed and approximately 20% of macrophages were infected with on average 4 amastigotes each. Infectivity of *L. mexicana* promastigotes to peritoneal exudate cells changed during growth *in vitro*. Morphotypes 1 and 3 infected approximately 70% of macrophages with on average 9 amastigotes. Morphotype 2 promastigotes infected almost 50% fewer macrophages and the mean number of amastigotes per macrophage was also reduced from 9 to 5.

Differentiation studies were conducted to investigate the stimuli responsible for transformation between the different promastigote forms. Heat-inactivated foetal calf serum (HIFCS) was found to contain a dialysable component which was essential for differentiation to morphotype 1 promastigotes. Moreover, increasing the amount of this component by increasing the quantity of HIFCS in the culture medium caused promastigotes to retain morphotype 1 morphology for longer before transforming to the next stage. Altering the starting density also altered the progression. Metacyclogenesis and de-differentiation of metacyclics could also be induced by variation of the culture conditions. Sub-passage into conditioned medium resulted in generation of metacyclics earlier than in control cultures while purified morphotype 3 promastigotes of *L. major* sub-passaged into fresh culture medium transformed into morphotype 1 forms. These results provided evidence that the different *in vitro* morphotypes can be induced by changing environmental conditions and that the developmental cycle is not one way.

The results of this study show that three promastigote populations of both species of *Leishmania* exhibit distinct biochemical characteristics. The data overall and

the changes in particular, support the contention that each promastigote form is adapted to a distinct microenvironment in the sandfly host.

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List of Abbreviations

AAM	Amino acid mix
AcCoA	Acetyl-CoA
AM	Amastigote
ATP	Adenosine triphosphate
BALB/c	In bred strain of mice
C	Carbon
Ca^{2+}	Calcium ions
Citr	Citrate
CO_2	Carbon dioxide
CP	Cysteine proteinase
DHAP	Dihydroxyacetone phosphate
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Electrochemiluminescence
E-64	Trans-epoxysuccinyl-$_L$-leucylamido-(4-guanidino)butane
FBP	Fructose 2,6-bisphosphate
F6P	Fructose 6-phosphate
G1	Gap period before DNA synthesis
G2	Post-synthetic gap period
GAP	Glyceraldehyde 3-phosphate
GDH	Glutamate dehydrogenase
G-3-P	Glycerol 3-phosphate
G6P	Glucose 6-phosphate
gp63	Glycoprotein 63 kilodaltons
GPI	Glycosyl-phosphatidylinositol
HCl	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonicacid]
HIFCS	Heat-inactivated foetal calf serum
HK	Hexokinase
hplc (HPLC)	High pressure/performance liquid chromatography
HRP	Horse radish perioxidase
ICDH	Isocitrate dehydrogenase
KCl	Potassium chloride
LDH	Lactate dehydrogenase
LPG	Lipophosphoglycan
Mal	Malate
MP	Metacyclic promastigote
$MgCl_2$	Magnesium chloride
MΦ	Macrophage
MT	Morphotype
NaCl	Sodium chloride
N_2	Nitrogen gas

OPT	Thiol o-phthaldialdehyde-thiol
Oxac	Oxaloacetate
O_2	Oxygen
P	Promastigote
PA	Paramastigote
PEP	Phosphoenolpyruvate
PBS	Phosphate buffered saline
PK	Pyruvate kinase
PMSF	Phenylmethylsulfonylfluoride
PNA	Peanut agglutinin
Poly(a)	Polyadenylic acid
Pyr	Pyruvate
RNA	Ribonucleic acid
S	DNA synthesis
SDM	Schneiders' Drosophila Medium
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGH	Salivary gland homogenate
SM1	Simple medium 1
SM2	Simple medium 2
SP	Sub-passage
Succ	Succinate
SuccCoA	Succinyl-CoA
TBS	Tris buffered saline
UV	Ultraviolet
Z-Phe-Ala-CHN_2	Diazomethane Z-phenylalanine-alanine-CHN_2
1,3BPGA	1,3-bisphosphoglycerate
2-PGA	2-phosphoglycerate
3-PGA	3-phosphoglycerate

Declaration

The results presented in this thesis are my own except where stated otherwise. Assistance with enzyme assays and organic acid hplc was provided by Dr. Helen Denton, while any procedures with live animals were carried out by Biological Services.

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CHAPTER 1

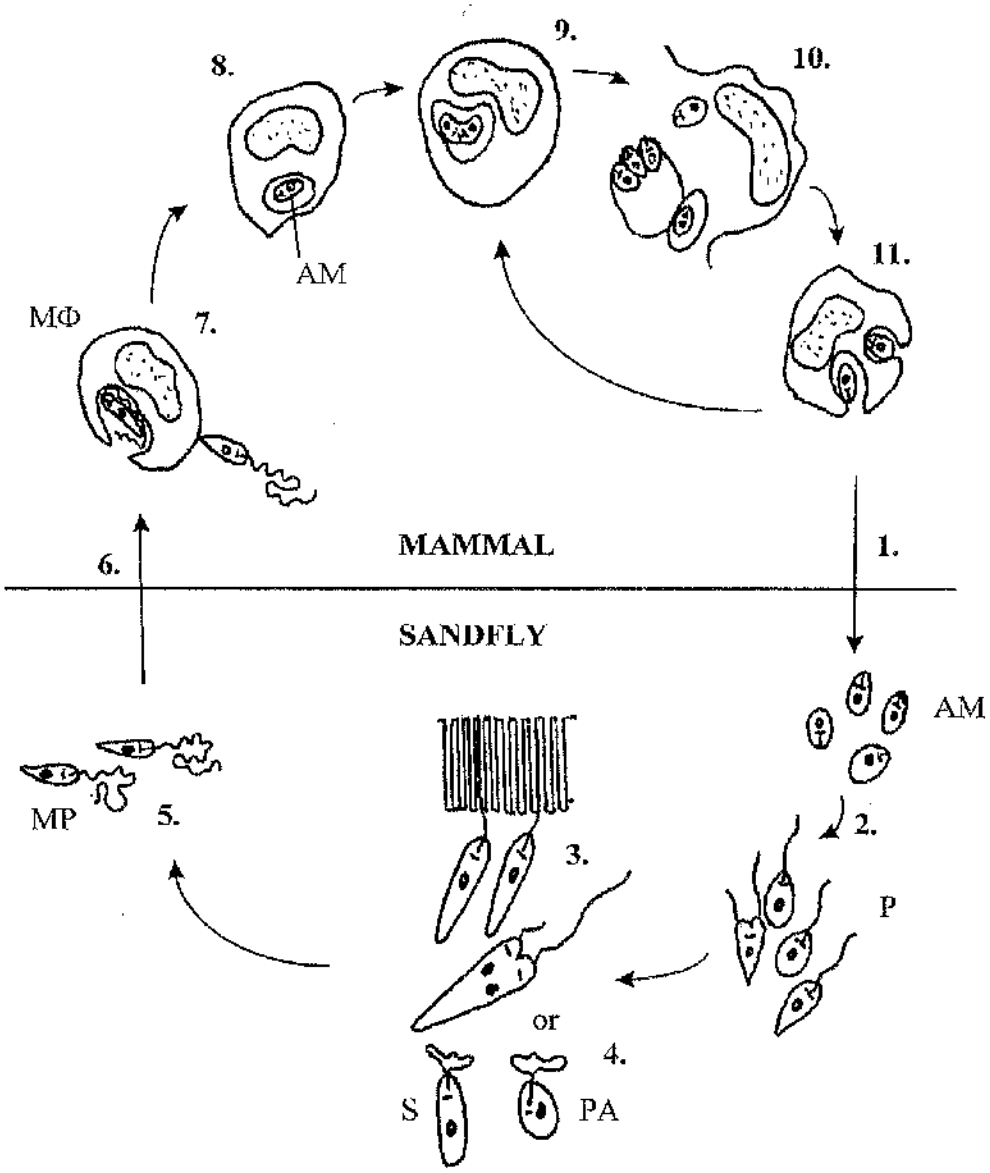
INTRODUCTION

1.1 Biology of *Leishmania mexicana and L. panamensis*

The leishmaniases are a group of diseases of vertebrates prevalent in tropical and neotropical areas of both the Old and New World which result from infection with protozoan parasites of the genus *Leishmania*. Clinically, these diseases range from single self-healing cutaneous lesions through grossly disfiguring cutaneous or mucocutaneous lesions to a more serious and commonly fatal, if untreated, visceral form of the disease [Marsden, 1984]. The disease type which results is dependent upon the infecting species, all of which are transmitted between vertebrate hosts by female sandflies. A schematic representation of the life-cycle, demonstrating the main parasite forms and their development in the vertebrate and invertebrate hosts is shown in Figure 1.1. The parasites responsible live and multiply as non-motile amastigotes within macrophages of the mammalian host's defence system and are transmitted between mammals by blood-sucking phlebotomine sandflies. After ingestion by a susceptible fly, transformation to flagellated promastigote stages occurs which undergo a period of multiplication and development within the sandfly midgut or hindgut, and then move anteriorly to the foregut where they occur as metacyclic promastigotes [Killick-Kendrick, 1979; 1986; Molyneux and Killick-Kendrick, 1987]. This third life-cycle stage is believed to represent the end-point of the life-cycle in the fly and is thought to be required for initiation of infection in a vertebrate host [Sacks and Perkins, 1984; 1985]. The life-cycle is completed when these metacyclics are injected into a susceptible vertebrate during blood feeding wherein they are taken up by macrophages

Figure 1.1: *Leishmania* life-cycle in the sandfly and in the mammalian host, a simplified scheme.

1. Infections in female sandflies are initiated when amastigotes (AM) are ingested during feeding on blood of an infected mammal; 2. differentiation to the promastigote (P) stage and subsequent multiplication within the blood meal which is encapsulated by the peritrophic membrane; 3. promastigotes attach to the midgut epithelium by insertion of their flagellum between the microvilli; 4. promastigotes migrate and attach to the hindgut by means of hemidesmosomal plaques which form between promastigote flagella and the cuticular lining (it is uncertain which parasite forms are found in this region, paramastigotes (PA) [see Molyneux and Killick-Kendrick, 1987] and spatulate-shaped promastigotes (S) [Walters, 1993] are shown here); 5. forward migration to the anterior midgut and foregut and development in these sites (not shown) which culminates in the generation of metacyclic promastigotes (MP), the end-point of development in the insect; 6. inoculation of metacyclics into the skin during blood feeding; 7. attachment and phagocytosis of metacyclics by a macrophage (MΦ); 8. differentiation of promastigotes into amastigotes within the phagolysosome; 9. multiplication of amastigotes; 10. rupture of heavily parasitized macrophage and release of amastigotes; 11. phagocytosis of amastigotes by a macrophage.


8.
9.
10.
11.
AM
МФ
7.
MAMMAL
1.
6.
SANDFLY
AM
2.
P
3.
or
4.
S
PA
MP
5.

prior to differentiation to the amastigote stage. This is a considerably simplified description of the life-cycle, particularly of the developmental stages in the invertebrate host.

Although all *Leishmania* species are morphologically similar, many species have been identified and classified [reviewed by Lainson and Shaw, 1987] through the application of clinical, biological and serological criteria [Schmidt and Roberts, 1989]. One such biological criterion involves the developmental cycle in the sandfly vector for which two distinct developmental patterns have been identified. Subsequent to excretion of the residual blood meal by the sandfly, released promastigotes generally follow one of two migratory routes. They either move posteriorly and attach to the hindgut or remain in the abdominal midgut where they attach to the epithelial lining. In both cases the infection subsequently proceeds with anterior migration of promastigotes to the cardia and foregut. The developmental route undertaken was found to be characteristic of the *Leishmania* species involved and was proposed as an adequate criterion for classification purposes [Lainson and Shaw, 1979; 1987]. The only mammalian infective leishmanias found to develop in the hindgut, a characteristic common to lizard leishmanias, are members of the *L. braziliensis* complex. All others are thought to undergo an equivalent period of development in the abdominal midgut prior to anterior migration. The distinct and consistant behavioural pattern reportedly adopted by promastigotes of the *L. braziliensis* complex warrented Lainson and Shaw to firstly isolate them in a non-taxonomic category, the Peripylaria [1979], which they subsequently upgraded to the sub-genus status of *Viannia* [1987]. Classification within this sub-genus required the life-cycle in the sandfly to "include a prolific and prolonged phase of development as rounded or stumpy paramastigotes and promastigotes attached to the wall of the hindgut (pylorus and/or ileum) by flagellar hemidesmosomes, with

later migration to the midgut and foregut". In parallel the group Suprapylaria and then the *Leishmania* sub-genus were created for those *Leishmania* species which were limited to the midgut and foregut of the alimentary tract (for the purpose of this thesis I will use the terminology devised in 1979 by Lainson and Shaw, that is Peripylaria and Suprapylaria). All mammalian leishmanias, other than the *L. braziliensis* complex, were at the time classified in the category Suprapylaria. However, as more host-parasite combinations are investigated it is becoming apparent that colonization of the hindgut may not be restricted to those species of *Leishmania* classified in the section Peripylaria. In several cases, flagellates of supposed suprapylarian species have been observed both free and attached to the hindgut [Schlein, 1986; Anez *et al.*, 1989; Walters *et al.*, 1993]. Free parasites may indicate expulsion of promastigotes from the sandfly gut. However, attached forms, such as those of *L. major* observed in association with the pylorus and ileum of 21% of *Lutzomyia longipalpis* [Walters *et al.*, 1993], are less easy to explain and should not be overlooked. It has been reported that the maintenance procedures of laboratory-reared sandflies could affect promastigote behaviour in the sandfly gut, specifically resulting in hindgut development by suprapylarian species [Anez *et al.*, 1989]. Both the holding temperature and sugar solution imbibed by sandflies were found to influence the migration and establishment of *L. amazonensis* in the gut of *Lu. youngi*. Specific combinations of these parameters resulted in atypical invasion of the hindgut by what were considered to be suprapylarian parasites. Another explanation for this unusual behaviour was proposed by Schlein [1986] who suggested that promastigotes adopt a hindgut locality to evade potentially harmful digestive enzymes which are secreted in the midgut. Clearly the developmental patterns defined by Lainson and Shaw [1979; 1987] are not inflexible and hindgut development may occur with a range of *Leishmania* species in a range of hosts, albeit some of them unnatural.

The reason why two different patterns of development occur in the sandfly is not yet fully understood, but it is thought that hindgut development represents a primitive characteristic, a remnant from when *Trypanosomatidae* were intestinal parasites of arthropods [Hoare, 1948; Adler, 1964; Baker, 1965]. As with modern day arthropod parasites, such as *Leptomonas,* it is likely that primitive leishmanial promastigote stages occupied the lumen of the insect gut, principally the posterior station with encysted amastigote-like forms released in the faeces. These infections may have passed to higher organisms, most likely to lizards, via predation. Infection of reptiles in this manner would initially require parasite survival in the alimentary tract prior to successful invasion of the blood and tissues. Evolution of forward migrating promastigotes to establish colonization of the anterior sandfly gut and allow transmission during feeding could have resulted in the infection of a number of mammalian hosts, those affected depending on the feeding habits of particular sandflies. Consequently, it has been proposed that promastigotes of suprapylarian species are evolutionarily more advanced than peripylarian species in that hindgut development is no longer necessary for the production of infective promastigotes.

The importance of the hindgut developmental phase to production of metacyclic promastigotes and subsequent transmission of *L. braziliensis* complex is unclear and yet to be confirmed. Indeed, reports by Johnson and Hertig [1970] and Walters *et al.* [1989a] following an investigation of *L. panamensis* infection suggested that hindgut development may not be essential to the establishment of infective forms of this parasite. Although primary movement of flagellates was posteriorly following peritrophic membrane breakdown, anterior migration to the cardia region of the midgut was also observed without any midgut developmental phase. The subsequent development of the promastigotes which exhibited forward migration without first

migrating to the hindgut was not mapped, thus it remains unclear whether colonization of the anterior alimentary canal of the sandfly can result from development of these cells alone. It was noted, however, that primary colonization of anterior sites was subsequent to the hindgut phase [Walters *et al.*, 1989a]. This discrepancy which challenges the efficacy of the classifications erected by Lainson and Shaw [1979; 1987] may be an artefact of unnatural holding conditions for sandflies and highlights the limitations of such experiments carried out in the laboratory. The results of similar studies should thus be interpreted with caution since experimental infections and maintenance of sandflies in the laboratory may not represent what occurs in a natural infection. Basic factors such as sandfly nutrition have yet to be fully elucidated, and yet are presumably highly influential to promastigotes developing in the gut.

Presuming that hindgut development is a naturally occurring phenomenon which is characteristic of promastigotes of the *L. braziliensis* complex, several hypotheses have been put forward as to its functional significance. The reason proposed by Walters *et al.* [1989a] to explain development in the hindgut was that it serves to hold promastigotes within the gut during expulsion of the blood meal and hence provides a reservoir of infection comparable to the midgut reservoir in suprapylarian species. This perhaps reflects differences in promastigote surface molecules or in receptors on the midgut lining which are important for attachment of suprapylarian promastigotes to the midgut epithelia. Another hypothesis is that promastigotes in the peripylaria grouping adopt a hindgut locality to 'escape' potentially harmful digestive proteinases [Schlein, 1986]. This, however, seems unlikely since comparison of maximum proteinase activity in blood sucking diptera [Akov, 1972; Hardy *et al.*, 1983] with the chronology of *L. panamensis* development in the sandfly [Walters *et al.*, 1989a] indicates that promastigotes are exposed to relatively high activities during development in the blood

meal. Thus the reason for hindgut development of *L. braziliensis* complex promastigotes remains unclear. More study is required if this phenomenon is to be understood and adaptation of growth conditions in order to produce the hindgut promastigotes *in vitro* would be a major help.

The two species which are the main subjects of this study represent examples of both types of promastigote development since *L. panamensis*, a member of the *L. braziliensis* complex, is a peripylarian species and *L. mexicana*, a member of the *L. mexicana* complex, is a suprapylarian species. The other human infective *Leishmania* species which are included in these complexes are listed in Table 1.1. The development of these species, in addition to many others, has been carefully followed in a number of sandfly vectors and such studies have identified that the life-cycle of mammalian leishmanias in the sandfly outlined above is a considerably simplified description of both the developmental route and of the morphological forms which occur at different sites along the sandfly gut [reviewed by Molyneux and Killick-Kendrick, 1987]. It is clear that promastigotes undergo an elaborate migratory route and sequence of transformations resulting in several different morphological forms which appear to congregate at different sites along the gut length. The reason for this and whether each form is biochemically distinct has yet to be confirmed, however, it is thought that the different morphological forms occur "in response to the differing physiological conditions in the various microhabitats within the alimentary tract of the sandfly" [Killick-Kendrick, 1979]. Interestingly, morphological changes can also be induced in promastigotes grown in culture simply by alteration of the osmotic potential of their surrounding medium [Darling and Blum, 1990]. Addition of water, glucose, fructose, mannose or proline caused promastigotes to round up, a morphological change which was accompanied by the rapid release of alanine and other ninhydrin-positive

L. braziliensis complex	*L. mexicana* complex
L. braziliensis	*L. mexicana*
L. panamensis	*L. amazonensis*
L. guyanensis	*L. garnhami*
L. peruviana	*L. pifanoi*
	L. venezuelensis

Table 1.1: Human infective *Leishmania* species which comprise the *L. braziliensis* and *L. mexicana* complexes, adapted from Walton [1987]. The sub-species were raised to species status in the taxonomic revision by Lainson and Shaw [1987], a classification alteration that has been gradually accepted by others. This system, one of dinomenclature, is used here.

substances [Darling *et al.*, 1990]. It is thought that this serves to prevent excess swelling and potential damage of promastigotes during their development in the sandfly gut. Presumably the conditions in the sandfly gut change as the blood meal is digested and excreted and as sugar meals are taken. Perhaps such changes result in the observed morphological changes *in situ* by modification of the osmotic potential of the gut environments. This has yet to be clarified but raises the important issue of how similar cultured promastigotes are to those in the sandfly. This is a question that has been raised previously, notably by Killick-Kendrick [1979] in his review of the biology of *Leishmania* in phlebotomine sandflies where he reported that the changes that occur in the fly "are not readily demonstrable in cultures". In contrast to this, several studies on promastigotes grown *in vitro* have identified an apparent developmental sequence of different morphological types [Christophers *et al.*, 1926; Bates, 1994; Charlab *et al.*, 1995]. How similar these forms are to those that occur in the fly, both morphologically and biochemically and whether each is biochemically distinct are questions which should be addressed. Also, do the morphological forms which occur in culture follow in the same chronological order as those *in vivo* and if so is this due to similarities between culture conditions and those in the sandfly gut or does it occur because of a genetically programmed sequence of morphological forms?

Promastigotes of *L. panamensis* and *L. mexicana* can be grown in culture and it may be possible by following such development to correlate the *in vitro* forms with those which occur *in vivo*. Moreover, comparison of these two species may reveal important differences which can be related to adaptations for development in the hindgut and midgut. Comprehensive studies of *in vivo* development of *L. mexicana* and *L. panamensis* in experimentally-infected laboratory reared sandflies, both natural and unnatural host-parasite combinations, have been carried out by light and electron

microscopy. These include *L. mexicana* differentiation in *Lu. abonnenci* [Walters *et al.*, 1987] (an unnatural host), *Lu. diabolica* (presumed natural vector) and *Lu. shannoni* (thought to be an unnatural host) [Lawyer *et al.*, 1987] and *L. panamensis* development in *Lu. gomezi* (natural host) [Walters *et al.*, 1989a] and *Phlebotomus papatasi* (unnatural host) [Walters *et al.*, 1992]. Three of these studies [Walters *et al.*, 1987; Walters *et al.*, 1989a; Walters *et al.*, 1992] have been summarized, in a review of *Leishmania* differentiation in the sandfly by Walters [1993]. This review also includes a paper on the differentiation of *L. chagasi* in *Lu. longipalpis* [Walters *et al.*, 1989b] which was only mentioned briefly here. In all four studies considered in this introductory section, several different promastigote types, based on morphology, were identified.

1.2 Life-cycles in the sandfly

1.2.1 The sandfly gut

A schematic representation of a sandfly in saggittal section is given in Figure 1.2. The nomenclature and anatomical divisions of the gut are the same as those of Gemetchu [1974] and Walters *et al.* [1987].

In brief, the alimentary canal is composed of three defined regions, namely the foregut, midgut and hindgut. Each region is then sub-divided further. The foregut is comprised of the proboscis, cibarium, pharynx, crop, oesophagus and the stomodeal valve. The midgut is sub-divided into two regions, the anterior or thoracic midgut and the posterior or abdominal midgut, and terminates at the pyloric valve. The region posterior to the pyloric valve constitutes the hindgut which includes the anterior and posterior intestines. The former of these regions is composed of the pylorus, ileum and colon and the latter the rectal sac which contains the rectal papillae and the rectum proper that leads to the anus. The pylorus, also called the hind-triangle in early

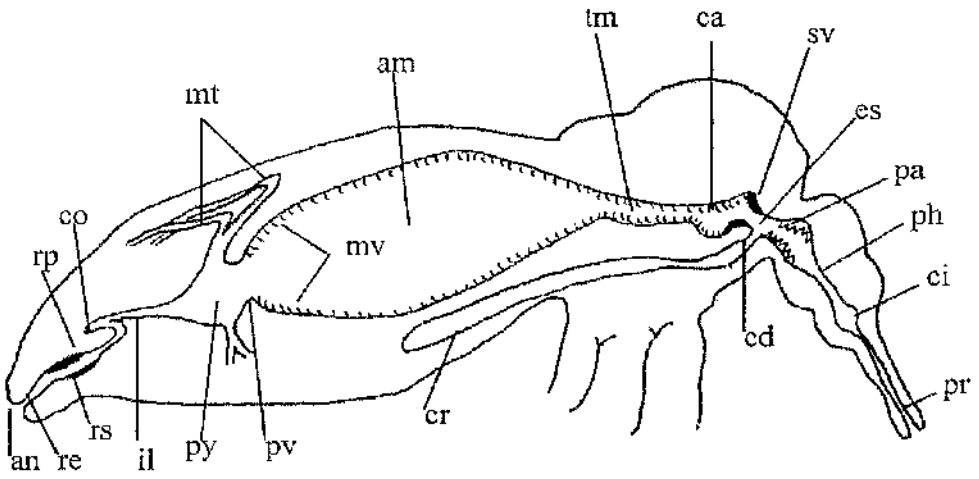


Figure 1.2: Schematic drawing of a sandfly in saggital section, modified from Walters *et al.*, 1987. The drawing shows the morphological and anatomical divisions of the gut, proboscis (pr), cibarium (ci), anterior pharynx (pa), oesophagus (es), crop duct (cd), crop (cr), stomodeal valve (sv), cardia (ca), thoracic midgut (tm), abdominal midgut (am), microvilli(mv), pyloric valve (pv), malpighian tubules (mt), pylorus or hind-triangle (py), ileum (il), colon (co), rectal sac (rs), rectal papillae (rp), rectum proper (re), anus (an).

literature [Hertig and McConnell, 1963; Johnson *et al.*, 1963], occurs directly after the pyloric valve and comprises the relatively spacious area in the anterior intestine into which the malpighian tubules empty.

The nature of the gut lining changes along its length. Both the foregut and hindgut are lined with cuticular intima while the midgut lining consists of epithelia cells with microvilli.

1.2.2 Promastigote migration and morphological forms of *L. panamensis* and *L. mexicana in vivo.*

It became clear from very early studies using light microscopy that promastigote development within the sandfly involved a complex migratory route which was paralleled by the sequential development of several different morphological forms which appeared to congregate at different sites along the gut length [Shortt, 1928; Adler and Theodor, 1931]. Subsequently, more detailed examinations revealed that this pattern of development was essentially conserved for mammalian infective *Leishmania* species but detected two different migratory routes which were characteristic of the species involved [reviewed by Killick-Kendrick, 1979 and Molyneux and Killick-Kendrick, 1987]. As discussed in section 1.1 development in the hindgut or midgut has become the basis for separation of those species which infect mammals into two groupings, the peripylaria and suprapylaria, respectively. Advances in identification of different forms of *Leishmania* in the sandfly gut have necessitated updating of the terminology used to accommodate all of the different forms.

1.2.2.1 Terminology

Walters proposed a novel system of nomenclature in 1993 (Table 1.2) based on her observations of a number of *Leishmania* species in different sandfly hosts. In this review she challenges the terms nectomonad and haptomonad which were resurrected by Killick-Kendrick *et al.* in 1974 to describe the morphology of distinct promastigote forms found in *L. amazonensis* infected *Lu. longipalpis*. Instead she ascribes these terms to each form identified in order to differentiate between whether they are attached (haptomonad) or free-swimming (nectomonad). This nomenclature is used during the following discussion.

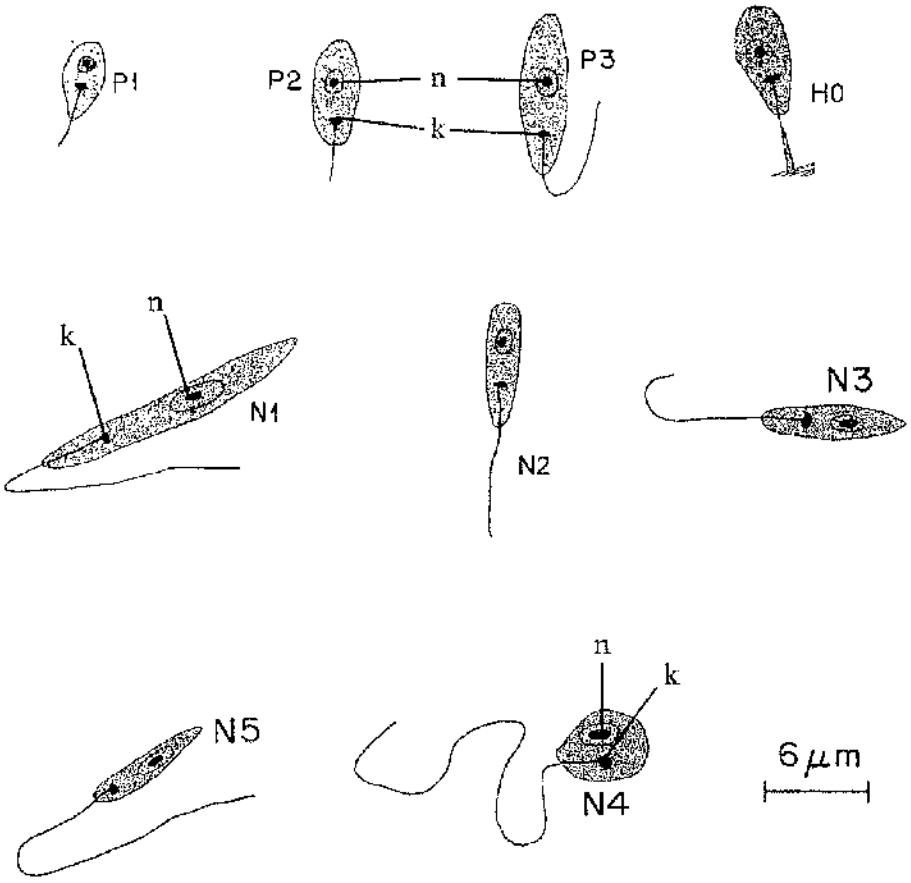
In Walter's review a total of 14 *Leishmania* forms were identified along the gut length of infected sandflies. These are listed in Table 1.2, modified from Walters [1993], and include the ingested amastigote stage (AM). Schematic drawings of the morphologically distinct promastigote forms are shown in Figure 1.3. The first three promastigote terms, P1, P2 and P3, were erected to describe the initial promastigote stages which occur in the blood meal. These forms have attracted little interest but have been identified in a number of studies where they are usually referred to as procyclics. Morphologically, these promastigotes are stumpy in appearance having small rounded cell bodies and underdeveloped flagella. A total of 5 different unattached forms were identified in the gut lumen; N1-N5. The prefix denotes the unattached nectomonad phase. Four of these were also found to have an attached or haptomonad phase; H1-H4.

Promastigotes [Woodcock, 1914; Hoare and Wallace, 1966; Wallace, 1977] and paramastigotes [Janovy *et al.*, 1974] conform to definitions previously designated, according to the position of the kinetoplast relative to the nucleus. In promastigotes the kinetoplast is anterior to the nucleus whereas in paramastigotes the kinetoplast is lateral to the nucleus. Both promastigotes and paramastigotes have been identified in the

Table 1.2: *Leishmania* forms in the sandfly gut and their developmental sites as designated by Walters [1993]. ✓ and ✗ indicate positive and negative identification in that developmental site, respectively, while ?, signifies a developmental site which requires clarification.

NAME		LOCATION IN THE GUT				
		Blood meal	Midgut	Hindgut	Foregut	Stomodeal Valve
AM	Amastigote	✓	✗	✗	✗	✗
P1	First stumpy promastigote	✓	✗	✗	✗	✗
P2	Second stumpy promastigote	✓	✗	✗	✗	✗
P3	Third stumpy promastigote	✓	✗	✗	✗	✗
Unattached nectomonads						
N1	Elongate nectomonad promastigote	✓	✗	✓	✗	✗
N2	Spatulate nectomonad promastigote	✓	✗	✓	✗	✗
N3	Short nectomonad promastigote	✗	✓	✗	?	✗
N4	Nectomonad paramastigote	✗	✓	✗	?	✗
N5	Metacyclic nectomonad promastigote	✗	✓	✗	✓	✗
Attached haptomonads						
H0	Pear-shaped haptomonad promastigote	✗	✗	✓	✓	✗
H1	Elongate haptomonad promastigote	✗	✓	✗	✗	?
H2	Spatulate haptomonad promastigote	✗	✗	✗	✗	✗
H3	Short haptomonad promastigote	✗	✗	✗	✗	✓
H4	Haptomonad paramastigote	✗	✗	✓	✓	✗

Figure 1.3: Schematic drawing of the morphologically different promastigote forms which occur in the alimentary tract of an infected sandfly vector. These drawings were taken from Walters [1993] and represent a summary of the promastigotes which were identified by this author and her colleagues in a number of different *Leishmania*-sandfly combinations; P1, P2, P3 are stumpy promastigotes in the blood meal, N1 is an unattached elongate promastigote commonly found towards the end of blood meal digestion and subsequently in the abdominal midgut of suplapylarian infections, N2 represents spatulate-shaped promastigotes, unique to peripylarian species, which predominate late blood meals and the hindgut, N3 are short promastigotes commonly found in the cardia and foregut, N4 are paramastigotes and also occur in the cardia, N5 represents the metacyclic promastigote of suprapylarian *Leishmania* species, H0 is a pear-shaped attached promastigote identified in the foregut. The prefix N and H distinguish between unattached and attached forms, respectively. N2, N3 and N4 also occur as attached forms (H2, H3 and H4) showing modified flagella similar to H0. The nucleus (n) and kinetoplast (k) of some cells are labelled.


P1
P2
n
k
P3
H0
k
n
N1
N2
N3
N5
n
k
N4
6 μm

sandfly intestine with various cell body shapes, a feature which has proven useful for the classification of different promastigote types. Spatulate-shaped promastigotes are broader at the posterior end and more slender at the anterior end than elongate, short and metacyclic promastigotes which are all spindle shaped. Paramastigotes can be rounded, oval or pear-shaped.

One additional haptomonad form was found for which no nectomonad phase was identified. These promastigotes were pear-shaped (H0) and were thought to be equivalent to the "haptomonad" promastigotes found in a variety of studies of different *Leishmania* species in the sandfly [Killick-Kendrick *et al.*, 1974; Killick-Kendrick *et al.*, 1977; Killick-Kendrick, 1979; Killick-Kendrick *et al.*, 1979; Molyneux and Killick-Kendrick, 1987; Killick-Kendrick *et al.*, 1988].

1.2.2.2 Differentiation to the promastigote stage and its development in the blood meal

Infections in susceptible female phlebotomine sandflies are initiated when amastigote stages (AM) are ingested during blood feeding on an infected vertebrate. The infected blood is taken directly into the midgut where it becomes encapsulated by the peritrophic membrane, a chitin lattice embedded in a protein-carbohydrate matrix which is excreted by the midgut epithelia [Gemetchu, 1974]. It is within this sac-like structure that blood digestion takes place, coincident with amastigote differentiation to the extracellular flagellated promastigote stage. The preliminary events of this differentiation are a matter for debate and have yet to be fully elucidated for all species of *Leishmania*. However, it has become evident that the behaviour is not only parasite species-dependent but is also determined by the species of sandfly and the combination of *Leishmania* species and invertebrate host. In some cases, *L. mexicana* in a number of

sandfly hosts [Strangeways-Dixon and Lainson, 1966] and *L. panamensis* in a natural [Walters *et al.*, 1989a] and unnatural [Walters *et al.*, 1992] vector, the preliminary division sequence took place during transformation with amastigotes undergoing morphological changes prior to division of aflagellated intermediates. Differentiation of *L. mexicana* occuring in unison with division was also observed *in vitro* [Hart *et al.*, 1981b; Bates, 1994]. In both instances, cells were found in the process of division before the morphological or biochemical changes were completed and it was concluded that division was an obligatory step in transformation. Contradictory results were reported from other studies. With some species, *L. donovani* in *P. argentipes* [Shortt, 1928] and *L. chagasi* in *Lu. longipalpis* [Lainson and Shaw, 1988], differentiation was not evident until amastigotes had undergone at least one division. In addition, the strain of parasite may also be an important determinant of the initial behavioural pattern in the fly. Evidence that parasite strain is influential to the division and differentiation of amastigote forms ingested by the sandfly was attained by comparison of two different strains of *L. chagasi* in *Lu. longipalpis*. Lainson and Shaw [1988] observed division prior to differentiation while Walters *et al.* [1989b] noted that division occurred only after initial differentiation of amastigotes. Thus it appears that the preliminary events differ depending upon the exact nature of the parasite and host.

Early promastigote forms produced from differentiation of amastigotes have small rounded cell bodies and short flagella (less than the body length) giving them a stumpy appearance. Some authors choose to call these stages procyclics [for example, Lawyer *et al.*, 1990], a term used previously to describe those stages of some *Trypanosoma* which divide in the midgut of the insect vector. As development proceeds the stumpy promastigotes become progressively larger such that three distinct types were identified (P1, P2 and P3). The behaviour of these stages within the blood

meal has been followed in a few studies. It appears that they concentrate at the periphery of the blood meal, adjacent to the peritrophic membrane [Walters *et al.*, 1987; Walters *et al.*, 1989a], a digestive interface which is likely to contain many possible substrates including carbohydrates, amino acids and lipids. Presumably the highly nutritious nature of their surroundings is at least partially responsible for the prominant multiplicative nature of these forms. In all four studies reviewed by Walters [1993], the stumpy promastigotes were reported to exist in pairs or in rosettes, the cells attached via their flagella.

Differentiation of stumpy promastigotes into promastigotes with a more elongate cell body shape generally occurred subsequent to multiplication of stumpy promastigotes but prior to breakdown of the peritrophic membrane. *L. mexicana* promastigotes in *Lu. shannoni* and *Lu. diabolica* were described as "long, slender, highly motile promastigotes" of the size 8-10 μm long and 2 μm wide (N1) [Lawyer *et al.*, 1987]. Non-dividing promastigotes of two different morphological types were described in the blood meal of *Lu. gomezi* infected with *L. panamensis*, prior to membrane breakdown; short spatulate-shaped promastigotes (N2) (5-8 μm body length) and longer 'nectomonad' promastigotes (N1) (13-19 μm body length) [Walters *et al.*, 1989a]. Although of dissimilar body length and posterior shape, these two promastigote types were similar in respect to width, relative kinetoplast-nuclear distance and relative length of the free flagellum.

1.2.2.3 Migration and promastigote morphology directly after release from the peritrophic membrane

Migration of promastigotes, the developmental site occupied and promastigote morphology and behaviour at this site following breakdown of the peritrophic

membrane has been proposed as an adequate method of differentiating between *L. braziliensis* complex and *L. mexicana* complex infections [Lainson *et al.*, 1977]. As discussed in section 1.1, *L. braziliensis* migrate to the hindgut while *L. mexicana* remain in the abdominal midgut.

1.2.2.3.1 Development in the hindgut

Colonization and multiplication of flagellates in the hindgut of the sandfly vector has been considered as an important, if not essential, part of the life-cycle of parasites of the *L. braziliensis* complex [Killick-Kendrick *et al.*, 1977; Lainson *et al.*, 1977; Lainson *et al.*, 1979]. Therefore it was not surprising that hindgut development was a feature of *L. panamensis* in both a natural (*Lu. gomezi*) [Walters *et al.*, 1989a] and an unnatural (*P. papatasi*) [Walters *et al.*, 1992] sandfly host. A schematic representation of this development, taken from Walters 1993, is depicted in Figure 1.4. However, the morphology of the forms found in this site and their apparant non-dividing state (only very few dividing forms were identified) in these two studies are inconsistant with the classification criteria proposed by Lainson and Shaw [1987]. Moreover, on two separate occasions colonization of both the hindgut and the cardia occurred concurrently, suggesting that hindgut development by flagellates may not be essential to the generation of the infective forms responsible for disease transmission via sandfly bite [Johnson and Hertig, 1970; Walters *et al.*, 1989a].

Leishmania species of the sub-genera *Viannia*, by definition, develop and multiply in the sandfly hindgut as rounded or stumpy paramastigotes or promastigotes attached to the gut wall by hemidesmosomes [Lainson and Shaw, 1987]. In accordance with this definition "short broad" or "round to oval" flagellates were observed by light microscopy in early studies of parasites of the *L. braziliensis* complex in the sandfly

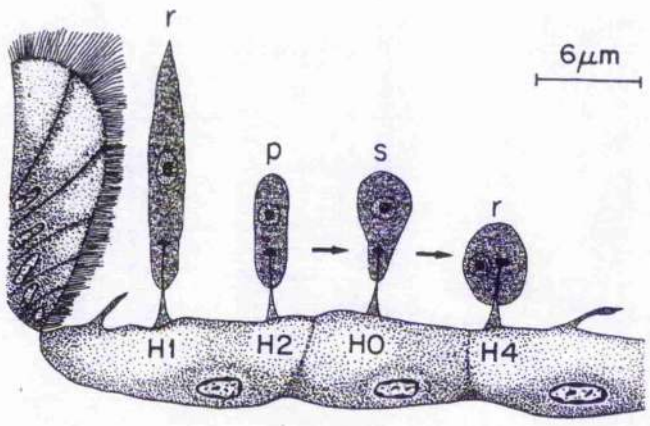


Figure 1.4: Schematic representation of development in the hindgut based on light and electron microscopy. The morphological forms and hypothesised differentiation sequence (arrows) of *L. panamensis* attached to the cuticle of the pylorus region of the hindgut of *Lu. gomezi*, 3-16 days post-infection. Relative prevalence of each form is indicated as primary (p), secondary (s) or rare (r). H0, pear-shaped haptomonad promastigote; H1, elongate haptomonad promastigote; H2, spatulate haptomonad promastigote; H4, haptomonad paramastigote. Hindgut tissue is not to scale. This diagram is modified from Walters [1993].

hindgut [Hertig and McConnell, 1963; Johnson *et al.*, 1963; Lainson *et al.*, 1973]. When the ultrastructure of morphologically similar flagellates of *L. braziliensis* in *Lu. longipalpis* was investigated by electron microscopy [Killick-Kendrick *et al.*, 1977], it became clear that these cells were not all promastigotes. Based on the relative positions of the kinetoplast and nucleus the hindgut forms were identified as paramastigotes and sphaeromastigotes, some 'typical' promastigotes were also observed. All three cell types were capable of division. In contrast, paramastigotes were only occasionally found in the hindguts of *Lu. gomezi* infected with *L. panamensis*. The predominant morphological form was the spatulate-shaped promastigote which was also identified in the blood meal just before breakdown of the peritrophic membrane. Elongated nectomonads (N1), also seen in the blood meal, were present for only a short period of time in the hindgut subsequent to migration from the posterior midgut. These forms were presumed to transform to spatulate-shaped promastigotes shortly after attaching to the cuticular lining (H2). Two other cell types were observed attached in the hindgut; pear-shaped promastigotes (H0) and paramastigotes (H4). However, both were greatly outnumbered by spatulate-shaped forms (H2) which predominated throughout the hindgut, including the pylorus/ileum, anterior rectum and rectal sac/rectal papillae. This was the first report of such extensive colonization of the hindgut by peripylarian parasites. Previously flagellates, of various sub-species, appeared to be restricted to the pylorus/hind-triangle regions of the hindgut [Johnson *et al.*, 1963; Hertig and McConnell, 1963; Johnson and Hertig, 1970; Young *et al.*, 1987] and were on occasion found to occupy the ileum [Lainson *et al.*, 1977; Killick-Kendrick *et al.*, 1977]. In one exceptional study, *L. braziliensis* in *Lu. longipalpis* and *Lu. renei,* flagellates were observed as far back in the gut, but not including, the rectal papillae [see review by Schlein, 1986].

Another feature of *L. panamensis* development in *Lu. gomezi* that is inconsistant with the definition of *Viannia* species involves multiplication of hindgut forms. In *Lu. gomezi*, these stages appeared to be essentially non-dividing and the "extensive proliferation" in the hindgut expected of a *Viannia* species of *Leishmania* was not observed [Walters *et al.*, 1989a]. The only indication of division was of parasites in the anterior rectum which had expanded kinetoplast DNA, a feature which has been associated with replication in trypanosomes [Englund *et al.*, 1982].

Promastigotes of *L. panamensis* in the unnatural host *P. papatasi* behaved in a similar but distinct fashion to *L. panamensis* in *Lu. gomezi* following release from the peritrophic membrane. An equivalent migratory route and progression of morphological forms were adopted, however the parasites were notably larger than in the natural host. The preliminary and predominant morphological form identified in the hindgut was the spatulate-shaped promastigote which, following migration from the posterior midgut, attached to the pylorus, ileum and colon by means of hemi-desmosomal plaques. Transformation to pear-shaped promastigotes was reported after four days, these forms replacing spatulate-shaped promastigotes as the major cell type, and some paramastigotes were observed after eight days. Although division forms were observed, flagellates occupying the hindgut niche did not undergo extensive multiplication.

Thus it appears that as more *Leishmania*-sandfly interactions are examined in greater detail, the behaviour of promastigotes within the hindgut deviates further from the definitions erected by Lainson and Shaw [1987]. Clearly the isolation of *L. braziliensis* complex within a separate group from other species based on development in the hindgut still apparently applies. However, it may be useful to re-evaluate the other behavioural criteria and update the definitions based on the findings of recent

studies.

1.2.2.3.2 Development in the abdominal midgut

The corresponding developmental phase of suprapylarian species takes place in the abdominal/posterior midgut. Following their release from the peritrophic membrane, "long slender" or "elongated nectomonad" promastigotes (N1) of *L. mexicana* were initially free in the midgut lumen. Walters *et al.* [1987] observed that some of the free nectomonads were "organized in longitudinal arrays surrounding bloodmeal fragments", a phenomenon also reported by Killick-Kendrick *et al.* [1974] when examining *L. amazonensis*-infected *Lu. longipalpis*. It is unclear whether these "longitudinal arrays" are a common feature of suprapylarian midgut infections since they are not reported in other studies, including *L. mexicana* in *Lu. diabolica* and *Lu. shannoni* by Lawyer *et al.* [1987]. Other promastigotes of *L. mexicana* attach to the midgut epithelia via flagellar interdigitation with the microvilli. This attachment appears common to many other species of suprapylarian leishmanias [Shortt *et al.*, 1926; Killick-Kendrick *et al.*, 1974; Warburg *et al.*, 1986; Walters *et al.*, 1989b] and has been generally accepted as part of the life-cycle for all suprapylarian species. In some studies this interaction may involve intracellular associations between some promastigote flagella and the cytoplasm of the midgut epithelial cells [Killick-Kendrick *et al.*, 1974; Walters *et al.*, 1989b]. Killick-Kendrick *et al.* [1974] observed promastigote flagella within epithelia cells, below the microvilli, an observation which led them to speculate that parasites may be capable of passing through into the coelomic cavity. This observation was confirmed by Walters *et al.* [1989b] when they studied the life-cycle of *L. chagasi* in *Lu. longipalpis*.

Whether or not multiplication of nectomonad promastigotes occurs in the

abdominal midgut is unclear and may well be species-dependent. *L. mexicana* nectomonads in *Lu. abonnenci* [Walters *et al.*, 1987] were reportedly non-dividing while in *Lu. shannoni* and *Lu. diabolica* division forms were not mentioned [Lawyer *et al.*, 1987]. Rapid multiplication of *L. amazonensis* [Killick-Kendrick *et al.*, 1974] and *L. chagasi* [Walters *et al.*, 1989b] nectomonads in *Lu. longipalpis* was observed, which resulted in distention of the entire gut in the former study.

1.2.2.4 Migration to and promastigote morphology in the thoracic midgut and cardia

Forward migration from the hindgut or the abdominal midgut by flagellates to the thoracic midgut and cardia appears to be a common feature of mammalian leishmanias in the sandfly host [Killick-Kendrick *et al.*, 1979]. Subsequent multiplication in these regions often leads to them becoming grossly swollen with parasites which form a "plug" at the anterior end of the cardia and stomodeal valve which marks the entrance to the foregut [Adler and Theodor, 1926b; Shortt *et al.*, 1926; Killick-Kendrick *et al.*, 1974; Walters *et al.*, 1987]. However, the morphological forms which occur and their ability to divide and to attach to the epithelial lining of this region appear characteristic of the species and even sub-species of *Leishmania*. A schematic representation of promastigote development in the lumen of the thoracic midgut and cardia is shown in Figure 1.5.

Exactly which type of *L. panamensis* promastigote migrates from the hindgut to their next development site, the cardia, of *Lu. gomezi* is unclear. Spatulate-shaped promastigotes, as seen in the hindgut (H2) and the blood meal (N2), were found in the cardia region from as early as three days post-infection, coincident with breakdown of the peritrophic membrane and initial invasion of the hindgut. These cells were thought

to have migrated directly from the abdominal midgut independent of any hindgut developmental phase. However, primary colonization of the cardia region of *Lu. gomezi* did not occur until approximately six days subsequent to infection, coincident with a massive migration of parasites in an anterior direction from the hindgut. Unfortunately, the promastigotes which migrated to the cardia following breakdown of the peritrophic membrane were not followed by Walters *et al.* [1989a], although gross expansion of this population of cells would have been obvious and difficult to overlook. Thus it seems likely that with this species hindgut promastigotes are important for establishment of infection in the sandfly foregut.

Based on the dual locality of spatulate-shaped promastigotes, Walters *et al.* [1989a] suspected this form to be responsible for migration from the hindgut to the thoracic midgut/cardia. However, this may be incorrect since three other morphologically distinct forms of *L. panamensis* with free flagella were found to inhabit the lumen of the cardia. These were: Type A (N3), short promastigotes (6-11 μm body length with flagella 1·4-1·6 times the body length); Type B (N5), short promastigotes (7-9 μm body length with flagella 2·1-2·9 times the body length); and paramastigotes (N4) (4-6 μm body length with flagella > 3 times the body length). All of these presumably developed, either directly of indirectly, from spatulate-shaped promastigotes (N2). All of these forms are included in Figure 1.3 except for Type B promastigotes which are similar to metacyclics (N5) of suprapylarian species but have a more rounded cell body. Short promastigotes (N3) predominated in the cardia and were often observed in division, a feature which prompted Walters *et al.* [1989a] to propose these cells as the "second major multiplicative reservoir of infection in *Lu. gomezi*". In addition, these promastigotes were considered to be "similar in some respects to the short promastigotes (N3 and H3) of *L. mexicana*" observed in the midgut of the

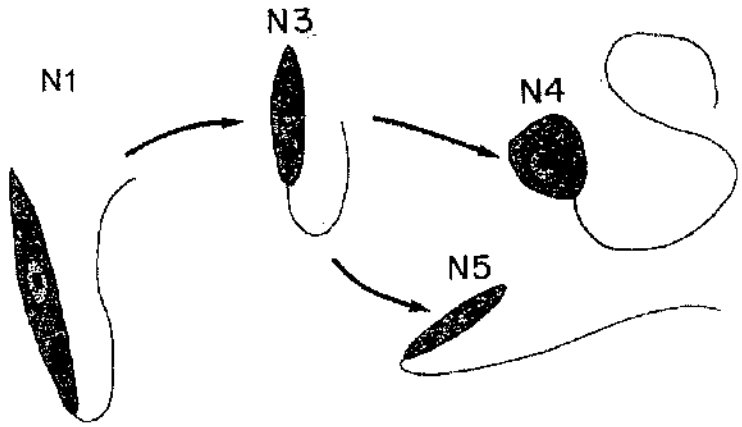


Figure 1.5: Schematic representation of development in the cardia. Morphological forms and proposed differentiation sequence (arrows) of *Leishmania* parasites in the cardia region of the midgut, 2-18 days post-infection. Based on light and electron microscopy of parasites in two natural (*L. panamensis/Lu. gomezi, L. chagasi/Lu. longipalpis*) and two unnatural (*L. mexicana/Lu. abonnenci, L. major/Lu. longipalpis*) life-cycles. N1, elongate nectomonad promastigote; N3, short nectomonad promastigote; N4, nectomonad paramastigote; N5, metacyclic nectomonad promastigote. This diagram is copied from Walters [1993].

experimental host *Lu. abonnenci* [Walters *et al.*, 1987].

Short promastigotes (N3 or H3) and paramastigotes (N4 or H4) appear to be a common feature of many species of *Leishmania* in the cardia of infected sandflies, both suprapylaria and peripylaria. Promastigotes morphologically similar to the short promastigotes of *L. mexicana* have been described so far for *L. chagasi* [Walters *et al.*, 1989b] and *L. panamensis* [Walters *et al.*, 1989a], while paramastigotes of the same species and *L. major* [Warburg and Schlein, 1986; Warburg *et al.*, 1986] have been identified in the cardia region of infected sandflies. Both types of flagellate were also found in the unnatural combination of *L. panamensis* in *P. papatasi*, but were retained within the peritrophic sac [Walters *et al*, 1992]. The short promastigotes of *L. mexicana* and *L. chagasi* were thought to originate from elongate nectomonads (N1), either prior or subsequent to their migration from the abdominal midgut and were the primary dividing forms in the cardia of *Lu. abonnenci* [Walters *et al.*, 1987] and *Lu. longipalpis* [Walters *et al.*, 1989b], respectively. Unlike the morphologically similar promastigotes of *L. panamensis* in *Lu. gomezi*, short promastigotes of *L. mexicana*, *L. chagasi* and *L. major* [Warburg *et al.*, 1986] attached via flagellum interdigitation with the epithelial microvilli of the thoracic midgut. In all three cases flagella were observed not only between the microvilli but were also reported to penetrate epithelial cells. Killick-Kendrick *et al.* [1974] reported a similar finding for the flagella of elongate promastigotes of *L. amazonensis* in the abdominal midgut of *Lu. longipalpis* and for *L. braziliensis* in the thoracic midgut region of *Lu. wellcomei* [Killick-Kendrick *et al.*, 1977]. The relevance of this remains unclear, however it is likely that piercing epithelial cells would release potential energy substrates for use by leishmania.

The "long, slender, highly motile" midgut promastigotes (N1 and H1) of *L. mexicana* in *Lu. diabolica* and *Lu. shannoni* also underwent differentiation to generate

short promastigote forms [Lawyer *et al.*, 1987]. However, these promastigotes were also described as "broad", suggesting a distinct promastigote type from the short promastigotes previously discussed. Whether these promastigotes are equivalent to the haptomonad forms of *L. amazonensis* [Killick-Kendrick *et al.*, 1974; Killick-Kendrick, 1979] or *L. major* [Warburg *et al.*, 1986; Killick-Kendrick *et al.*, 1988] is unclear and yet to be confirmed. Although morphologically similar, the "short broad" promastigotes of *L. major* appeared capable of division [Lawyer *et al.* 1987] unlike haptomonads of *L. amazonensis* [Killick-Kendrick *et al.*, 1974; Killick-Kendrick, 1979].

1.2.2.5 Development in the foregut

The foregut of the sandfly is lined with cuticular intima and is sub-divided into the stomodeal valve, oesophagus, crop, pharynx, cibarium and proboscis. The stomodeal valve marks the entrance and is the initial site of colonization by flagellates in the foregut. It is believed that, establishment of infection of the stomodeal valve is by promastigotes generated in the cardia which are forced forwards as the parasite population expands. Rapid division of promastigotes in the cardia often causes the anterior midgut to become swollen and distended with parasites which form a "plug" extending into the stomodeal valve region of the foregut [Adler and Theodor, 1926b; Shortt *et al.*, 1926; Killick-Kendrick *et al.*, 1974; Walters *et al.*, 1987]. The parasites have a definite orientation within the "plug", their flagella are directed forwards towards the head of the fly and where they touch the cuticular epithelium of the stomodeal valve they become attached [Adler and Theodor, 1926b; Killick-Kendrick *et al.*, 1974; Warburg *et al.*, 1986; Walters *et al.*, 1987]. This attachment is often accompanied by differentiation and examination of flagellates colonizing the stomodeal valve region of the sandfly foregut has identified a number of morphological forms in the different

associations studied. These are depicted in Figure 1.6.

Common to several *Leishmania*-sandfly combinations are forms which morphologically resemble the haptomonads (H0) of *L. amazonensis* described in the foregut of *Lu. longipalpis* by Killick-Kendrick *et al.* [1974]. These promastigotes were the sole occupants of regions anterior to the abdominal midgut and were described as short (<12 μm) and electron lucid with stumpy modified flagella and closely positioned, relative to each other, kinetoplast and nucleus. Studies in which equivalent morphological forms were identified attached to the stomodeal valve include *L. panamensis* in *Lu. gomezi* [Walters *et al.*, 1989a] and *L. mexicana* in *Lu. diabolica* and *Lu. shannoni* [Lawyer *et al.*, 1987]. However, in contrast to the haptomonads of *L. amazonensis* which were non-proliferative, the "pear-shaped" and "short broad" promastigotes of *L. panamensis* and *L. mexicana*, respectively, were capable of division.

Pear-shaped haptomonad promastigotes (H0) of *L. panamensis* were accompanied on the stomodeal valve of *Lu. gomezi* by haptomonad paramastigotes (H4) [Walters *et al.*, 1989a], a form previously reported to be restricted in locality to the oesophagus and pharynx regions of the foregut and to the hindgut [Killick-Kendrick, 1979; Molyneux and Killick-Kendrick, 1987]. Neither of these forms were detected on the valve in the unnatural association of *L. mexicana* and *Lu. abonnenci*, although both forms existed further forwards in the gut. The only form found at the stomodeal valve in this study was the short promastigote (N3) in the haptomonad phase (H3). In her review of *Leishmania* differentiation, Walters [1993] proposes that short promastigotes (N3) migrate to the valve from the cardia region (in natural host/parasite associations) and attach to its surface before differentiating into pear-shaped forms (H0) at variable rates.

In many studies [Killick-Kendrick, 1979; Lawyer *et al.*, 1987; Walters *et al.*,

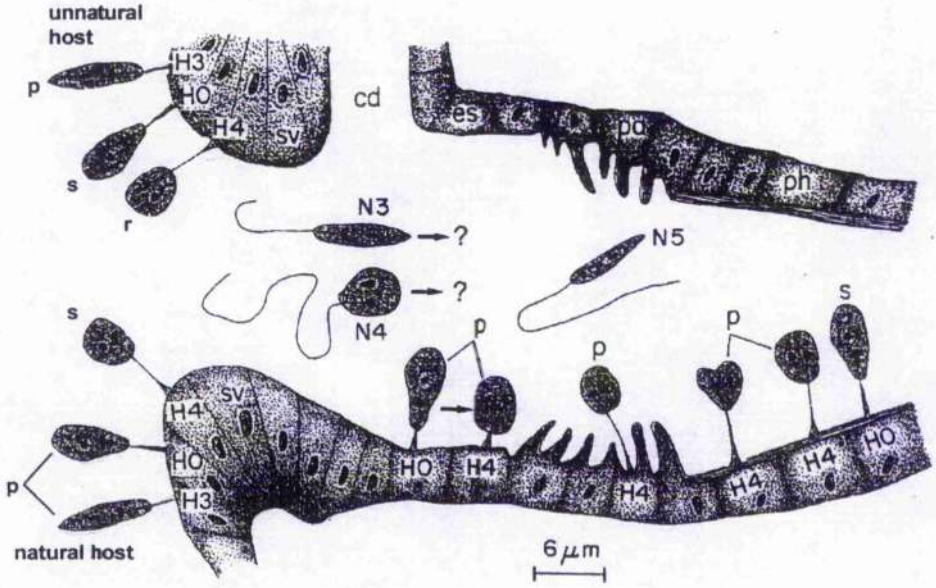


Figure 1.6: Schematic drawing representing *Leishmania* development in the foregut, stomodeal valve, oesophagus and pharynx, of the sandfly vector, 3-18 days post-infection. Based on light and electron microscopic examination of parasites in these regions in two natural (*L. panamensis*/*Lu. gomezi*, *L. chagasi*/*Lu. longipalpis*) and two unnatural (*L. mexicana*/*Lu.abonnenci*, *L. major*/*Lu. longipalpis*) life-cycles. Relative prevalence of attached forms is indicated as primary (p), secondary (s) or rare (r). H3, short haptomonad promastigote; H4, haptomonad paramastigote; H0, pear-shaped haptomonad promastigote; N3, short nectomonad promastigote; N4, nectomonad paramastigote; N5, metacyclic nectomonad promastigote; sv, stomodeal valve; cd, crop duct; es, oesophagus; pa, pharyngeal armature; ph, anterior pharynx. Foregut tissue is not drawn from scale. Figure is modified from Walters [1993].

1987; Walters *et al.*,1989a; Walters *et al.*, 1989b] of different host-parasite combinations, the promastigotes in the cardia and at the stomodeal valve were found to be embedded in a thick gel-like substance of unknown origin or functional significance. It has been suggested that this may act to help parasites maintain their position within the gut. Detection of a similar gel-like matrix in cultures of promastigotes [Christophers *et al.*, 1926; Schnur *et al.*, 1972; Handman *et al.*, 1982] suggest that it must be of parasite origin. Subsequent analysis of promastigote types along the gut length using monoclonal antibodies raised against lipophosphoglycan (LPG), which plays a role in parasite attachment to the midgut epithelia, confirmed this and identified what appeared to be promastigote LPG within epithelia cells [Davies *et al.*, 1990]. It was concluded that the excreted LPG corresponds to the gel-like matrix which occurs *in vitro* and *in vivo*.

Anterior to the stomodeal valve are the oesophagus and the pharynx regions of the foregut which are also lined with cuticular intima. Figure 1.6 illustrates the different parasite forms found attached to the surface and free in the lumen. Primary colonizers of the oesophagus in the parasite-host associations of *L. mexicana*/*Lu. abonnenci* [Walters *et al.*, 1987] and *L. panamensis*/*Lu. gomezi* [Walters *et al.*, 1989a] were pear-shaped/oval haptomonad promastigotes (H0) and pear-shaped/oval/round haptomonad paramastigotes (H4) [Walters, 1993]. Both of these cell types were also located on the stomodeal valve of *Lu. gomezi* infected with *L. panamensis*. The relative proportions of these two cell types changed to being predominantly haptomonad paramastigote forms (H4) in the pharynx of both parasite-host combinations. Also present in the oesophagus and pharynx regions of *Lu. gomezi* were short promastigotes, Types A (N3) and B (N5), of *L. panamensis*.

In a variety of studies, including *L. mexicana* in *Lu. abonnenci* by Walters *et al.*

[1987], paramastigotes were considered to be the principal type of flagellate to colonize the oesophagus and pharynx [Killick-Kendrick *et al.*, 1977; Killick-Kendrick *et al.*, 1988; Molyneux *et al.*, 1975; Warburg *et al.*, 1986], although Lawyer *et al.* [1987] found them to be rare in the natural life-cycle of *L. mexicana* in *Lu. diabolica*. A similar finding was reported for *L. major* in *P. duboscqi* [Lawyer *et al.*, 1990]. In contrast, the dominant promastigote forms of *L. mexicana* anterior to the stomodeal valve of *Lu. diabolica* and *Lu. shannoni* were described as "short, slender, highly active promastigotes with long flagella", forms which morphologically resemble the infective metacyclic promastigotes observed in a number of other suprapylarian life-cycles [Killick-Kendrick, 1979; Warburg *et al.*, 1986; Lawyer *et al.*, 1987] and reviewed by Killick-Kendrick [1986]. Metacyclics of suprapylarian *Leishmania* species are characteristically small (body length ≅ 4-13 μm) and slender (body width ≅ 1-2 μm) and have a flagellum which is approximately twice the body length [Warburg *et al.*, 1986; Lawyer *et al.*, 1987; Killick-Kendrick, 1986]. They are commonly observed unattached in the gut lumen, especially the foregut, and are generally regarded as free-swimming and non-dividing. The responsibility for transmission is thought to lie solely with these cells since they are thought to be the only parasite forms to be deposited in the skin of the vertebrate during sandfly feeding [Adler and Theodor, 1929a; Adler and Theodor, 1931; Adler and Ber, 1941; Warburg and Schlein, 1986]. Walters *et al.* [1989a] identified forms which they considered to be metacyclics of *L. panamensis*, a peripylarian *Leishmania* species, in the natural host *Lu. gomezi*. These promastigotes, which they called Type B promastigotes, were within the size range but often had a longer flagellum and were more rounded posteriorly than the suprapylarian metacyclics. Parasites similar in morphology to these putative metacyclics were also described by Almeida *et al.* [1993] in stationary phase cultures of *L. braziliensis*. Other metacyclic

characteristics exhibited by these *in vitro* cultivated promastigotes included modification of LPG as indicated from the development of a new lectin agglutination profile, enhanced complement resistance and increased infectivity *in vivo*. Promastigotes of *L. mexicana* fitting the description of the "proboscis or infective forms" described by Killick-Kendrick [1979] were not detected in the foregut of *Lu. abonnenci* [Walters *et al.*, 1987]. Indeed, very few parasites were observed in the pharynx and none at all in the cibarium/proboscis regions of this species of sandfly. Meagre colonization of these anterior regions and apparent lack of infective metacyclic promastigotes suggests that *Lu. abonnenci* is a poor vector of *L. mexicana* and probably does not serve as a natural host in the wild.

Surprisingly, the locality of metacyclogenesis or of storage of resultant metacyclics in an 'infective' sandfly are unknown. It is clear, however, that there is a sequential development in the sandfly from an essentially non-infective promastigote form to an infective form and that these so called metacyclics may develop in the midgut of sandflies from as early as 3 days post-infection as was reported for *L. major* in *P. papatasi* [Sacks and Perkins, 1984; 1985]. Sandfly infectivity as judged by the rate of growth and size of resultant *L. major* lesions in BALB/c mice increased progressively with time and flies were deemed optimally infective 7-10 days after the infecting blood meal, by which time promastigotes have often advanced beyond the stomodeal valve into the head region of the fly. Maybe metacyclic precursors are stimulated to differentiate into metacyclics by a combination of factors, and those which do not differentiate die and are degraded. In electron micrographs of *L. amazonensis* [Killick-Kendrick *et al.*, 1977] and *L. mexicana* [Walters *et al.*, 1987] paramastigotes in various stages of degeneration were found attached to the surface of the pharynx. They were intermixed with apparently healthy intact paramastigotes. Perhaps the surviving

paramastigotes represent a sub-population of cells which have already been stimulated to undergo metacyclogenesis by declining energy reserves, imbibed sugars and acidic pH (see section 1.3).

It is clear that development of *Leishmania* promastigotes within the sandfly gut is an elaborate process which involves periods of proliferation, attachment and migration paralleled by changes in promastigote morphology. Consequently, several distinct promastigote forms have been identified in numerous *Leishmania* species, the morphology of which reflects the region of the gut in which they occur. The physiological significance of the differences in parasite attachment and morphology in different parts of the insect gut remains to be clarified, but is suspected to relate to dissimilar conditions prevailing in the various gut regions.

1.3 Conditions encountered by promastigotes in the sandfly gut

As promastigotes develop within the sandfly gut they encounter a variety of different microenvironments. The environmental changes to which they are subjected during migration in addition to those which are a result of sandfly digestion and the parasite's energy metabolism are thought to be responsible for the numerous promastigote morphotypes which arise during the period spent in the sandfly gut. Unfortunately, little research has been carried out on this aspect of sandflies. Various information, however, is available within the literature on the physiology of a wide range of insects, including many blood feeding diptera. These data could potentially be applied to sandflies. The dearth of information on sandfly physiology is due mainly to the small size of these insects as well as to difficulty in maintaining laboratory-reared colonies. Some of these difficulties have been overcome and a few groups of workers have investigated such things as sandfly diet [Ewart and Metcalf, 1956; Auclair, 1963;

Lewis and Domoney, 1966; Ready, 1978; Young *et al.*, 1980; Schlein, 1986], sandfly digestion [Dillon and Lane, 1993] and promastigote development (discussed in section 1.2). A short, but dated, review which discusses the biology of phlebotomine sandflies is also available [Killick-Kendrick, 1978].

Leishmaniasis is transmitted by female sandflies rather than males since females require additional protein, supplied by regular blood meals, for egg production. Between blood feeds their diet is supplemented with sugar and amino acids from plant sources and from aphids [Ewart and Metcalf, 1956; Auclair, 1963; Lewis and Domoney, 1966; Ready, 1978; Killick-Kendrick, 1979; Young *et al.*, 1980; Schlein, 1986]. Thus the environment for *Leishmania* development within the gut consists of two different nutrient media and their respective digestive enzymes. The activity of these enzymes as well as adsorption of nutrients from the gut is influenced by the concentration of hydrogen ions, a factor which has also been implicated as important for promastigote development *in vitro*. It has been suggested that the pH of the alimentary canal of insects can be correlated with insect feeding habits. In a review on insect digestion [House, 1965], three papers were referred to which reported that the hindgut is usually less acidic than the midgut in omnivorous and carnivorous species [Grayson, 1951; 1958; Swingle, 1931], although it was also proposed that hindgut pH would be determined by the decomposition of different foods or by the function of the malpighian tubules [Grayson, 1951]. In contrast, other authors from the same review [House, 1965], found gut pH to be a result of various factors. Waterhouse [1949] and Strivastava and Strivastava [1961] concluded that pH tends to be more a characteristic of the taxonomic group than of the feeding habit. Microorganisms have also been implicated in affecting gut hydrogen ion concentration [Wigglesworth, 1927 also cited in House, 1965]. Clearly, the pH values within the different gut regions and how they are affected by

digestion and by parasite metabolism have yet to be fully determined. Changing the pH at which promastigotes are cultivated *in vitro* has been shown to influence their development and morphology. Specifically, acidification of *in vitro* culture medium was reported to initiate differentiation of *L. mexicana* promastigotes thereby generating a morphologically homogeneous population of promastigotes which were characterised on biochemical and morphological criteria as metacyclics forms [Bates and Tetley, 1993]. A similar change in pH may occur *in vivo*, resulting from the accumulation of organic acids secreted by vast numbers of promastigotes in the sandfly cardia and foregut, and could act as a trigger of metacyclogenesis within the sandfly. Advances in techniques permitting the measurement of pH of very small volumes coupled with improvements in sandfly maintenance should allow many questions regarding the effect of gut pH on promastigote development in the sandfly to be addressed.

Interestingly some insects, including sandflies, are able to discriminate between food materials and many blood-sucking diptera dispatch highly proteinaceous food directly to the midgut while sugary liquids first undergo a period of storage in the crop. This process is thought to act as a defence against bacterial infection since food taken into the crop is mixed with an antibacterial factor [Schlein, 1986]. Thus ingested blood, which may contain amastigotes, is taken directly into the abdominal midgut where it becomes encapsulated by the peritrophic membrane and digestion is initiated coincident with amastigote differentiation. It is thought that the rapid drop in temperature experienced by amastigotes on ingestion by the sandfly participates in their stimulation to transform to the promastigote stage, as occurs *in vitro*. The promastigotes which result appear to congregate at the periphery of the blood meal a digestive interface presumably rich in nutrients. As digestion proceeds this microenvironment is likely to change quite dramatically, in terms of both surrounding gases and blood composition as

the meal is digested by host enzymes. High levels of proteinase activity [Gooding, 1975] are induced on blood uptake since proteins constitute approximately 95% of the blood meal [Albritton, 1952]. Such enzymes are thought to be important effectors of *Leishmania* selection since their production appears to be reduced by naturally transmitted species of *Leishmania* but not by species which fail to be transmitted [Schlein and Romano, 1986]. Morphological changes to promastigotes have been detected towards the end of blood digestion and it is thought that this differentiation arises in response to changes within the blood meal.

Following digestion, the blood meal remnant together with the peritrophic membrane are passed to the hindgut and expelled. Promastigotes which have escaped through breaks in the membrane are now free in the gut lumen and attach to its surface in order to prevent their own expulsion. Peripylarian *Leishmania* species migrate and attach to the cuticular lining of the hindgut while suprapylarian species attach via lectin associations with the epithelia of the abdominal midgut. The morphology and behaviour of promastigotes differ, perhaps reflecting differences in their surroundings.

It is thought that promastigotes in the midgut will have access to the sugars and amino acids from plant sap and honeydew which are reported to supplement the sandfly diet between blood feeds [Ewart and Metcalf, 1956; Auclair, 1963; Lewis and Domoney, 1966; Ready, 1978; Killick-Kendrick, 1979; Young *et al.*, 1980; Schlein, 1986]. Surprisingly, these foodstuffs do not move through the alimentary canal as an uninterrupted flow down a digestive gradient, but instead may be distributed and redistributed back and forth especially between the ventriculus and the crop and the proventriculus. It is thought that sugars ingested by the sandfly not only serve as potential energy substrates but may also play a role in detachment of promastigotes from the epithelial lining. This detachment is also mediated through the modifications

to the promastigote surface which accompany metacyclogensis [reviewed by McConville and Ferguson, 1993]. Interestingly, the generation of metacyclics in the sandfly and subsequent disease transmission is also thought to by influenced by blood meal expulsion and ingestion of sugars (see below).

Promastigotes developing in a hindgut environment are subject to both peristaltic movement of gut contents and secretions from malpighian tubules. The malpighian tubule-hindgut-rectum system plays the most important role in the removal of nitrogenous end products in the majority of insects [Stobbart and Shaw, 1973]. Uric acid is generally the dominant nitrogenous component of the excreta of terrestrial insects. It is broken down to yield allantoin and allantoic acid. Small quantities of ammonia, urea and amino acids may also be excreted. Some arthropod species achieve removal of uric acid and/or its derivatives from the haemolymph via storage in the body fat or by storage together with excretion by the intestine. In the later stages of its formation the excretory fluid becomes acidic and water and salts are reabsorbed from it. These processes are associated with the precipitation of free uric acid and occur mostly in the rectum and also in the proximal region of the malpighian tubules, having the direct result of acidifying these areas. The malpighian tubules are probably also able to excrete other organic substances. Additional organic molecules of low molecular weight may enter the tubules passively, any of metabolic importance being reabsorbed subsequently in the hindgut and rectum.

Thus the amount of nutrients available to promastigotes in the hindgut will be limited, although not necessarily negligible. The identity of all the substrates available for use in this region remains to be determined, although analysis of the excreta from *Rhodnius prolixus*, the vector of *Trypanosoma cruzi*, detected a variety of amino acids. Interestingly, Howard *et al.* [1991] reported increased growth, in terms of promastigote

numbers, when culture media was supplemented with human urine. This result occurred for a number of species, including those thought not to develop in areas containing nitrogenous waste. The reason for improved growth under these conditions and the physiological significance of the effect therefore remains to be established.

Subsequent to development in the hindgut or abdominal midgut both peripylarian and suprapylarian promastigotes move forwards in the gut to take up residence in the thoracic midgut and the foregut. The stimuli responsible for this migration has not yet been determined, but in suprapylarian species it may be associated with competition with sugars for binding sites on the midgut epithelia coupled with changes to lectins on the promastigote surface [reviewed by McConville and Ferguson, 1993]. Whether or not parasites, other than metacyclics (discussed below), in the cardia and foregut have an endogenous energy store or are directly reliant upon plant material imbibed by the sandfly between blood meals is unclear, however their immotility is suggestive that conditions are not optimal. Limited energy substrates may be one reason for the observed immotility, although it could also be caused by increasing acidity from promastigote digestive products or simply a direct consequence of the thick gel-like substance in which the cells are embedded.

The stimuli for metacyclogenesis in the sandfly and *in vitro* have yet to be fully determined, although several factors are thought to be involved. In cultures, it is thought to be triggered, at least in part, by the exhaustion of a particular nutrient or set of nutrients which slows down multiplication causing what is known as the stationary phase of growth. A corresponding decrease in promastigote growth rate is thought to be experienced by promastigotes in the cardia region of the sandfly gut which were described as immotile yet viable [Lawyer *et al.*, 1987; Walters *et al.*, 1987; Walters *et al*, 1989]. It has been suggested that these cells have reached the stationary phase of

growth, a state perhaps resulting from expulsion of the blood meal, which has also been linked to the generation of metacyclics. However, studies on cultivated promastigotes in the stationary phase of growth have identified two possible endogenous energy reserves, carbohydrate [Keegan and Blum, 1992] and lipid [Mallinson and Coombs, 1989], which are either absent or present in reduced amounts, respectively, in log phase cells. It is likely that these are degraded for energy generation by metacyclics in the sandfly foregut but may also be utilised during differentiation to the amastigote stage. The occurrence of such an energy source in metacyclics could account for their rapid movement in nutrient free medium and in the sandfly foregut. Furthermore, sugar meals imbibed subsequent to excretion of the blood meal would presumably be available to promastigotes in the cardia and the foregut regions, suggesting that nutrient depletion as such may not be the trigger of metacyclogenesis. It has been proposed that the sugars which supplement sandfly diet between blood meals are involved in metacyclogenesis [reviewed by Schlein, 1986]. It is thought that these sugars are required for transmission of *Leishmania* by bite, a conclusion derived from laboratory experiments which found that inclusion of raisins in the diet of *P. argentipes* facilitated the successful transmission of *L. donovani*. However, this did not always result in consistent transmission due to a lack of other components ingested in the wild. Comparison of laboratory infected flies which had been released and recaptured with those which were kept in the laboratory identified differences in *L. infantum* promastigote location in *P. ariasi* [Killick-Kendrick, 1981]. Those maintained in the laboratory were found only in the midgut while released flies had promastigotes in the foregut and were deemed infective. Another contributing factor to *in vitro* metacyclogenesis, which is also relevant to rapidly multiplying and respiring parasites in the cardia, is acidification of their surrounding environment. Culture of

promastigotes *in vitro* under acid condition, pH 5·5, has been reported to result in homogeneous populations of *L. mexicana* metacyclics [Bates and Tetley, 1993]. Organic acid production and secretion by vast numbers of promastigotes within the confines of the fly gut is likely to acidify their environment, perhaps triggering metacyclogenesis.

Sandfly saliva has been shown to directly influence promastigote development in the anterior parts of the alimentary canal [Charlab and Ribeiro, 1993; Charlab *et al.*, 1995]. Salivary gland homogenates (SGH) were shown to exhibit a dual effect on cultured promastigotes of *L. amazonensis*, inhibiting growth and inducing differentiation. Comparison of control cultures with those which included SGH demonstrated a difference in promastigote size, the treated cultures containing more elongate promastigotes which resembled the slender nectomonads (N1) which predominate immediately prior to blood meal expulsion and in the midgut. When these effects were investigated further it was clear that they were related to the phase of promastigote growth since log phase cells were less sensitive to SGH than stationary phase cells. It is suspected that the growth arrest observed *in vitro* represents a mechanism for routing parasites to a differentiation step within the sandfly vector.

Changes in substrate availability, gaseous conditions, pH and osmolarity are all thought to contribute to the transformation of promastigote stages within the sandfly vector. Changes in morphology are likely to be accompanied by alterations in biochemistry, especially metabolism, as promastigotes migrate to different regions within the sandfly gut. Little information is available on the passage of food along the sandfly alimentary tract, from ingestion to excretion, thus correlating environmental conditions with the different promastigote types observed at various regions along the gut length [Walters, 1993] is a challenging project.

Clearly the exact environmental conditions experienced by promastigotes in the different regions of the gut are unknown. Information on the available substrates, osmolarity, pH and gaseous conditions could allow development of a method for *in vitro* culture of pure populations of the different promastigote forms. Biochemical analysis of these cells would then be possible and so the adaptations of each stage may be uncovered.

1.4 *In vitro* cultivation

One of the aims of current research on *Leishmania* is to obtain a better understanding of the biochemical basis of adaptation and survival of each stage in the parasite's life-cycle. Ideally, characterisation should be carried out upon pure populations of cells isolated from natural hosts. However, this is rarely possible for a variety of reasons and this has necessitated the development of other ways of obtaining large quantities of pure parasites. Several methods have been devised which have permitted the purification of promastigotes from sandflies [Stephenson *et al.*, 1987] and amastigotes from vertebrate hosts [Childs *et al.*, 1976; Brazil, 1978; Infante *et al.*, 1980; Hart *et al.*, 1981a; Saraiva *et al.*, 1983; Meade *et al.*, 1984; Glaser *et al.*, 1990]. None of these are ideal and low yields or contamination with host materials means that most of the methods are impractical as sources of parasites for study. In recent years the focus of attention has shifted from purification of parasites from natural or experimental hosts to the further development of axenic culture techniques. Determination of the conditions required for axenic culture have yielded useful information on parasite biochemisty and cell biology. Whether *in vitro* cultured parasites are identical to those which occur *in vivo* in natural hosts has yet to be confirmed, although, comparisons have identified several biological, biochemical and morphological similarities.

1.4.1 Promastigotes

Most studies, biochemical and otherwise, have been carried out upon promastigotes obtained through *in vitro* culture in a range of media at 25°C-28°C [Chang and Hendricks, 1985]. Since no "universal" culture medium in which all the different leishmanias will grow is available, several have been developed. Initially complex media such as NNN medium which were biphasic and based on blood-agar were devised [Wenyon, 1926]. An important feature of these media is that they would support transformation of amastigote stages of some *Leishmania* species to promastigotes and could sustain subsequent growth of promastigotes. More recently developed complex biphasic media, for example Evans' modified Tobie's medium [Evans *et al.*, 1984], are based on this early one but contain a considerable number of additional components. Modification in this way has produced media which have proven successful for the isolation of a great variety of leishmanias from both the Old and the New World [Evans, 1987]. Complex monophasic media, eg. LIT, have also been devised [Carmargo, 1964].

For routine promastigote culture, complex media have been replaced in many laboratories by commercially available semi-defined liquid culture media which are usually supplemented with heat-inactivated foetal calf serum (HIFCS). These are frequently based on insect cell culture media, such as Schneiders' and Graces', and have been shown to support the growth of *Leishmania* [Childs *et al.*, 1978; Hendricks *et al.*, 1978]. In addition, transformation of amastigotes to promastigotes of some species of *Leishmania* (eg. *L. mexicana*) will occur in these media. However, biphasic media are still required for some of the more fastidious *Leishmania* species, notably *L. braziliensis*, *L. panamensis* and *L. guyanensis*. For survival and multiplication of almost all species of *Leishmania*, HIFCS is an essential supplement to these media.

However, several chemically defined media have also been developed and these are particularly useful in studies of the parasite's nutritional requirements. REI of Steiger and Steiger [1976] was the first medium in which all chemical components were known and which supported the growth of human infective *Leishmania* species, in this case *L. donovani* and *L. braziliensis*. Subsequently others have been devised, for example RE111 [Steiger and Steiger, 1977], HOSMEM [Berens and Marr, 1978], REIX and REX [Steiger and Black, 1980], DME2 [Iovannisci and Ullman, 1983] and MD-29 [Melo *et al.*, 1985]. All of these defined media, except for MD-29, were used to grow only one or two species of *Leishmania*. In contrast MD-29 was used for the growth of a representative range of species and strains.

One vital consideration which must be taken into account when relating the results of *in vitro* studies on promastigotes to those forms and events which occur *in vivo* is whether the *in vitro* cultivated promastigotes are representative of the forms which occur in the sandfly. It has generally been assumed that promastigotes produced by *in vitro* cultivation are essentially similar if not identical to the *in vivo* forms. This view has persisted, despite studies of promastigote life-cycles within the insect vector as detailed in section 1.2. It is thought that the changes in promastigote form *in vivo* occur in response to differing physiological conditions in the various microhabitats within the alimentary tract of the sandfly [Killick-Kendrick, 1979] and as such probably parallel and reflect other adaptations, including biochemical features. If this view is correct, it seems likely that cultured promastigotes would differ from the sandfly forms since *in vitro* conditions are unlikely to be the same as those in the fly intestine. However, sequential development of promastigotes has also been reported to occur *in vitro* and at least three different morphological forms have been identified which resemble some of those described in the sandfly [Christophers *et al.*, 1926; Bates, 1994; Charlab *et al.*,

1995].

Christophers *et al.* [1926] carried out an in-depth investigation of *in vitro* promastigote development, following the morphology of *L. donovani* amastigotes initiated into promastigote culture conditions over a period of 10 days. Early flagellate forms (which he designated Type 2) occurred shortly after inoculation of amastigotes (designated Type 1) and developed into what were described as dividing stumpy forms (designated Type 3). These took the form of short pear-shaped, oval or spindle-shaped promastigotes with a well developed flagellum (usually twice or three times the body length) which did not appear until around the second day of culture but persisted throughout. At the same time, "fusiform multiplicative forms" (designated Type 4) appeared, of which there were two types; (a) elongate oval or torpedo-shaped forms and (b) spigot-shaped forms. Both types were slender in appearance with the body length exceeding three times the breadth and differed from each other in the general shape of the cell body. Promastigotes of Type 4a exhibited a cell body which was similar in shape both anteriorly and posteriorly while Type 4bs possessed a more attenuated posterior end. Type 4 promastigotes were the commonest forms to be seen by Christophers *et al.* [1926] in their study of *L. donovani* promastigotes *in vitro* and both Types a and b had a tendency to group together to form clumps. Type 4a were observed to form small groups in early cultures while Type 4b produced large aggregations of cells. As cultures progressed other cell types emerged, but Type 4, particularly a, promastigotes predominated from about day 4 of culture until the study terminated on the tenth day. A fifth group of cell types, "mature flagellates", became apparent at around day 5 and remained until day 10 of culture, the final observation point. With a body length of five to six times their breadth these were the longest of the 4 Types of flagellates to be described. Again this group was sub-divided into several

morphological categories: (a) large stout forms; (b) large attenuated forms; (c) smaller forms with the same general characters as (b); and (d) extremely slender forms. Types 5d were rare and were not seen until day 8 of culture. These forms probably correspond to the infective metacyclic promastigotes which are now thought to occur in stationary phase cultures of promastigotes [for example, Mallinson and Coombs, 1989; Almeida *et al.*, 1993]. Two other cell Types were described, Type 6 - aflagellates and Type 7 - degenerative forms. Aflagellates were rare before day 8 and increased progessively thereafter. Three types of degenerative form were identified and appeared in cultures as early as day 4. Their numbers remained consistantly low throughout the study. In summary, *in vitro* development of *L. donovani* promastigotes involved the sequential development of different morphological forms. Early forms, stumpy dividing promastigotes, were replaced by more elongate promastigotes which predominated from day 4 onwards. As cultures progressed a third promastigote Type was identified which included the longest promastigotes to be described in addition to very slender forms which did not appear until relatively late on. Subsequently, parasites were aflagellates or degenerative forms.

A similar sequence of developmental forms was reported to occur in cultures of *L. mexicana* promastigotes [Bates, 1994]. Three morphologically distinct groups of promastigotes were identified during the course of the complete axenic life-cycle although it is noted that these were arbitrary divisions in a continual sequence. Early flagellates resembled those promastigotes identified within the blood meal in the sandfly abdominal midgut in that they were broad and rounded in shape and had short flagella. These are also morphologically similar to the stumpy promastigotes of *L. donovani* except that these promastigotes of *L. donovani* had well developed flagella [Christophers *et al.*, 1926]. These forms of *L. mexicana* were gradually replaced in

neutral HOMEM medium by long slender promastigotes which predominated in cultures in the late-log phase of growth. These forms are believed to be equivalent to the suprapylarian midgut infections in the sandfly. The third category of promastigotes was comprised of characteristically small slender forms typical of anterior midgut and/or foregut promastigotes in infective sandflies. These forms are thought to be the metacyclic promastigotes which are responsible for disease transmission and usually occur in stationary phase cultures of promastigotes. The proportion of *L. mexicana* metacyclics relative to the other promastigote forms could be enhanced by acidification of the culture medium, a modification which reportedly generated a homogeneous population of metacyclics [Bates and Tetley, 1993].

1.4.2 Amastigotes

Axenic *in vitro* cultivation of amastigote-like forms has proved relatively difficult to achieve when compared with promastigote culture, but has now been reported for a reasonable number of species, including *L. pifanoi*, *L. panamensis*, *L. braziliensis*, *L. donovani*, *L. mexicana*, *L. major*, *L. infantum* and *L. tropica* [Pan, 1984; Eperon and McMahon-Pratt, 1989; Doyle *et al.*, 1991; Bates *et al.*, 1992; Pan *et al.*, 1993] and was reviewed by Bates [1993a]. Despite many failed attempts to generate axenically cultured amastigotes, investigations persisted as it was realised that a successful system would provide a valuable source of parasites in the form responsible for the disease in the mammalian host. Such a source would be essentially limitless as well as free from host contamination and as such would permit detailed study of the biological and biochemical processes associated with this developmental stage, aspects which had been little studied due to a lack of adequately pure material. Consequently, identification of parasite unique features could provide potential drug and/or vaccine

targets.

It is suspected that transformation between parasite stages is triggered by changes in their surrounding environment that occur naturally *in vivo*. Consequently, the majority of attempts at axenic amastigote cultivation have been through transformation of promastigote forms by manipulation of the culture conditions. These manipulations were based on known changes which could potentially act as environmental stimuli during this transition. Differentiation of flagellated metacyclic promastigotes into aflagellated amastigotes occurs upon their injection into the mammalian host, a transition which along with many other environmental changes is accompanied by an increase in temperature. In this regard, preliminary attempts at axenic culture concentrated on heat-shock as a biologically relevant stimulus for transformation of stationary phase promastigotes into amastigote-like forms. This had some degree of success since promastigotes of various species subjected to elevated temperatures had a tendency to round up and become more amastigote-like in morphology. However, they rarely survived for more than a few days and were incapable of growth or division [see Pan *et al.*, 1993]. Furthermore, examination of these heat-shocked organisms at the molecular level revealed that compared with axenic amastigotes they were metabolically inactive [see Pan *et al.*, 1993]. Increasing the temperature gradually achieved transformation of promastigotes of four species of *Leishmania* to amastigote-like forms which responded favourably to subsequent serial passage [Pan, 1984; Eperon and McMahon-Pratt, 1989; Doyle *et al.*, 1991]. In addition to temperature, Pan's studies of *L. pifanoi* differentiation concluded that medium acidity was an important stimulus. These organisms were adapted for growth and appeared to require both elevated temperatures as well as low pH (<6) for transformation. Subsequent investigation has indicated that although both of these parameters are

important for transformation and subsequent growth of axenic amastigotes, the optimal conditions vary depending on the species of *Leishmania* involved. For example, species of *Leishmania* which cause cutaneous disease were found in general to prefer lower temperatures (28°C-32°C) than those species responsible for visceral pathology (37°C-39°C).

Persistant attempts to achieve axenic cultivation of amastigotes have extended our knowledge of the conditions required for transformation of promastigotes to amastigotes and for the subsequent growth of these amastigotes and it appears that this transition is a very sensitive one and not dependent on only temperature and pH [Pan *et al.*, 1993]. The selection and age of the culture medium as well as the batch, and indeed the process of heat-inactivation, of foetal calf serum appear to be critical. In addition, it has been shown that some lines of axenic amastigotes have enhanced sensitivity to antibiotics when compared with promastigotes, and that their absence during and subsequent to differentiation is therefore potentially beneficial. Thus, differentiation to and subsequent growth of extracellular amastigote-like organisms from promastigote forms is fraught with complications and no single procedure appears to be universally successful with all species and strains. A novel approach for axenic cultivation of *L. mexicana* amastigotes was adopted by Bates *et al.* [1992]. In contrast to others, these authors attempted to grow, in culture, amastigotes isolated from lesions rather than from the *in vitro* transformation of promastigotes. This method would presumably by-pass the requirement for essential signals necessary for transformation and hence one would imagine be subject to fewer complications. The conditions selected for growth included a temperature of 32°C, a pH of 5·5, plus a nutritionally-rich medium, Schneiders' Drosophila Medium, supplemented with 20% HIFCS. These conditions supported growth and division of forms morphologically similar to the inoculated lesion

amastigotes. On more detailed examination, at the ultrastructural, biochemical and biological level, the amastigote-like forms were found to be similar, but not identical to lesion amastigotes, but significantly distinct from promastigotes. The differences are thought to simply reflect adaptation of lesion amastigotes to inappropriate culture conditions, and suggest that further refinement is necessary.

1.4.3 Complete *in vitro* life-cycle

Design of a complete developmental sequence for *Leishmania* in axenic culture would provide the opportunity not only to investigate and compare the main life-cycle stages of this parasite but also to examine the differentiation between stages. Through manipulation of culture conditions such a system has been demonstrated for *L. mexicana* [Bates, 1994] whereby the resulting sequence of morphological forms resembles that observed in the natural life-cycle of the parasites. Briefly, amastigotes isolated from CBA mice were isolated and inoculated into promastigote culture medium (HOMEM [Berens *et al.*, 1976] supplemented with 10% (v/v) HIFCS, at pH 7·5) at 25°C and the resulting promastigote forms allowed to multiply until in the late-log phase of growth. At this time these forms were sub-passaged into culture medium developed for generation of homogeneous populations of metacyclic promastigote forms [Bates and Tetley, 1993] (Schneiders' Drosophila medium supplemented with 20% (v/v) HIFCS at pH 5·5), and allowed to progress until they reached the stationary phase of growth. Resultant metacyclics were then sub-passaged into the same medium but were subjected to a temperature shift from 25°C to 32°C, conditions which are reported to support long-term axenic growth of amastigote-like forms [Bates *et al.*, 1992]. Thus through manipulation of culture conditions the three main stages in the *L. mexicana* life-cycle (amastigotes, multiplicative promastigotes and metacyclic

promastigotes) can be obtained in large quantity for biological, biochemical, immunological and molecular characterisation. In addition, the *in vitro* life-cycle provides a unique system for the examination of differentiation between the different stages.

1.5 Differentiation

Stage differentiation of protozoan parasites, such as higher eukaryotic cells, frequently occurs in response to changes in the immediate environment [Shiels *et al.*, 1994], but can also be intrinsically determined within the cell [Turner *et al.*, 1995]. The cell responds by alteration of gene expression such that morphological and endogenous biochemical changes occur, consequently altering the function and developmental potential of the parent cell [Newton and Ohta, 1990]. Thus, the new cell type differs and can usually be distinguished from the original cell by phenotypic markers, either biochemical and/or morphological. At the molecular level, it is probable that the cell type is determined by regulatory genes which when translated in the cytoplasm become proteins which are capable of maintaining transcription of these same genes following their migration into the nucleus.

In animal development, differentiated cells arise by means of one of two fundamental mechanisms, asymmetric cell division involving an unequal segregation of developmental potential [discussed by Horvitz and Herskowitz, 1992] or via cell interactions which involves the interaction of two different cell types resulting in the generation of a third cell type [see review by Gurdon, 1992]. The first of these mechanisms includes a division step whereby two dissimilar daughter cells are produced and is exemplified in early egg development where it serves to generate early embroyonic cells that differ in their content of materials inherited from the fertilised egg

[Gurdon, 1992]. Asymmetric divisions are also common throughout nematode development, the pattern of which has been demonstrated to be important by the generation of mutations that lead to developmental defects [Horvitz and Herskowitz, 1992]. The second mechanism by which cell differentiation is initiated in metazoans involves cell interactions [Gurdon, 1992]. This is particularly important in vertebrate embryonic induction and is thought to be the single most important mechanism leading to differences between cells and to the organisation of cells into tissues and organs. Unicellular eukaryotic and prokaryotic organisms are also sensitive to external influences and respond with adaptive changes in their gene expression patterns. These modifications can cause profound changes of their developmental states such as in the *Leishmania* life-cycle.

1.5.1 Differentiation of *Leishmania*

Leishmania parasites undergo a very complex life-cycle which involves periods of development and proliferation as distinct morphological and biochemical forms in two very different hosts. The aflagellated amastigote stage resides in the relatively stable environment of the parasitophorous vacuole of macrophages in the vertebrate host while the flagellated promastigote forms are free living in the digestive tract of the sandfly vector. Differentiation between amastigotes and promastigotes is induced by exogenous changes in the cells surroundings with the consequence that each life-cycle stage is specifically adapted for survival in its immediate environment, adaptations which are reflected in their differing morphology and biochemistry. Of many possible changing environmental factors, temperature and pH appear to be key elements influencing transformation between these stages, as indicated from studies on *in vitro* cultivation [reviewed by Zilberstein and Shapira, 1994].

Due to ease of *in vitro* cultivation [Berens *et al.*, 1976] the most studied stage of *Leishmania* is the promastigote which occupies the digestive tract of the sandfly, wherein it undergoes a complex migratory route involving the sequential development of several different morphological forms. The route taken and the forms which occur are dependent on the species of sandfly, the strain of *Leishmania* as well as sandfly nutrition subsequent to infection and it is clear that the promastigotes undergo a number of significant morphological and biochemical changes throughout their development in the invertebrate host (see sections 1.2 and 1.6). These modifications may well arise in response to different environmental conditions as the promastigotes migrate through different regions of the gut and as sandfly diet fluctuates in relation to the gonotrophic cycle. Alternatively, or in addition to induced differentiation, it is possible that alterations to promastigote morphology are governed intrinsically within the cells. In support of this possibility the findings that similar changes in form have been reported to occur in *in vitro* cultures of *L. donovani* [Christophers *et al.*, 1926], *L. mexicana* [Bates, 1994] and *L. amazonensis* [Charlab *et al.*, 1995] and it is unlikely that cultured promastigotes are exposed to the same environmental conditions as their *in vivo* counterparts. Presumably *in vitro* cultivation provides a relatively more stable environment which is subject to gradual changes in contrast to conditions *in vivo* which will alter dramatically with each sandfly feed.

While it remains to be determined whether or not all of the different promastigote morphological forms represent distinct developmental stages, a vast body of evidence confirms that at least two morphologically and biochemically distinct promastigote stages exist in the sandfly as well as in *in vitro* cultures. These consist of the dividing long slender 'nectomonad' promastigotes (N1), supposedly typical of midgut infections of suprapylarian species, and non-dividing short slender-bodied free-

swimming metacyclic promastigotes (N5) which are found predominately anteriorly and in the mouthparts of the sandfly, in the case of *L. major* and *L. mexicana* [reviewed by Sacks, 1989]. Although it is clear that several different morphological forms occur in *in vitro* cultures, it is these two stages that are recognised and predominate in late-log phase cultures and stationary phase cultures, respectively, although their numbers depend on the sub-passage record and culture conditions applied. It has been shown through modification of culture conditions that metacyclogenesis can be induced by environmental changes, the key element of which appears to be acidic growth conditions. Cultivation of *L. mexicana* promastigotes at acidic pH (pH 5·5) produced a homogeneous population of metacyclic-like forms [Bates and Tetley, 1993] in contrast to neutral starting conditions which yields a mixed population of promastigotes, including metacyclic forms. This finding was supported by Howard *et al.* [1987] who attributed the high numbers of *L. donovani* metacyclics obtained through cultivation in a modified Grace's medium to the acidic conditions. Furthermore, incubation of mid-log phase *L. major* promastigotes in naturally acidified 'spent medium' resulted in a more rapid production of metacyclic forms [Sacks and Perkins, 1985; Mallinson and Coombs, 1989], although other factors, such as nutrient depletion or a soluble differentiation factor, were not ruled out in this study. The mechanism by which acidic pH induces differentiation is unknown. One hypothesis suggested that it acts as a density-dependent regulator of parasite numbers, promoting differentiation either by selection or induction [Bates and Tetley, 1993] and that this phenomenon may occur *in vivo* due to high parasite numbers and build up of acidic metabolic end products [Marr, 1980] in the thoracic midgut and foregut regions of the sandflies [Lawyer *et al.*, 1987; Walters *et al.* 1987]. Whether this infact happens is unclear as the pH value of the sandfly midgut is unknown as yet. However if it is similar to that found in another bloodsucking insect,

the mosquito, for which the reported value was > pH 8·5 [reviewed by Zilberstein and Shapira, 1994] then the hypothesis would fail.

Slender promastigotes in the late-log phase of growth and metacyclic promastigotes differ morphologically and biochemically, exhibiting adaptations which are thought to reflect the parasites surrounding environment *in vivo*. In addition, metacyclic promastigotes are pre-adapted, both morphologically and biochemically, for survival in the vertebrate host. One such biochemical adaptation involves extensive modification to the parasites surface. The major surface molecule lipophosphoglycan (LPG) has been identified on promastigotes of all *Leishmania* species studied so far [Sacks, 1992] and with some at least it undergoes considerable alteration during metacyclogenesis. These changes are thought to contribute to development within the sandfly as well as to establishment of infections in the mammalian host (see section 1.6.4.2).

Several other promastigote forms have been identified in the sandfly but have not been studied due to inadequate isolation procedures. One of these forms, the stumpy promastigote or procyclic, resembles promastigotes which predominate in early cultures. Whether this is a distinct promastigote stage requires further investigation as does its similarity to its *in vivo* counterpart, the blood meal promastigote form. Characterisation of biochemical features which are likely to be unique to this stage and comparison with late-log phase promastigotes and metacyclic promastigotes should provide evidence as to how similar or different these three stages are.

Obligate hindgut development of peripylarian species of *Leishmania* in the sandfly has been questioned recently by Walters *et al.* [1993]. However, it is clear that following breakdown of the peritrophic membrane the majority of promastigote forms migrate in a posterior direction and attach to the cuticular lining of the hindgut.

Moreover, a promastigote form equivalent to the midgut nectomonads of suprapylarian species has not been identified and it appears that peripylarian promastigotes migrate through this region on route to the thoracic midgut and stomodeal valve without stopping. The morphology and behaviour of hindgut forms have received significant attention since their initial identification by Johnson *et al.* in 1963. Biochemical characterisation, however, has not been attempted, presumably due to difficulties of isolation and purification. It is possible that the hindgut stages could be obtained through modification of culture regimes to mimic putative hindgut conditions. However, this may prove a very difficult task, especially as phenotypic markers for these forms are unavailable. Clearly, the biochemical adaptations of hindgut promastigotes and their similarities to and differences from other *in vivo* and *in vitro* forms is unknown and requires study.

1.6 Biochemical adaptations of *Leishmania*

Differentiation between parasite stages involves changes in gene expression which are paralleled by biochemical modifications. These alterations are commonly in response to changes in the parasite's immediate environment to which it must adapt biochemically to survive. Several examples of such adaptations have been identified in both *in vivo* and *in vitro* cultivated *Leishmania* parasites and consequently studies of biochemical features have focused upon comparison of log phase and stationary phase promastigotes, or purified metacyclics if possible, with each other and with amastigotes. Very few studies have distinguished between morphologically distinct promastigotes in the early-log phase and late-log phase of growth, despite their apparent resemblance to forms observed *in vivo*. It would be predicted that these promastigote forms will differ significantly from each other and from metacyclics and amastigotes in ways which can

be related to their particular *in vivo* microenvironments. Comparative studies on amastigotes, metacyclics and multiplicative promastigotes have identified several biochemical distinctions providing phenotypic markers which are stage-specific. These include changes in energy metabolism, proteinase and nuclease activities and surface molecules.

1.6.1 Energy metabolism

Although the energy generating pathways of *Leishmania* parasites have been under investigation for many years, significant gaps in our knowledge still remain. Comparison of those pathways which have been elucidated with mammalian energy metabolism has identified some distinct features providing targets and potential targets for chemotherapeutic attack. These include compartmentation of glycolysis and the formation of toxic methylglyoxal as a by-product of D-lactate synthesis. However, most of these studies have been confined to culture-generated promastigotes in the log phase of growth rather than amastigotes or metacyclic promastigotes, mainly because both of these latter stages were relatively difficult to obtain in sufficient quantity for metabolic investigation. Advances in purification procedures for metacyclics of *L. major* [Sacks *et al.*, 1985] and amastigotes of *L. mexicana* [Hart *et al.*, 1981b] now make it feasible for these stages to be investigated at a metabolic level. Moreover, recent developments in *in vitro* culture techniques have provided metacyclics of *L. mexicana* [Bates and Tetley, 1993] and amastigotes and amastigote-like cells of *L. pifanoi, L. panamensis, L. braziliensis, L. donovani, L. mexicana, L. major, L. infantum* and *L. tropica* [Pan, 1984; Eperon and McMahon-Pratt, 1989; Doyle *et al.*, 1991; Bates *et al.*, 1992; Pan *et al*, 1993], permitting interspecies comparisons. Investigation of these purified or culture grown metacyclics and amastigotes in the manner already

carried out for promastigotes will increase our understanding of the metabolic changes that occur as the parasites adapt to changing environmental conditions and may ultimately provide targets for innovative therapeutic strategies.

Preliminary comparison of multiplicative promastigotes, metacyclics and amastigotes suggests that the metabolic pathways of energy generation are generally similar in each stage and include glycolysis, the citric acid cycle and a cytochrome containing respiratory chain within the mitochondrion [Glew *et al.*, 1988]. However, several distinctions have become apparent. These include the preferences for energy substrates, which is reflected in the activities of the enzymes responsible for their degradation, and in the metabolic end-products which are generated.

Although glucose is taken up and oxidised by both multiplicative promastigotes [reviewed by Marr, 1980] and amastigotes, it is thought not to serve as the primary fuel source of either stage [Glew *et al.*, 1988]. Promastigotes apparently rely substantially on amino acid degradation for energy generation [Marr, 1980] while amastigotes appear to favour oxidation of fatty acids [Hart *et al.*, 1981c; Coombs *et al.*, 1982; Hart and Coombs, 1982]. The preferred energy substrate of metacyclic promastigotes has yet to be determined, although stationary phase promastigotes, containing metacyclics, were reported to oxidise a variety of compounds at a lower rate than promastigotes in the log phase of growth. These include acetate, glycerol [Keegan and Blum, 1990], leucine [Blum, 1991], proline [Blum, 1996] and alanine [Keegan and Blum, 1990; Blum, 1996]. Differential use of glucose has also been reported, however the results of these studies are conflicting, perhaps reflecting the different species investigated. Mukkada *et al.* [1974] and Blum [1996] found glucose oxidation to be higher in stationary phase promastigotes of *L. tropica* and *L. donovani*, respectively, in contrast to Keegan and Blum [1990] who reported a higher rate of glucose oxidation by log phase

promastigotes of *L. major*. Evidence is also available which suggests that metacyclic promastigotes possess endogenous energy reserves of carbohydrate [Keegan and Blum, 1992] and lipid [Mallinson and Coombs, 1989] which are degraded when exogenous substrates are limited. The relevance of these observations to substrate preference *in vivo* is unclear, however these substrates reflect putative *in vivo* nutrient availability as promastigotes in the blood meal are likely to be surrounded by amino acids originating from blood proteins whereas metacyclics may have access to sugars ingested from plant sources and amastigotes reside within host cells which are responsible for uptake and degradation of the triacylglycerols contained in lipoproteins [Glew *et al.*, 1988].

Those promastigotes which, subsequent to excretion of the blood meal, develop in the hindgut, abdominal and thoracic midgut and foregut regions are unlikely to encounter such nutrient-rich conditions as those held within the peritrophic membrane, although the ones which remain anterior to the hindgut will be exposed to frequent meals containing sugars and amino acids [Ewart and Metcalf, 1956; Auclair, 1963; Lewis and Domoney, 1966; Ready, 1978; Killick-Kendrick, 1979; Young *et al.*, 1980; Schlein, 1986]. The changes in nutrients available to promastigotes developing in the gut as sandflies switch from blood to meals from plants and aphids containing sugars and amino acids may account for the apparent correlation observed between the phase of *L. tropica* promastigote growth and the rate of glucose catabolism [Mukkada *et al.*, 1974]. There is also apparent correlation with the transport system responsible for uptake of glucose and two key glycolytic enzymes (phosphofructokinase and pyruvate kinase) being maximally expressed in stationary phase cells. Thus glucose uptake and catabolism occurs at a faster rate in culture-generated promastigotes of *L. tropica* which are similar in morphology to promastigotes in the thoracic midgut and foregut of the fly than in log phase promastigotes which resemble stages in the blood meal. Although

these results tie in with putative nutrient availability in these regions of the gut, this should not become accepted as the general situation as a subsequent study monitoring the rate of glucose uptake in defined and semi-defined media by promastigotes of *L. braziliensis* and *L. donovani* detected high rates of uptake throughout the growth cycle [Steiger and Meshnick, 1977]. Indeed it seems that carbohydrate catabolism may be important for all forms, although it is possible that the glucose which is consumed by the early culture forms largely goes to providing intermediates of glycolysis and the citric acid cycle which stimulate the transamination reactions involved in amino acid catabolism.

The relevance of these findings to the *in vivo* situation has yet to be confirmed, but it is possible that early promastigotes, those dividing in the blood meal, use proline and other amino acids preferentially to carbohydrate. Exhaustion of amino acids may then lead to transformation to a more elongate promastigote (typical of late-log phase promastigotes), breakdown of the peritrophic membrane and switching from oxidation of proline and other amino acids to catabolism of a different substrate, perhaps carbohydrate from subsequent sugar meals. This hypothesis fits with most of the currently available data, but these are very limited in number and require confirmation.

It is unknown which *in vitro* culture form, if any, corresponds to the hindgut promastigote of peripylarian *Leishmania* species. Its morphology and behaviour *in vivo* appear to be variable (see section 1.2.2.3.1), perhaps reflecting differences in the hindgut environment between flies. Conditions in this region are as yet unknown although it is presumably relatively barren when compared to anterior gut regions since most nutrients are absorbed by the fly in the abdominal and thoracic midguts and it is mostly waste matter that passes to the hindgut, either directly from the abdominal midgut or from the malpighian tubules. Potential energy sources for these hindgut

promastigote stages have yet to be identified (see section 1.3).

It is evident that the substrate utilisation and preference of the different life-cycle stages are yet to be determined. Results for *in vitro* cultured promastigotes are contradictory, which may reflect comparisons of unequivalent promastigote populations. It may be insufficient to group promastigotes simply by phase of growth as these usually contain several distinct promastigote forms and it is likely to prove beneficial to work on promastigote cultures which are at least dominated by one form. The variable results so far obtained show that more thorough investigations are needed.

Despite the gathering, albeit inconclusive, evidence for substrate selection by the different life-cycle stages it is clear that each is capable of catabolising three main energy substrates (glucose, amino acids and fatty acids) via pathways similar to, but distinct from, those which occur in mammals. Some of these are outlined in Figure 1.7. The complete complement of metabolic pathways involved in energy generation by *Leishmania* parasites is far from clear and many gaps in our knowledge still prevail. The energy metabolism of other vectorially transmitted trypanosomatids, particularly *Trypanosoma brucei*, is further advanced and provides information in areas which have yet to be researched or are uncertain for *Leishmania*. Some of the known stage-specific adaptations, as well as the common pathways of energy metabolism of *Leishmania* and how they differ from mammalian energy, are briefly discussed below.

1.6.1.1 Carbohydrates

Carbohydrate utilisation by *in vitro* grown promastigotes has been studied in considerable depth and is the subject of several reviews including Marr [1980], Cazzulo [1992] and Blum [1993a; b]. Most of these consider promastigotes to be one developmental stage and fail to make any distinction between multiplicative

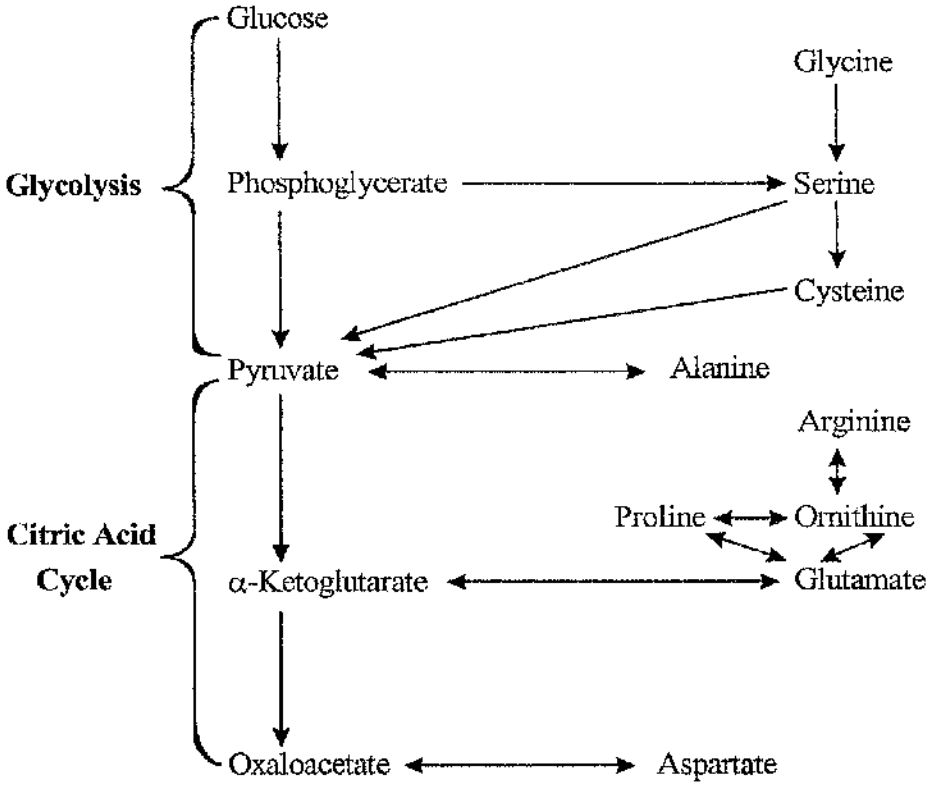


Figure 1.7: Interconversion of some amino acids and intermediates of carbohydrate metabolism. Fatty acid catabolism also is involved with breakdown products entering at the level of dihydroxyacetone phosphate (a glycolytic intermediate) and acetyl CoA (which feeds into the citric acid cycle).

promastigotes and metacyclic promastigotes. This is probably because few comparative studies have been carried out. The limited data which are available, however, suggest that metacyclic promastigotes of *L. donovani* oxidise glucose at a faster rate than promastigotes in the log phase of growth [Blum, 1996], consistant with the report, mentioned previously, that two key glycolytic enzymes are maximally expressed in the metacyclic stage [Mukkada *et al.*, 1974]. This pattern of glucose use may be species-dependent since investigation of glucose utilisation by *L. major* promastigotes reported that glucose oxidation occurred at a faster rate in log phase promastigotes than in stationary phase promastigotes [Keegan and Blum, 1990]. Amastigotes have been less extensively investigated than promastigotes, although it is known that they also catabolise glucose. One study which did include amastigotes compared the activity of several glycolytic enzymes of this stage of *L. mexicana* with those of promastigotes of the same species [Coombs *et al.*, 1982]. It was apparent that several of the glycolytic enzymes were less active in the mammalian stage than in the insect stage, which is perhaps related to the lower rate of energy generation and use by the intracellular stage in the life-cycle. For instance, most promastigotes are highly motile and for this would require energy not consumed by non-motile amastigotes. However, lower activities of enzymes may also suggest that glycolysis is not the only means of energy generation in amastigotes (see section 1.6.1.3). Investigations on promastigotes have collectively resulted in partial elucidation of the metabolic pathways involved in energy generation from catabolism of glucose [reviewed, for example, by Cazzulo, 1992; Blum, 1993a; b]. They have also provided limited data on how these pathways are modified by promastigotes during growth *in vitro* and in response to changing gaseous conditions.

Catabolism of glucose to pyruvate by promastigotes of *Leishmania* occurs via the glycolytic pathway (Figure 1.8), as it does in mammals. However, glycolysis in

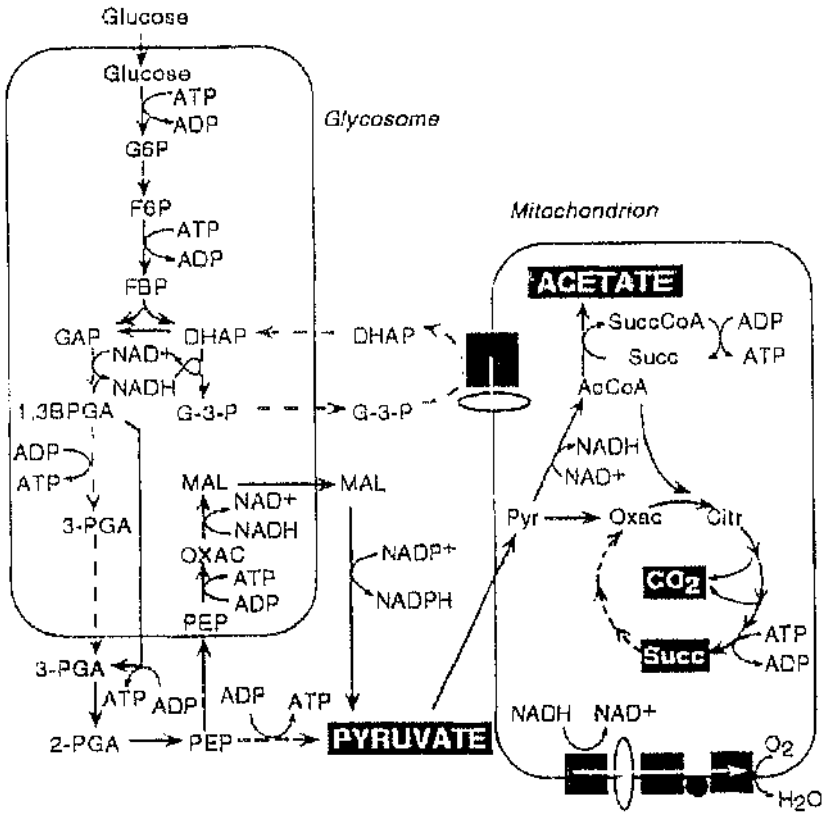


Figure 1.8: Main pathways of carbohydrate catabolism by *Leishmania* promastigotes. End-products are shown in boxes and dashed lines indicate relatively minor pathways. The enzyme complexes involved in electron transport are indicated by squares and the ubiquinone/ubiquinol pool is indicated by ellipses. Abbreviations: AcCoA, acetyl CoA; Citr, citrate; DHAP, dihydroxyacetone phosphate; FBP, fructose 2,6-bisphosphate; F6P, fructose 6-phosphate; GAP, glyceraldehyde 3-phosphate; G-3-P, glycerol 3-phosphate; G6P, glucose 6-phosphate; Mal, malate; Oxac, oxaloacetate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Succ, succinate; SuccCoA, succinyl-CoA; 1,3BPGA, 1,3-bisphosphoenolpyruvate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate.

trypanosomatids, including *Leishmania*, differs from conventional mammalian glycolysis in that the initial stages (from glucose to 3-phosphoglycerate) do not take place in the cell cytosol but are confined within organelles known as glycosomes [Opperdoes and Borst, 1977; Opperdoes, 1991; Michels *et al.*, 1997]. The final three glycolytic reactions, from 3-phosphoglycerate to pyruvate, take place in the cell cytosol. The end-product of these reactions, pyruvate, has a number of potential fates including excretion into the surrounding medium, transamination to alanine or transport into the mitochondrion where it is further degraded (see Figure 1.8). Within this organelle pyruvate is first oxidatively decarboxylated by pyruvate dehydrogenase to acetyl-CoA, which can then be further degraded to succinate and carbon dioxide by the citric acid cycle. A large part of the acetyl-CoA, however, does not enter the citric acid cycle, but is converted to acetate. Other end-products of glucose catabolism are D-lactate and glycerol which are excreted in addition to pyruvate, succinate, acetate, alanine and carbon dioxide (CO_2).

Thus *Leishmania* promastigotes have an energy metabolism in which a small part of the consumed carbohydrate is completely oxidised to carbon dioxide via the citric acid cycle, but in which also large amounts of partly oxidised products are produced from glucose. A small proportion of the pyruvate is also transaminated to alanine which is then excreted. Although several partly oxidised products are excreted by *Leishmania* promastigotes, these parasite stages do not possess a fermentative metabolism. The succinate produced during aerobic incubations of *Leishmania* promastigotes is mainly generated via an oxidative pathway involving part of the citric acid cycle (from oxaloacetate via citrate to succinate) and NADH oxidation by a respiratory chain in which oxygen acts as the final electron acceptor.

Leishmania promastigotes are reported to possess a classical respiratory chain

upon which they are strongly dependent for their energy production. Thus, incubation of promastigotes of several *Leishmania* species under anoxic conditions resulted in severe inhibition of glucose degradation which in turn caused inhibition of promastigote motility and proliferation [Van Hellemond and Tielens, 1997; Van Hellemond *et al.*, 1997]. A similar metabolic arrest under anaerobic conditions in the absence of CO_2 was reported for promastigotes of *L. major* by Darling *et al.* [1989b]. However, in this latter study this was apparently reversed and glucose consumption could be returned to the same rate as under aerobic conditions simply by inclusion of 5% CO_2 in the gas phase. In contrast, subsequent studies of glucose catabolism by promastigotes of several *Leishmania* species incubated under anaerobic conditions in the presence of CO_2 [Van Hellemond and Tielens, 1997; Van Hellemond *et al.*, 1997] found that although promastigotes have the ability to survive for periods of time without respiring, they have only a limited capacity for anaerobic functioning and die after prolonged inhibition of the respiratory chain. The promastigotes in their study were able to withstand at least 40 hours of metabolic arrest, induced by either starvation or lack of O_2. This attribute is likely to play an important role in promastigote survival within the variable conditions in the sandfly alimentary tract (see section 1.3).

1.6.1.2 Amino acids

Despite evidence highlighting the importance of amino acids as energy substrates for *Leishmania* parasites, particularly promastigotes, most studies have concentrated upon elucidation of the pathways involved in glucose catabolism (see section 1.6.1.1). However, some investigations on amino acid metabolism have been carried out and these have principally studied the requirement for amino acids by promastigotes during growth in culture.

Several amino acids have been reported to be essential to cultivation of promastigotes. Amino acids which could not be deleted from culture medium without affecting the growth of *L. tarentolae* promastigotes included proline, arginine, histidine, tryptophan, phenylalanine, serine, tyrosine, threonine, valine, leucine and lysine [Krassner and Flory, 1971]. All other amino acids could be removed, individually, without adversely affecting continuous culture. *L. braziliensis* and *L. donovani* required a similar but extended complement of amino acids [Steiger and Steiger, 1977]. Cysteine, glutamine, isoleucine and methionine were also deemed essential to growth of these species, while serine was not. These amino acids are thought to be mainly required for biosynthetic purposes, rather than or in addition to energy generation. Interestingly, alanine has been identifed as an important osmolyte of *Leishmania* promastigotes, apparently guarding against lysis resulting from acute changes in osmolarity [see below and reviewed by Blum, 1996].

Most of the studies regarding amino acid metabolism by *Leishmania* have focussed on proline utilisation since this amino acid is present in large quantities in haemolymph of some insects [in the tsetse fly, for example, Bursell, 1963] and is thought to serve as a major energy substrate in insect flight metabolism [Raghupathi and Campbell, 1969]. By monitoring promastigote growth in various culture media, it has been demonstrated that proline serves as an important energy source for cultured promastigotes of *L. tarentolae* [Krassner, 1969] and *L. donovani* [Krassner and Flory, 1972], although it was found to be dispensable for *L. tarentolae* provided ten other amino acids were present (arginine, histidine, tryptophan, phenylalanine, serine, tyrosine, threonine, valine, leucine and lysine). Proline was also implicated as one of fourteen amino acids required in addition to glucose for continuous cultivation of *L. donovani* and *L. braziliensis* promastigotes [Steiger and Steiger, 1977]. Clearly the

nutritional requirements of *L. donovani* differ in the aforementioned studies, a contradiction which may result from comparison of different parasite strains or different morphological forms. In this regard it has been demonstrated recently [Blum, 1996] that proline uptake and oxidation vary with increasing culture age. Log phase promastigotes of *L. donovani* consumed and oxidised proline from the culture medium at a rate of four-five times that of stationary phase promastigotes, indicating lower use by metacyclic promastigotes than by multiplicative promastigotes.

Closer inspection of proline metabolism by *L. tarentolae* [Krassner, 1969], *L. donovani* [Krassner and Flory, 1972] and *L. braziliensis* [Zeledon and de Monge, 1967] with a view to elucidation of the catabolic pathways involved have demonstrated the presence of the proline oxidase system in these *Leishmania* species.

The metabolism of two other amino acids, arginine [Bera, 1987; Blum, 1992] and leucine [Blum, 1991], which were reportedly essential for cultivation of several *Leishmania* species [Krassner and Flory, 1971; Steiger and Steiger, 1977] have been studied in promastigotes of *L. donovani*. Arginine catabolism occurs via the γ-guanidinobutyramide pathway [Bera, 1987; Blum, 1992], a reaction sequence which is also found in fungi of the *Actinomyces* group. In contrast to arginine, leucine degradation is thought to occur by a pathway similar to that of mammalian cells [Blum, 1991]. Catabolism is initiated by transamination to α-ketoisocaproate which on entry to the mitochondrion is converted to acetyl-CoA and acetoacetate by a series of degradative steps. Investigation of the pathways which occur subsequent to this in promastigotes of *L. donovani* indicate that all acetoacetate is converted to acetyl-CoA, some of which is then oxidised to CO_2 while the remainder is utilised for biosynthesis of fatty acids and other compounds. Studies on another *Leishmania* species, *L. mexicana*, have also implicated leucine as an important biosynthetic precursor of sterols [Ginger *et*

al., 1995]. Comparison of the rate of leucine oxidation in log phase and mid-stationary phase promastigotes of *L. donovani* showed that leucine oxidation decreases during this transition, similarly to acetate, alanine, glycerol and glucose catabolism by *L. major* [Keegan and Blum, 1990]. This is consistant with a switch in metabolism from use of exogenous substrates to mobilisation and catabolism of stored carbohydrate [Keegan and Blum, 1992] and lipid [Mallinson and Coombs, 1989] and perhaps a lower rate of biosynthesis in the non-replicating metacyclic forms.

The role of alanine in promastigote metabolism is not yet fully understood despite significant attention in recent years. It was found that extracellular alanine was oxidised by *L. panamensis* promastigotes at a similar rate to glucose [Keegan *et al*, 1987; Darling *et al.*, 1989a] and studies on the intracellular reserves of amino acids revealed an alanine pool within promastigotes and high levels of alanine aminotransferases in addition to other transaminases [Chaterjee and Ghosh, 1957; Le Blanq and Lanham, 1984; Simon *et al.*, 1983] accounting for formation of this pool. Intracellular interactions between glucose and alanine were investigated by Darling *et al.* [1989a] by means of radioactively labelled glucose. Labelling prior to incubation with promastigotes allowed the pathway of glucose metabolism to be studied. With time, label was observed in pyruvate and then alanine. A similar route was demonstrated, from pyruvate to alanine, following degradation of radioactively labelled proline via the proline oxidase system by promastigotes of *L. tarentolae* [Krassner, 1969] and *L. donovani* [Krassner and Flory, 1972]. Also using radioactive label, it was possible to monitor the rapid uptake and incorporation of exogenous alanine into the endogenous pool. Variation of the gaseous conditions during promastigote incubation in glucose resulted in changes to the intracellular pool. The reserve in *L. major* was partially consumed under 95% O_2/5% CO_2 while pO_2 reduction to 6% resulted in alanine

production [Keegan and Blum, 1990]. A small net production was also observed in promastigotes of *L. panamensis* in air [Darling *et al.*, 1989a]. These observed changes under varying pO_2 prompted the suggestion that control of alanine production or consumption is under the control of a low affinity oxygen sensor.

Further work [Darling and Blum, 1990; Darling *et al.*, 1990] has linked alanine metabolism with survival of promastigotes under hypo- and hyper-osmotic stress. *L. major* were observed to change shape rapidly from long slender promastigotes to short rounded promastigotes by addition of glucose (5·6 mM), 2-deoxyglucose (5·6 mM), fructose (5·6 mM), mannose (5·6 mM) or proline (10 mM). This shape change could be prevented or stimulated through raising or lowering the osmolarity of the surrounding medium. Accompanying conditions of hypo-osmotic stress was the release of alanine and other ninhydrin-positive substances from promastigotes which underwent rapid shape change, becoming shorter and more rounded. Consequently, Darling *et al.* [1990] proposed that alanine release serves to prevent excess swelling in conditions of hypo-osmotic stress. A similar role for alanine has been suggested for *Giardia intestinalis* [Park *et al.*, 1995]. The mechanisms of cell signalling leading to alanine production and release are unknown, however, a study by Darling and Blum [1990] suggests that the shape change does not involve any of the common second messengers such as divalent calcium ions (Ca^{2+}). Subsequent investigation indicated that protein kinases and arachidonic acid are involved [reviewed by Blum, 1996].

A closer inspection of the changes which occur when promastigote are subjected to osmotic stress revealed alterations to the flow of carbon along several metabolic pathways, including those which involve alanine. Investigation of alanine oxidation under hyper-osmotic stress revealed that these conditions result in an inhibition of metabolism. Since similar results were observed with [1-^{14}C]glutamate, [1(3)-

[^{14}C]glycerol, [1-^{14}C]acetate, [6-^{14}C]glucose and [1-^{14}C]leucine it appears that acute hyper-osmotic stress causes an inhibition of mitochondrial oxidation consequently preventing use of cytoplasmic alanine. Hypo-osmotic stress has a different effect on alanine metabolism, resulting in its consumption from intracellular pools in addition to its excretion into the surrounding medium. It is thought these conditions increase the rate of flux through the citric acid cycle which is consistant with an increase in the rate of CO_2 formation and decrease in the rates of pyruvate and acetate formation. Clearly, further studies on the effects of changing osmolarity on the metabolism of glucose and other substrates are required for complete elucidation of the pathways which are affected in addition to their relevance *in vivo*.

1.6.1.3 Fatty acids

Fatty acids appear to be important energy substrates of some life-cycle stages of *L. mexicana*, particularly amastigotes which oxidised fatty acids at a rate approximately 10-fold higher than promastigotes [Hart and Coombs, 1982]. Exhaustive use of non-esterified fatty acids by both promastigotes and amastigotes was observed in this study with both stages exhibiting a high capacity for their use. Amastigotes appeared to catabolise fatty acids completely, producing CO_2, whereas in promastigotes [Beach *et al.*, 1979] the non-essential fatty acids were incorporated into the cell.

Similarly, to Hart and Coombs [1992], Blum [1987] detected an increase in the consumption rate of fatty acids during the *in vitro* transformation of *L. panamensis* promastigotes to amastigotes as stimulated by heat-shock. Interestingly, Blum also noticed that the consumption rate of two fatty acids, laurate and ecruate, varied with promastigote culture age. Promastigotes in the late-stationary phase of growth oxidised both of these fatty acids at a rate of approximately two-fold that of promastigotes in

earlier growth phases. In relation to this, it is interesting to note that promastigotes in the stationary phase of growth contain numerous large lipid droplets which are present but substantially reduced in number in promastigotes in the log phase of growth [Mallinson and Coombs, 1989]. Perhaps these represent an energy store for use by infective metacyclics when nutrient availability is poor or are required by the parasite during transformation to the amastigote stage.

Little is known concerning the pathways involved in fatty acid oxidation by *Leishmania* parasites despite their apparent utilisation by both amastigote and promastigote stages of *L. mexicana*. In mammalian cells, degradation occurs in the mitochondrion via a series of reactions known collectively as the β-oxidation pathway. Several enzymes of this pathway have also been identified in *L. mexicana*, some of which appear to be glycosome-related, despite evidence that a large proportion of fatty acid oxidation occurs in the mitochondrion [Hart and Coombs, 1982; Hart and Opperdoes, 1984].

1.6.2 Proteinases

Stage-regulation of proteinase activities has been reported for several *Leishmania* species, notably *L. mexicana* [see Coombs and Mottram, 1997]. Qualitative and quantitative changes have been demonstrated to occur during the life-cycle [North and Coombs, 1981; Pupkis and Coombs, 1984; Lockwood *et al.*, 1987] and these have provided phenotypic markers of amastigotes, metacyclics and multiplicative promastigotes of this species. Many of the cysteine proteinases have been characterised in detail and it is thought that the amastigote enzymes are crucial for survival and multiplication of the mammalian stage of the parasite in macrophages and are thus potential drug targets. Several of the multiple, high-activity enzymes which are present

in amastigotes are also thought to occur in metacyclics but are apparently absent or of low activity in multiplicative promastigotes. In addition, metacyclic promastigotes contain a stage-specific cysteine proteinase activity. Although these enzymes have been well characterised, their function *in vivo* has yet to be fully elucidated.

In contrast to *L. mexicana*, the proteinases of *L. panamensis* have been little studied. Developmental changes such as those which occur in *L. mexicana* would suggest that each form is biochemically distinct and detection of stage-specific enzyme activities would provide phenotypic markers of the stages.

1.6.3 Nucleases

Nuclease activities of the *in vitro* life-cycle stages of *L. mexicana* were analysed by substrate SDS-PAGE and shown to undergo developmental regulation [Bates, 1994]. The two forms of the 3'-nucleotidase/nuclease which were detected varied in activity in the parasite stages investigated and exhibited stage-specific profiles for amastigotes (lesion and axenically cultivated), multiplicative promastigotes and metacyclic promastigotes. One of the aforementioned activities was reported to be exclusive to the promastigote stage. Furthermore, its activity was considerably reduced in metacyclic promastigotes when compared with multiplicative promastigotes. Its apparent surface location and its similarity in molecular weight and inhibitor sensitivity to a 3'-nucleotidase/nuclease isolated from *L. donovani* promastigotes [Campbell *et al.*, 1990] led to the proposal that the *L. mexicana* enzyme probably functions in purine salvage from the sandfly host. This is consistant with the finding that *Leishmania* parasites are unable to synthesise the purine ring *de novo* and are dependent upon an exogenous supply from the host [Steiger and Steiger, 1977; Marr, 1991; Berens *et al.*, 1995]. Furthermore, reduced activity in the metacyclic form ties in with reports that this stage

is non-dividing since the requirement for precursors of DNA would be significantly lower than for multiplicative promastigotes. Interestingly, enzymes with similar activities or properties to these nucleases have yet to be identified in mammalian cells, making them potential candidates for chemotherapeutic attack.

1.6.4 Surface molecules

The parasite surface plays a crucial role in many host-parasite interactions both in the sandfly and in the vertebrate host. Investigation of surface molecules by comparison of different life-cycle stages has identified developmental changes for which the functional significance have been studied. Two such developmentally-regulated surface molecules are the metalloproteinase gp63 and lipophosphoglycan (LPG). Each exhibit alteration, either differential expression or modification, as the parasites transform between developmental stages.

1.6.4.1 Metalloproteinase gp63

The glycoprotein proteinase, gp63, has been identified in a number of *Leishmania* species [Bouvier *et al.*, 1987] and has been claimed to have potential as a vaccine for protection against leishmaniasis [Frommel *et al.*, 1990]. In promastigote stages, the molecule is membrane-bound [Bouvier *et al.*, 1987] and is distributed over the entire parasite surface, including the flagellum [Fong and Chang, 1982; Russell and Wilhelm, 1986; Kweider *et al.*, 1987; Etges and Bouvier, 1991; Coombs and Mottram, 1997]. Differential expression during *in vitro* and *in vivo* growth has been demonstrated for *L. braziliensis* [Kweider *et al.*, 1987] and *L. major* promastigotes [Davies *et al.*, 1990] and it was shown that development of infective metacyclics of these species is accompanied by a progressive increase in the expression of this antigen on the parasite

surface. A similar progression was observed *in vitro* for promastigotes of *L. mexicana* but not for promastigotes of *L. chagasi* [Kweider *et al.*, 1987], indicating that this is not a common occurrence in all species of *Leishmania*. Nevertheless, gp63 has been shown to occur on the promastigote surface of all species of *Leishmania* so far examined [Bouvier *et al.*, 1987] and is thought to play a crucial role in uptake and survival in the vertebrate macrophage. Lectin interactions between glycan residues of gp63 and mannosyl-fucosyl receptors on the macrophage surface act to target the parasite to its host cell [Blackwell *et al.*, 1985], while recognition of the inactivated form of the complement component 3 (C3bi), thought to be produced from cleavage of C3b by gp63, on the surface of promastigotes by the complement receptor 3 (CR3) of the phagocyte is thought to trigger the parasite's uptake [Russell and Wright, 1988]. gp63 is thought to perform another role while promastigotes develop in the sandfly gut [reviewed by Schlein, 1993]. Promastigotes within the blood meal concentrate in "nests" close to the peritrophic membrane and with time the dark colour of the blood haemoglobin surrounding these nests disappears. It is thought that this clearing results from digestion of the haemoglobin by parasite proteinases, possibly gp63, and aids promastigote escape from the peritrophic membrane. *In vitro* studies have reported that secretion of parasite-derived chitinolytic enzymes (see section 1.6.5), which are capable of degrading the chitinolytic framework of the peritrophic membrane, is inhibited in the presence of haemoglobin. Thus the metalloproteinase gp63, which has broad substrate specificity and will hydrolyse haemoglobin *in vitro*, potentially facilitates promastigote release from the peritrophic membrane - an essential step without which promastigotes would probably be expelled along with the residual blood meal.

The presence of gp63 and its location in amastigote stages of *Leishmania* are a matter for debate. Initial studies carried out to locate gp63 in the amastigote stage were

negative or inconclusive [Etges and Bouvier, 1991], despite identification of abundant, constitutively expressed messenger RNA [Button *et al.*, 1989; Wilson and Hardin, 1990]. Frommel *et al.* [1990], however, were able to demonstrate gp63 in both amastigote and promastigote stages by *in situ* fluorescence and Western blot analysis. Comparison of the molecules recognized by the latter technique highlighted a small size difference between the gp63 of amastigotes and promastigotes which these authors proposed was due to differences in post-translational modifications. This hypothesis may also explain previous failed attempts to detect amastigote gp63 using certain monoclonal antibodies [Chang *et al.*, 1986]. More recently, a study on amastigote gp63 of *L. mexicana* identified that the protein is expressed at lower levels, than in promastigotes, is soluble with an acidic pH optimum and is located in lysosomes [Ilg *et al.*, 1993]. The soluble nature of the protein in this parasite stage reflects the lack of a glycosyl-phosphatidylinositol (GPI)-anchor and accounts for the size difference between amastigotes and promastigotes discussed above [see Coombs and Mottram, 1997].

1.6.4.2 Lipophosphoglycan

LPG expression appears to be largely restricted to promastigote stages and is found only in small quantities or is undetectable in amastigotes [reviewed by McConville and Ferguson, 1993]. It comprises the major surface molecule of promastigotes where it forms a densely organised glycocalyx which plays a variety of roles to promote promastigote survival in the sandfly gut [reviewed by Sacks *et al.*, 1994]. It is therefore not surprising that LPG has been identified on promastigotes of all *Leishmania* species so far examined [Sacks, 1992]. It is thought that its presence on the surface of promastigotes of suprapylarian *Leishmania* species which occupy the sandfly midgut is critical for establishment of infection since the LPG is believed to mediate

promastigote attachment in this region and so prevents their expulsion with the blood meal remnant. Identification of lectin-like receptors on the sandfly midgut epithelia that act like haemagglutinins could possibly be candidate receptors for LPG [Pimenta *et al.*, 1992; Jacobson, 1995; Jacobson and Doyle, 1996], although this has yet to be fully investigated. Although present on late-log phase promastigotes and metacyclic promastigotes, the LPG has been shown to undergo considerable alteration during metacyclogenesis. In both of these promastigotes the LPG molecule is comprised of linear chains of phosphorylated oligosaccharide repeat units, anchored to the membrane via a glycosyl-phosphatidylinositol anchor [McConville *et al.*, 1990]. During metacyclogenesis of *L. major*, there is an approximate doubling in the number of repeat units per molecule and a change in the terminal sugar residue on the side branches. The terminal sugar galactose is replaced by arabinose. This change has facilitated the purification of *L. major* metacyclics since all non-infective promastigotes are agglutinated by the lectin peanut agglutinin (PNA). This application has permitted an in-depth study of pure populations of PNA- cells of *L. major* (believed to be metacyclic forms).

Modification of LPG is also thought to be important for development of promastigote forms in the midguts of infected sandflies. Interactions between LPG and the midgut epithelia facilitate binding of promastigotes in this region. It has been proposed that modifications to the LPG associated with metacyclogenesis alter this binding potential such that promastigotes are unable to attach, encouraging forward migration with the consequence that differentiated forms are in a position to be regurgitated and inoculated during the next blood meal.

The putative roles for LPG are multi-fold. It is thought that extension of LPG during metacyclogenesis is involved in protection of metacyclic forms from attack by

the vertebrate host immune system. The densely packed elongated chains act as a physical barrier inhibiting antibody access to surface molecules, including gp63 [Karp *et al.*, 1991] and therefore aid in evasion from immunological attack. Furthermore, changes in LPG appear to have a modulatory effect on the binding of promastigotes to receptors on human macrophages [McConville *et al.*, 1992] a characteristic which appears to be a key factor contributing to their increased infectivity. The metacyclic specific changes promote complement activation and C3 deposition in a non-lethal manner, such that opsonization of promastigotes leads to attachment and uptake via macrophage receptors [Sacks, 1992].

1.6.5 Chitinases

Chitinases are enzymes which hydrolyse chitin, a (1-4)-β homopolymer of N-acetylglucosamine. Such activities have been detected in culture supernatants of promastigotes of several species of *Leishmania* and it is thought that they are also produced by promastigotes *in vivo* [see review by Shahabuddin and Kaslow, 1993]. Whether, like the proteinases and nucleases of *L. mexicana*, these enzymes exhibit stage-specificity has yet to be confirmed although roles have been suggested for parasite chitinases in both establishment of early infections in the fly and in transmission to the vertebrate host during feeding [reviewed by Shahabuddin and Kaslow, 1993]. Hydrolysis of the peritrophic membrane by chitinases secreted by promastigotes in the blood meal is thought to permit their release into the lumen of the midgut, hence preventing expulsion along with the digested blood. Moreover, there is also evidence to suggest that chitinases released by promastigotes in the stomodeal valve region of the gut facilitate parasite transmission through hydrolysis and damage to the main valve of the feeding system. This hinders fly feeding and results in regurgitation and

redistribution of parasites from the midgut to the food pumps and proboscis. Thus during feeding there is a constant supply of parasites for the repeated infective probes.

Clearly there remain many gaps in our knowledge of the biochemistry of *Leishmania* parasites, particularly with amastigotes and metacyclic promastigotes. Little is known of the different promastigote stages which occur in the sandfly. Until procedures are developed which permit isolation and characterisation of these stages, investigations rely upon *in vitro*-generated promastigotes. Identification of a similar sequential development of different morphological forms *in vitro* and *in vivo* provides some confidence of similarity between these stages, however this has yet to be confirmed. Development of the apparently complete life-cycle *in vitro* for *L. mexicana* provides a valuable means of obtaining large amounts of cells free from host contamination for metabolic and biochemical study. Characterisation of *in vitro* forms should clarify whether each is biochemically distinct, while the choice of parameters which are investigated may allow correlation of *in vitro* and *in vivo* forms. It is hoped that investigation of these cells will elucidate the adaptations of each stage and determine potential targets for chemotherapeutic attack.

1.7 Aims of project

The overall general aim of this project was to elucidate how the different developmental stages of leishmanias are adapted, at the molecular level, to their particular microenvironments. Preliminary studies identified at least three distinct promastigote forms in *in vitro* cultures of *L. panamensis* and *L. mexicana* which morphologically resemble promastigotes at different localities in an infected sandfly gut. One main aim was to investigate the possibility that these morphologically

different forms are in fact biochemically distinct promastigote stages. If so, this would lead to the development of biochemical criteria for identification of each form. A second aim was to provide information on the adaptations of each form for its particular living environment in the fly. One form of particular interest is the hindgut promastigote, apparently unique to peripylarian species of *Leishmania* such as *L. panamensis*. Comparison of *L. panamensis* with *L. mexicana* was the approach adopted in an attempt to identify biochemical differences that could be correlated to hindgut development. The final aim was to provide information on the stimuli responsible for differentiation of the different forms in order to gain some insight into the mechanisms involved and provide further data on whether or not they are distinct developmental stages in the life-cycle.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Reagents

Unless otherwise stated, the reagents used were of analytical grade from Sigma (Poole, UK). In experiments where the precise source or grade of a reagent might affect the reproducibility of experiments, details are given in the text.

2.2 Parasites

The *Leishmania* species and lines used in this study were *L. mexicana* (MNYC/BZ/62/M379), *L. panamensis* (strain 1, MHOM/PA/71/LS94 and strain 2, MHOM/PA/67/BOYNTON, both kindly donated by Simon Croft) and *L. major* (MHOM/SA/83/RKK2). All manipulations of live parasites were carried out in a class II safety cabinet which had been sterilised with U.V. light and 70% (v/v) ethanol. A side fastening laboratory coat and latex or vinyl gloves were routinely worn.

2.2.1 Promastigotes

2.2.1.1 Transformation of lesion amastigotes to promastigotes

Promastigotes of *L. mexicana* and *L. panamensis* used in all studies were initially obtained through transformation of lesion amastigotes in crude preparations (section 2.2.2.2.1). *L. mexicana* amastigotes were transformed to promastigotes in 10 ml cultures in 25 cm^2 flat bottomed flasks (Gibco Life Technologies) inoculated at 10^6 ml^{-1} at 25°C in HOMEM medium [Berens *et al.*, 1976] supplemented with 10% (v/v) heat-inactivated foetal calf serum (HIFCS, from Labtech) in the absence of

antibiotics and with air as the gas phase. This medium will be subsequently referred to as complete HOMEM medium. *L. panamensis* was found to be more fastidious than *L. mexicana* and would not transform under these conditions. Promastigotes of this species were obtained through transformation subsequent to inoculation of 0·2-0·5 ml of crude lesion preparation (see 2.2.2.2) containing approximately $2x10^5$ amastigotes into the liquid phase of 3° Evans' modified Tobie's medium [Evans *et al.*, 1984] and incubated at 25°C with air as the gas phase. Freshly-transformed promastigotes of *L. mexicana* and *L. panamensis* were sub-passaged once in complete HOMEM medium before cryopreservation (see section 2.2.5). These stabilated cultures provided a source of freshly transformed parasites for all subsequent work.

L. major promastigotes were cultured directly from stabilated cultures of freshly-transformed amastigotes.

2.2.1.2 Routine culture of promastigotes

L. panamensis, *L. mexicana* and *L. major* promastigotes were cultured axenically at 25°C in complete HOMEM medium in 25 cm^2 flat bottomed culture flasks with air as the gas phase. The flasks were used flat to maximise the surface area for gas exchange. Ten ml stock cultures of promastigotes were sub-passaged routinely when in the mid-late-log phase of growth (approximately 10^7 ml^{-1}) to give a starting density of $5x10^5$ cells ml^{-1}. Some experimental cultures involved different starting populations and densities, as specified in the text. Cultures which had been sub-passaged about 15 times were discarded and new cultures set up using cryopreserved promastigotes.

2.2.1.3 Identification of promastigote forms

The morphology of promastigotes during *in vitro* culture was routinely determined by microscopic observation of parasites in thin smears which were air-dried, methanol-fixed and Giemsa-stained (section 2.3). Individual cultures were set up at $5x10^5$ promastigotes ml^{-1} from stock cultures in the mid-log phase of growth (ca. $8x10^6$ ml^{-1}) and monitored daily through to stationary phase. The latter was defined to start when the cell density was equal to or less than that on the previous day and with the majority of promastigotes still motile. Three morphologically distinct promastigote forms (morphotypes 1, 2 and 3) were initially identified by light microscopy at x1000 and cell body lengths and widths were measured using a calibrated eye piece graticule (1 unit = 1·25 μm). Promastigotes with bent cell bodies or daughter flagella were excluded. After classification into distinct morphotypes, careful examination of several cultures for these three forms enabled judgements to be made on when each form would predominate and so facilitated their production for study.

For these investigations, 50 ml cultures of *L. panamensis* and 100 ml cultures of *L. mexicana* were set up in complete HOMEM medium using an inoculum of mid- to late-log phase cells (ca. 10^7 ml^{-1}) to a density of $5x10^5$ ml^{-1}. These cultures were used to provide populations of the different promastigote forms. Autoclaved flat bottomed, glass bottles were used and these were positioned at a slight angle from flat in order to prevent culture from reaching the lid and yet to provide a large surface area for gas exchange. Cells were harvested on day 1/2 for populations containing morphotype 1 promastigotes and day 3/4 for morphotype 2s. Morphotype 3 populations were cells in the stationary phase of growth. Cultures were examined by phase contrast light microscopy and populations were harvested (section 2.2.6) if abundant in the appropriate promastigote form.

2.2.1.4 Metacyclic promastigotes

2.2.1.4.1 Cultures of metacyclic promastigotes

The method for producing metacyclic promastigotes of *L. mexicana* reported by Bates and Tetley [1993] was used in some studies. This involved sub-passage of mid-log phase promastigotes into Schneiders' Drosophila Medium (Gibco Life Technologies) which was supplemented with 20% (v/v) HIFCS and titrated to pH 5·5 with 1 M hydrochloric acid (this medium is designated as complete SDM). These cultures were incubated at 25°C with air as the gas phase until they reached the stationary phase of growth. For most studies, however, stationary phase cultures of promastigotes of low sub-passage grown in complete HOMEM medium were used. These proved more satisfactory than the SDM cultures, as long as promastigotes that had not been serially sub-passaged more than 10 times were used.

2.2.1.4.2 Purification of metacyclic promastigotes of *L. major*

Metacyclic promastigotes of *L. major* were purified using a slightly modified version of the peanut agglutination method reported by Sacks *et al.* [1985]. Briefly, promastigotes in the stationary phase of growth were washed twice in sterile phosphate buffered saline pH 7·4 (PBS, 0·01 M phosphate buffer, 2·7 mM KCl, 0·137 M NaCl, pH 7·4) before resuspension at 1-3x10^8 ml^{-1} (final density) in 0·9 mls of PBS and final addition of 0·1 ml of filter-sterilised peanut agglutinin (1 mg ml^{-1} stock). The cell suspension was then left at room temperature for approximately 30 minutes to allow for agglutinated cells to settle. Subsequently the supernatant was carefully removed, leaving the pellet intact, and the volume was made up to 10 mls with PBS. The cells were then washed twice in PBS (1600 g for 10 minutes) with the cell densities being determined after the first wash.

2·2·2 Lesion amastigotes

2.2.2.1 Initiation of infections in mice

Infections in female BALB/c mice were initiated through sub-cutaneous inoculation of stationary phase promastigotes of *L. panamensis* or *L. mexicana* grown in complete HOMEM medium or complete SDM in 25 cm^2 culture flasks with air as the gas phase. The cells were pelleted by centrifugation at 1600 g for 10 minutes and resuspended in incomplete HOMEM medium (containing no HIFCS) at $5x10^7$ cells ml^{-1} immediately prior to inoculation of 0·1 ml into the shaven rump of a 6-8 week old mouse.

2.2.2.2 Isolation of amastigotes from lesions

2.2.2.2.1 Crude preparations

The most frequently used procedure for isolation of amastigotes resulted in a crude preparation contaminated with host cells. Such a preparation was used for transformation of amastigotes to promastigotes (section 2.2.1.1). The procedure was carried out asceptically and 25 $\mu g\ ml^{-1}$ and gentamicin sulphate was included in initial cultures to limit bacterial growth. Culled mice were drenched in 70% (v/v) ethanol before the lesion was excised. Once removed, lesion tissue was cut away from any skin and pressed through a piece of fine gauze, using a 5 ml syringe plunger, into 10 ml of incomplete HOMEM medium. Macrophages and other large host cell debris were disrupted by shearing the suspension by repeated passage through a 26 gauge needle and the released amastigotes were counted using a haemocytometer and either pelleted, after one wash in incomplete HOMEM medium, and stored at -70°C or inoculated into medium for transformation to promastigotes (section 2.2.1.1).

2.2.2.2.2 Amastigote purification

Relatively pure preparations of *L. mexicana* amastigotes were obtained using the method described by Hart *et al.* [1981a]. Briefly, this involved excision of the lesion, as for crude preparations (section 2.2.2.2.1), which was rinsed in PSGEMKA (a suspension medium comprising phosphate buffer, sodium chloride, albumin, glucose, EDTA, $MgCl_2$ and KCl) rather than HOMEM medium, and then chopped up and carefully disrupted before grinding. Saponin at a final concentration of 125 $\mu g\ ml^{-1}$ was then added to the suspension to lyse remaining red blood cells and the amastigotes were washed three times at 1250 g for 15 minutes each in PSGEMKA. The final purification step, before final washing (3 times at 1250 g for 15 minutes), involved passing the cells through a Sephadex G25 (Pharmacia Biotech) column. Before the final wash, the cell density was determined and then pellets containing known numbers of amastigotes were either used immediately or stored at -70°C.

2.2.3 Axenic amastigotes

A variety of conditions were used in attempts to achieve axenic cultures of amastigotes of *L. panamensis*. Stationary phase promastigote cultures in complete HOMEM medium (pH 7·5 and pH 5·5) and complete SDM (pH 7·0 and pH 5·5) were sub-passaged to a starting density of $5x10^5$ cells ml^{-1} into HOMEM medium or Schneiders' medium supplemented with 20% (v/v) HIFCS which had been adjusted to pH 4·0, pH 4·5, pH 5·0, pH 5·5, pH 6·0 and pH 7·5 in flat bottomed 25 cm^2 culture flasks and incubated at 32°C, 34°C, or 37°C. Parasites were also cultured under different gaseous conditions, air or 95% air/5% CO_2, and concentrations of HIFCS, 10%, 20% and 30% (v/v). Growth of amastigotes directly from crude preparations of lesion amastigotes was also attempted. These were inoculated at $10^6\ ml^{-1}$ into complete

SDM containing 15 μg ml^{-1} gentamicin sulphate and incubated at 32°C in flat bottomed culture flasks with air as the gas phase. Cell morphology was examined using phase contrast light microscopy and densities determined using a haemocytometer.

Axenic cultures of *L. mexicana* amastigotes were obtained by 2 means; through direct culture of crudely harvested lesion amastigotes (section 2.2.2.2.1 and above) and by differentiation of stationary phase promastigotes cultured in complete HOMEM medium. Promastigotes or amastigotes were inoculated to a starting density of 10^6 ml^{-1} into complete SDM containing 15 μg ml^{-1} gentamicin sulphate and incubated at 32°C in flat bottomed culture flasks with air as the gas phase. Cryopreserved amastigotes (section 2.2.5) were also used to initiate axenic cultures under the same conditions. When amastigotes reached the late-log phase of growth they were sub-passaged into fresh medium to a starting density of 10^6 ml^{-1} following breakdown of clumps by shearing through a 26 gauge needle.

2.2.4 Determination of cell densities

Cell densities were determined using an Improved Neubauer Haemocytometer. With the exception of promastigotes, all counts were carried out on live cells. Promastigotes were fixed in 4% (v/v) formaldehyde in PBS immediately prior to counting. After a sample was loaded onto the haemocytometer it was left for a few minutes to allow the cells to settle, but not long enough for the chambers to dry out. At least 100 cells were counted, except for when cultures were less dense than $2x10^6$ ml^{-1}. In these cases all promastigotes in the 25 squares of 16 were counted.

2.2.5 Cryopreservation of parasites

Lesion amastigotes of *L. mexicana* and recently transformed promastigotes of *L. mexicana* and *L. panamensis* were cryopreserved in liquid nitrogen as follows. Cultures of mid-late log phase promastigotes or crudely harvested lesion amastigotes were mixed with an equal volume of complete HOMEM medium supplemented with 14% (v/v) dimethylsulfoxide (DMSO) or glycerol. One ml aliquots were distributed into cryotubes which were wrapped in cotton wool, placed inside a polystyrene box and cooled slowly by placing in a -70°C freezer overnight. The frozen cells were then cooled further in liquid nitrogen and stored in liquid nitrogen until required.

On removal from liquid nitrogen, cells were quickly transferred to the safety cabinet which had been pre-warmed using a bunsen. The tubes were swabbed with 70% (v/v) ethanol and the caps were loosened to prevent pressure build up. The heat from the bunsen caused the stabilates to thaw rapidly and revived cells were immediately diluted 10-fold with the appropriate culture medium. Promastigotes were cultured directly but amastigotes were washed (1250 g for 10 minutes) once in this medium to remove DMSO or glycerol.

2.2.6 Harvesting of parasites

Cells in axenic culture were routinely washed three times (1250 g for 15 minutes) in incomplete HOMEM medium, PBS, 0·25 M sucrose or HEPES saline (10 mM HEPES, 145 mM NaCl, pH 7·2). The wash medium used depended on the study undertaken and is stated in the appropriate sections, where deemed important. Washed parasites were used immediately or pelleted by centrifugation at 1600 g for 15 minutes in a microfuge and stored at -70°C.

J774G8 cells which were uninfected or infected with amastigotes of *L. panamensis* were harvested as follows. Attached macrophages were dislodged from the flask surface using a cell scraper and the resulting cell suspension was centrifuged at 1250 g for 15 minutes in 0·25 M sucrose. The pellet of infected cells which formed was sheared through a 26 gauge needle several times to break open remaining macrophages and to break up clumps of amastigotes while the pellet of uninfected macrophages was left intact. The volume of both pellets, one containing intact uninfected macrophages and the other amastigotes and macrophage debris, was made up to 5 mls with 0·25 M sucrose and a differential cell count was made using an Improved Neubauer Haemocytometer. The cells were then pelleted at 1250 g (15 minutes) and stored at -70°C.

2.3 Staining parasites

Aliquots of cultures (0·5-1 ml) were centrifuged at 1600 g for 5 minutes in a microfuge and the resulting pellets resuspended in approximately 50 µl of spent medium before preparation of thin smears using approximately 10 µl of this concentrated culture. These were quickly air dried and fixed in absolute methanol prior to staining for 10 minutes in 10% (v/v) Giemsa's stain in 10 mM phosphate buffered saline, pH 7·2.

2.4 Dialysis of HIFCS

HIFCS (ca. 30 ml) was pippetted asceptically into lengths of Visking Tubing (cut off of ca. 10 kDa) which had been knotted at one end. The other ends were subsequently tied such that the resulting bags was relatively flaccid. These were placed individually into beakers containing ca. 250 ml of autoclaved ultrapure water (see

section 2.10.2.1.2) which were covered with tinfoil and incubated with a magnetic stirrer overnight at 4°C. The next day the water was decanted and replaced by fresh autoclaved ultrapure water and the procedure repeated. On the second day the bags were removed and the dialysed HIFCS was filter sterilised (0.2 µm filter), aliquotted and stored at -70°C. Frozen aliquots were defrosted when required.

2.5 Determination of medium osmolarity

Medium osmolarity was measured using a Westcor 5500 Vapor Pressure Osmometer. Promastigote cultures were initiated at $5x10^5$ ml^{-1} in complete HOMEM medium and allowed to progress to the stationary phase of growth. Samples of fresh (before addition of promastigotes) and spent medium, from days 1, 2, 3, and 5 of culture, were collected. Promastigotes were removed by centrifugation at 1600 g for 5 minutes in a microfuge and the spent media were drawn off using Pasteur pipettes, taking care not to disturb the cell pellet. A sample of each medium was checked by light microscopy to ensure efficient removal of promastigotes and the media were frozen at -70°C until analysed. Fresh complete HOMEM medium which was diluted with double distilled deionised water was also analysed.

2.6 SDS-PAGE

2.6.1 Production of cell extracts

Homogenates of parasites were routinely produced by addition of cold (4°C) 0·25% (v/v) Triton X-100 (± proteinase inhibitors depending on the analysis, see appropriate section) to frozen cell pellets with subsequent gentle aspiration with a Gilson pipette tip. The resulting lysates were divided into pellet and supernatant fractions. This involved centrifugation of homogenates at 1600 g for 10 minutes in a

microfuge, the supernatants being removed and stored separately. Pellets were resuspended in chilled 0·25% (v/v) Triton X-100 to the same volume as was used for the initial lysis and passed through a 26 gauge needle to ensure adequate mixing.

Parasite samples were kept on ice and mixed with an equal volume of double strength gel sample buffer, either reducing (in all cases except for nuclease gels) or non-reducing (nuclease gels), [Hames, 1990] prior to electrophoresis.

2.6.2 Protein gels

Cell homogenates were generated as in section 2.6.1 with one change to the procedure. The proteinase inhibitors trans-epoxylsuccinyl-$_L$-leucylamido-(4-guanidino)butane (E-64), phenylmethylsulfonylfluoride (PMSF) and 1,10-phenanthroline were included in the 0·25% (v/v) Triton X-100, at 10 μM, 1 mM and 10 mM respectively, to prevent hydrolysis of proteins.

Proteins in pellet and supernatant fractions were separated by electrophoresis using 10% (w/v) acrylamide minigels and were stained with Coomassie Blue. Gels were subsequently destained with a solution containing 12·5% (v/v) methanol and 10% (v/v) acetic acid. Apparent molecular masses were deduced by addition of molecular mass markers to the gel prior to electrophoresis.

2.6.3 Proteinase detection using substrate SDS-PAGE

Proteinases in cell lysate supernatants, lacking proteinase inhibitors, were analysed following separation in 10% (w/v) SDS-PAGE gels containing 0·2% (w/v) co-polymerized porcine gelatin as a proteinase substrate [Lockwood *et al.*, 1987]. After electrophoresis the gels were incubated for 30 minutes in 2·5% (v/v) Triton X-100 on a shaking table at room temperature to remove SDS and re-nature the enzymes. This was

followed by an incubation period (2 hours for *L. mexicana* and approximately 20 hours for *L. panamensis*) at 37°C with gentle agitation in the appropriate buffer. Sodium acetate buffer at pH 5·5 including 1 mM DTT was used routinely for *L. mexicana* proteinases. A range of buffers and pH values were initially tested to find an optimum for proteinases of *L. panamensis*; glycine pH 3·5, acetate pH 3·5, acetate pH 4·5, acetate pH 5·5, sodium hydrogen maleate pH 5·5, sodium hydrogen maleate pH 6·5, phosphate pH 6·5, phosphate pH 7·5, tris pH 7·5, tris pH 8·5, hydrogen borate pH 8·5 and hydrogen borate pH 9·5. Subsequently, hydrogen borate pH 8·5 was used routinely. Following gelatin hydrolysis, gels were fixed and stained with Coomassie Blue and destained in a solution containing 12·5% (v/v) methanol and 10% (v/v) acetic acid [Lockwood *et al.*, 1987]. Apparent molecular masses were deduced by addition of molecular mass markers to the gel prior to electrophoresis. To enable partial characterisation of *L. panamensis* proteinases, the proteinase inhibitors E-64, PMSF and phenanthroline were included in the incubation buffer at final concentrations of 10 μM, 1 mM and 10 mM, respectively. One gel slice was incubated in buffer plus 5% (v/v) methanol to control for those inhibitors dissolved in this organic solvent (PMSF and phenanthroline). The effect of adding 1 mM DTT to the final incubation buffer was also tested.

2.6.4 Nuclease detection using substrate SDS-PAGE

A 1:1 mixture of non-reducing sample buffer with cell supernatants (section 2.6.1) including proteinase inhibitors (section 2.6.2) were boiled for 2-3 minutes prior to loading on a gel containing co-polymerized polyadenylic acid (poly(A)) at 0·3 mg ml^{-1} final concentration in the resolving gel. Each lane was loaded with the equivalent of $9x10^6$ promastigotes. After electrophoresis, gels were washed by gentle agitation in

0·1% (v/v) Triton X-100/100 mM HEPES, pH 8·5, to enable renaturation of enzyme activity, then incubated at 37°C in the same buffer for a further 1-2 hours. Each gel was then fixed in 10% (v/v) acetic acid for 10 minutes, washed in distilled water for 3 x 10 minutes, stained with 0·2% (w/v) Toluidine Blue in 10 mM HEPES, pH 8·5, for 15 minutes, and destained with distilled water [Bates, 1993b].

2.7 Protein determinations

Protein determinations were carried out on cell pellets lysed in the presence of proteinase inhibitors (see section 2.6.1). In addition, amastigote cysteine proteinases were inhibited *in vitro* by incubation in incomplete HOMEM medium in the presence of the diazomethane Z-phenylalanine-alanine-CHN_2 (Z-Phe-Ala-CHN_2), at 30 $\mu g\ ml^{-1}$ for 30 minutes immediately prior to harvesting (section 2.2.6).

Protein concentrations were estimated spectrophotometrically (Titertek Multiscan® MCC/340) using the Pierce BCA Protein Assay Reagent kit (microtitre assay, as per the manufacturer's instructions) and were calculated from standard curves, produced using the computer programme Grafit [Leatherbarrow, 1992], for each individual assay. Standard curves were generated using the supplied solution of bovine serum albumin which was diluted with sodium phosphate buffer.

2.8 Generation of polyclonal antisera

Polyclonal antisera were raised to whole cell homogenates of different life-cycle stages of *L. panamensis* and *L. mexicana*. The antibodies were raised in BALB/c mice to promastigote morphotypes 1, 2 and 3 of both species plus axenic and lesion amastigotes of *L. mexicana*. Rabbits were used to generate antibodies to

promastigote morphotypes 1, 2 and 3 and purified lesion amastigotes of *L. mexicana*. All *L. mexicana* cells were incubated with the diazomethane, Z-phe-ala-CHN_2, at 30 $\mu g\ ml^{-1}$ for 30 minutes prior to harvesting which involved three washes in incomplete HOMEM medium. This incubation was to inhibit cysteine proteinase activity and hence maximise protein content. *L. panamensis* cells were not pre-incubated with inhibitor prior to use.

Cell lysates were produced either by freeze thaw (x3) or by sonication (3 bursts of 10 seconds at approximately 15 microns on a Soniprep 150 sonicator) of pellets resuspended in HOMEM medium. For injection of rabbits *L. mexicana* promastigote and amastigote frozen pellets were lysed at the following cell densities; promastigote morphotypes 1 and 2 at $1{\cdot}25x10^8\ ml^{-1}$, morphotype 3 promastigotes at $2{\cdot}5x10^8\ ml^{-1}$, and lesion amastigotes at $6{\cdot}25x10^8\ ml^{-1}$ in a total volume of 400 µl chilled HOMEM medium. Immunization of mice involved injection of *L. mexicana* and *L. panamensis* parasite lysates of the following cell densities; promastigotes of *L. mexicana* morphotypes 1 and 2 at $2{\cdot}5x10^8\ ml^{-1}$, *L. mexicana* morphotype 3 promastigotes and axenic amastigotes at $10^9\ ml^{-1}$, *L. panamensis* morphotype 1 promastigotes at $6x10^8\ ml^{-1}$, *L. panamensis* morphotype 2 promastigotes at $4x10^8\ ml^{-1}$ and *L. panamensis* morphotype 3 promastigotes at $8{\cdot}5x10^8\ ml^{-1}$. Frozen cell pellets were resuspended in 0·1 ml of chilled HOMEM medium prior to sonication or freeze thaw. Samples were then checked by microscopy to ensure lysis. An equal volume of Freund's Adjuvant was applied and vortexed thoroughly (5-10 minutes) until the sample was completely emulsified. Complete Adjuvant was used for primary injections and incomplete Adjuvant thereafter. Mice were given a total of 4 injections at fortnightly intervals and rabbits three injections at monthly intervals.

Two weeks after the third injection test bleeds were taken for analysis using dot blots. Taking these results into account, the rabbit blood was collected at this time. The mice required a further immunisation to boost the antibody titre. Again test bleeds were taken and dot blots carried out. The blood was then extracted from the mice by cardiac puncture.

2.9 Immunoblotting

2.9.1 Dot blots

Dot blots were carried out on all antisera raised in mice and in rabbits. Cell pellets of the appropriate antigen were lysed in 0·25 % (v/v) Triton X-100 at 10^9 cells ml^{-1}. Prior to electrophoresis, lysate supernatants were mixed with reducing sample buffer and boiled for 2 minutes. Two microlitre aliquots of each sample were 'dotted' onto nitrocellulose strips, pre-labelled in pencil, and allowed to dry. A sufficient number of dots of each antigen were applied to test the antisera at a range of concentrations. These strips were then incubated for 2 hours in blocker [5% (w/v) dried milk, 10% (v/v) horse serum in Tris Buffered Saline (TBS - 20 mM Tris base, 137 mM sodium chloride, pH 7·6) containing 0·1% (v/v) Tween] at 4°C on a shaking table. Following this incubation the strips were washed four times (15 minutes each) in TBS Tween. Solutions of primary antibodies were prepared in blocker at 1:99, 1:499, 1:999, 1:1999, 1:4999 and 1:9999. Two microlitres of the correct antibody solution were applied to the nitrocellulose, directly onto the antigen. These were incubated at 4°C in humid conditions for 2 hours. The strips were subsequently washed in TBS Tween before addition of the secondary antibody. An anti-mouse (Bio-Rad) or an anti-rabbit (Promega) horse radish peroxidase (HRP) conjugate was used for mouse and rabbit antisera, respectively. These were applied in blocker and

incubated for one hour, after which time the strips were again washed in TBS Tween (4 x 15 minute washes). Any signal was detected by ECL (Amersham).

2.9.2 Western Blotting

Reduced and denatured supernatant proteins separated on 10% (w/v) acrylamide mini-gels by SDS-PAGE (section 2.6.2) were blotted onto nitrocellulose paper in 20 mM Tris/150 mM glycine/20% (v/v) methanol for 60 minutes at 100 volts (Bio-Rad mini-transblotter). Following protein transfer the nitrocellulose was blocked overnight with 5% (w/v) dried milk (Boots) in TBS Tween ± 10% (v/v) heat-inactivated horse serum, depending on the primary antibody used, and then incubated for 2 hours at 4°C on a shaking table in the same solution with primary antiserum. Horse serum was only excluded from the blocker when polyclonal antibodies to *L. mexicana* were used. The concentration of primary antiserum depended on which was applied; antibodies against cysteine proteinases were used at 1/500, polyclonal antisera raised in rabbits against *L. mexicana* promastigotes were used at 1/5000 and the monoclonal antibody raised against gp63 [Wallace and McMasters, 1987] was used at 1/2000. Blots were washed 4x15 minutes with TBS Tween prior and subsequent to incubation with the secondary antibody. The length of this incubation varied depending on the antibody used, see the appropriate sections. Signal detection was with ECL (Amersham).

2.9.2.1 Preparation of *L. mexicana* polyclonal antisera for immunoblotting

Final bleeds from the rabbits were taken and placed in glass universals. Clotting was permitted at room temperature and after 4 hours a glass pasteur pipette was used to detach the clot from the surface of the universal and the blood was

placed at 4°C overnight. This allowed the clot to shrink and aided maximum serum recovery. The supernatant was then transferred to centrifuge tubes and centrifuged for 5 minutes at 1250 g to remove remaining host cells. Aliquots of antisera with no other additions were frozen at -70°C.

Attempts to generate stage-specific antisera involved precipitation of antibody-antigen complexes (thereby removing 'common' antibodies). This was accomplished by mixing each antiserum individually with each promastigote morphotype lysate (10^9 ml^{-1}) in the ratio 1:100. These were rapidly shaken overnight at 4°C. Prior to final dilution (1:50) with blocker (5% w/v dried milk in TBS Tween) for incubation with the nitrocellulose, the antibody-antigen complexes were removed by centrifugation at 1600 g for 10 minutes at room temperature in a microfuge.

2.10 Metabolic labelling

2.10.1 Incubations

Promastigotes of *L. panamensis* and *L. mexicana* and axenic amastigotes of *L. mexicana* were harvested by centrifugation (1250 g for 20 minutes) and washed twice in sterile PBS. The resulting pellets of promastigotes and axenic amastigotes were resuspended to ca. $5x10^6$ ml^{-1} or $1{\cdot}4x10^7$ ml^{-1}, respectively, and subsequently incubated at 25°C in 10 mls of methionine-deficient medium, MEM with Earle's salts (Gibco Life Technologies), supplemented with 2x L-glutamine (20x stock from Gibco Life Technologies) and 10% (v/v) dialysed HIFCS. After approximately 90 minutes, 30 μCi (ca. 6 μl) of [^{35}S]-methionine (EXPRE^{35}S^{35}S protein labelling mix at 1000 Ci per mmole from NEN Dupont) were added to each of the promastigote cultures and 100 μCi (ca. 20 μl) to the amastigote culture, which were incubated for

a further 150 minutes. Cells were harvested and washed once in PBS by centrifugation, 1250 g for 15 minutes, and stored as pellets at -70°C.

2.10.2 Detection of metabolic label

Frozen pellets were lysed, within one week of labelling, at $2x10^8$ ml^{-1} in 0·25% (v/v) Triton X-100 with 1 mM PMSF, 10 mM phenanthroline and 10 μM E-64. Centrifugation of these for 5 minutes (1600 g) at 4°C provided supernatant and pellet fractions. The pellet fractions were resuspended in lysing solution (equal volume to supernatant) using a syringe and a 26 gauge needle. Samples were mixed 1:1 with double strength sample buffer, boiled for 2 minutes, and 10 μl of each sample were loaded onto a 12% (w/v) acrylamide mini-gel for SDS-PAGE. Subsequently gels were stained with Coomassie Blue, destained (section 2.6.2), and dried before being exposed to X-Ray film.

2.11 Changes in metabolite concentrations during short term incubations in simple media

2.11.1 Incubations

Eight simple media were used (one set of four at pH 7·0 and one set at pH 5·5), each of which was supplemented with 10% (v/v) dialysed HIFCS (section 2.4), as below;

1. 10 mM HEPES/10 mM MES/145 mM sodium chloride (buffered saline)
2. Buffered saline with 10 mM glucose
3. Buffered saline with 2% (v/v) Amino Acid Mix (AAM)

AAM: 1:1, v/v of MEM amino acids solution (without glutamine) (50x) and MEM non-essential amino acids (100x) (both amino acid solutions were purchased from Gibco Life Technologies)

4. Buffered saline with 10 mM glucose and [2%, v/v] AAM

Promastigote cultures, of *L. mexicana* and *L. panamensis*, judged by microscopic observation to be abundant in each of the three promastigote forms, purified lesion amastigotes (section 2.2.2.2.2) and axenic amastigotes (section 2.2.3) of *L. mexicana* were washed with buffered saline at 4°C (2 x 1250 g for 15 minutes) prior to each incubation. Cells resuspended in buffered saline after the first wash were counted and following the second wash they were resuspended in the appropriate simple medium (ca. 0·75-1·0 ml) at 10^8 cells ml^{-1} and transferred to sterile 8 ml flat sided tubes (Nunc, Gibco Life Technologies). Promastigotes were incubated only in media at pH 7·2 at 25°C, while amastigotes were incubated at both pH 7·2 and pH 5·5 at 32°C with 95% air/5% CO_2 as the gas phase. Various different gases were applied to investigate their effect on amino acid and glucose metabolism during the two hour incubation of promastigotes at 25°C. Air was displaced from the gas phase by gassing at high speed with either oxygen free N_2, argon or 95% air/5% CO_2. Tubes containing promastigotes to be incubated with air were fitted with loose caps, the lids on tubes purged with other gasses were tightly closed. One set of experiments for each promastigote form of *L. panamensis* was carried out under the aforementioned three gas phases, whereas promastigotes of *L. panamensis* and *L. mexicana* incubated with air as the gas phase were carried out in triplicate on separate occasions. After 2 hours, the cells in each culture were checked visually by microscopic observation for motility and apparent viablity and the spent media were

harvested by centrifugation at 1600 g for 10 minutes. Each was checked by microscopy to ensure that cells had been removed. The supernatants were stored at -70°C until analysed (sections 2.11.2 and 2.11.3).

2·11·2 Analyses using high pressure liquid chromatography (hplc)

2.11.2.1 Amino acids

2.11.2.1.1 Extraction of media samples

Protein was precipitated by addition of media aliquots to four volumes of cold methanol (BDH, HiPerSolv grade) for 10 minutes at 4°C and removed by centrifugation (1600 g for 10 minutes in a microfuge) and filtration (0·2 μm).

2.11.2.1.2 Chromatography

Samples were analysed for amino acids using the hplc technique of Zuo and Coombs [1995] which is briefly described below. All reagents were of hplc grade and where appropriate were prepared using ultra pure water (18·0 megohm cm.) produced from tap water by a Purite Select Analyst HP 100. OPT-thiol (o-phthaldialdehyde-thiol) reagent was prepared as described by Joseph and Marsden [1986]. A Rainin Dyn Microsorb C18 4·6x150 mm column was used in conjunction with two solvents. Solvent A was 0·02 M sodium acetate-methanol-tetrahydrofuran (85-11-4, v/v) and was prepared and filtered on the day of use. Solvent B was acetonitrile. Both solvents were thoroughly degassed with helium prior to use. A constant flow rate of 1·3 ml min^{-1} and a solvent gradient of 1-18% solvent B over 13 minutes, 18-30% solvent B over 11 minutes, and 30-0% solvent B over 2 minutes, with 4 minutes subsequent re-equilibration with solvent A, were applied. Detection of amino acids was with a Gilson Model 121 Fluorometer. Amino acids in the

simple media, as detected by hplc, were identified by comparing the retention times of peaks with those of known standards and quantified by use of standard calibration curves generated using the computer program Grafit [Leatherbarrow, 1992].

2.11.2.2 Organic acids

2.11.2.2.1 Extraction of media samples

Organic acids were extracted from media samples using a technique modified from Guerrant *et al.* [1992]. The following components were vortexed for 1 minute; 200 µl sample, 0·12 g NaCl, 40 µl 18 N H_2SO_4 and 1 ml hplc grade diethyl ether. The mixture was then centrifuged at 1400 g for 1 minute. The upper ether layer was then removed using a glass pasteur pipette and transferred to a separate tube containing 100 µl of 0·1 N sodium hydroxide. These were vortexed and centrifuged as before. Subsequently the upper ether fraction was discarded and the tubes allowed to stand open at room temperature in the fume hood for 15 minutes to allow residual ether to evaporate. The remaining material was either analysed immediately or stored at -70°C.

2.11.2.2.2 Chromatography

A Hamilton PRP-X300 ion exclusion column (7 µm bore size, 250 x 4·1 mm) was used in conjunction with a single eluent, H_2SO_4, pH 2·7 (produced by titrating ultrapure water with 18 N H_2SO_4)which was thoroughly degassed with helium prior to use. Twenty microlitres of filtered extracted samples were individually applied to the system when the solvent flow was 2 ml min^{-1}. The total run time was 10 minutes and detection was at 210 nm on a Gilson U.V. detector (Model 115). Unknowns were identified by comparing retention times with those of reagent grade standards.

2.11.3 Determination of glucose concentrations

The glucose concentration in fresh and spent media was assayed spectrophotometrically on a Titertek Multiscan® MCC/340. It was quantitated enzymatically by a linked enzyme reaction involving $NADP^+$ reduction and determining the change in absorbance at 340 nm [Bergmeyer *et al.*, 1983]. Concentrations were extrapolated from standard curves generated using known amounts of glucose. All assays were carried out in a final volume of 250 µl in microtitre plates (50 µl sample volume); incubation was at 37°C. The standard assay contained: 50 mM HEPES (pH 7·6); 90 mM KCl; 3 mM $MgCl_2$; 1 mM ATP; 1 mM $NADP^+$; 0·5 U hexokinase (type III from bakers yeast, Sigma # H-5000); 0·5 U glucose 6-phosphate dehydrogenase (type VII from bakers yeast, Sigma # G-7877); and sample.

2.11.4 Determination of glycerol concentrations

Glycerol assays were performed in the same manner as for quantitation of glucose (section 2.11.3). The standard assay contained: 150 mM glycine, 650 mM hydrazine (pH 9·8); 1·5 mM $MgCl_2$; 5 mM ATP; 1 mM NAD^+; 3 U glycerophosphate dehydrogenase (type I from rabbit muscle, Sigma # G-6751); 3 U glycerol kinase (*Cellulomanas* species, Sigma # G-6142); and sample.

2.12 Changes in exogenous glucose and amino acid concentrations during promastigote growth *in vitro* in complete HOMEM medium

Daily media samples were taken from cultures of *L. mexicana* and *L. panamensis* promastigotes inoculated at $5x10^5$ ml^{-1} in complete HOMEM medium. Cells densities were determined using a haemocytometer before removal of cells by centrifugation at 1600 g for 10 minutes. The resulting spent media were stored at

-70°C until analysed for amino acid (section 2.11.2.1) and glucose content (section 2.11.3).

2.13 Organic acid production by promastigotes of *L. mexicana* and *L. panamensis* promastigotes during *in vitro* growth

Cultures of *L. mexicana* and *L. panamensis* promastigotes were initiated at $5x10^5$ ml^{-1} in complete HOMEM medium and incubated at 25°C with air as the gas phase. Cell free media samples were obtained by centrifugation (1600 g for 10 minutes) of aliquots of the culture immediately after promastigote inoculation and when cultures reached the stationary phase of growth. These samples were stored at -70°C until analysed for organic acid content.

2.14 Intracellular amino acid pool

The endogenous amino acids of *L. mexicana* promastigote morphotypes 1, 2 and 3 (harvested by washing 3 times in HEPES saline by centrifugation at 1,250 g for 15 minutes, at 4°C) were identified and quantitated by hplc. A sample of morphotype 3 promastigotes which had been incubated with the cysteine proteinase inhibitor Z-PheAla-CHN_2 at 30 μg ml^{-1} for 30 minutes prior to harvesting was also analysed subsequent to harvesting as for the other promastigote samples. Frozen cell pellets were lysed at 10^9 ml^{-1} osmotically by addition of ultra pure water (see section 2.10.2.1.2) containing 0·1 mM PMSF, 1 mM phenanthroline, 1 μM pepstatin and 10 μM E-64. The homogenates were then extracted with methanol (section 2.11.2.1.1) prior to chromatography (section 2.11.2.1.2).

2.15 Enzyme assays

All assays contained soluble cell extract (the supernatant fractions resulting from centrifugation of cell homogenates) and were carried out at 37°C in a final volume of 1 ml. Cell extracts were prepared by lysis of frozen cell pellets in ice cold 50 mM HEPES, pH 7·4, containing 20% (v/v) glycerol, 10 mM phenanthroline, 10 μM E-64, 1 mM PMSF and 1 μM pepstatin. Reactions were monitored spectrophotometrically at 340 nm on a Philips PU 8720 visible/U.V. Scanning Spectrophotometer and initiated by the addition of substrate at saturating concentrations. To confirm the specificity of the reaction, control assays were carried out lacking one or more of the assay components. Experiments were also conducted to ensure a linear relationship between reaction rate and sample size over the normal working range. All pyruvate kinase assays were carried out using freshly prepared cell extracts; other enzyme assays were performed on extracts which had been stored at -70°C. Unless stated in the text all enzymes were assayed by standard spectrophotometric techniques as described by Bergmeyer *et al.* [1983].

2.15.1 Pyruvate kinase

The standard assay contained: 50 mM HEPES, pH 7·5; 1 mM DTT; 7·5 mM $MgCl_2$; 75 mM KCl; 0·28 mM NADH; 10 mM phosphoenolpyruvate; 2 U lactate dehydrogenase; 5 mM ADP and crude enzyme.

2.15.2 Hexokinase

Activity was measured using a coupled enzyme reaction involving reduction of NADP by glucose 6-phosphate dehydrogenase [Jenkins and Thompson, 1989]. The standard assay contained: 100 mM Tris HCl, pH 7.3; 6·6 mM $MgCl_2$; 15 mM glucose;

1 mM ATP; 0.33 mM $NADP^+$; 1·4 U glucose 6-phosphate dehydrogenase.

2.15.3 Lactate dehydrogenase

The standard assay contained: 100 mM Tris HCl, pH 7·4; 200 mM NaCl; 0·28 mM NADH; 1·6 mM pyruvate; and crude enzyme.

2.15.4 Isocitrate dehydrogenase (NAD^+ and $NADP^+$-dependent)

The standard assay contained: 50 mM HEPES (pH 7·5); 2 mM $MnCl_2$; 6·7 mM isocitrate; 0·4 mM $NADP^+$ or NAD^+; and crude enzyme. 2 mM ADP was included in assays for the NAD^+-dependent enzyme.

2.15.5 Glutamate dehydrogenase

Reaction mixtures contained: 100 mM Tris ethanolamine buffer, pH 8·0; 0·5 mM NADH; 320 mM ammonium sulphate; and sample. The reaction was initiated with 6·6 mM α-ketoglutarate.

2.16 DNA content analysis

The DNA content of *L. mexicana* promastigotes was monitored during the *in vitro* growth cycle. Promastigotes were set up under routine conditions (section 2.2.1.2), counted and harvested on days 1, 2, 3, 4 and 6 of growth in complete HOMEM medium. Two individual cultures that had been through a different number of sub-passages, 2 and 8, were assessed. Harvesting involved one wash (centrifugation at 1250 g for 15 minutes) in sterile PBS and cell pellets were subsequently resuspended in a small volume of the same buffer before fixation in 70% (v/v) methanol in PBS at room temperature for 2 hours. Subsequently samples were pelleted, resuspended in

2 ml of PBS and incubated with RNase and propidium iodide, both at a final concentration of 10 μg ml^{-1}, for 1 hour. Samples were then analysed on a Becton Dickinson FACScan. For each reading 10,000 promastigotes were counted.

2.17 Infectivity of stationary phase promastigotes of *L. mexicana* and *L. panamensis* to J774G8 cells

The macrophage-like cell line J774G8 was cultured in RPMI (Gibco Life Technologies) supplemented with 20% (v/v) or 5% (v/v) HIFCS in 0·4 ml tissue culture chamber slides (Nunc, Gibco Life Technologies). The cultures were initially incubated at 32°C overnight in 95% air/5% CO_2 to allow the cells to settle and adhere to the slides. The medium was then removed and promastigotes were added in complete RPMI at a ratio of approximately 10 promastigotes:1 macrophage. A period of 3 hours was allowed for attachment and phagocytosis of promastigotes, before removal of medium containing unbound cells. RPMI supplemented with 20% or 5% HIFCS was then added. After this initial 3 hour incubation and every subsequent 24 hours, one slide was washed, fixed with Giemsa's stain. Cells were viewed using light microscopy.

2.18 Infectivity of the different promastigote morphotypes to peritoneal exudate cells

Peritoneal macrophages were removed from culled female BALB/c mice by peritoneal lavage. This involved injection of 5 ml of chilled RPMI supplemented with 10% (V/V) HIFCS (complete RPMI medium) into the peritoneal cavity followed by gentle finger massage to detach cells from this region. The resulting cell suspension was then drawn off using a 5 ml syringe fitted with a 26 gauge needle. Care was taken to leave the gut intact and so prevent bacterial

contamination. Macrophages were counted using an Improved Neubauer Haemocytometer and the volume adjusted with complete RPMI medium to give a final macrophage density of $5x10^5$ ml^{-1}. Four hundred microliter aliquots of this culture were placed into the wells of a sterile 8 chamber slide (Nunc, Gibco Life Technologies) and incubated at 32°C under 95% air/5% CO_2 overnight. Unattached cells were removed the next day and the chambers were rinsed once with complete RPMI medium prior to addition of parasites at a parasite:macrophage ratio of 1:1. A 4 hour incubation period at 32°C under 95% air/5% CO_2 was allowed for parasite attachment and infection. At the end of this time promastigotes free in the medium were removed and the wells were rinsed 3 times with complete RPMI medium. Addition of 0·4 ml of complete RPMI medium was the final manipulation before incubation for 7 days at 32°C under 95% air/5% CO_2. When this incubation period was complete the culture medium was removed, the chambers dismantled and the slides were quickly air dried prior to fixation in absolute methanol and staining with Giemsa's stain (section 2.3).

2.19 Transformation studies

Various modifications to the promastigote culture conditions were carried out to determine their effects on promastigote growth and morphology. A control culture was routinely set up at $5x10^5$ ml^{-1} in complete HOMEM medium using an identical promastigote starting population and culture vessel for comparison with experimental cultures. The vessel varied depending on the experiment carried out. Flat bottomed culture flasks (25 cm^2) were used in studies involving variation of promastigote starting densities (*L. panamensis*), continuous sub-passage of morphotype 1 promastigotes

(*L. panamensis* and *L. mexicana*), comparison of whole and dialysed foetal calf serum (*L. mexicana*, *L. panamensis* and *L. major*) and addition of supplements to culture medium supplemented with dialysed foetal calf serum (*L. panamensis*), sub-passage into spent (morphotype 2) HOMEM medium and differentiation of purified morphotype 3 promastigotes of *L. major*. All other studies involving promastigotes were set up in microtitire plates which were incubated under humid conditions to prevent the cultures drying out. Investigations of the differentiation of promastigotes of *L. mexicana* to axenic amastigotes were also carried out in multiwell plates (Nunc, Gibco Life Technologies), but the ones used comprised 6 large wells (5 ml) rather than 96 small wells (250 µl). On the appropriate day, subsequent to initiation of the experiments, cell samples were removed and Giemsa-stained smears produced (section 2.3). Promastigote morphology was assessed using these smears through measurements of cell body length and breadth and the proportions of each promastigote morphotype calculated according to the size definitions detailed in Table 3.1. Other features of the transformation experiments were more variable and are therefore discussed in detail separately below.

2.19.1 Starting density

Early-log phase promastigotes of *L. mexicana* were pelleted at 1250 g for 10 minutes and subsequently resuspended at $2x10^7$ ml^{-1} in complete HOMEM medium before sub-passage to densities of $5x10^5$ ml^{-1}, $5x10^6$ ml^{-1}, $1x10^7$ ml^{-1} and $2x10^7$ ml^{-1} in the same culture medium, in duplicate. On day 2 post-inoculation, a sample of each mini-culture was taken for the production of a Giemsa-stained smear. The lengths and breadths of fifty promastigotes were measured per culture.

L. panamensis promastigotes in the late-log/early stationary phase of growth were pelleted by centrifugation (1,250 g for 15 minutes) and resuspended in half of the initial culture volume of complete HOMEM medium before sub-passage to densities of $1x10^5$ ml^{-1}, $5x10^5$ ml^{-1}, $1x10^6$ ml^{-1}, and $1x10^7$ ml^{-1}. Daily counts were carried out and Giemsa-stained smears were produced daily, from day 1 - day 6, and 100 promastigotes were measured per culture.

2.19.2 Continual sub-passage of morphotype 1 promastigotes

Promastigotes of *L. mexicana* and *L. panamensis* in the mid- to late-log phase of growth were subjected to identical experimental conditions. Both cultures were serially sub-passaged at initial densities of $5x10^5$ ml^{-1} in complete HOMEM medium (final culture volume 10 ml) on the first day post-inoculation on three successive days. The first culture set up was subsequently followed for a further two consecutive days (days 2 and 3) and the second culture was assessed on the second day of growth. One hundred promastigotes were measured from Giemsa-stained smears produced from each culture, except for *L. mexicana* on the third consecutive sub-passage for which only 33 promastigotes were found.

2.19.3 Dialysed HIFCS

Promastigotes of *L. mexicana* and *L. major* in the late-log/early stationary phase of growth were sub-passaged into HOMEM medium supplemented with 10% (v/v) complete or dialysed HIFCS. Individual 10 ml cultures were set up for each species at $5x10^5$ ml^{-1} and samples were taken on the first 4 days of growth for cell counts and Giemsa-stained smears. All four smears of *L. mexicana* promastigotes were analysed

for the proportions of each promastigote morphotype while only that produced on the first day was assessed for *L. major*.

2.19.4 Dialysed HIFCS plus supplements

Four 5 ml cultures of *L. panamensis* were set up at $5x10^5$ ml^{-1} in HOMEM medium supplemented with 10% (v/v) dialysed HIFCS subsequent to the addition of 0·01 mg ml^{-1} hemin, 0·01 mg ml^{-1} haemoglobin or 10 μM acetylcholine (1000-fold dilutions of stock solutions. Note hemin was dissolved in 5 M NaOH). A control culture was also set up to the same density with an identical starting population of promastigotes but in complete HOMEM medium.

2.19.5 Variation of HIFCS concentration

L. mexicana promastigotes in the late-log phase of growth were sub-passaged to the density of $5x10^5$ ml^{-1} into HOMEM medium which was supplemented with various quantities of HIFCS (5%, 10%, 20% and 50%). In addition one culture was prepared in 100% HIFCS. Five μl of stock culture were pipetted into individual wells in a microtitre plate subsequent to the addition of 245 μl of the appropriate culture medium. Cell samples were assessed for morphology in Giemsa-stained smears on the third and fourth day post-inoculation. Fifty promastigotes were measured per culture except for promastigotes in 100% HIFCS on day 4 for which only 43 promastigotes were found on the slide.

2.19.6 Conditioned media

Promastigotes of *L. mexicana* and *L. major* in the late-log phase of growth were sub-passaged to the density of $5x10^5$ ml^{-1} (final culture volume of 10 ml) in freshly

prepared complete HOMEM medium and morphotype 2 conditioned medium (complete HOMEM medium which had been harvested from late-log phase cultures by centrifugation at 1600 g for 10 minutes). Cell densities and Giemsa-stained smears were prepared on the first four days of culture.

Conditioned medium from morphotype 3 promastigote cultures was prepared in the same way as morphotype 2 conditioned medium (see above). Two aliquots of a *L. mexicana* early-log phase promastigote culture were centrifuged (1250 g for 10 minutes) and resuspended at $2x10^7$ ml^{-1} in either complete HOMEM medium or morphotype 3 spent medium. These were subsequently aliquotted into wells of a microtitre plate (96 well) and the volume made up to 200 µls with the appropriate medium. Samples were removed for the production of Giemsa-stained smears on the second day post-inoculation. From these 50 promastigotes were assessed for their cell body length and breadth measurements and the proportion of each promastigote morphotype was calculated.

2.19.7 Differentiation of morphotype 3 promastigotes of *L. major*

Metacyclic promastigotes of *L. major* were purified using a slightly modified version of the technique developed by Sacks [1985] (section 2.2.1.4.2). Subsequent to removal of metacyclic forms (unagglutinated promastigotes present in the supernatant), they were washed in sterile PBS. After one wash (1,250 g for 10 minutes) the cell density was determined and the promastigotes were subsequently resuspended to $6x10^6$ ml^{-1} in HOMEM medium. The cell densities were then reduced 10-fold by addition of HOMEM medium and then a further 10% by addition of HIFCS, so giving a density of $5x10^5$ ml^{-1}. Two 10 ml aliquots of this culture of purified metacyclics of

L. major were placed in 25 cm^2 flat bottomed culture flasks and the densities and morphology (using Giemsa-stained smears) monitored on a daily basis (including day 0).

2.19.8 Induction of metacyclogenesis of *L. mexicana*

Late-log phase promastigotes of *L. mexicana* were pelleted and resuspended in complete HOMEM medium or complete SDM to give a final density of $6{\cdot}6x10^6$ ml^{-1}. 180 µls of these cultures were then added to wells of a tissue culture plate (96 well) which contained 20 µl of test solution; water, aphidicolin, kindly donated by Brian Shiels, (to give final concentrations of 2·5 µg ml^{-1}, 1 µg ml^{-1}, 0·75 µg ml^{-1}, 0·5 µg ml^{-1}, 0·25 µg ml^{-1}, 0·125 µg ml^{-1} and 0·0625 µg ml^{-1}, stock aphidicolin dissolved in 100% DMSO) or DMSO (to give final concentrations of 2%, 1·5%, 1%, 0·75%, 0·5% and 0·25%). The final cell density was $3{\cdot}3x10^5$ ml^{-1}. Promastigote densities and morphology were subsequently monitored at appropriate time points using standard procedures.

2.19.9 Differentiation of promastigote morphotypes 1, 2 and 3 of *L. mexicana* to axenic amastigotes

Early-log, late-log and stationary phase populations of promastigotes of *L. mexicana* were sub-passaged to 10^6 ml^{-1} into complete SDM (final culture volume of 5 ml) and incubated at 32°C under air. Because of their low densities, early-log phase cultures were initially pelleted (1,250 g for 10 minutes) before being resuspended to the appropriate density in medium. Triplicate cultures were set up and samples were removed on consecutive days of growth for cell density determinations and the production of Giemsa-stained smears. Length and breadth measurements of 100 cells

per sample were made on days 2 and 9 post-inoculation and compared with those made on Giemsa-stained purified lesion amastigotes.

CHAPTER 3

RESULTS

3.1 Growth & Morphology

3.1.1 Amastigotes

3.1.1.1 Lesion amastigotes

Infections in mice were set up to provide a source of lesion amastigotes and freshly transformed promastigotes of *L. panamensis* strain 2 for various investigations. Sub-cutaneous injection of stationary phase promastigotes of *L. panamensis* variably produced small cutaneous lesions on the rump of female BALB/c mice. On occasions the lesions formed at the base of the tail rather than at the site of inoculation on the rump. This is consistent with the characteristic of this species of *Leishmania* to disseminate and associate with the lymph nodes. All lesions remained relatively small in size, becoming ulcerated with time. Isolation of amastigotes using crude procedures (section 2.2.2.1) proved successful (see Figure 3.1, panel A), but yields were very low and passage of these between mice failed to produce any visible infections. Initial attempts to transform lesion amastigotes to promastigotes using liquid culture medium (complete HOMEM medium) were futile, however promastigotes were obtained when the biphasic Modified Tobie's medium was used. Subsequent culture of promastigotes was possible in liquid medium provided it was supplemented with a suitable batch of heat-inactivated foetal calf serum (HIFCS).

Lesions resulting from infection of mice with *L. mexicana* arose more quickly and were substantially bigger than those of *L. panamensis* (not shown). Consequently, larger quantities of *L. mexicana* lesion amastigotes were obtained. These were purified, using the method of Hart *et al.* [1981a], for biochemical investigation. A light

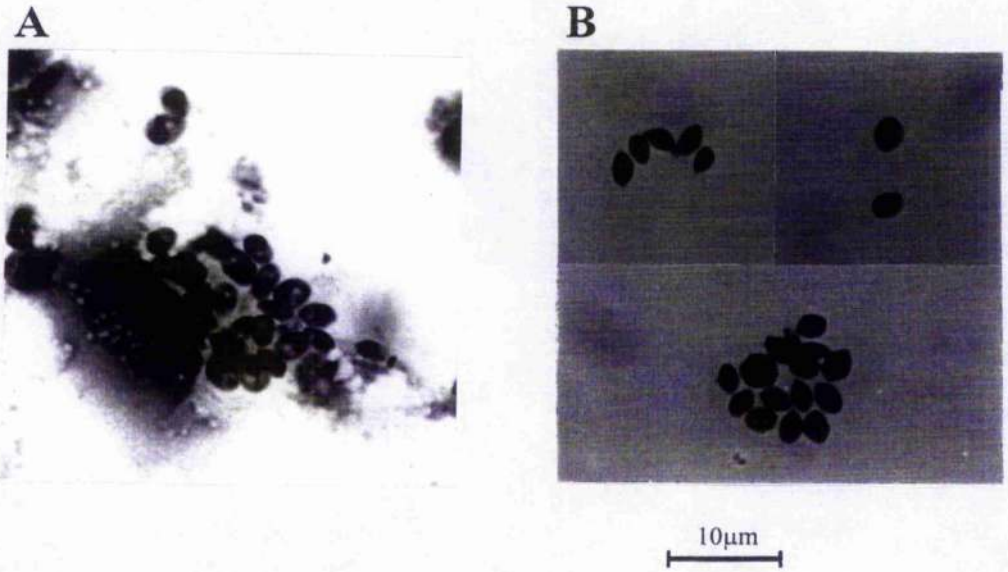


Figure 3.1: Light micrographs of Giemsa-stained smears of *L. panamensis* (Boynton) amastigote forms isolated from infected mice (Panel A) and transformed *in vitro* from stationary phase promastigotes (Panel B).

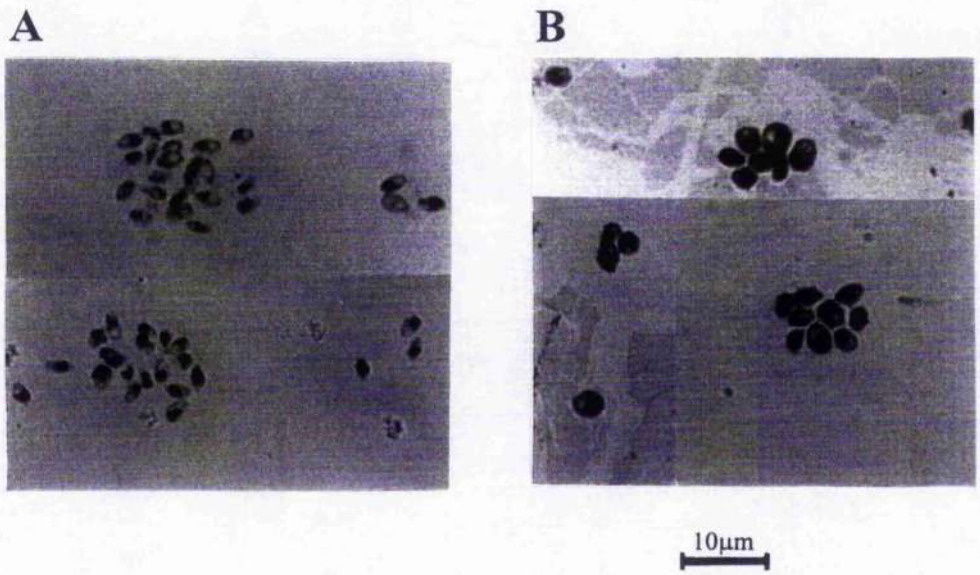


Figure 3.2: Light micrographs of Giemsa-stained smears of *L. mexicana* amastigote forms isolated from infected mice (Panel A) and transformed *in vitro* from stationary phase promastigotes (Panel B).

micrograph of Giemsa-stained lesion amastigotes of *L. mexicana* is shown in Figure 3.2, panel A. In contrast to *L. panamensis*, amastigotes of *L. mexicana* isolated in crude preparations (section 2.2.2.2.1) transformed to promastigotes readily in complete HOMEM medium at 25°C and motile flagellated forms were observed as early as 24 hours after inoculation. Moreover, isolated lesion amastigotes could be cultured directly as axenic amastigotes in complete SDM at 32°C (see section 3.1.1.2.2) and were robust enough to withstand cryopreservation. However, these stabilated amastigotes took longer to transform to promastigotes after inoculation of the thawed stabilates into liquid medium and were more sensitive to gentamicin sulphate than untreated lesion amastigotes or cryopreserved promastigotes. Death of revived cryopreserved lesion amastigotes inoculated into culture media containing 25 $\mu g\ ml^{-1}$ gentamicin sulphate was avoided by reducing the gentamicin sulphate concentration to 15 $\mu g\ ml^{-1}$.

3.1.1.2 Axenic amastigotes

Since yields of amastigotes of *L. panamensis* from cutaneous lesions in mice were poor, axenic cultivation of amastigotes was attempted following the procedures previously developed for *L. mexicana* [Bates *et al.*, 1992; Bates, 1994]. *In vitro* cultivation would potentially provide a valuable source of the mammalian stage of the parasite in large numbers and free from host contamination for biochemical characterisation. These potential advantages also prompted axenic cultivation of *L. mexicana* amastigotes. Two different approaches were adopted, transformation of stationary phase promastigotes (containing metacyclic-like cells) and direct culture of lesion amastigotes.

3.1.1.2.1 Transformation of promastigotes

In vitro transformation to amastigotes of stationary phase promastigotes of culture grown *L. panamensis* and *L. mexicana* was attempted through modification of the culture conditions. The two lines of *L. panamensis* used responded differently to exposure to 32°C (not shown). Line 1 multiplied and could be sub-passaged, however these forms differed in morphology from Line 2 (Boynton) amastigotes isolated from cutaneous lesions in mice. In contrast, Line 2 transformed to forms morphologically very similar to those that occur in mice (compare cells in Figure 3.1, panels A and B), but the *in vitro* form could not be grown for more than a few days. Variations in medium (HOMEM and SDM), batch of HIFCS, percentage of HIFCS (10%, 20% and 30%, v/v), temperature (30°C, 32°C, 34°C and 37°C), medium pH (ranging from pH 4·0 to pH 7·5, at intervals of 0·5 pH unit) and the composition of the gaseous phase (air and 95% air/5% CO_2) had no beneficial effects in terms of achieving viable populations of axenic amastigotes which would survive and grow upon sub-passage, whilst retaining amastigote-like morphology. However, the proportion of aflagellates obtained and their subsequent survival and growth were sensitive to some of these variations. For example, SDM at acidic pH gave denser cultures containing larger proportions of aflagellates than HOMEM medium under equivalent conditions. It was also found that parasites were sensitive to temperature and died at 34°C and above, whereas they remained as promastigotes below 32°C.

Transformation of *L. mexicana* stationary phase promastigotes and subsequent growth as axenic amastigotes (Figure 3.2, panel B) was achieved by following the method of Bates [1994], with the exception that the initiating promastigote population were grown in complete HOMEM medium at neutral

starting pH. Nonetheless, they contained a high proportion of metacyclic-like cells as judged on morphological criteria. The resulting *in vitro* forms morphologically resembled amastigotes isolated from infected mice although on occasion the *in vitro* aflagellates were slightly larger (compare Figure 3.2, panels A and B). A subsequent experiment which involved measurement of lesion and axenic amastigotes revealed no clear difference in size (Figure 3.40). Survival and growth of the axenic aflagellate forms was strictly dependent on the batch of HIFCS used. Different batches were tried, the most successful serum permitted survival for 4 sub-passages.

3.1.1.2.2 Direct culture from lesion amastigotes

Axenically grown amastigotes of *L. mexicana* were also obtained through direct culture of isolated lesion amastigotes [Bates *et al.*, 1992] and cryopreserved lesion amastigotes. Again this was strictly dependent upon an appropriate batch of HIFCS and also produced only short term (up to 4 sub-passages) cultures. Furthermore, cryopreserved amastigotes exhibited increased sensitivity to gentamicin sulphate (see section 3.1.1.1). Direct culture of axenic amastigotes from isolated lesion amastigotes of *L. panamensis* was unsuccessful.

3.1.2 Infectivity of stationary phase promastigotes to J774G8, a macrophage-like cell line

Infection of and survival of *Leishmania* parasites within an *in vitro* grown macrophage-like cell line was attempted to test for infectivity of the parasites and ultimately to provide promastigotes which had been recently transformed from amastigotes for preliminary studies on growth, morphology and biochemistry. It was

concluded that this source of amastigotes would be useful as lesions in mice were relatively slow growing. Infection of the macrophage-like cell line, J774G8, with stationary phase promastigotes of both *L. panamensis* strains and *L. mexicana* was attempted. Giemsa-stained smears showed uptake of promastigotes by the macrophages and subsequent changes in the parasite's morphology, the promastigotes becoming more amastigote-like (not shown). However, these rounded forms of *L. panamensis* strains 1, with short or completely retracted flagella, did not survive for more than a few days. This may reflect loss of infectivity in this particular strain since uptake, transformation and subsequent survival of *L. panamensis* Boynton and *L. mexicana* amastigotes within the macrophages did occur. For this reason all subsequent studies with *L. panamensis* were carried out on the Boynton strain (strain 2). In addition, all studies involving promastigotes of *L. mexicana*, *L. panamensis* and *L. major* were carried out with promastigotes relatively recently transformed from lesion amastigotes (< 15 sub-passages *in vitro*).

3.1.3 Promastigotes

Promastigote survival and subsequent multiplication was also strictly dependent on the foetal calf serum batch. Once a batch which sustained rapid growth and high cell densities was identified, it was used routinely to supplement the growth medium. The batch of foetal calf serum used for *L. panamensis* was different from that used for *L. mexicana* and *L. major*. Example growth curves for promastigotes of these three species are shown in Figure 3.3. *L. mexicana* promastigotes consistantly grew initially at a slightly faster rate than the other two species, although all three species investigated took approximately the same length of time, on average 5-6 days, to reach the maximum cell densities achieved -

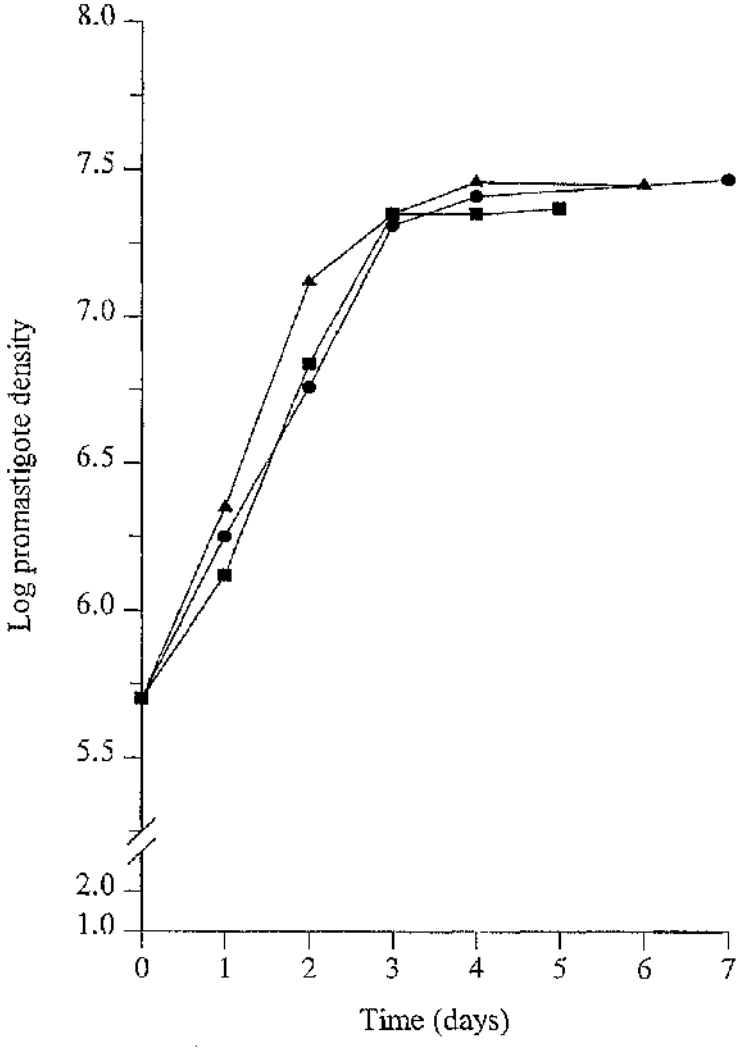


Figure 3.3: Typical growth curves of *L. mexicana* (triangles), *L. panamensis* (squares) and *L. major* (circles) promastigotes in complete HOMEM medium. Cultures were initiated at $5x10^5$ ml^{-1} with promastigotes in the mid- to late-log phase of growth and cell densities were determined daily using a haemocytometer.

$2\text{-}3x10^7$ ml^{-1} (log 7·3-log 7·5). From observations of each species set up under standard conditions, it was possible to estimate when each phase of growth would occur. The first two days of growth when promastigote densities were below $3\text{-}4x10^6$ ml^{-1} (log 6·5-log 6·6) typically corresponded to the early-log phase of growth. Mid-log phase of growth, $4\text{-}8x10^6$ ml^{-1} (log 6·6-log 6·9), was routinely reached by days 2-3, while late-log growth phase, $8x10^6$ ml^{-1}-$3x10^7$ ml^{-1} (log 6·9-log 7·5), did not occur until days 3-4. Stationary phase of growth, when there was no overall increase in cell numbers and probably reflecting cessation of division, was usually reached by days 5-6 when promastigotes had reached a density of $2\text{-}3x10^7ml^{-1}$ (log 7·3-log 7·5).

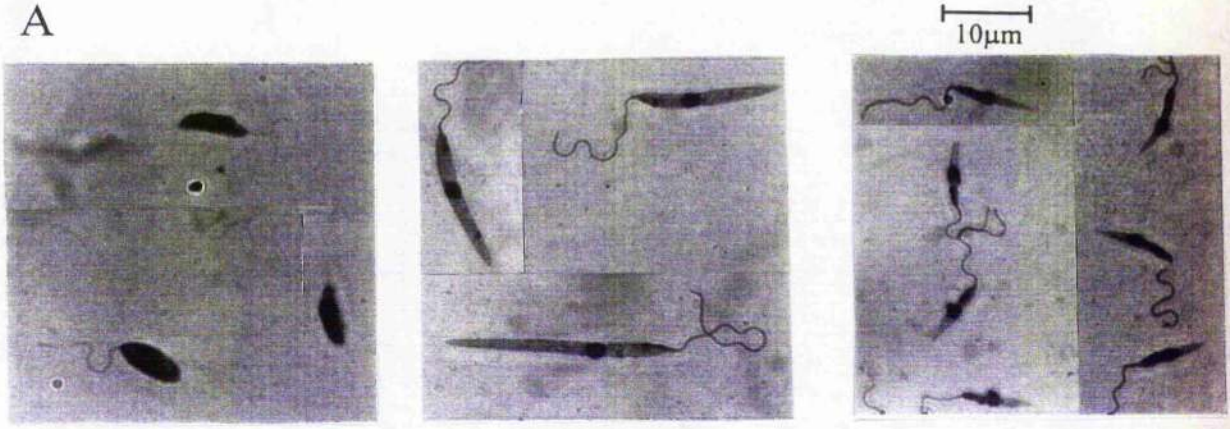
When promastigote morphology was examined on consecutive days of *in vitro* culture, a pattern of development emerged which was consistantly observed with promastigotes of all three *Leishmania* species investigated. It was apparent that cultures initiated with promastigotes consistently underwent serial transformations such that several distinct morphological forms could be distinguished and that the occurrence of these forms was related to the phase of the *in vitro* growth cycle. When body length and width measurements of promastigotes in each of the growth phases were considered in combination, three categories of promastigote, designated morphotypes 1 (MT1), 2 (MT2) and 3 (MT3), of promastigote were classified for all three species of *Leishmania*. The size criteria for each species and promastigote morphotype are shown in Table 3.1 while light micrographs of the different forms of each species and the relationship between the different promastigote morphotypes and their appearance in *in vitro* cultures are demonstrated in Figures 3.4-3.6. The overall changes were very similar with each of the three species. Promastigotes with rounded cell bodies (morphotype 1) predominated in early-log phase and were

Species/Type	Morphotype 1	Morphotype 2	Morphotype 3
L. mexicana	≥ 2·5 μm wide ≤ 12·5 μm long	< 2·5 μm wide > 12·5 μm long	< 2·5 μm wide ≤ 12·5 μm long
L. panamensis	≥ 2·5 μm wide ≤ 6·25 μm long	< 2·5 μm wide > 6·25 μm long	< 2·5 μm wide ≤ 6·25 μm long
L. major	≥ 2·5 μm wide ≤ 10 μm long	< 2·5 μm wide > 10 μm long	< 2·5 μm wide ≤ 10 μm long

Table 3.1: Size criteria of the three main promastigote morphotypes identified in *in vitro* cultures of *L. mexicana*, *L. panamensis* and *L. major*. Measurements of Giemsa-stained promastigotes were made using a calibrated eye piece graticule by bright field microscopy at x1000.

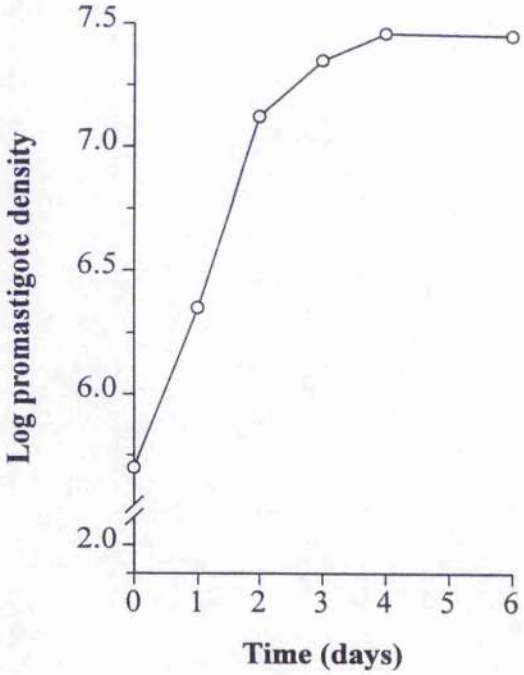
Figure 3.4: Growth and morphological development of *L. mexicana* promastigotes in *in vitro* culture. Light micrographs of Giemsa-stained smears of *L. mexicana* promastigotes (A) demonstrating the three different morphotypes and their proportions (C): morphotype 1 promastigotes, closed bars; morphotype 2 promastigotes striped bars; morphotype 3 promastigotes, open bars at different phases of growth of a typical promastigote culture (B): Each promastigote morphotype was characterised by its body length and breadth measurements in Giemsa-stained smears, the definitions of which are outlined in Table 3.1. One hundred promastigotes were examined for each daily sample from each culture.

A



Morphotype 1 Morphotype 2 Morphotype 3

B



C

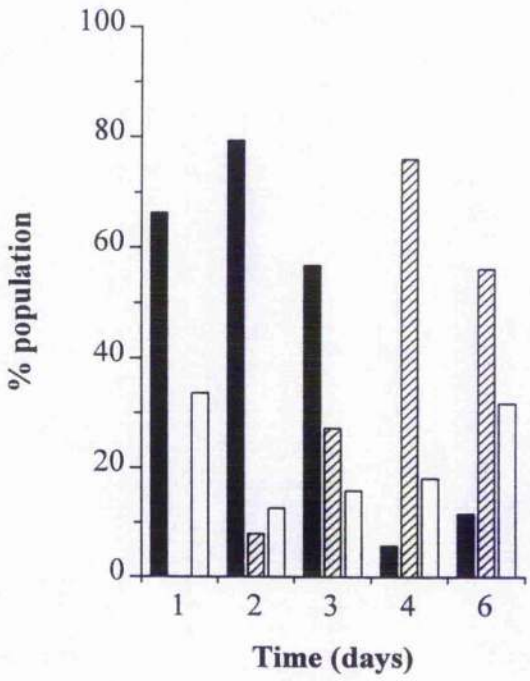
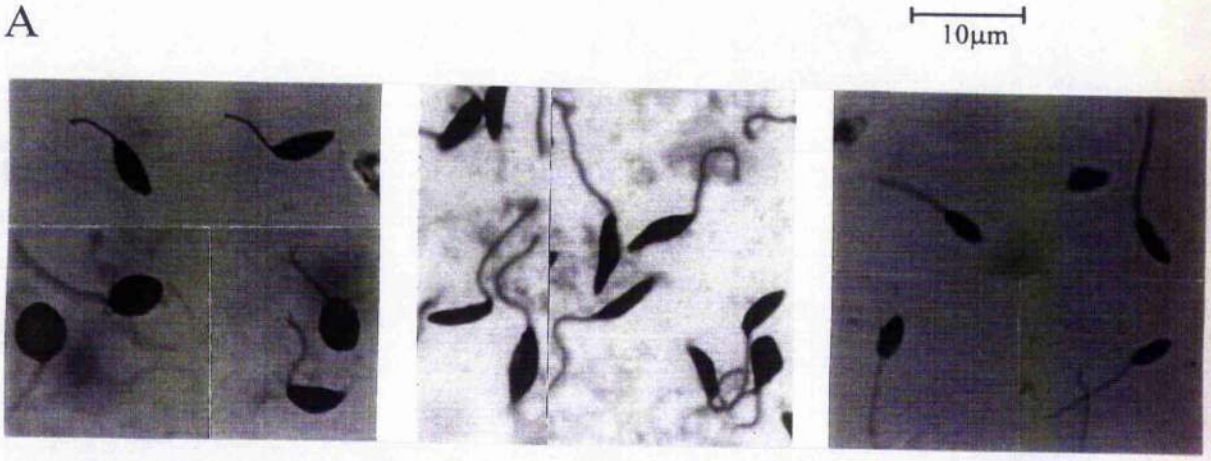


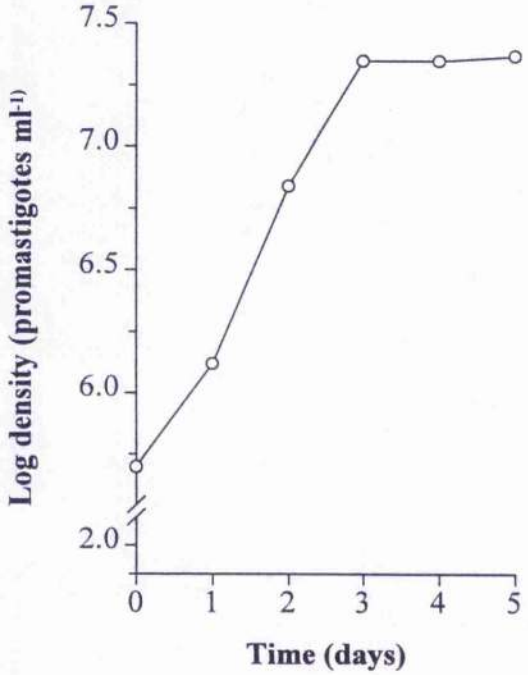
Figure 3.5: Growth and morphological development of *L. panamensis* promastigotes in *in vitro* culture. Light micrographs of Giemsa-stained smears of *L. panamensis* promastigotes (A) demonstrating the three different morphotypes and their proportions (C): morphotype 1 promastigotes, closed bars; morphotype 2 promastigotes striped bars; morphotype 3 promastigotes, open bars at different phases of growth of a typical promastigote culture (B): Each promastigote morphotype was characterised by its body length and breadth measurements in Giemsa-stained smears, the definitions of which are outlined in Table 3.1. One hundred promastigotes were examined for each daily sample from each culture.

A



Morphotype 1 Morphotype 2 Morphotype 3

B



C

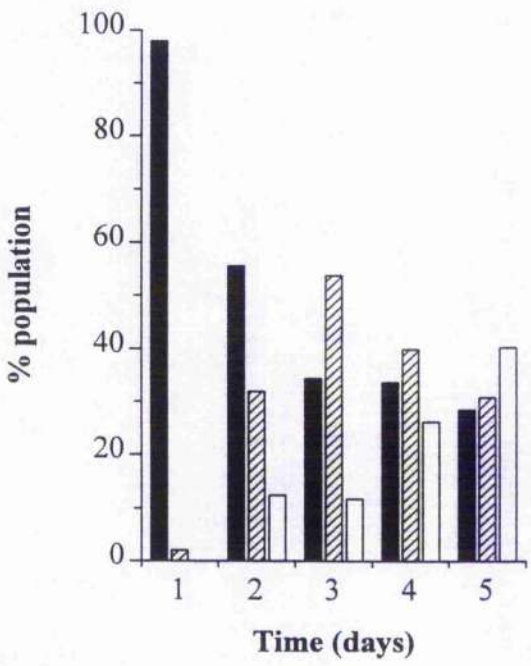
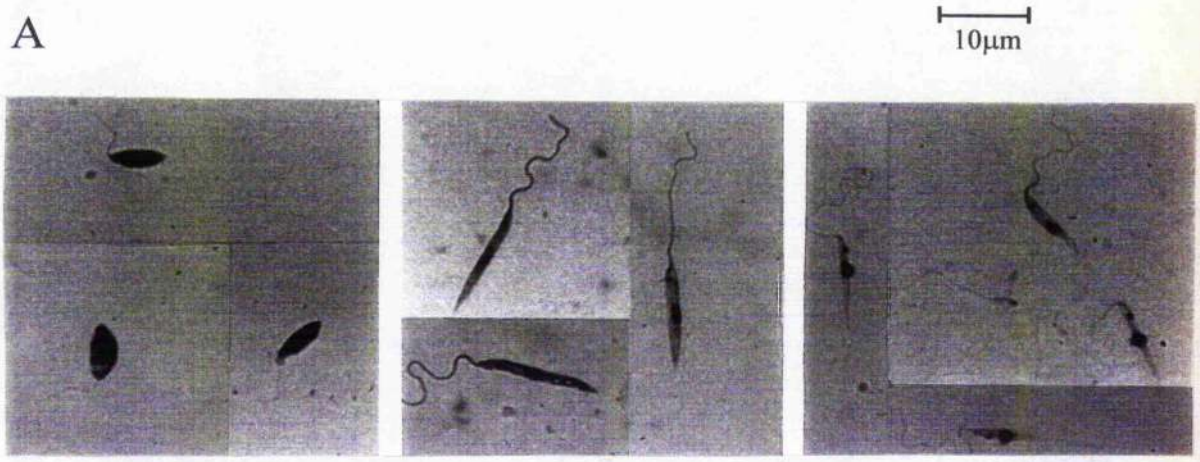


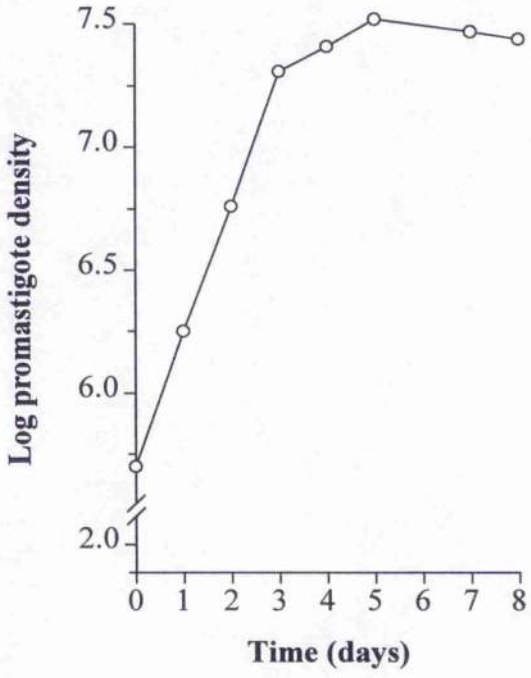
Figure 3.6: Growth and morphological development of *L. major* promastigotes in *in vitro* culture. Light micrographs of Giemsa-stained smears of *L. major* promastigotes (A) demonstrating the three different morphotypes and their proportions (C): morphotype 1 promastigotes, closed bars; morphotype 2 promastigotes striped bars; morphotype 3 promastigotes, open bars at different phases of growth of a typical promastigote culture (B): Each promastigote morphotype was characterised by its body length and breadth measurements in Giemsa-stained smears, the definitions of which are outlined in Table 3.1. One hundred promastigotes were examined for each daily sample from each culture.

A

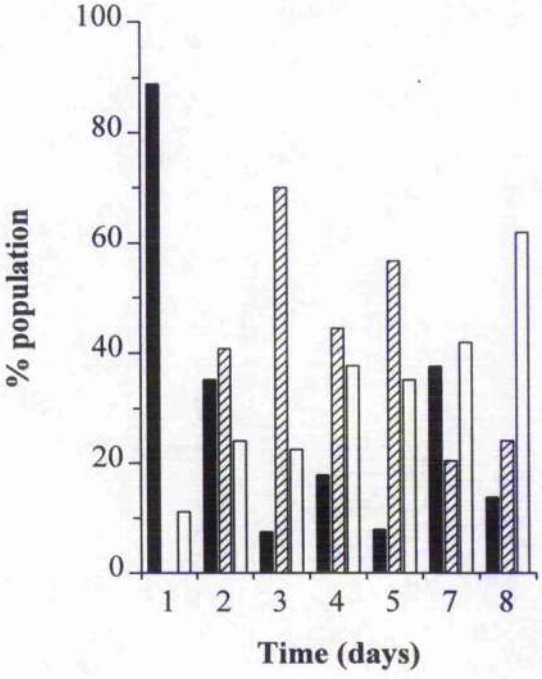


Morphotype 1 Morphotype 2 Morphotype 3

B



C



gradually replaced by longer, more slender promastigotes (morphotype 2) which reached maximum numbers in late-log phase. These elongate promastigotes reduced in number with the advent of stationary phase when a third promastigote morphotype (morphotype 3) with a characteristic short slender cell body occurred. Although each species produced promastigotes of all three morphotypes, there were clear differences in lengths of equivalent morphotypes (Table 3.1). *L. mexicana* promastigotes were longest whereas *L. panamensis* were the shortest. Promastigotes intermediate in size between morphotypes 1 and 2 predominated in cultures in the mid-log phase of growth. This 'intermediate' (MTi) category was also defined consisting of cells greater than or equal to 2·5 μm wide but also greater than 6·2 μm, 10·0 μm or 12·5 μm long for *L. panamensis*, *L. major* and *L. mexicana*, respectively.

To investigate more fully the apparent differences in morphology of promastigotes of *L. mexicana* in early-log phase, late-log phase and stationary phase cultures, a quantitative analysis of their cell body lengths and widths was performed from Giemsa-stained smears. Figure 3.7 illustrates the frequency distribution of promastigote body lengths and breadths observed in a typical culture of *L. mexicana* produced from each of the growth phases. In the experiment presented, which is typical of many analysed but was at sub-passage 15, the mean promastigote body lengths and widths of these three populations were 8·3 units (10·4 μm) x 2·0 units (2·5 μm), 13·3 units (16·6 μm) x 1·5 units (1·9 μm) and 10·7 units (13·7 μm) x 1·4 units (1·8 μm), respectively. When another stationary phase culture of lower sub-passage (SP6), which contained a higher proportion of metacyclic-like cells, was assessed the mean body length and width were reduced to 7·9 units (9·9 μm) and 1·1 units (1·4 μm), respectively. A comparison of the frequency distribution of the two stationary phase cultures is shown in Figure 3.8.

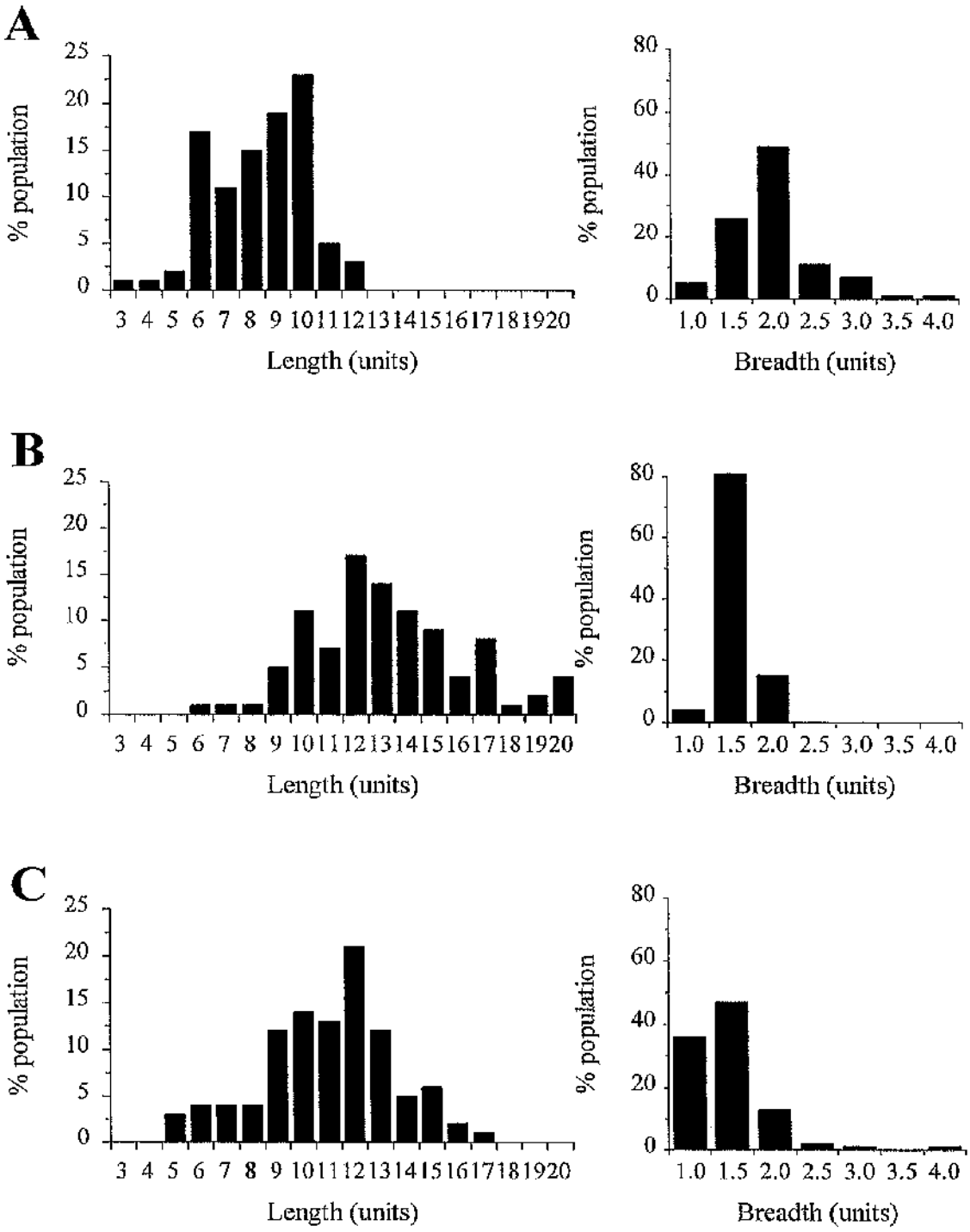


Figure 3.7: Comparison of the body lengths and breadths of *L. mexicana* promastigotes in different phases of the *in vitro* growth cycle: A, early-log phase (day 1); B, late-log phase (day 4); C, stationary phase (day 6). Promastigotes in the mid- to late-log phase of growth were initiated into complete HOMEM medium at $5x10^5$ ml^{-1} and allowed to progress until the stationary phase of growth. Cell counts and Giemsa-stained smears were produced daily to monitor growth and morphology. One hundred promastigotes were measured for each group. Each unit = 1·25 μm.

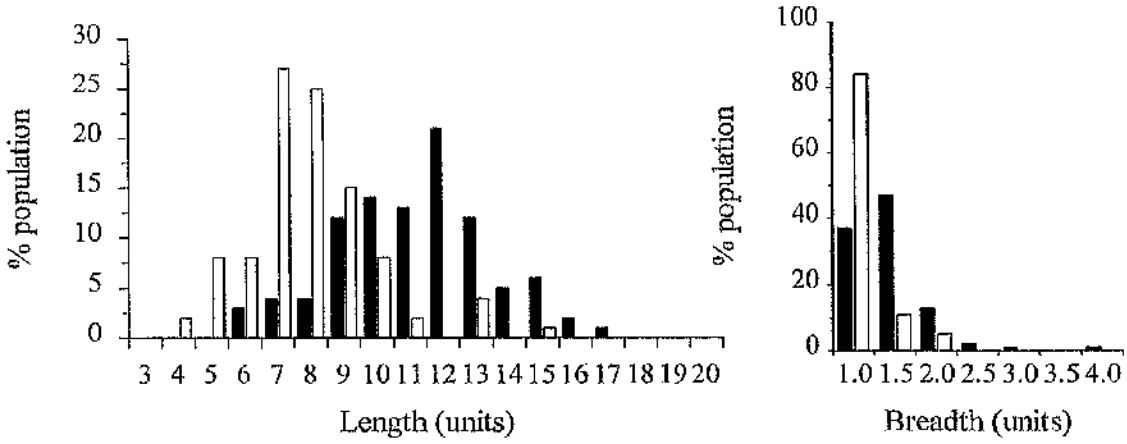


Figure 3.8: Comparison of the frequency distributions of promastigote body lengths and widths of two stationary phase cultures of *L. mexicana*. The culture represented by the open bars contained a higher proportion of metacyclic-like promastigotes than that represented by the closed bars. One hundred promastigotes were measured for each culture.

Promastigotes which morphologically resemble the three main promastigote morphotypes (MT1, MT2 and MT3) have been identified in the alimentary tract of infected sandflies. Promastigotes classified in the morphotype 1 category are similar to those described in early sandfly infections, rapidly multiplying within the blood meal in the peritrophic membrane. Morphotype 2 forms of *L. mexicana* and *L. major* are similar to the promastigotes of suprapylarian species that multiply within the midgut of the sandfly and attach, via their flagella, to the microvilli. Such forms have not been described from studies of sandflies infected with *L. panamensis*. An equivalent stage is thought to be located attached to the chitinous lining of the hindgut. The slender morphotype 2 culture forms of *L. panamensis* could be biochemical equivalents of these hindgut forms. Unfortunately the precise nature of these forms remains unknown, although they have been described as spatulate like. Such forms were not however obtained under any of the *in vitro* conditions used in my experiments. Morphotype 3 promastigotes of *L. mexicana* and *L. major* are thought to represent the infective metacyclic promastigotes, a morphologically and biochemically distinct promastigote stage which has yet to be defined for *L. panamensis* although is presumed to exist. Stationary phase cultures of *L. panamensis* contained promastigotes of two main morphologies: small bodied cells with long flagella and large, rounded bodied cells also with long flagella. Both of these resemble the morphological descriptions of the promastigotes identified in stationary phase cultures of *L. braziliensis* [Almeida *et al.*, 1993]. The latter of these two forms in cultures of *L. panamensis* was present in large amounts in late-log/early stationary phase cultures and decreased when promastigote densities began to decline (late stationary phase/decline phase). The proportion of small bodied promastigotes increased at this time and were therefore classified as morphotype 3

promastigotes of this species. Since the rounded promastigotes which occurred in late-log/early stationary phase cultures never outnumbered other morphotypes they were not investigated further.

3·2 Medium osmolarity

Promastigote morphology is known to alter depending upon the osmolarity of the surrounding medium [reviewed by Blum, 1996]. To test whether this could be a contributing factor to the observed morphological changes, media samples were taken from cultures of *L. panamensis* promastigotes on different days and analysed for osmolarity after removal of cells by centrifugation. The results are shown in Table 3.2. Dilution of fresh medium with distilled, deionised water gave readings expected for 2- and 4-fold dilutions, that is approximately a half and a quarter that of the stock solution, respectively. The osmolarity was very similar in all the media samples tested, fresh medium and spent medium from cultures at early-log phase through to late-log/early stationary phase of growth. Thus, the different promastigote forms of *L. panamensis* which occur during growth in *in vitro* culture are not simply brought about by changes in medium osmolarity.

3.3 Stage-Specific Characteristics

To determine whether the three different promastigote morphotypes of *L. mexicana* and *L. panamensis* were biochemically distinct, and if so to identify features which are characteristic of each, various properties were analysed. The studies were undertaken on populations of promastigotes which were not homogeneous but which were judged by eye to contain a very large proportion of

Medium	Osmolarity (mmol/kg)
Fresh HOMEM with 10% FCS	329 ± 2·3
Fresh HOMEM with 10% FCS, diluted 2 fold	159 ± 1·5
Fresh HOMEM with 10% FCS, diluted 4 fold	78 ± 1·7
Spent HOMEM, Day 1	336 ± 2·6
Spent HOMEM, Day 2	328 ± 6·4
Spent HOMEM, Day 3	330 ± 2·3
Spent HOMEM, Day 5	329 ± 2·0

Table 3.2: Measurement of fresh and spent medium osmolarity during growth of *L. panamensis* promastigotes *in vitro*. Spent medium samples were harvested from a routine culture by centrifugation on the days stated. Each sample was analysed three times and the means ± S.D. are presented.

Species / Cell Type	Protein / 10^8 cells (mg)
L. mexicana	
Morphotype 1 promastigotes	0·58 ± 0·12 (2)
Morphotype 2 promastigotes	0·65 ± 0·03 (2)
Morphotype 3 promastigotes	0·51 ± 0·06 (3)
Axenic amastigotes	0·20 ± 0·02 (3)
L. panamensis	
Morphotype 1 promastigotes	0·47 ± 0·11 (2)
Morphotype 2 promastigotes	0·44 ± 0·08 (2)
Morphotype 3 promastigotes	0·48 ± 0·08 (2)

Table 3.3: Protein content of whole cell homogenates of each promastigote morphotype of *L. mexicana* and *L. panamensis* and axenic amastigotes of *L. mexicana*. Protein content is presented as milligrams per 10^8 cells as estimated using the Pierce BCA kit. Sample concentrations were calculated by comparison with a standard curve constructed using the supplied solution of bovine serum albumin. The numbers in parenthesis indicate the number of cell pellets analysed.

the appropriate promastigote morphotype. This approach was unavoidable as methods for separating the individual morphotypes were not available. As the main aim was to identify positive characteristics of each form, the use of enriched populations was satisfactory if not ideal.

Morphotype 1 promastigotes consisted of early-log phase promastigotes harvested approximately 24 hours subsequent to inoculation at $5x10^5$ ml^{-1}. Cultures set up in an identical fashion but left until day 3/4 of growth when promastigotes were in the late-log phase of growth were representative of morphotype 2 forms. Biochemical studies carried out on promastigotes of morphotype 3 were more specifically on stationary phase cells of low sub-passage (less than 10) grown in complete HOMEM medium. The proportions of each promastigote form in a typical culture of *L. mexicana* and *L. panamensis* as assessed from Giemsa-stained smears prepared daily are detailed in Figures 3.4C and 3.5C, respectively.

3.3.1 Protein content

The total protein content of the different promastigote morphotypes of *L. panamensis* and *L. mexicana* and axenic amastigotes of *L. mexicana* are presented in Table 3.3. The protein content remained relatively constant during *L. panamensis* promastigote growth *in vitro* with all morphoyypes containing ca. 0·44-0·48 mg protein 10^8 $cells^{-1}$. In contrast, the protein content of *L. mexicana* promastigotes differed depending on the promastigote morphotype examined. Morphotype 3 promastigotes contained the least amount of protein, 0·51 mg protein 10^8 $cells^{-1}$, while morphotype 2s contained the most, 0·65 mg protein 10^8 $cells^{-1}$. Morphotype 1 promastigotes were intermediate between the other two morphotypes and contained 0·58 mg protein 10^8 $cells^{-1}$. Axenic amastigotes of *L. mexicana* contained 0·2 mg

protein 10^8 cells^{-1}, approximately one third that of promastigotes.

3.3.2 Protein Profiles

3.3.2.1 SDS-PAGE analysis

Coomassie stained protein profiles of the different promastigote morphotypes of *L. mexicana* and *L. panamensis* were compared following protein separation by SDS-PAGE (Figure 3.9). Supernatant and pellet fractions of cells lysed by aspiration of frozen cell pellets with 0·25% (v/v) Triton X-100 were analysed separately and whole cell lysates were not examined.

When equal numbers of promastigotes were compared, it was consistantly found that the different samples varied in terms of the overall amount of protein they contained (not shown). Supernatant lysates of both species and pellet lysates of *L. mexicana* promastigotes followed a similar trend; morphotype 1 promastigotes produced much more intensely stained profiles than either of the other two forms, while morphotype 2 promastigotes gave banding intensity intermediate between morphotypes 1 and 3. This indicates that morphotype 1 promastigotes contained higher quantities of soluble proteins than promastigote morphotypes 2 or 3 while morphotype 3 promastigotes contained the lowest amounts. A cocktail of proteinase inhibitors was included during cell lysis, so the differences observed were unlikely to have been due to differential proteolysis. The same result was observed for *L. mexicana* insoluble proteins. *L. panamensis* pellet fractions produced a different gradation of staining intensity. The results indicated that morphotype 2 promastigotes contained larger quantities of insoluble proteins than promastigote morphotypes 1 or 3 and that morphotype 3 promastigotes contained intermediate amounts.

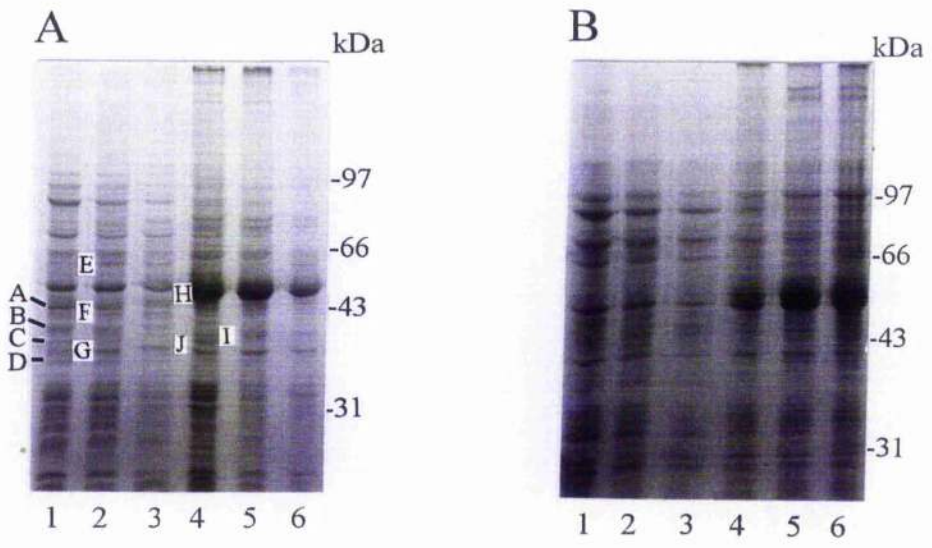


Figure 3.9: Protein profiles of *L. mexicana* (panel A) and *L. panamensis* (panel B) *in vitro* promastigote forms. Panel A, lanes 1-3 contain soluble extracts of *L. mexicana* promastigote morphotypes 1, 2 and 3, respectively, and lanes 4-6 are pellet extracts, in the same order. Each lane contains the equivalent of 10^7 promastigotes, except for lane 1 which contains $2{\cdot}5x10^6$ promastigotes. Panel B contains *L. panamensis* promastigotes, sample identities as for panel A. Each lane contains the equivalent of 10^7 promastigotes, except for lane 5 which contains $5x10^6$ promastigotes. The positions of molecular mass standards are indicated. For an explanation of differential loading and labelled bands refer to section 3.3.2.

Comparisons of individual protein bands could be made more easily by analysing unequal numbers of promastigotes (see Figure 3.9). In general and as expected, the protein profiles of the three promastigote morphotypes of *L. mexicana* were rather similar. However, several proteins appeared to vary quantitatively when the profiles were compared. Most of these changes followed a similar trend, morphotype 1 promastigotes containing the largest amounts and morphotype 3s the smallest - which reflects the differences in soluble protein content of the three stages. Four of the most prominent bands which changed in this way are labelled on the Figure (panel A, lane 1): A (ca. 43 kDa), B (ca. 40 kDa), C (ca. 38 kDa) and D (ca. 36 kDa). In contrast, at least three protein bands appeared to be most abundant in morphotype 2 promastigotes (E, ca. 60 kDa; F, ca. 42 kDa and G, ca. 37 kDa). Pellet lysates of *L. mexicana* also produced very similar protein profiles but again quantitative variations in individual protein bands were observed. The most obvious change was in one protein band of approximately 46 kDa in size (H). This protein was present in similar amounts in promastigote morphotypes 1 and 2 but the quantity was significantly reduced in morphotype 3 forms. Two other insoluble proteins, labelled I (ca. 39 kDa) and J (ca. 38 kDa), appeared to undergo some regulation in the different promastigote forms. A larger amount of I was present in morphotype 2 promastigotes than in either of the other two promastigote forms, while J was most abundant in morphotype 1 promastigotes.

The protein profiles of *L. panamensis* promastigotes were much more similar than those of *L. mexicana* and are shown in Figure 3.9 (panel B). No clear-cut changes in proteins that occurred during *in vitro* growth were identified by the procedure used.

3.3.2.2 Stage-specific protein synthesis

Promastigotes of *L. mexicana* and *L. panamensis* in different phases of the *in vitro* growth cycle and axenic amastigotes of *L. mexicana* were incubated with radioactive methionine in an attempt to demonstrate stage-specific protein synthesis. Radioactively-labelled cell pellets were lysed in the presence of proteinase inhibitors and separated into supernatant and pellet fractions by centrifugation, each fraction was analysed by SDS-PAGE. Whole cell lysates were not investigated.

The results showed that there were clear differences in the overall quantity of methionine-containing proteins being synthesized in the different forms of *L. mexicana* during the 2 hour incubation period. These differences were reflected in the intensity of the signal detected in autoradiographs (not shown). Supernatant fractions of morphotype 1 promastigotes consistantly exhibited much higher quantities of radioactivity than equivalent fractions of the other two forms, while pellet fractions of promastigote morphotypes 1 and 2 separated by SDS-PAGE gave autoradiograph profiles which were similar in intensity. Morphotype 3 pellet fractions gave more intense banding profiles than similar fractions of the other two forms. Profiles of the various promastigote forms of *L. panamensis* were much more consistant in terms of the strength of the radioactive signal detected, although the pellet fraction of morphotype 2 promastigotes incorporated a higher quantity of radioactivity than either fraction of the other two forms, suggesting a higher level of synthesis of membrane-associated protein in these promastigotes.

The results of a preliminary experiment which compared synthesis of soluble proteins by promastigote morphotypes 1 and 2 of *L. mexicana* are shown in Figure 3.10. Each lane contained equivalent cell numbers, demonstrating the difference in amount of protein synthesis by these two promastigote stages. Comparison of

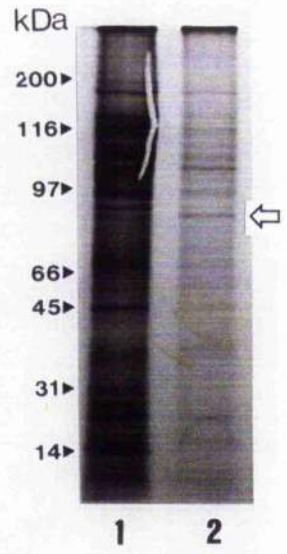
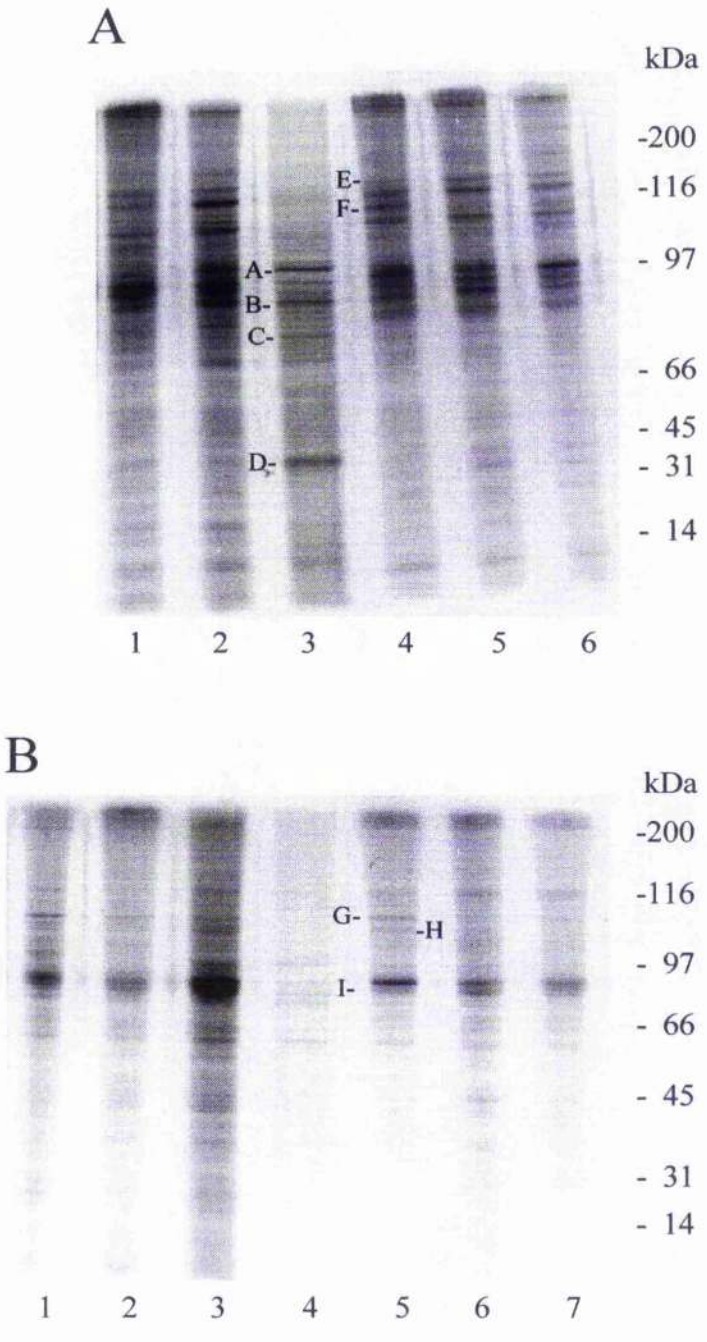


Figure 3.10: Protein synthesis in *L. mexicana* morphotype 1 (lane 1) and morphotype 2 (lane 2) promastigotes. Autoradiograph demonstrating ^{35}S-methionine labelled proteins in supernatant fractions of early-log (morphotype 1) and late-log (morphotype 2) phase promastigotes. Both lanes contain the equivalent of 10^6 cells. Closed arrows mark the positions of molecular mass standards while the open arrow labels the most prominant differentially expressed protein.

individual protein bands of this species, following separation by SDS-PAGE, revealed at least one protein (arrowed), of ca. 80 kDa, which was produced in larger quantities by morphotype 2 forms than by morphotype 1s. The other prominant bands appeared to be synthesised by both stages, although in greater amounts by morphotype 1 promastigotes.

Subsequent experiments were carried out to compare protein synthesis by promastigote morphotypes 1, 2 and 3 of *L. panamensis* and morphotypes 2 and 3 and axenic amastigotes of *L. mexicana*. Unequal cell numbers were loaded in order to produce relatively balanced profiles thereby permitted comparison of individual protein bands. The results are presented in Figure 3.11. Although very similar protein profiles were exhibited for all three promastigote forms of the same species, various changes in expressed proteins occurred during growth and development of promastigotes in *in vitro* culture. The most significant differences are labelled. Comparison of soluble protein profiles of *L. mexicana* promastigote morphotypes 2 and 3 identified that the main differences were quantitative, most proteins being produced in greater quantities in morphotype 3 promastigotes (compare lanes 1 and 2, panel A). Transformation of morphotype 3 promastigotes to axenic amastigotes resulted in several changes in protein synthesis as identified by this procedure (compare lane 3 with 2, panel A). The changes which were detected were principally apparently quantitative, most proteins being synthesised in lesser quantities in axenic amastigote than in morphotype 3 promastigotes (despite loading 10 times as many amastigotes). However, two proteins, (C, ca. 78 kDa; D, ca. 33 kDa) were detected in higher quantities in axenic amastigote samples than in morphotype 3 promastigote samples while two others were present in relatively equivalent amounts (A, ca. 98 kDa and B, ca. 90 kDa).

Figure 3.11: Stage-specific protein synthesis. Autoradiographs demonstrating ^{35}S-methionine-labelled protein profiles of supernatant fractions of *L. mexicana* promastigote morphotypes 2 and 3 (panel A, lanes 1 and 2, respectively) and *L. panamensis* promastigote morphotypes 1, 2 and 3 (panel A, lanes 4, 5 and 6, respectively) and axenically cultivated amastigotes of *L. mexicana* (panel A, lane 3). The volume of lysate was adjusted to allow comparison of proteins in the different forms. Lanes 1, 2, 4, 5 and 6 contain the equivalent of 10^6 cells, while lane 3 contains 10^7 cells. Radioactively labelled pellet fractions of the same samples in the same order (lanes 2, 3, 4, 5, 6 and 7) but preceded by early-log phase promastigotes of *L. mexicana* (lane 1) are presented in panel B. Lane 1 contains the equivalent of $2{\cdot}5x10^5$ cells, lanes 2, 3, 5, and 7 the equivalent of 10^6 cells, lane 3, 10^7 cells and lane 6, $5x10^5$ cells. The positions of molecular mass standards are indicated. Protein bands of particular interest are marked (A-I).


A
kDa
-200
-116
- 97
- 66
- 45
- 31
- 14
E-
F-
A-
B-
C-
D-
1 2 3 4 5 6
B
kDa
-200
-116
- 97
- 66
- 45
- 31
- 14
G-
-H
I-
1 2 3 4 5 6 7

When synthesis of insoluble proteins of *L. mexicana* promastigotes was compared it was clear that most differences again were apparently quantitative (Figure 3.11, panel B). Morphotype 1 promastigotes synthesised greater quantities of most of the detectable proteins than did the other two forms. Morphotype 2 promastigotes contained lowest amounts. Axenic amastigotes produced a very weakly stained profile, indicating that insoluble proteins were synthesised in smaller quantities than soluble proteins. When insoluble proteins of axenic amastigotes and promastigotes were compared it was found that many of the most abundant proteins were common to both stages.

Examination of methionine-containing proteins synthesised by *L. panamensis* promastigotes detected differential expression of several proteins during *in vitro* growth (Figure 3.11, panel A lanes 4-6 and panel B lanes 5-7). Two soluble proteins were notably different (panel A). Protein E (ca. 150 kDa), was synthesised in greater quantities by morphotype 2 promastigotes than morphotypes 1 and 3, while protein F (ca. 115 kDa), was produced in larger amounts by morphotype 1 promastigotes than either of the other two promastigote forms. Synthesis of membrane bound proteins (panel B), those retained in the pellet fraction, of promastigote morphotypes 2 and 3 were comparable. Morphotype 1 promastigotes, however, produced two proteins in particular in greater quantities than the other two forms. These are labelled G and H and were ca. 113 kDa and 112 kDa in size, respectively. One smaller protein (I, ca. 90 kDa), was notably synthesised in larger amounts by morphotype 2 forms than by morphotypes 1 or 3.

3.3.2.3 Western blotting analysis

3.3.2.3.1 Antibodies raised to whole cell homogenates of *L. mexicana*

The antisera which were raised in rabbits against whole cell homogenates of promastigote morphotypes 1, 2 and 3 of *L. mexicana* were used to probe soluble extracts of each of these stages following separation by SDS-PAGE. Examples of the recognition profiles for each stage are shown in Figure 3.12. Pre-immune sera detected no background bands and are therefore not presented. Each immune-serum recognized a similar array of molecules in all three of the promastigote forms. When profiles were compared no molecules were identified which were clearly specific to one of the promastigotes morphotypes. The banding profiles produced from probing morphotype 1 and morphotype 2 promastigotes with serum raised against morphotype 1 promastigotes were very similar (panel A, lanes 1 and 2), although at least three molecules appeared to be present in larger quantities in morphotype 2 promastigotes than in morphotype 1 promastigotes while two different proteins were more abundant in morphotype 1 lysates. Fewer molecules were detected in soluble lysates of morphotype 3 promastigotes with the same immune-serum (panel A, lane 3), however those bands which were recognised were also present in the other two forms. The antiserum raised against morphotype 2 promastigotes (panel B) recognised similar but distinct profiles to that detected with the morphotype 1 antiserum (panel A). None of the high mobility bands (< 45 kDa) which were detected in promastigote morphotypes 1 and 2 with anti-morphotype 1 antiserum were recognised while some of the lower mobility bands (> 97 kDa) present in morphotype 2 and morphotype 3 promastigotes appeared to be absent in morphotype 1 forms. In addition, a number of molecules (45-66 kDa) recognised by the serum raised against morphotype 2 promastigotes appeared to be differentially expressed in the different promastigote morphotypes. A larger number of

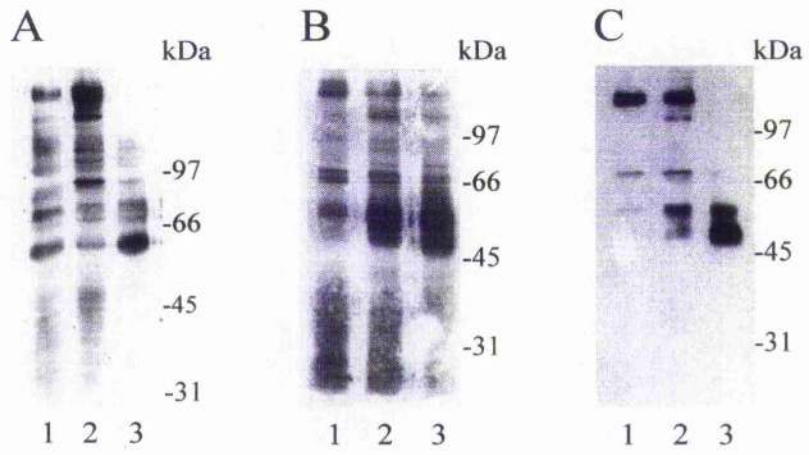


Figure 3.12: Western blots of *L. mexicana* promastigote soluble lysates probed with antisera raised in rabbits against each of the different promastigote morphotypes. Reduced and denatured soluble lysates of morphotype 1 (lane 1), morphotype 2 (lane 2) and morphotype 3 (lane 3) promastigotes were probed with anti-morphotype 1 (panel A), anti-morphotype 2 (panel B) and anti-morphotype 3 (panel C) antisera. Each lane contains the equivalent of 10^6 promastigotes and the positions of molecular mass standards are indicated.

bands in this size range were detected in morphotype 3 promastigotes (panel B, lane 3) than in either of the other two forms (panel B, lanes 1 and 2). An intermediate quantity were present in morphotype 2 promastigotes. Anti-morphotype 2 serum detected only one molecule, of approximately 66 kDa, in morphotype 1 promastigotes which did not appear to be present, or if present was at considerably lower quantities, in promastigotes of morphotypes 2 or 3. Antiserum which was raised against the morphotype 3 promastigote stages of *L. mexicana* (panel C) recognised fewer molecules in all of the three stages than either of the other two antisera, however, the most prominant bands detected were also recognised by the anti-morphotype 1 and anti-morphotype 2 immune sera.

Generation of partially stage-specific antisera was attempted by reacting soluble extracts of promastigotes rich in each morphotype with the antisera generated in rabbits. Based on a preliminary experiment with promastigotes of morphotypes 1 and 2, a 1:100 dilution of immune serum with promastigote lysate supernatant (10^9 cells ml^{-1}) was sufficient to remove the majority of common antigens (not shown). Supernatant lysates of each promastigote morphotype were probed following SDS-PAGE with antisera generated from the reaction of each antigen type with a different serum type. The results are shown in Figure 3.13. Molecules were only detected when lysates were probed with serum originating from morphotype 3 antiserum (panels E and F). A doublet of approximately 47 and 50 kDa was detected by serum raised against morphotype 3 promastigotes which had been modified to remove antibodies to antigens which were also present in promastigotes of morphotypes 1 and 2.

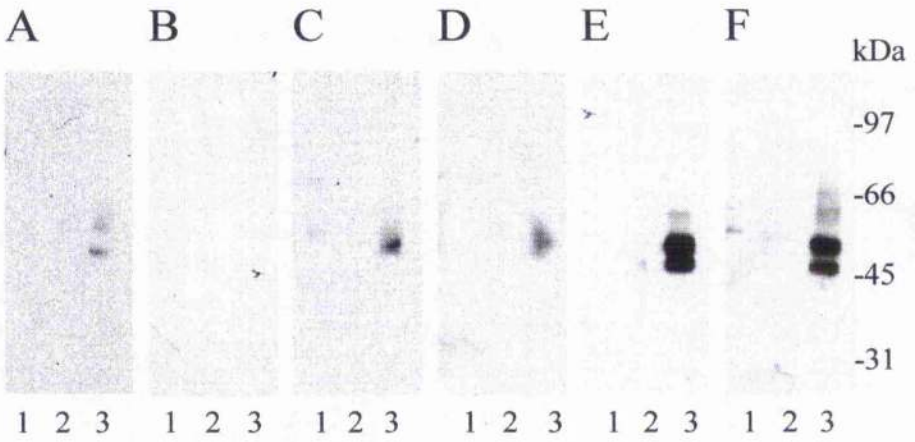


Figure 3.13: Western blots of *L. mexicana* promastigote soluble lysates probed with partially stage-specific antisera. Reduced and denatured soluble lysates of morphotype 1 (lanes 1), morphotype 2 (lanes 2) and morphotype 3 (lanes 3) promastigotes were probed on Western blots with antisera which were raised in rabbits against each of the different promastigote morphotypes (MT) which had undergone subsequent modification by antigen-antibody complexing: panel A, anti-(MT2+anti-MT1); panel B, anti-(MT3+anti-MT1); panel C, anti-(MT1+anti-MT2); panel D, anti-(MT3+anti-MT2); panel E, anti-(MT1+anti-MT3); panel F, anti-(MT2+anti-MT3). Partially stage-specific antisera were produced as follows. Supernatant lysates of each promastigote morphotype (lysed at 10^9 ml^{-1} and separated by centrifugation at 3,000 g for 10 minutes) were vigorously shaken overnight in the presence of the different antisera in the ratio 99:1. Complexes were removed by centrifugation and the supernatants used to probe Western blots. Each lane contains the equivalent of 10^6 cells and the positions of molecular mass standards are indicated.

3.3.2.3.2 Antibodies raised to cysteine proteinases of *L. mexicana*

Antibodies raised against three cysteine proteinases (CPs) of *L. mexicana* were used to probe soluble samples of the three promastigote morphotypes of *L. panamensis* and *L. mexicana* and lesion amastigotes of *L. mexicana*. These enzymes exhibit stage regulation in *L. mexicana* [reviewed by Coombs and Mottram, 1997] and have been previously studied in log-phase promastigotes, metacyclics and amastigotes. Their occurrence in relation to the three promastigote morphotypes which occur *in vitro* (other than morphotype 3/metacyclic forms of *L. mexicana*) had not yet been studied for either *L. mexicana* or *L. panamensis*.

3.3.2.3.2.1 Anti-*cpb* antisera

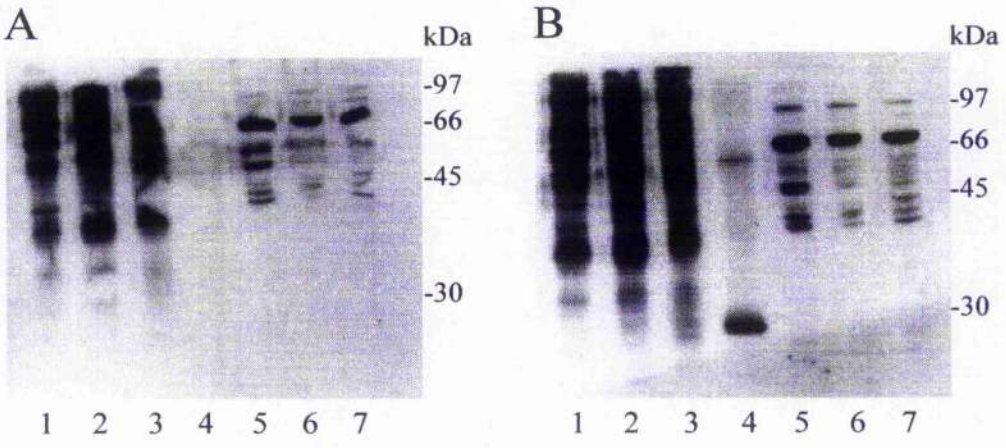
Soluble extracts of *L. mexicana* and *L. panamensis* promastigotes and lesion amastigotes of *L. mexicana* were probed using Western blotting techniques with antisera which had been raised in rabbits against the reduced and denatured forms of Type I CPs (encoded by the cpb gene array) of *L. mexicana* [Robertson and Coombs, 1994]. Figures 3.14 and 3.15 show the data with two different antisera (anti-type B enzyme and and anti-type C enzyme). The immune antisera (panels B) reacted similarly although different bands were detected by pre-immune sera (panels A). The immune antisera detected two proteins (24 and 27 kDa) in lesion amastigote samples of *L. mexicana* which were not recognised by pre-immune sera. The predominant molecule recognised in amastigotes (about 24 kDa) was also detected in morphotype 3 promastigotes together with a protein of marginally higher mobility. The antisera failed to recognise any of these proteins in extracts of the other two promastigote morphotypes.

Extracts of *L. panamensis* promastigotes were probed in Western blots with the same antisera (Figures, 3.14 and 3.15). No proteins of equivalent molecular mass to

Figure 3.14 (top): Western blot of various promastigote forms of *L. mexicana* (lanes 1-3) and *L. panamensis* (lanes 5-7) promastigotes and of *L. mexicana* lesion amastigotes (lane 4) probed with anti-type B CP antiserum (panel B): lanes 1 & 5, morphotype 1 promastigotes; lanes 2 & 6, morphotype 2 promastigotes; lanes 3 & 7, morphotype 3 promastigotes. Each lane was loaded with the equivalent of 10^7 cells. Panel A was probed in parallel with pre-immune serum.

Figure 3.15 (middle): Western blot of various promastigote forms of *L. mexicana* (lanes 1-3) and *L. panamensis* (lanes 6-8) promastigotes and of *L. mexicana* lesion amastigotes (lane 4) probed with anti-type C CP antiserum (panel B): lanes 1 & 6, morphotype 1 promastigotes; lanes 2 & 7, morphotype 2 promastigotes; lanes 3 & 8 morphotype 3 promastigotes; lane 5 contains no sample. Each lane was loaded with the equivalent of 10^7 cells. Panel A was probed in parallel with pre-immune serum.

Figure 3.16 (bottom): Western blot of various promastigote forms of *L. mexicana* (lanes 1-3) and *L. panamensis* (lanes 5-7) promastigotes and of *L. mexicana* lesion amastigotes (lane 4) probed with antisera raised against the mature domain of Type II CP of *L. mexicana* (panel B): lanes 1 & 5, morphotype 1 promastigotes; lanes 2 & 6, morphotype 2 promastigotes; lanes 3 & 7, morphotype 3 promastigotes. Each lane was loaded with the equivalent of 10^7 cells. Panel A was probed in parallel with pre-immune serum.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kDa
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-30
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B
kDa
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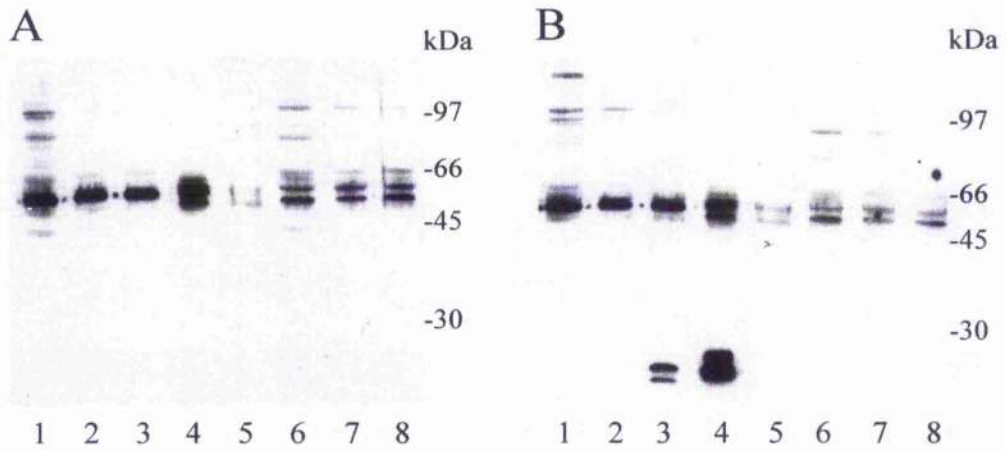
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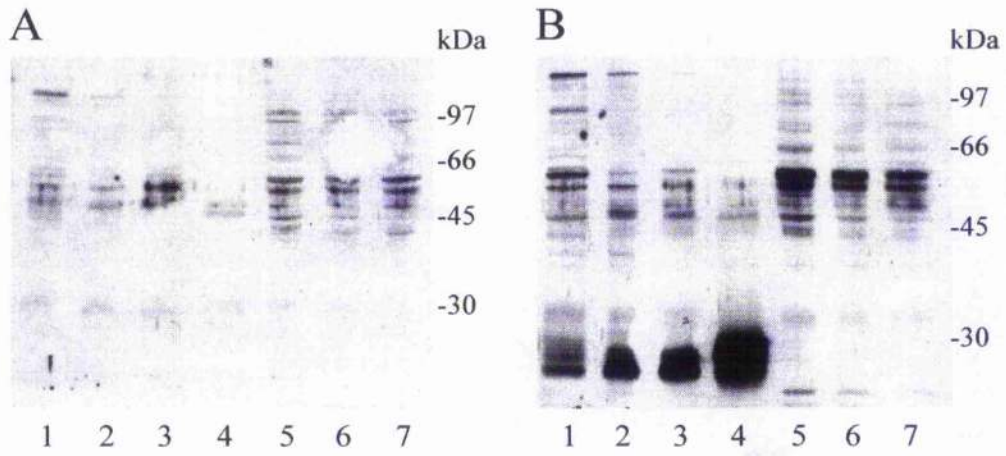
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B
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-45
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1 2 3 4 5 6 7

those specifically detected in *L. mexicana* were detected in any of the three promastigote forms. However, two bands of higher mobility (between 14 and 24 kDa) were recognised by anti-type B CP antiserum (Figure 3.14, panel B) on one occasion. These were at a higher level in morphotype 1 promastigotes than in morphotype 2s. Morphotype 3 promastigotes contained lowest levels.

3.3.2.3.2.2 Anti-*cpa* antisera

Soluble extracts of *L. mexicana* and *L. panamensis* promastigote morphotypes 1, 2 and 3 and lesion amastigotes of *L. mexicana* were probed with antisera raised against the Type II CPs mature domain of *L. mexicana* [Mottram *et al.*, 1992] following separation by SDS-PAGE and blotting onto nitrocellulose (Figure 3.16). Pre-immune sera detected background bands (panel A). The immune antisera recognised two additional protein bands, 24 kDa and 27 kDa, in all three promastigote forms and purified lesion amastigotes of *L. mexicana*. Both proteins were present in equal amounts in lesion amastigotes but in all promastigote forms the 24 kDa band predominated. Morphotype 1 promastigotes contained considerably reduced amounts, while lesion amastigotes contained more of both proteins than did the other two promastigote forms.

The antisera failed to recognise proteins of equivalent sizes (24 kDa and 27 kDa) in *L. panamensis* promastigotes, although 2 bands of higher mobility (between 14-24 kDas) were detected (Figure 3.16). These were both at higher levels in morphotype 1 promastigotes than in morphotypes 2 and 3, the latter promastigote form containing the least.

3.3.2.3.3 Antibodies raised to gp63

Parasite lysates at 10^9 ml^{-1}, were obtained by aspiration of frozen cell pellets with 0·25% (v/v) chilled Triton X-100 containing a cocktail of proteinase inhibitors. The homogenates were then separated by centrifugation at 3,000 g for 10 minutes at room temperature using a microfuge to partially and crudely isolate membrane (pellet fractions) and cytosolic (supernatant fractions) components. The monoclonal antibody 235 [Wallace and McMasters, 1987] failed to recognise any proteins in Western blots of either extracts of *L. panamensis* promastigotes (not shown). However, a signal was detected in lysates of *L. mexicana* promastigotes and crudely harvested lesion amastigotes after identical treatment (Figure 3.17). This signal consisted of a doublet banding pattern at approximately 66 kDa molecular mass in promastigotes and a more complex series of bands in amastigotes. The majority of signal was confined to the particulate fraction of lysates of both amastigote and promastigote stages, although it was also detected in the soluble fraction. Comparison of the abundance of the protein (assuming that signal strength was directly proportional to protein quantity) identified that the quantities varied depending on the promastigote morphotype as well as the nature of the sample analysed. The largest and smallest quantities were detected in insoluble extracts of promastigotes of morphotypes 1 and 2, respectively, while soluble extracts of promastigotes of morphotypes 2 and 3 contained highest and lowest amounts, respectively.

The same antibody recognised a number of bands in a crudely harvested pellet of lesion amastigotes. The most intense signal was emitted by a doublet (ca. 58 and 60 kDa) slightly smaller in molecular mass than the promastigote bands. These proteins are clearly distinguishable in soluble extracts. At least another two

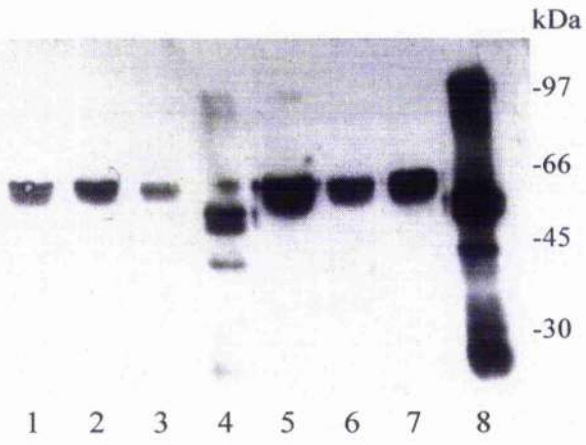


Figure 3.17: Western blot of *L. mexicana* promastigote and lesion amastigote pellet and supernatant lysates probed with the mab 235 raised against gp63. Supernatant samples of morphotype 1 promastigotes (lane 1), morphotype 2 promastigotes (lane 2), morphotype 3 promastigotes (lane 3) and crudely harvested amastigotes (lane 4). Pellet samples of the same lysates are loaded in lanes 5-8. The positions of molecular mass standards are indicated.

faster moving bands were recognised as well as some slower ones. All were present in larger amounts in the particulate fraction than in the soluble fraction. Only one of these proteins (ca. 43 kDa) failed to be recognised when a purified lesion amastigote lysate was probed (not shown).

3.3.3 Proteinases

The proteinases of *L. mexicana* log-phase and stationary promastigotes and amastigotes have been studied and characterised in depth. The results of this study confirm and expand upon proteinase data previously published [Bates *et al.*, 1994] on these forms and are shown in Figure 3.18. The proteinases of early-log phase promastigotes (morphotype 1) in addition to comparison of incubation length (1-5 hours and 2-5 hours, panels A and B, respectively) are also presented here. Amastigotes exhibited multiple proteinases, the most active of which were between 22-24 kDa molecular mass. Several lower mobility bands ranging from 24 kDa-60 kDa were also active. A third group of bands in the size range 70-100 kDa was also detected. Morphotype 3 promastigotes exhibited a slightly different banding profile from amastigotes including a proteinase thought to be metacyclic specific (ca. 21 kDa) [Robertson and Coombs, 1992]. This band was also present in soluble lysates of morphotype 2 promastigotes, perhaps indicating the presence of some metacyclic forms. In general, the proteinase profiles of promastigote morphotypes 2 and 3 were very similar, although the enzymes were less active in morphotype 2s. Morphotype 1 promastigotes exhibited the lowest proteinase activity of all four life-cycle stages investigated. Only the low mobility bands (70-100 kDa) were detected in gels where gelatin hydrolysis was permitted for 1-5 hours. When this incubation was lengthened to 2-5 hours, proteinases of higher molecular mass (between 24-60

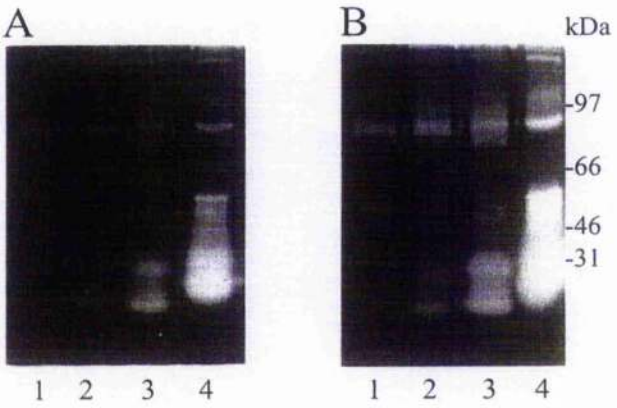


Figure 3.18: Gelatin SDS-PAGE analysis of supernatant extracts of purified lesion amastigotes and the various promastigote forms of *L. mexicana*: lanes 1, morphotype 1 promastigotes; lanes 2, morphotype 2 promastigotes; lanes 3, morphotype 3 promastigotes; lanes 4, lesion amastigotes. Gels A and B were incubated for 1·5 hours and 2·5 hours, respectively, with sodium acetate buffer pH 5·5 including 1 mM DTT at 37°C. Each lane contains the equivalent of 10^7 cells. The positions of molecular mass standards are indicated.

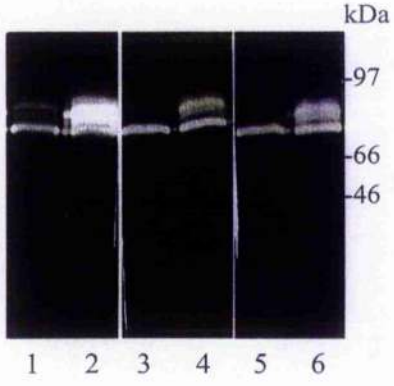


Figure 3.19: Gelatin SDS-PAGE analysis of stationary phase promastigotes of *L. panamensis* grown in HOMEM, pH 7·0 (odd numbered lanes) and SDM pH 5·5 (even numbered lanes) and comparison of incubation buffers. The following buffers were used: glycine, pH 3·5 (lanes 1 and 2); tris pH 8·5 (lanes 3 and 4); hydrogen borate, pH 8·5 (lanes 5 and 6). Each lane was loaded with the equivalent of $6x10^6$ promastigotes. The positions of molecular mass standards are indicated.

kDa) were also detected.

As many as 6 proteinases (between 66-90 kDa) were detected in soluble lysates of *L. panamensis* promastigotes by gelatin SDS-PAGE. However, some of these activities proved variable and were not always detected. A variety of buffers within the pH range 3·5-9·5 were tested in order to determine the pH optimum of these enzyme activities towards gelatin in stationary phase promastigotes grown in complete HOMEM medium at neutral pH and complete SDM medium at pH 5·5. The results with buffers which permitted highest proteinase activities are presented in Figure 3.19. Glycine buffer at pH 3·5 and hydrogen borate (H_3BO_3) or Tris at pH 8·5 gave the most active profiles in promastigotes grown in both media. Interestingly, differences in proteinase activity were detected between the stationary phase promastigote populations which were cultivated in the two different media (discussed below). All subsequent proteinase gels were incubated with H_3BO_3 at pH 8·5.

The proteinase profiles of soluble lysates of the three different promastigote morphotypes of *L. panamensis* grown in HOMEM medium pH 7·0 were compared by gelatin SDS-PAGE (Figure 3.20). No stage-specific proteinases were identified. However, growth of *L. panamensis* promastigotes in medium developed to maximise the numbers of metacyclics (morphotype 3 promastigotes) in *L. mexicana* cultures (complete SDM medium) resulted in a gradation of enzyme activities, those of stationary phase cells (morphotype 3) being more active than late-log phase (morphotype 2) promastigotes which were more active than promastigotes in the early-log phase (morphotype 1) of growth (not shown). Consistant with this, comparison of proteinases in stationary phase cells grown in these two media detected differences in the activity of the enzymes (Figure 3.19). Greater gelatin

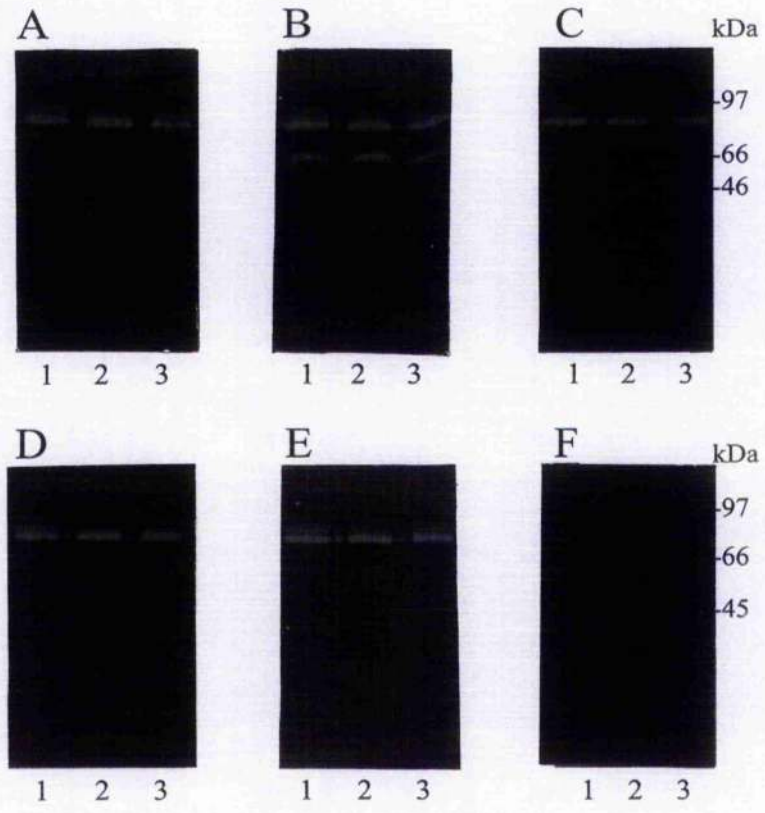


Figure 3.20: Activities of soluble proteinases of morphotype 1, morphotype 2 and morphotype 3 promastigotes of *L. panamensis* grown in HOMEM pH 7 towards co-polymerized gelatin subsequent to electrophoresis: lane 1, morphotype 1 promastigotes; lane 2, morphotype 2 promastigotes; lane 3, morphotype 3 promastigotes. Each gel slice was incubated under different conditions. Hydrogen borate buffer at pH 8·5 was used consistantly and contained no additions (panel A), 1 mM DTT (panel B), 10 μM E-64 (panel C), 5% methanol (panel D), 1 mM PMSF (panel E) or 10 mM 1,10-phenanthroline (panel F). Each lane was loaded with the equivalent of 10^7 promastigotes. The positions of molecular mass standards are indicated.

hydrolysis was observed in stationary phase promastigotes cultured in the metacyclic medium.

Partial characterisation of the proteinases active during growth of *L. panamensis* promastigotes *in vitro* was attempted using a variety of proteinase inhibitors and dithiothreitol (DTT), an activator of cysteine proteinases (Figure 3.20). All four proteinases detected in control gels (panels A & D) were inhibited by 1, 10-phenanthroline (panel F), an inhibitor of metallo-proteinases, while E-64 (panel C) and PMSF (panel E) had no apparent effect. One additional enzyme, of approximately 66 kDa, was detected in all three promastigote morphotypes when gels were incubated in the presence of 1 mM DTT (panel B). The activity was not apparent in the absence of DTT. This is indicative of a cysteine proteinase activity.

Amastigotes of *L. panamensis* were obtained, for analysis of proteinase content, by infection of J774G8 cells with stationary phase promastigotes. These cultures were incubated at 32°C for 7 days and then harvested and analysed by substrate SDS-PAGE following crude isolation of amastigotes (Figure 3.21). An uninfected culture of J774s was also analysed as a control. One proteinase activity, also detected in the three different promastigote forms, was detected in the soluble lysate of infected cells which was not present in uninfected macrophages.

3.3.4 Nucleases

Analysis of promastigotes and amastigotes of *L. mexicana* by substrate SDS-PAGE revealed four forms of nuclease (Figure 3.22, panel A). Three of these have been described previously: a band of apparent molecular mass 40 kDa and a doublet of 29/31 kDa [Bates, 1994]. All were found to be active in pellets rich in morphotype 2 promastigotes (lane 2) while only the doublet occurred in morphotype

Figure 3.21: Gelatin SDS-PAGE analysis of uninfected J774G8 cells (lane 1), *L. panamensis* amastigotes crudely isolated from J774G8 cells (lane 2) and morphotype 1 promastigotes of *L. panamensis* (lane 3). Lanes containing parasites were loaded with the equivalent of $5x10^6$ cells and those with J774G8 cells, $7{\cdot}6x10^5$. The positions of molecular mass standards are indicated.

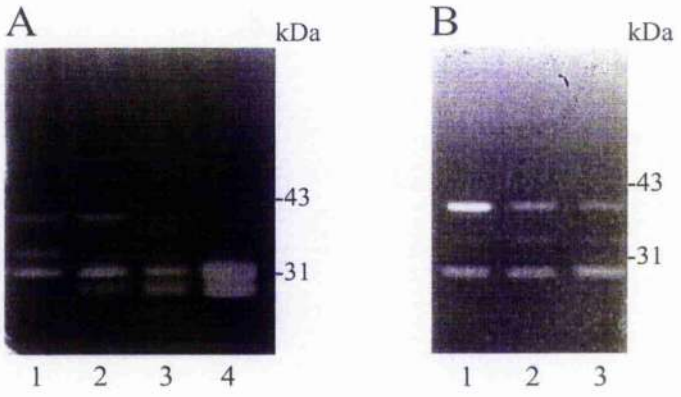


Figure 3.22: Analysis of nuclease profiles of *L. mexicana* and *L. panamensis* promastigote forms and lesion amastigotes of *L. mexicana* by substrate SDS-PAGE. Panels A and B, lanes 1-3 contain soluble extracts of morphotype 1, morphotype 2 and morphotype 3 promastigotes of *L. mexicana* and *L. panamensis*, respectively. Lane 4 of panel A contains soluble lysate of purified lesion amastigotes. Each lane was loaded with the equivalent of 10^7 cells. The positions of molecular mass standards are indicated.

3 promastigotes (lane 3) and lesion amastigotes (lane 4). Each of these doublet bands consisted of two individual nuclease activities (four in total), in amastigotes at least (not shown), which were distinguished when the period allowed for digestion was reduced. Morphotype 1 promastigotes (lane 1) exhibited a banding profile distinct from that of any of the other stages. In addition to the 40 kDa enzyme and the slower moving of the doublet, 31 kDa, a third nuclease of approximately 33 kDa was detected. The results suggest that it is a morphotype 1 specific nuclease. The 29 kDa band was also present, but at low activity. The same is true of the 33 kDa band in morphotype 2 promastigotes, perhaps reflecting the presence of some morphotype 1 cells present in this heterogeneous population.

Soluble lysates of *L. panamensis* promastigotes morphotypes, 1, 2 and 3, were also analysed for nuclease activity by substrate SDS-PAGE. The results are shown in Figure 3.22, panel B. Three nucleases, of approximate sizes 42, 34 and 24 kDa, which had the ability to hydrolyse poly(A) under the incubation conditions applied were detected. No stage-specific nucleases were detected and the banding profiles, as regards activity of the nucleases, were comparable with the exception of the 40 kDa nuclease activity. This enzyme exhibited enhanced activity in morphotype 1 promastigotes when compared with morphotypes 2 and 3 (compare lane 1 with lanes 2 and 3). Its activity in the latter two forms was similar.

3.3.5 Changes in metabolite concentrations during short term incubation of parasites in simple media

3.3.5.1 Amino acid uptake and production by the different promastigote morphotypes of *L. mexicana* and *L. panamensis*

One potential environmental difference experienced by the different promastigote forms *in vivo* is nutrient availability. Short term incubations in buffered saline supplemented with either glucose alone, a mixture of amino acids or a combination of glucose and amino acids were performed to determine whether this variation in the environment is reflected in differences in uptake of these nutrients by each of the *in vitro* promastigote forms. Promastigotes of both species which had been washed three times then resuspended in simple media (buffered saline supplemented with amino acids or glucose and amino acids) or culture medium lacking any other additions were non-motile, however they became active almost immediately after the addition of whole or dialysed HIFCS. Analysis of substrate utilisation and product formation by the three promastigote morphotypes of *L. panamensis* and *L. mexicana* was carried out on active promastigotes, the simple incubation media (buffered saline with amino acids or amino acids and glucose) containing 10% (v/v) dialysed HIFCS. Promastigotes of both species survived and remained active during the two hour incubations in this basal medium when it was supplemented with amino acids alone (SM 1) or amino acids and glucose (SM 2). When no potential energy substrates were added, promastigotes were immotile at the end of the two hour incubation period, although there was some flagella movement in a large proportion of promastigotes. No changes in promastigote morphology were observed during the incubations.

Although 20 amino acids were present in the amino acid mixture used (AAM), only 12 could be quantified by the hplc procedure used. Histidine/glutamine and valine/methionine co-eluted. Of all the amino acids detected, only lysine was inconsistant, appearing in some chromatograms and not others. Quantitation of glycine was abandoned since glycine solutions of equivalent molarity, prepared and analysed at the same time, produced quite different, although linear, standard curves. A different and more complicated method of pre-column derivitization is required to detect cysteine and proline. Application of solutions of individual amino acids allowed the designation of estimated retention times (Table 3.4) which permitted their identification in solutions containing a mixture of amino acids. Figure 3.23 shows a typical chromatogram of the standard amino acid solution which was used for quantitation purposes. Standard curves were produced using the computer programme 'Grafit' [Leatherbarrow, 1992] for each set of samples using freshly defrosted aliquots of the standard amino acid solution. Examples of some typical standard curves are presented in Figure 3.24. The same programme allowed quantitation of amino acids in pre- and post-incubation media.

The changes in amino acid concentrations during two hour incubations of early-log (morphotype 1), late-log (morphotype 2) and stationary (morphotype 3) phase promastigotes of *L. mexicana* and *L. panamensis* in both simple media (SM 1 and SM 2) are given in Tables 3.5 and 3.6, respectively. Analysis of pre- and post-incubation media revealed both qualitative and quantitative differences in utilisation and production of amino acids between the three promastigote morphotypes of *L. mexicana*, both in the presence and absence of glucose. A gradation of use was observed, the largest quantities consumed by morphotype 1 promastigotes and the smallest by morphotype 3 promastigotes. Utilisation by morphotype 2

Amino Acid	Retention Time (minutes)
Aspartate	1·9
Glutamate	2·6
Asparagine	5·9
Serine	7·0
Threonine	9·9
Arginine	11·0
Alanine	12·0
Tyrosine	13·9
Tryptophan	19·9
Phenylalanine	20·4
Isoleucine	20·9
Leucine	21·4

Table 3.4: Typical retention times for amino acids separated by hplc.

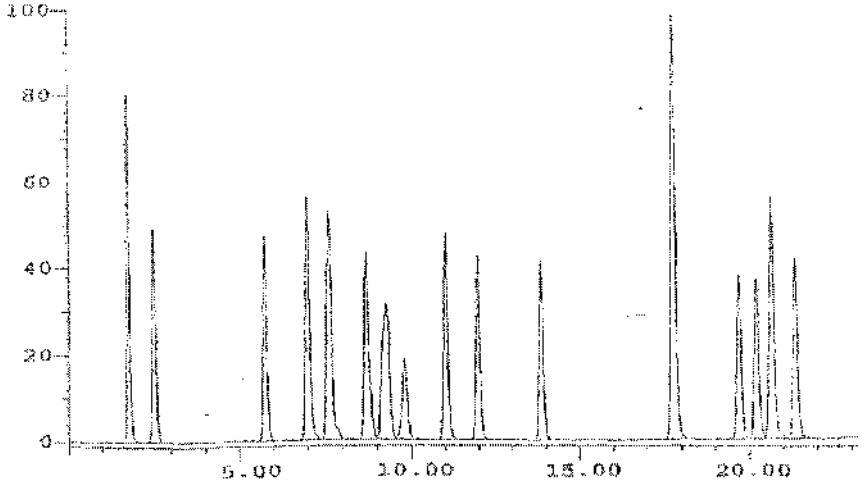


Figure 3.23: A typical chromatogram demonstrating separation of amino acids by hplc.

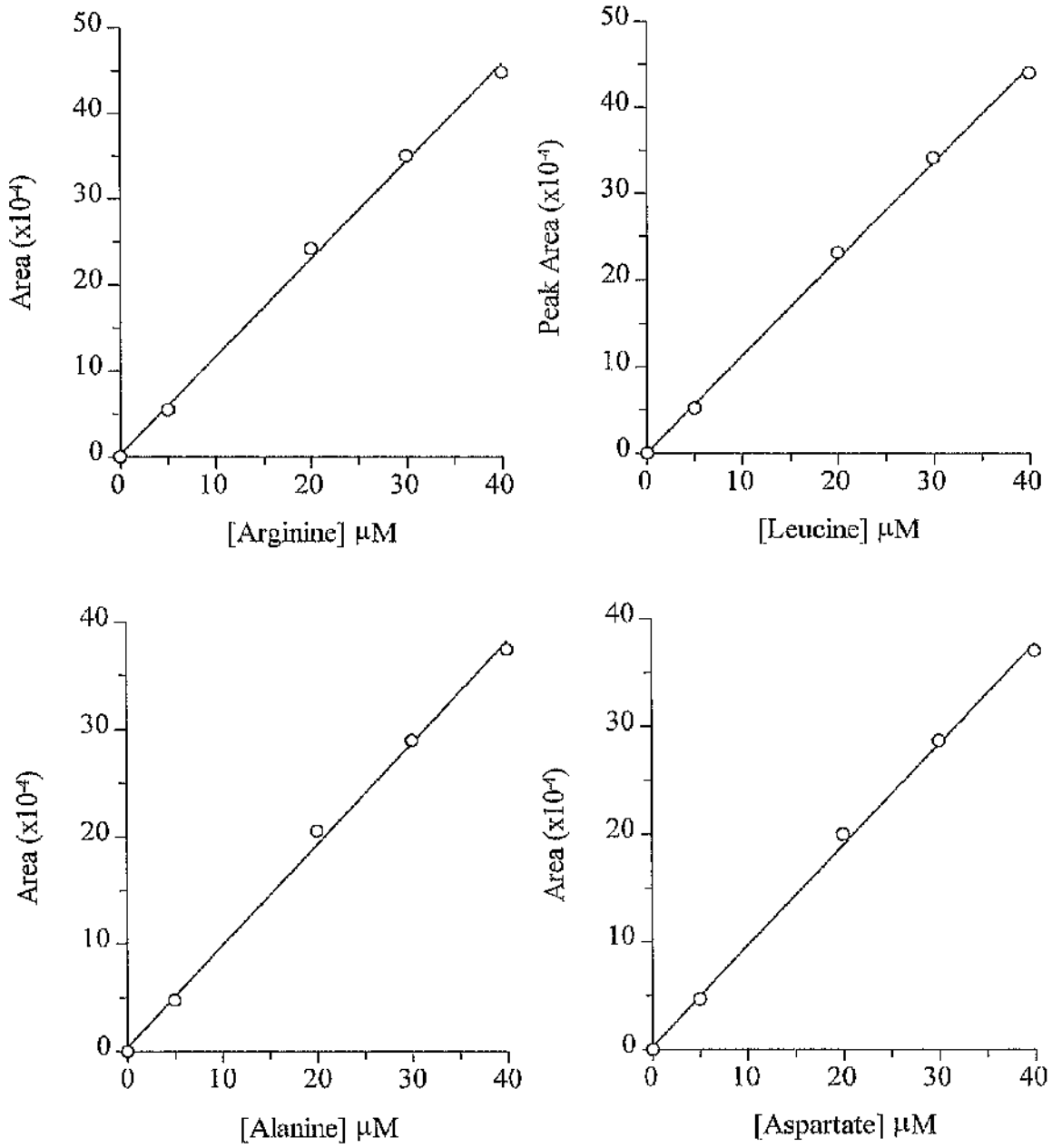


Figure 3.24: Some typical amino acid standard curves generated by Grafit for quantitation of amino acid concentrations in unknown samples.

AMINO ACID	INITIAL CONC. μM	SM 1 Changes over 2 hours MT1	MT2	MT3	INITIAL CONC. μM	SM 2 Changes over 2 hours MT1	MT2	MT3
ASPARTATE	113 ± 23	−99 ± 1	−62 ± 15	−30 ± 4	109 ± 27	−99 ± 1	−67 ± 1	−25 ± 13
GLUTAMATE	118 ± 9	−98 ± 3	−60 ± 19	−29 ± 8	107 ± 9	−98 ± 2	−65 ± 2	−28 ± 8
ASPARAGINE	89 ± 14	−98 ± 2	−45 ± 15	−20 ± 3	85 ± 5	−98 ± 2	−53 ± 2	−16 ± 7
SERINE	116 ± 11	−97 ± 4	−67 ± 16	−20 ± 7	106 ± 14	−91 ± 14	−64 ± 14	−15 ± 5
THREONINE	217 ± 23	−83 ± 5	−21 ± 12	− 9 ± 7	192 ± 16	−77 ± 2	−27 ± 2	− 2 ± 6
ARGININE	295 ± 41	−73 ± 12	−49 ± 35	−16 ± 6	290 ± 25	−63 ± 21	−35 ± 21	− 9 ± 14
ALANINE	119 ± 9	−95 ± 1	0 ± 7	+ 4 ± 13	105 ± 6	−37 ± 24	+92 ± 24	+85 ± 34
TYROSINE	112 ± 9	−42 ± 36	− 2 ± 6	− 5 ± 1	100 ± 3	−19 ± 6	− 1 ± 6	− 3 ± 1
TRYPTOPHAN	29 ± 3	−92 ± 14	−38 ± 32	−32 ± 12	24 ± 3	−94 ± 10	−63 ± 10	−43 ± 15
PHENYLALANINE	121 ± 12	−28 ± 4	− 7 ± 14	− 4 ± 4	107 ± 7	−18 ± 6	− 1 ± 6	− 1 ± 6
ISOLEUCINE	220 ± 27	−66 ± 16	−17 ± 10	− 6 ± 10	200 ± 19	−45 ± 15	−16 ± 15	−17 ± 15
LEUCINE	207 ± 17	−69 ± 19	−23 ± 14	−12 ± 2	186 ± 9	−55 ± 18	−26 ± 18	−14 ± 4

Table 3.5: Amino acid changes during suspension of *L. mexicana* promastigotes in simple media. Percentage change in amino acid concentrations during two hour incubations of *L. mexicana* promastigotes in two simple media (SM 1 and SM 2) are presented. Pre-incubation concentrations of detectable amino acids as measured by hplc are shown for both media. In addition SM 2 contained 10 mM glucose. The starting concentration or percentage change ± S.D. (n=3) are presented.

AMINO ACID	INITIAL CONC. μM	SM 1 Changes over 2 h MT1	MT2	MT3	INITIAL CONC. μM	SM 2 Changes over 2 h MT1	MT2	MT3
ASPARTATE	133 ± 26	−87 ± 13	−74 ± 12	−88 ± 8	120 ± 11	−86 ± 14	− 80 ± 6	− 84 ± 4
GLUTAMATE	115 ± 14	−70 ± 18	−61 ± 11	−60 ± 8	110 ± 7	−67 ± 30	− 66 ± 6	− 57 ± 3
ASPARAGINE	106 ± 16	−51 ± 21	−32 ± 10	−39 ± 3	102 ± 6	−57 ± 17	− 42 ± 4	− 31 ± 6
SERINE	127 ± 25	−52 ± 21	−40 ± 5	−40 ± 5	118 ± 9	−45 ± 19	− 23 ± 5	− 23 ± 2
THREONINE	225 ± 33	−79 ± 11	−62 ± 6	−79 ± 2	219 ± 9	−72 ± 16	− 59 ± 3	− 73 ± 1
ARGININE	235 ± 42	−36 ± 10	−21 ± 8	−16 ± 17	299 ± 25	−51 ± 23	− 14 ± 6	− 13 ± 3
ALANINE	117 ± 10	+62 ± 30	+42 ± 14	+63 ± 21	115 ± 8	n.c.	+221 ± 37	+194 ± 0
TYROSINE	111 ± 15	−22 ± 13	− 9 ± 8	−13 ± 10	109 ± 7	−16 ± 8	− 10 ± 2	+ 1 ± 5
TRYPTOPHAN	30 ± 3	−23 ± 13	−17 ± 6	−16 ± 6	29 ± 1	−25 ± 5	− 19 ± 5	− 6 ± 1
PHENYLALANINE	118 ± 18	−21 ± 7	− 6 ± 6	−10 ± 12	116 ± 6	−22 ± 10	− 9 ± 3	− 1 ± 4
ISOLEUCINE	216 ± 29	−31 ± 21	−13 ± 8	−22 ± 8	213 ± 12	−20 ± 13	− 4 ± 12	− 12 ± 1
LEUCINE	206 ± 30	−38 ± 23	−19 ± 7	−30 ± 8	200 ± 11	−46 ± 8	− 24 ± 8	− 22 ± 1

Table 3.6: Amino acid changes during suspension of promastigotes of *L panamensis* in simple media. Percentage change in amino acid concentrations during short term incubations of *L. panamensis* promastigotes in two simple media (SM 1 and SM 2) are presented. Pre-incubation concentrations of detectable amino acids as measured by hplc are shown for both media. In addition SM 2 contained 10 mM glucose. The starting concentration or percentage change ± S.D. (n=3) are presented. n.c., not calculated.

promastigotes was intermediate between the other two promastigote forms. Morphotype 1 promastigotes of *L. mexicana* consumed considerable quantites of all of the detectable amino acids. In contrast, use by morphotype 2 promastigotes was reduced with levels of tyrosine, alanine and phenylalanine remaining relatively stable. Morphotype 3 promastigotes used smaller quantites of all of the amino acids than the other two forms. Aspartate, glutamate, asparagine, serine and tryptophan were consumed, while levels of threonine, arginine, tyrosine, phenylalanine, isoleucine and leucine remained consistant throughout the incubations.

The profiles for each promastigote form of *L. panamensis* are much more alike and are distinct from those of *L. mexicana*. During the two hour incubation all three promastigote morphotypes consumed large quantities of five of the detectable amino acids, in the presence and absence of glucose; aspartate, glutamate, asparagine, serine and threonine. Alanine was excreted. The remaining six amino acids changed little during the incubations.

Glucose appeared to have had little influence on amino acid uptake, although generally consumption was reduced in its presence. One exception to this was that alanine consumption and excretion was found to depend on glucose availability, the extent of which varied between *Leishmania* species and promastigote morphotypes. In the absence of carbohydrate, alanine was virtually exhausted by morphotype 1 promastigotes of *L. mexicana* whereas the amounts remained relatively stable throughout the two hour incubation with promastigote morphotypes 2 and 3. Consumption of alanine by morphotype 1s was reduced by approximately two thirds when glucose was available. Under the same conditions, promastigotes of morphotypes 2 and 3 excreted large quantities of alanine into the medium. All three promastigote morphotypes of *L. panamensis* behaved similarly when glucose was absent, excreting

large quantities of alanine. This release by morphotypes 2 and 3 was greatly enhanced in the presence of glucose.

Alteration of the gas phase from air to argon, nitrogen or 95% air/5% CO_2 had no apparent effect on the quantities of amino acids produced or consumed (not shown).

3.3.5.2 Glucose consumption and excretion of organic acids and glycerol by the different promastigote morphotypes of *L. mexicana* and *L. panamensis* during short term incubations in simple media

Pre- and post-incubation media were analysed for glucose, glycerol and organic acid content, however, insufficient quantities of these metabolites were consumed or produced during the 2 hour incubations for accurate quantitation by the procedures adopted.

3.3.5.3 Amino acid utilisation and production by purified lesion amastigotes of *L. mexicana*

Amino acid consumption by purified lesion amastigotes of *L. mexicana* during two hour incubations in simple media was investigated. Amastigotes were washed, subsequent to purification, and resuspended at 10^8 ml^{-1} or $3x10^8$ ml^{-1} in HEPES and MES buffered saline containing either amino acids (SM 1) or amino acids and glucose (SM 2), both of which were supplemented with 10% (v/v) dialysed HIFCS. Pre- and post-incubation media were analysed for amino acid content using hplc and the concentrations of individual amino acids were calculated. The percentage changes are presented in Tables 3.7 and 3.8.

The parasites consumed considerable quantities of most of the detectable amino acids available when resuspended in SM 1 at $3x10^8$ cells ml^{-1}. A higher percentage of

AMINO ACID	INITIAL CONC. µM	SM 1	INITIAL CONC. µM	SM 2
Aspartate	127	–59	104	–64
Glutamate	123	+ 2	115	–12
Asparagine	96	–28	84	–32
Threonine	225	–33	182	–36
Arginine	291	+ 2	295	–18
Alanine	209	–32	139	+ 4
Tyrosine	123	–16	104	–19
Tryptophan	33	n.c.	29	– 3
Phenylalanine	119	– 7	107	–17
Isoleucine	193	–17	178	–28
Leucine	213	–24	190	–35

Table 3.7: Amino acid utilisation and production by purified lesion amastigotes of *L. mexicana* resuspended for two hours in simple media (pH 7·0) at $3x10^8$ ml^{-1} in the presence (SM 2) and absence (SM 1) of 10 mM glucose, both of which were supplemented with 10% (v/v) dialysed HIFCS. Amino acid concentrations in pre- and post-incubation media from one experiment are presented. Utilisation and production were calculated as a percentage of this initial value. n.c., not calculated.

AMINO ACID	INITIAL CONC. μM	SM 1	SM 2
Aspartate	109 ± 25	−42 ± 4	−31 ± 7
Glutamate	119 ± 6	− 4 ± 2	− 1± 10
Asparagine	94 ± 3	− 4 ± 4	−11 ± 8
Threonine	204 ± 29	−24 ± 3	−12 ± 8
Arginine	291 ± 0	+ 4 ± 2	− 4 ± 8
Alanine	164 ± 64	−41 ± 1	− 8 ± 8
Tyrosine	117 ± 8	−13 ± 2	− 5 ± 8
Tryptophan	31 ± 3	− 6 ± 5	+ 1 ± 14
Phenylalanine	119 ± 0	− 5 ± 2	− 1 ± 9
Isoleucine	197 ± 6	− 7 ± 2	− 7 ± 11
Leucine	209 ± 6	−13 ± 2	− 8 ± 8

Table 3.8: Amino acid utilisation and production by purified lesion amastigotes of *L. mexicana* resuspended for two hours in simple media (pH 7·0) at 10^8 ml^{-1} in the presence (SM 2) and absence (SM 1) of 10 mM glucose, both of which were supplemented with 10% (v/v) dialysed HIFCS. Three separate cultures were set up from one amastigote purification and the spent media were analysed individually by hplc. The results presented represent mean ± S.D. (n=3).

aspartate was consumed than any of the other amino acids. Only glutamate, arginine and phenylalanine were used in negligable amounts, although tryptophan levels were not calculated. Inclusion of glucose acted to increase generally the quantities of amino acids consumed, including glutamate, arginine and phenylalanine. However, alanine was consumed only when glucose was not available in the medium, in its presence the exogenous concentration of alanine increased marginally.

Similar patterns of consumption were detected when the density of amastigotes was decreased from $3x10^8$ ml^{-1} to 10^8 ml^{-1}. Again, when consumption of individual amino acids was compared as a percentage of that available, aspartate was taken up in largest quantities. However, comparison of the concentrations of individual amino acids in the media identified that roughly equal amouns of aspartate and threonine were consumed and that in the absence of glucose alanine was consumed in the greatest amounts. In the presence of glucose, aspartate use remained high but the alanine concentration did not change.

3.3.5.4 Amino acid uptake and production by axenic amastigotes of *L. mexicana*

The utilisation and release profiles for axenic amastigotes differed considerably from those produced for purified lesion amastigotes incubated under identical conditions (compare Tables 3.8 and 3.9A). The percentage change in individual amino acids which resulted from short term incubations of axenic amastigotes in the two simple media at neutral or acidic pH are presented in Tables 3.9, A and B. Utilisation was negligible for all the amino acids detected in post-incubation media lacking glucose, at both pH 5·5 and pH 7·0, and also in the medium at pH 7·0 which included glucose. Indeed, in this latter medium the amounts of each amino acid, except for

A

AMINO ACID	INITIAL CONC. μM	SM 1 pH 7	INITIAL CONC. μM	SM 2 pH 7
Aspartate	103	− 3 ± 6	98	+ 3 ± 13
Glutamate	95	0 ± 8	91	+ 8 ± 14
Asparagine	92	− 1 ± 6	87	+ 6 ± 13
Serine	98	− 4 ± 7	90	+12 ± 26
Threonine	194	− 2 ± 16	186	+20 ± 40
Arginine	291	0 ± 25	282	+48 ± 19
Alanine	97	+36 ± 11	94	+48 ± 19
Tyrosine	95	+ 1 ± 9	91	+ 8 ± 13
Tryptophan	25	+ 1 ± 1	25	+ 1 ± 1
Phenylalanine	100	+ 3 ± 11	99	+ 7 ± 13
Isoleucine	186	0 ± 18	184	− 9 ± 23
Leucine	179	0 ± 18	175	−11 ± 24

B

AMINO ACID	INITIAL CONC. μM	SM 1 pH 5·5	INITIAL CONC. μM	SM 2 pH 5·5
Aspartate	98	−10 ± 1	101	−18 ± 0
Glutamate	90	− 5 ± 2	93	−12 ± 1
Asparagine	87	0 ± 1	90	− 4 ± 0
Serine	95	− 6 ± 2	93	− 7 ± 1
Threonine	182	0 ± 1	194	−17 ± 4
Arginine	279	+ 2 ± 0	295	−13 ± 5
Alanine	91	+ 3 ± 0	97	−23 ± 5
Tyrosine	89	+ 4 ± 0	96	− 5 ± 3
Tryptophan	24	+ 6 ± 3	26	+ 2 ± 1
Phenylalanine	94	+ 5 ± 0	102	− 6 ± 6
Isoleucine	180	− 0 ± 0	188	−12 ± 5
Leucine	169	− 1 ± 3	177	−14 ± 9

Table 3.9: Percentage change in amino acid concentrations during incubation of axenically cultured amastigotes of *L. mexicana* in HEPES and MES buffered saline supplemented with AAM/10% dialysed HIFCS (SM 1) or AAM/glucose/10% dialysed HIFCS (SM 2) adjusted to pH 7 (A) or pH 5·5 (B). Three individual cultures were set up in complete SDM from the same stationary phase promastigote culture and transformed to axenic amastigotes by temperature shift to 32°C. When in late-log phase of growth, axenically cultivated amastigotes were washed, resuspended at 10^8 ml^{-1} in the incubation media and incubated at 32°C for 2 hours. After this time the media were collected by removal of cells and analysed by hplc for amino acid content. Means ± S.D., n=3.

isoleucine and leucine, increased. Addition of glucose to media at pH 5·5 resulted in slightly elevated use of aspartate, glutamate, threonine, arginine, isoleucine, alanine and leucine. Alanine consumption and excretion was affected more than any other of the amino acids by modification of the incubation media. In the presence and absence of glucose at pH 7·0 alanine was excreted in large amounts. Acidification of the incubation media resulted in alanine uptake by aflagellates when glucose was available. In contrast, in the absence of glucose alanine concentration changed little during the incubation.

3.3.6 Changes in metabolite concentrations during promastigote growth *in vitro* in complete HOMEM medium

3.3.6.1 Amino acid utilisation and production by promastigotes of *L. mexicana* and *L. panamensis* during *in vitro* growth in complete HOMEM medium

Amino acid consumption and production during growth of promastigotes of *L. mexicana* and *L. panamensis* in complete HOMEM medium were investigated. The starting concentrations and the concentrations of the 12 detectable amino acids which remained in spent media on consecutive days of culture are presented in Tables 3.10 and 3.11. The *L. mexicana* promastigote cultures which were monitored were relatively slow growing in comparison with routine cultures and with cultures of *L. panamensis*, taking 10 days to reach the stationary phase of growth. The reason for this is unknown. Each of the amino acids monitored except alanine were gradually consumed throughout growth of both species of promastigotes *in vitro*. The concentration of alanine increased daily until stationary phase when it had

AMINO ACID	INITIAL CONC. μM	CONC AFTER 1 DAY	CONC AFTER 3 DAYS	CONC AFTER 4 DAYS	CONC AFTER 5 DAYS	CONC AFTER 6 DAYS	CONC AFTER 7 DAYS	CONC AFTER 8 DAYS	CONC AFTER 10 DAYS
ASPARTATE	100	86 ± 1	66 ± 5	44 ± 3	28 ± 4	21 ± 1	20 ± 3	19 ± 3	17± 1
GLUTAMATE	154	134 ± 5	144 ± 64	78 ± 6	48 ± 2	27 ± 2	10 ± 12	7 ± 1	17 ± 14
ASPARAGINE	76	68 ± 2	49 ± 2	38 ± 2	31 ± 5	26 ± 1	28 ± 5	33 ± 4	44 ± 2
SERINE	120	101 ± 2	81 ± 14	50 ± 6	32 ± 4	19 ± 1	26 ± 6	22 ± 6	29 ± 2
THREONINE	495	484 ± 15	466 ± 12	416 ± 11	386 ± 28	335 ± 9	249 ± 6	218 ± 8	192 ± 5
ARGININE	706	648 ± 23	592 ± 13	522 ± 19	481 ± 26	381 ± 3	224 ± 15	100 ± 15	31 ± 1
ALANINE	193	228 ± 9	426 ± 4	554 ± 17	792 ± 42	930 ± 14	937 ± 14	1088 ± 37	1388 ± 41
TYROSINE	252	242 ± 10	237 ± 5	221 ± 5	226 ± 11	224 ± 3	198 ± 5	188 ± 0	188 ± 1
TRYPTOPHAN	66	63 ± 3	59 ± 2	52 ± 2	44 ± 3	33 ± 1	22 ± 1	11 ± 1	n.d.
PHENYLALANINE	271	262 ± 8	257 ± 2	238 ± 2	245 ± 16	228 ± 6	215 ± 8	211 ± 1	213 ± 6
ISOLEUCINE	500	488 ± 12	484 ± 6	443 ± 9	441 ± 30	422 ± 28	359 ± 17	331 ± 4	309 ± 6
LEUCINE	466	452 ± 13	429 ± 6	389 ± 15	356 ± 24	322 ± 10	244 ± 11	207 ± 1	178 ± 5

n.d. not detected

Table 3.10: Changes in amino acid concentrations during growth of *L. mexicana* promastigotes in complete HOMEM medium. Spent media samples were collected daily from three different cultures of promastigotes initiated at $5x10^5$ ml^{-1} with an identical starting population of mid-log phase promastigotes. Complete HOMEM medium prior to inoculation of promastigotes was assayed to provide initial amino acid concentrations. Media samples were extracted and analysed by hplc for amino acid content. Data are mean concentrations ± S.D., n=3.

AMINO ACID	CONC. AT START	CONC AFTER 1 DAY	CONC AFTER 2 DAYS	CONC AFTER 3 DAYS	CONC AFTER 4 DAYS	CONC AFTER 5 DAYS
ASPARTATE	120 ± 6	99 ± 16	37 ± 4	17 ± 10	10 ± 6	18
GLUTAMATE	184 ± 8	163 ± 8	96 ± 13	28 ± 3	21 ± 4	22
ASPARAGINE	92 ± 8	85 ± 9	64 ± 9	58 ± 3	53 ± 8	49
SERINE	173 ± 81	168 ± 85	80 ± 32	64 ± 32	36 ± 10	38
THREONINE	589 ± 32	518 ± 28	319 ± 24	107 ± 14	34 ± 6	16
ARGININE	847 ± 52	756 ± 31	637 ± 34	499 ± 56	366 ± 15	289
ALANINE	249 ± 32	261 ± 32	398 ± 141	464 ± 198	538 ± 266	823
TYROSINE	294 ± 16	278 ± 7	259 ± 7	233 ± 12	231 ± 18	222
TRYPTOPHAN	72 ± 8	67 ± 7	59 ± 8	50 ± 3	57 ± 5	52
PHENYLALANINE	310 ± 24	300 ± 5	263 ± 8	234 ± 7	241 ± 14	240
ISOLEUCINE	595 ± 54	567 ± 17	512 ± 11	431 ± 20	387 ± 29	355
LEUCINE	574 ± 52	553 ± 79	409 ± 5	279 ± 15	186 ± 13	174

Table 3.11: Changes in amino acid concentrations during growth of *L. panamensis* promastigotes in complete HOMEM medium. Spent media samples were collected daily from three individual cultures of promastigotes of different subpassage numbers initiated at $5x10^5$ ml^{-1} with promastigotes in the mid-log phase of growth. The samples were analysed for amino acid content by hplc. Data are mean concentrations ± S.D., n=3, collected from inoculation through to stationary phase except for those in the final column, the results of which are from only one of these cultures (one analysis) which had not reached stationary phase by day 5.

reached concentrations some 2-3·5 times (*L. panamensis*) and 7 times (*L. mexicana*) the original amount. By this time considerable quantities of all of the other amino acids had been consumed by both species and aspartate and glutamate were virtually exhausted. One notable interspecies difference involved consumption of threonine and arginine. *L. panamensis* promastigotes consumed almost all exogenous threonine by stationary phase but arginine remained at approximately 30% of its original concentration. The reverse was found for *L. mexicana*, arginine was exhausted while considerable quantities of threonine remained.

3.3.6.2 Glucose consumption by promastigotes of *L. mexicana* and *L. panamensis* during *in vitro* growth in complete HOMEM medium

The changes in glucose concentration during growth of *L. mexicana* and *L. panamensis* promastigotes in complete HOMEM medium are given in Figures 3.25 and 3.26, respectively. From these it was clear that glucose was consumed by promastigotes of both species during *in vitro* growth. However, consumption was initially delayed, and was not apparent for the first 2-3 days of promastigote growth. A reduction in glucose concentration was not observed with the *L. panamensis* cultures until day 3, when promastigotes were approaching the late-log phase (10^7 ml^{-1}) of growth. *L. mexicana* promastigotes grew more slowly than *L. panamensis* reaching the late-log phase of growth at around days 5-6. As with *L. panamensis*, glucose was subsequently used at a steady rate until promastigotes entered the stationary phase of growth. In total 3 mM of glucose were consumed during the 5 days it took for promastigotes of *L. panamensis* to complete their growth in complete HOMEM medium. Over the same period of time, approximately the same quantity of glucose was consumed by *L. mexicana*

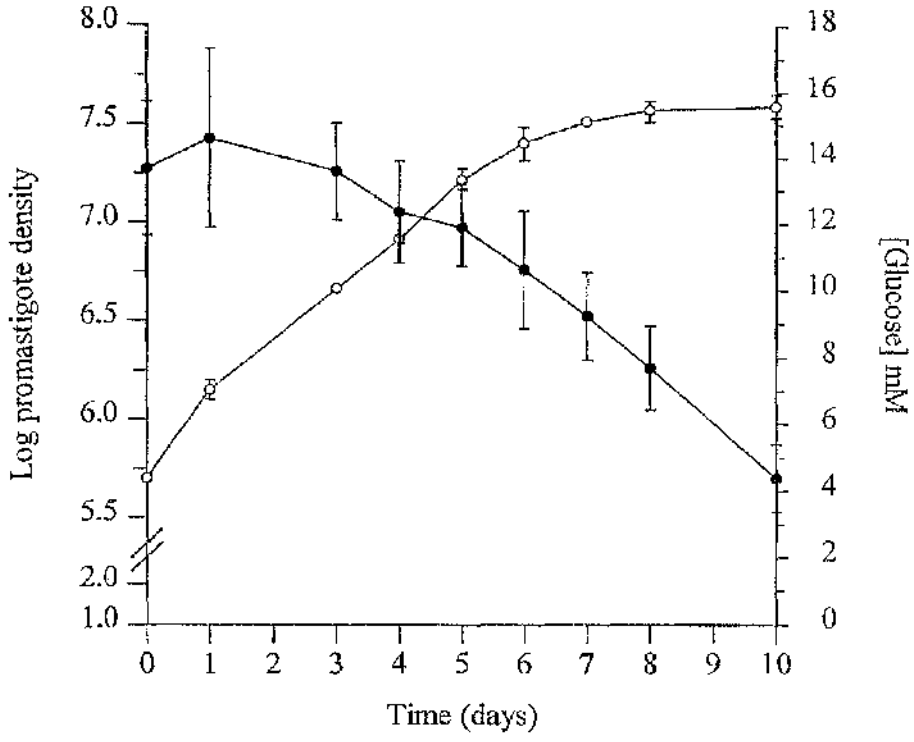


Figure 3.25: Changes in cell density (o) and glucose concentration (•) during growth of *L. mexicana* promastigotes in complete HOMEM medium. Cultures were initiated with an identical starting population at $5x10^5$ ml^{-1} and samples were taken daily for cell counts to determine the density in cells ml^{-1} and for determination of glucose concentration. Cell densities and glucose concentrations are means ± S.D., n=3.

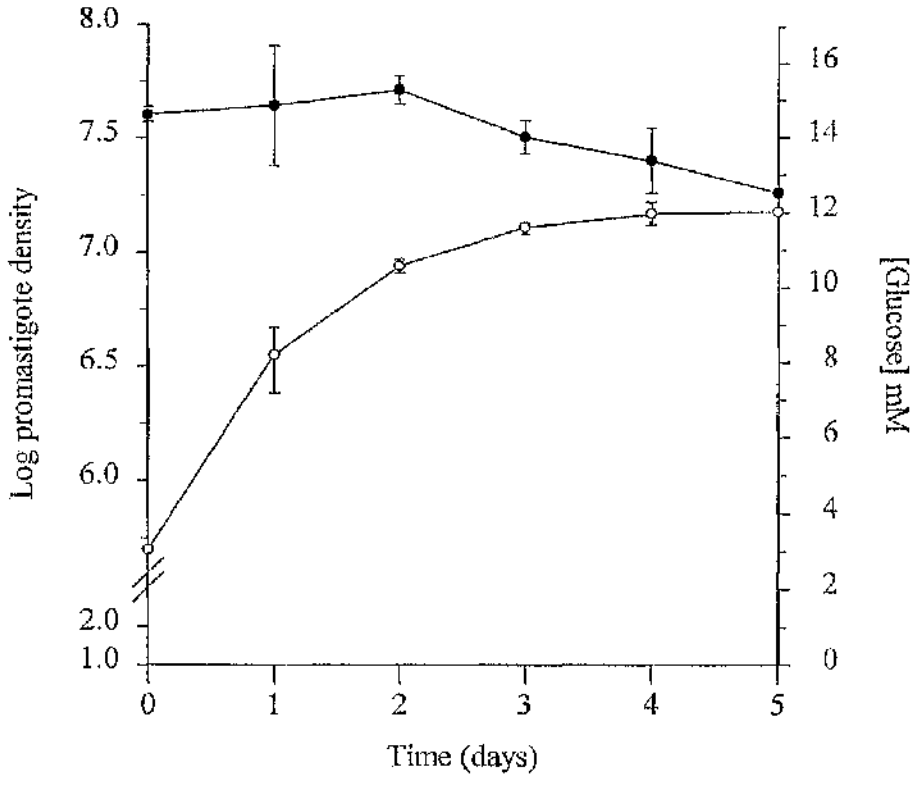


Figure 3.26: Changes in cell density (o) and glucose concentration (•) during growth of *L. panamensis* promastigotes in complete HOMEM medium. Three individual cultures were initiated with promastigotes in the late-log phase of growth at $5x10^5$ ml^{-1} and samples were taken daily for cell counts to determine the density in cells ml^{-1} and for determination of glucose concentration. With the exception of day 5 all values plotted are the means ± S.D.; n=3. Day 5 values were based on only one of the three cultures.

promastigotes, however, when this species reached stationary phase at day 10 approximately 10 mM glucose had been consumed.

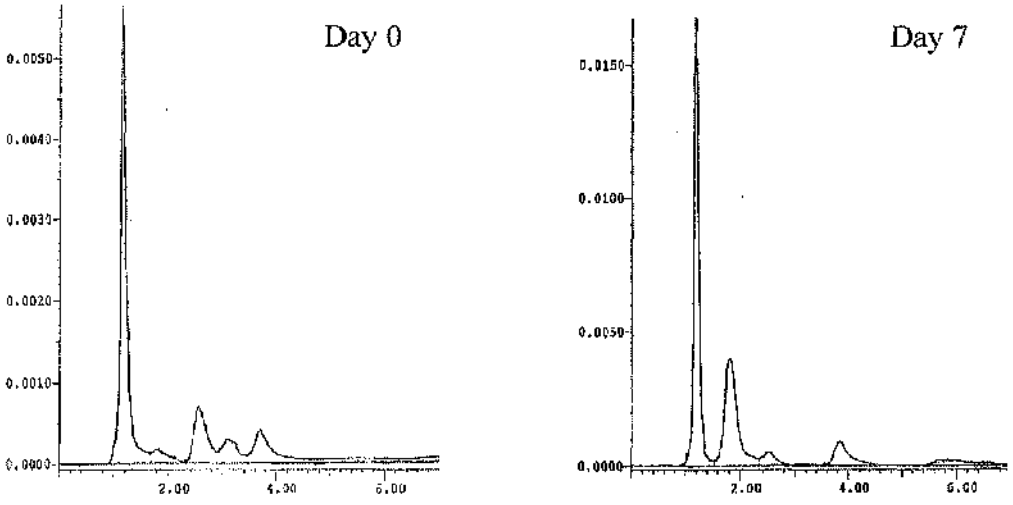
3.3.6.3 Organic acid use and production by promastigotes of *L. mexicana* and *L. panamensis* during *in vitro* growth in complete HOMEM medium

Media samples were collected from 7-day (*L. mexicana*) and 5-day (*L. panamensis*) promastigote cultures and analysed by hplc for organic acid content. Comparison was made with day 0 samples which were harvested immediately following promastigote inoculation. The hplc chromatograms (note that the scale of the y-axes differ) and tables showing retention times and peak areas are shown in Figures 3.27 and 3.28. Pyruvate, acetate and succinate were excreted by both *Leishmania* species during growth *in vitro*. In addition, lactate was produced by promastigotes of *L. panamensis* but appeared to be utilised by promastigotes of *L. mexicana*. Unfortunately insufficient quantities of organic acids were used or released by the promastigote morphotypes, during short term incubations in simple media, to allow accurate quantitation by hplc.

3.3.7 Enzymes of energy metabolism

Several enzymes were analysed in an attempt to identify differences in activities between the three different promastigote forms of *L. mexicana* and *L. panamensis* which correlated with the observed differences in amino acid and glucose utilisation. The enzyme activities detected in the parasite preparations are detailed in Table 3.12. The activities of several exhibited some stage specificity, in that they were enhanced or reduced in the different promastigote forms. All promastigote forms of *L. mexicana* and *L. panamensis* lacked lactate dehydrogenase (LDH) and NAD^+-linked isocitrate dehydrogenase (NAD^+-ICDH) but contained $NADP^+$-ICDH, pyruvate kinase (PK),

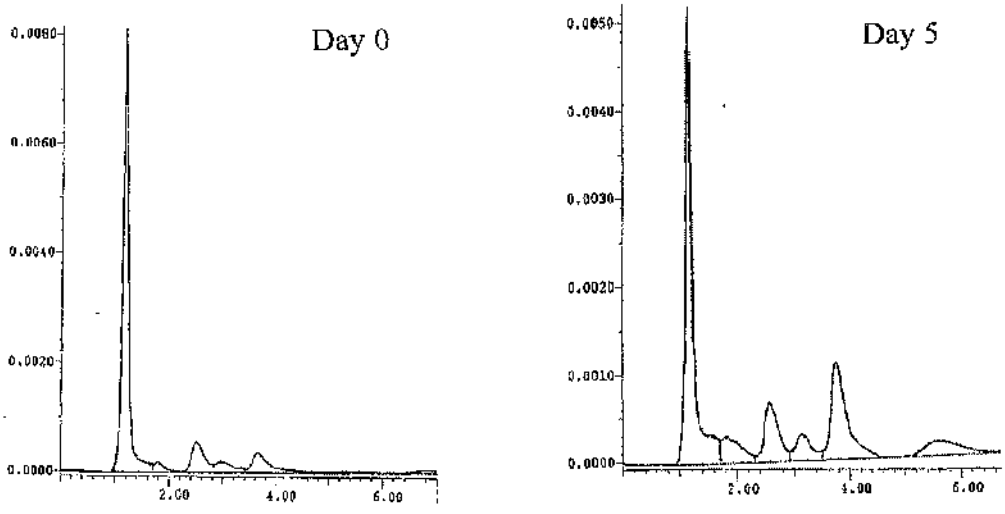
L. mexicana



Organic Acid	Retention Time (minutes)	Peak Area	
		Day 0	Day 7
Pyruvate	1·8	14,8873	273,3330
Lactate	2·5	49,1228	41,4043
Acetate	3·7	43,9310	83,9347
Succinate	5·9	7,2618	35,2212

Figure 3.27: Use and production of organic acids during growth of *L. mexicana* promastigotes in complete HOMEM medium. Cultures were initiated at $5x10^5$ ml^{-1} and media samples were harvested by centrifugation immediately following inoculation of promastigotes and on day 7 of growth when the culture had reached stationary phase. These samples were extracted and analysed for organic acid content by hplc. Organic acids were identified by comparison of retention times with known standards. The chromatograms and a table showing the retention times and peak areas of four organic acids in fresh and spent culture media are presented.

L. panamensis



Organic Acid	Retention Time (minutes)	Peak Area	
		Day 0	Day 5
Pyruvate	1·8	12,3789	30,9558
Lactate	2·5	39,2486	49,7413
Acetate	3·6	38,9801	92,8342
Succinate	5·4	9,9152	30,3668

Figure 3.28: Use and production of organic acids during growth of *L. panamensis* promastigotes in complete HOMEM medium. Cultures were initiated at $5x10^5$ ml^{-1} and media samples were harvested by centrifugation immediately following inoculation of promastigotes and on day 5 of growth when the culture had reached stationary phase. These samples were extracted and analysed for organic acid content by hplc. Organic acids were identified by comparison of retention times with known standards. The chromatograms and a table showing the retention times and peak areas of four organic acids in fresh and spent culture media are presented.

Enzyme Activity	*L. panamensis*			*L. mexicana*		
(nmol min^{-1} (mg protein)$^{-1}$)	MT 1	MT 2	MT 3	MT 1	MT 2	MT 3
Lactate dehydrogenase	n.d., < 1·5 (3)	n.d., < 1·5 (2)	n.d., < 1·5 (3)	n.d., < 0·5 (3)	n.d., < 0·5 (2)	n.d., < 0·5 (3)
$NADP^+$-isocitrate dehydrogenase	31·9 ± 6·6 (3)	26·6 ± 4·2 (2)	62·3 ± 13·2 (3)	256 ± 61 (3)	236 ± 8 (2)	190 ± 19 (3)
NAD^+-isocitrate dehydrogenase	n.d., < 2·0 (3)	n.d., < 2·0 (2)	n.d., < 2·0 (3)	n.d., < 2·0 (3)	n.d., < 2·0 (2)	n.d., < 2·0 (3)
Hexokinase	384 ± 100 (3)	322 ± 34 (2)	328 ± 20 (3)	507 ± 262 (3)	458 ± 155 (2)	603 ± 105 (3)
Pyruvate kinase	15·8 ± 1·1 (3)	23·0 ± 2·9 (2)	26·5 ± 6·8 (3)	858 ± 142 (3)	963 ± 30 (2)	590 ± 60 (3)
Glutamate dehydrogenase	1·0 ± 0·2 (3)	0·7 ± 0·1 (2)	0·5 ± 0·1 (3)	3·6 ± 1·0 (3)	3·3 ± 0·5 (2)	1·3 ± 0·1 (3)

means ± S.D., *n* in parentheses. n.d., not detected

Table 3.12: Activities of enzymes of energy metabolism in the different promastigote morphotypes of *L. mexicana* and *L. panamensis*.

hexokinase (HK) and glutamate dehydrogenase (GDH). *L. panamensis* morphotype 3 promastigotes contained approximately twice as much $NADP^+$-ICDH activity as did morphotype 1 or morphotype 2 forms. The activity of this enzyme in *L. mexicana* promastigotes was higher in promastigote morphotypes 1 and 2 than in morphotype 3 forms and was considerably greater (approximately 4-6 times greater depending on the promastigote Type) than that in the equivalent promastigote forms of *L. panamensis*. Similarly, HK, PK and GDH were all present at higher activity in extracts of *L. mexicana* promastigotes than in those of *L. panamensis* promastigotes. HK did not exhibit marked stage-regulation but was approximately twice as active in *L. mexicana* as in *L. panamensis*. PK did show some evidence of stage-regulation in both *Leishmania* species. In *L. panamensis* promastigotes PK activity was slightly reduced in morphotype 1 forms when compared with morphotypes 2 and 3. In contrast, morphotype 3 promastigotes of *L. mexicana* contained lower activity of PK than the other two forms of the same species. GDH followed the same pattern of activity in both species being at highest activity in morphotype 1 promastigotes and lowest in morphotype 3 promastigotes.

3.3.8 Intracellular amino acid pool of *L. mexicana* promastigotes

The amino acid concentrations detected in whole cell homogenates of *L. mexicana* promastigotes are given in Table 3.13. There were found to be differences in the concentrations of many of the amino acids between each of the three promastigote morphotypes, although most followed a similar trend. The most abundant amino acid which was detected in promastigote morphotypes 1 and 2 was arginine whereas alanine, which was detected in equivalent amounts in all three forms, was the most abundant amino acid in morphotype 3 promastigotes. All of the other detectable amino acids

Amino Acid	Morphotype 1	Morphotype 2	Morphotype 3
Aspartate	30	22	4
Glutamate	123	36	58
Asparagine	12	7	9
Serine	123	90	10
Threonine	207	90	33
Arginine	1036	1014	80
Alanine	634	685	583
Tyrosine	31	26	15
Tryptophan	37	12	11
Phenylalanine	31	27	15
Isoleucine	317	80	20
Leucine	234	60	27

Units = μM

Table 3.13: Concentrations of intracellular amino acids in the three promastigote morphotypes of *L. mexicana*. Single pellets of each morphotype were lysed at 10^9 cells ml^{-1} in the presence of proteinase inhibitors. The amino acids in each lysate were analysed once by hplc and the concentrations calculated by comparison with standard curves.

Species and promastigote morphotype	% Infected macrophages	Average number of amastigotes per macrophage
L. panamensis MT 1	25% ± 6	3.7 ± 0.8
MT 2	20% ± 14	4.4 ± 1.7
MT 3	25% ± 7	4.8 ± 0.5
L. mexicana MT 1	72% ± 16	8.7 ± 1.8
MT 2	22% ± 2	4.9 ± 1.0
MT 3	72% ± 6	9.1 ± 1.5

Table 3.14: Infectivity of promastigotes of *L. mexicana* and *L. panamensis* to peritoneal exudate cells as measured by the percentage of macrophages infected and the number of amastigotes per macrophage after incubation for 7 days. Experiments were carried out in multiwell tissue culture slides, and the figures presented represent means of three individual counts on three separate wells for each promastigote morphotype.

were present at highest levels in morphotype 1 promastigotes and lowest in morphotype 3s. The content of morphotype 2 promastigotes was in most cases intermediate between the other two forms.

3.3.9 Infectivity of the different promastigote morphotypes of *L. mexicana* and *L. panamensis* to peritoneal exudate cells

The infectivity of each promastigote morphotype of *L. mexicana* and *L. panamensis* to murine peritoneal exudate cells was studied. The numbers of infected macrophages and the average number of amastigotes per macrophage were compared for each promastigote form and are presented in Table 3.14. All three promastigote morphotypes of *L. panamensis* behaved similarly for both features analysed, with approximately 20% of the peritoneal exudate cells being infected with on average 4 amastigotes per macrophage. Infectivity of *L. mexicana* promastigotes changed during growth *in vitro*. Morphotypes 1 and 3 infected approximately 70% of macrophages. Morphotype 2 promastigotes, however, infected less than half as many peritoneal exudate cells and the mean number of amastigotes per macrophage was also reduced by approximately half.

3.3.10 DNA content analysis

To investigate a potential relationship between position in the cell cycle and promastigote morphology the DNA content of *L. mexicana* promastigotes harvested on consecutive days of *in vitro* growth was analysed by FACS. The growth curves for the cultures analysed and the proportions of promastigotes in G1, S and G2 phases of the mitotic cycle are shown in Figure 3.29. The proportion of promastigotes in the G1 phase remained high (>50%) throughout the growth cycle, although promastigotes in

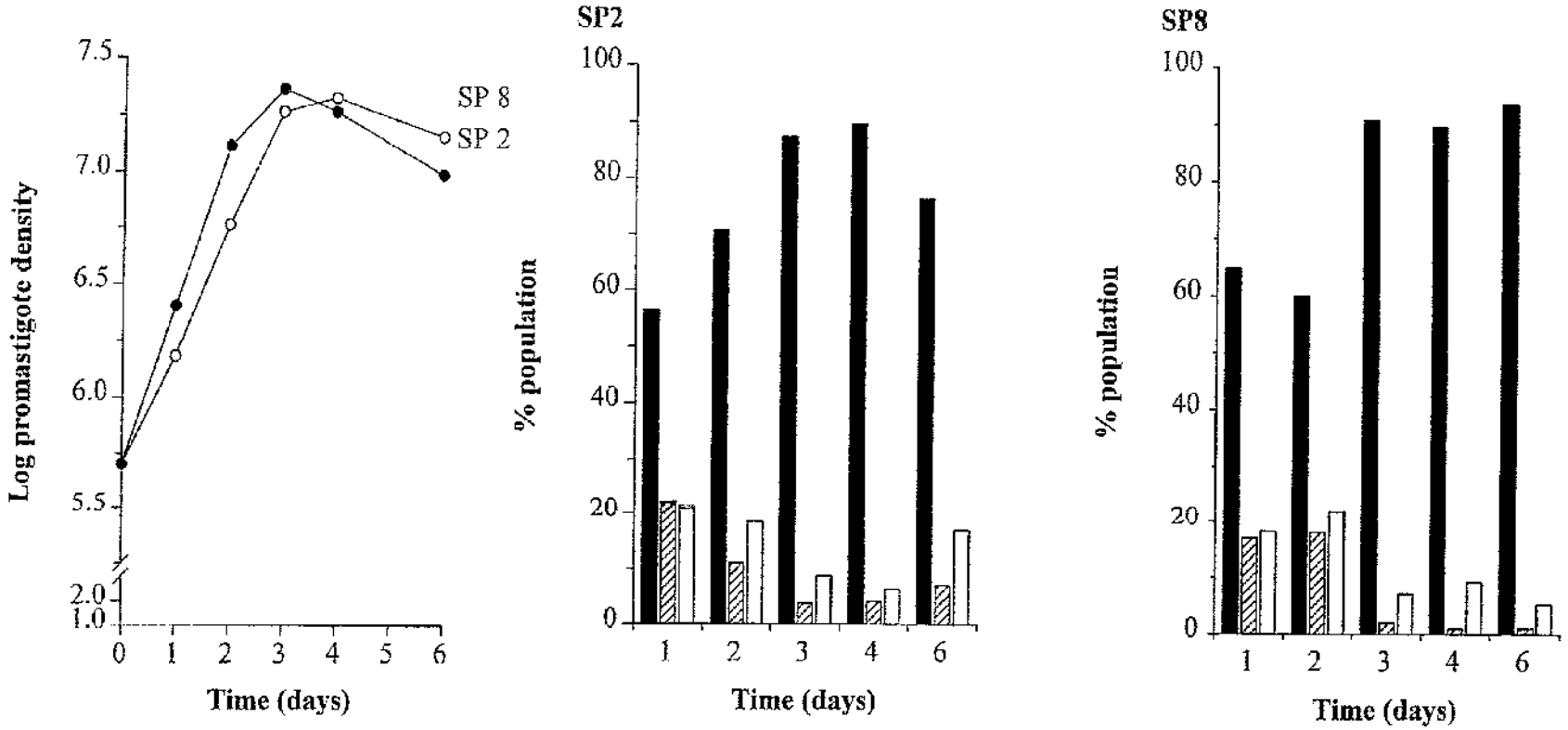


Figure 3.29: Growth curves of *L. mexicana* promastigotes, sub-passage (SP) 2 (•) and SP 8 (o), and the proportions of cells in the populations that were within G1 (closed bars), S (striped bars) or G2 (open bars) phases of the mitotic cycle. Samples of promastigotes were taken on days 1, 2, 3, 4 and 6 of growth, the density in cells ml^{-1} determined and the cells stained with propidium iodide and analysed for DNA content. Ten thousand cells for each sample were counted for the two cultures.

the early- and mid-log phases of growth contained a lower proportion than older cultures. The remaining promastigotes in the early- and mid-log phase cultures were comprised of rather similar proportions of promastigotes in the S and G2 phases of the cell cycle. Late-log phase and stationary phase promastigotes exhibited similar cell cycle profiles to each other and were comprised substantially of promastigotes in G1. A small proportion of the cells present were in S phase (<5%) or G2 phase (<10%).

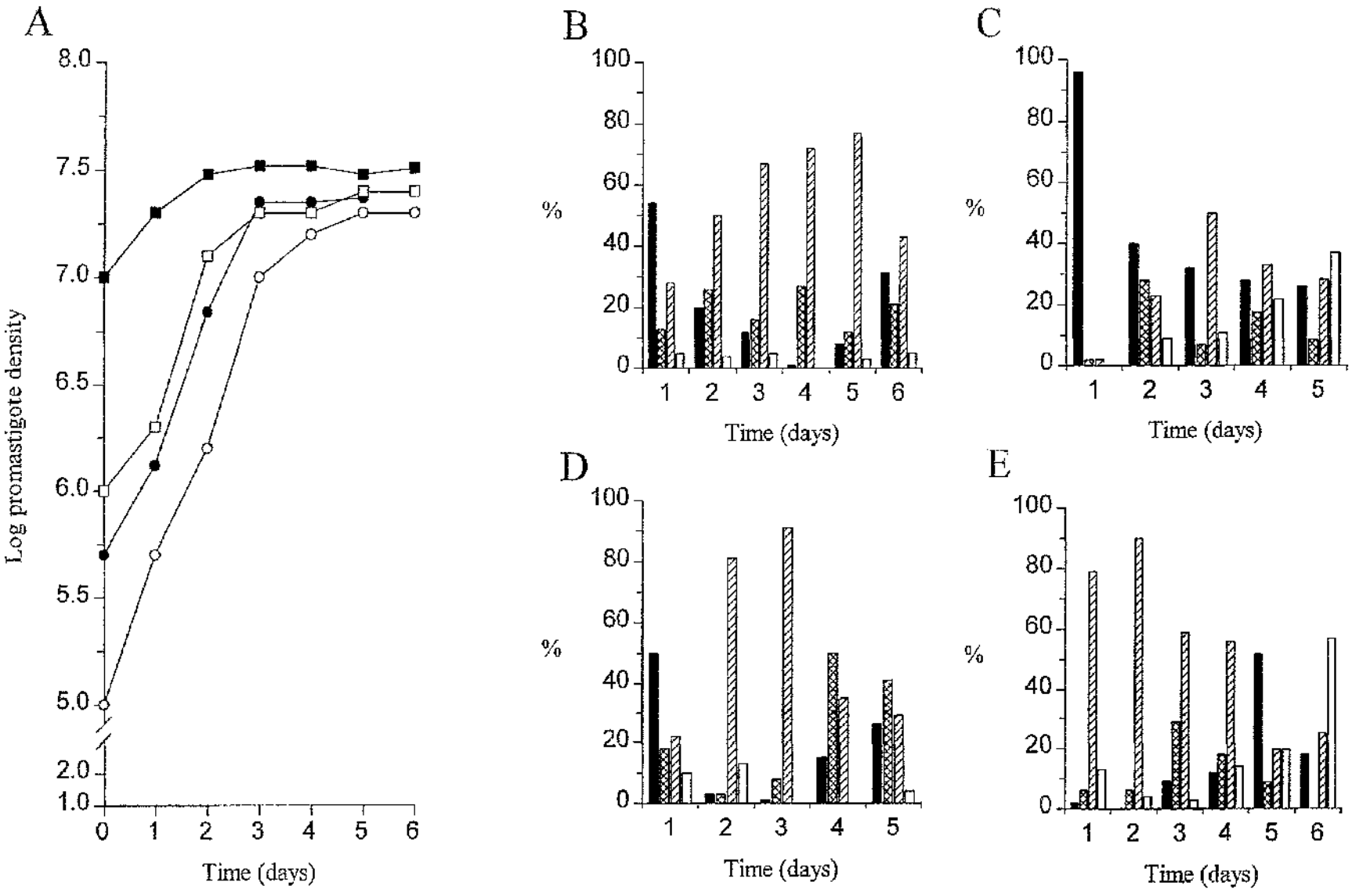
3.4 Transformation

Various experiments were conducted in an attempt to determine whether or not the promastigote morphotypes are distinct developmental forms. The occurrence of the different forms were monitored when culture conditions were modified to investigate their ability to transform back and also the trigger for transformation.

3.4.1 Variation in starting density

The growth of *L. panamensis* promastigotes inoculated at various starting densities and the proportions of each promastigote morphotype which occurred in the resulting populations are presented in Figure 3.30. Lower (10^5 ml^{-1}) and higher densities (10^6 ml^{-1} and 10^7 ml^{-1}) than routine control cultures ($5x10^5$ ml^{-1}) were investigated and it was found that changing the starting density affected the rate of progression through the developmental sequence, particularly influencing the timing of the appearance of promastigotes with morphology characteristic of morphotype 2 promastigotes. Comparison of the proportions of the different morphological forms in cultures identified that initiation at a higher starting density increased the rate of differentiation such that morphotype 2 forms occurred in higher numbers earlier than usual. Although morphotype 1 forms predominated day 1 cultures inoculated at 10^6 ml^{-1}, their numbers

Figure 3.30: Changes in cell densities (organisms ml^{-1}) of *L. panamensis* promastigote cultures initiated at 10^5 ml^{-1} (open circles), $5x10^5$ ml^{-1} (closed circles), 10^6 ml^{-1} (open squares) and 10^7 ml^{-1} (closed squares) and the proportions of each promastigote morphotype in the resulting populations (B, C, D and E, respectively); closed bars, MT1 promastigotes; hatched bars, MTi promastigotes; diagonally striped bars, MT2 promastigotes; open bars, MT3 promastigotes. Length and breadth measurements of 100 promastigotes in Giemsa-stained smears were made from one typical set of cultures and the percentage of each promastigote morphotype was estimated. Size criteria for each morphotype are listed in Table 3.1.


A
Log promastigote density
8.0
7.5
7.0
6.5
6.0
5.5
5.0
2.0
1.0
0 1 2 3 4 5 6
Time (days)
B
%
100
80
60
40
20
0
1 2 3 4 5 6
Time (days)
C
%
100
80
60
40
20
0
1 2 3 4 5
Time (days)
D
%
100
80
60
40
20
0
1 2 3 4 5
Time (days)
E
%
100
80
60
40
20
0
1 2 3 4 5 6
Time (days)

relative to the other morphological forms were substantially reduced when compared with control cultures, and by day 2, the developmental sequence had progressed such that morphotype 2 promastigotes were the most common form. These stages did not predominate in control cultures until day 3 of growth, coincident with entry into the late-log phase of growth. Subsequently, with the onset of stationary phase, the proportion of morphotype 2 forms decreased while the number of promastigotes with morphology typical of intermediates and morphotype 1 promastigotes increased. Morphotype 3 promastigotes were not produced in significant amounts. The culture inoculated at 10^7 ml^{-1} also exhibited accelerated development of morphotype 2 forms which comprised 79% of those promastigotes measured on day 1 of culture. Morphotype 1 promastigotes represented only 2% of the population at this time. By day 2 the number of promastigotes with morphology characteristic of morphotype 2 forms had increased further to 90%, but subsequently declined. Rounded promastigotes of morphotype 1 morphology predominated on day 5 but were outnumbered by morphotype 3 forms on day 6. Decreasing the starting density to 10^6 ml^{-1} resulted in a similar developmental pattern to control cultures. Day 1 cultures were predominated by morphotype 1 forms which were gradually replaced by morphotype 2 forms, slightly earlier than control cultures. By late-log phase, day 5, morphotype 2 forms reached maximum numbers and began to decline thereafter. This culture was not monitored until stationary phase which is perhaps reflected in the low number of morphotype 3 forms on day 6.

L. mexicana promastigote cultures which were initiated at various starting densities were analysed on day 2 of growth and the relative proportions of each promastigote morphotype calculated. Similarly to *L. panamensis*, the proportions of each promastigote morphotype varied depending on the starting density (Figure 3.31).

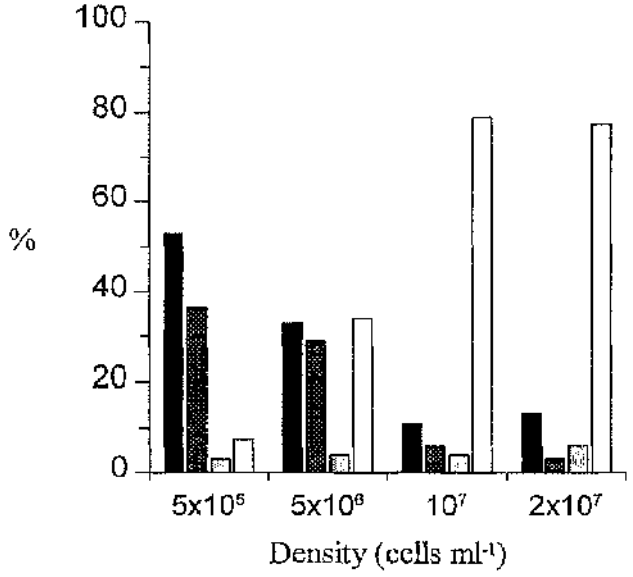


Figure 3.31: The average proportions of each promastigote morphotype in duplicate *L. mexicana* cultures 48 hours subsequent to initiation at different starting densities; closed bars, MT1 promastigotes; densley shaded bars, MTi promastigotes; lightly shaded bars, MT2 promastigotes; open bars, MT3 promastigotes. Length and breadth measurements were made on 50 promastigotes from each culture and the percentage of each form was estimated based on the size definitions outlined in Table 3.1.

Morphotype 1 promastigotes were the most prevalent promastigote form in cultures inoculated at $5x10^5$ ml^{-1}. Increasing the starting density to $5x10^6$ ml^{-1} resulted in a more evenly balanced population of cells, morphotype 2 forms being outnumbered by the others, each of which composed ca. 30% of the population. Cultures which were inoculated at higher densities still, 10^7ml^{-1} and $2x10^7$ ml^{-1}, behaved similarly to each other, but differently to those initiated at lower densities, in that by day 2 morphotype 3 promastigotes were the most prevalent form, accounting for approximately 80% of those promastigotes measured.

3.4.2 Continual sub-passage of morphotype 1 promastigotes

One day old cultures of *L. mexicana* and *L. panamensis* were repeatedly sub-passaged at $5x10^5$ ml^{-1} into complete HOMEM medium in order to ascertain whether morphotype 1 promastigotes could be continually maintained in *in vitro* culture or whether transformation to morphotype 2 promastigotes would occur regardless. The two species responded differently; *L. mexicana* promastigotes retained the morphology characteristic of morphotype 1 forms while *L. panamensis* promastigotes differentiated into morphotype 2 forms (not shown). With each sub-passage the number of morphotype 1 promastigotes of *L. mexicana* which were present in one day old cultures increased from 60% (1st sub-passage) to 88% (2nd sub-passage) and finally reached 97% on the third sub-passage, although the percentage of morphotype 1 promastigotes in this last case was estimated from approximately one third the number of cells than the others since the Giemsa-stained smear contained very few promastigotes. Promastigote morphology in cultures which were inoculated at $5x10^5$ ml^{-1} for the first (control) and second time was also assessed at subsequent time points. The proportion of morphotype 1 promastigotes in the control culture initially increased, from 59% to 77% when

analysed at 48 hours, but subsequently declined to 28% at 72 hours after inoculation. Those sub-passaged for a second time demonstrated a shift in predominance from promastigotes with morphotype 1 morphology (87% of the population at 24 hours) to forms intermediate in morphology between morphotype 1 and morphotype 2 promastigotes, MTis (47%) at 48 hours. In addition, the prevalence of morphotype 2 promastigotes had increased marginally.

In contrast to *L. mexicana*, with each sub-passage *L. panamensis* promastigotes progressed through the developmental cycle and did not retain morphotype 1 morphology. Promastigotes comprising the first sub-passage were predominated by morphotype 1 forms (49% of the population). When these were sub-passaged into complete HOMEM at $5x10^5$ ml^{-1} the resulting population of cells on day one of growth was predominated by the intermediate forms (comprising 53% of the population), although a large proportion (33%) of morphotype 1 promastigotes were also present. On repetition of this procedure using this day 1 promastigote culture, morphotype 2 promastigotes (comprising 57% of the population) were the most prevalent cell morphotype after one days growth.

3.4.3 Foetal calf serum

3.4.3.1 Dialysis of foetal calf serum

Comparison of promastigote growth and morphology in HOMEM medium which had been supplemented with 10% (v/v) complete or dialysed HIFCS demonstrated that dialysis not only halted growth but also altered the differentiation sequence. Growth curves for *L. mexicana* in these two media and the proportions of each promastigote morphotype which occurred in resultant populations are shown in Figure 3.32. The promastigote density in the culture containing dialysed HIFCS

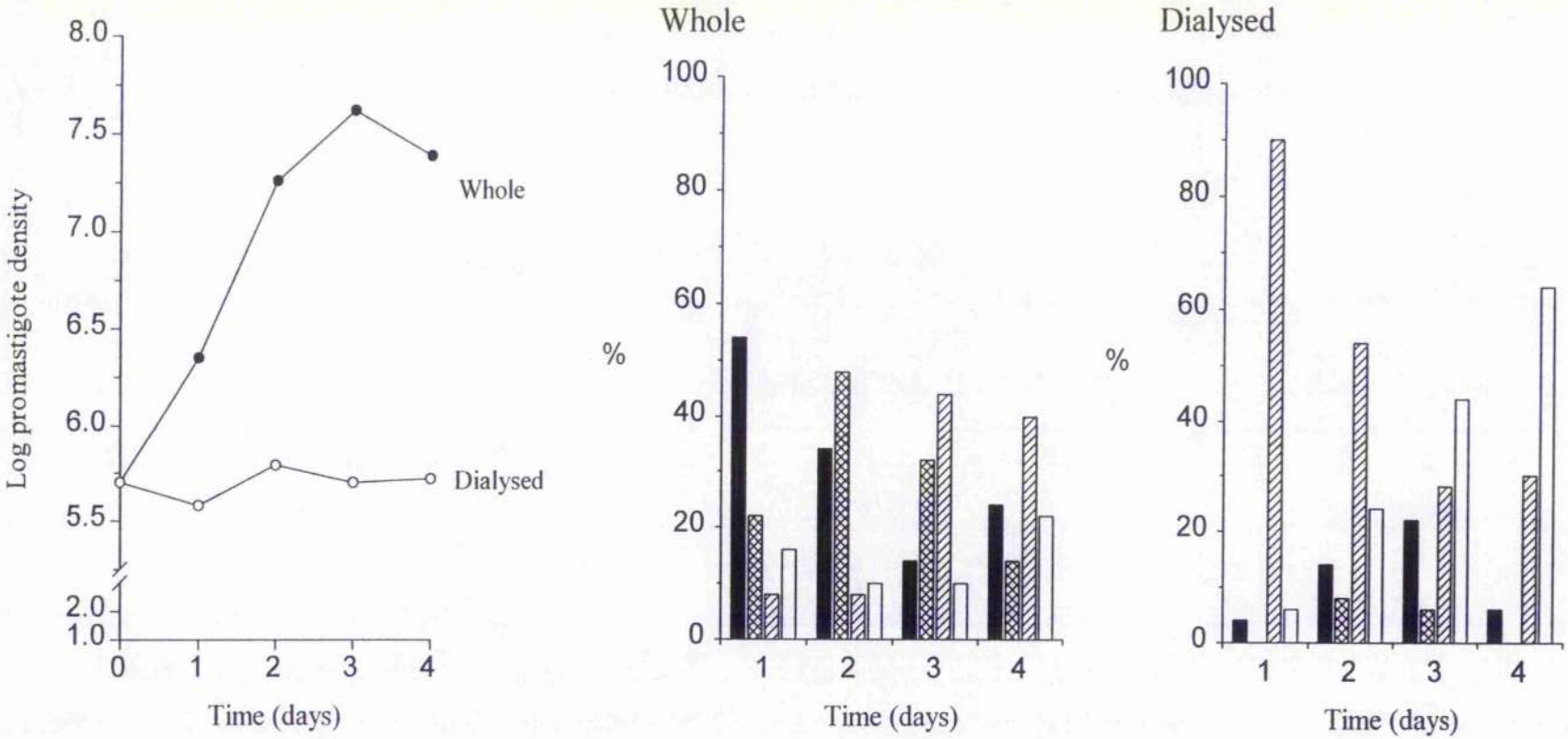


Figure 3.32: Changes in cell density (organisms ml^{-1}) of *L. mexicana* promastigotes cultured in medium supplemented with whole (•) or dialysed (o) heat-inactivated foetal calf serum and the proportions of each promastigote morphotype in the resulting populations: closed bars, MT1 promastigotes; hatched bars, MTi promastigotes; diagonally striped bars, MT2 promastigotes; open bars, MT3 promastigotes. The size criteria for each morphotype are defined in Table 3.1. Fifty cells were examined from a single but typical culture for each time point.

showed a slight increase over the first 2 days of the experiment, a rise equivalent to an approximate doubling of promastigote numbers. No further increase occurred subsequently and promastigote numbers began to decline. *L. major* promastigotes behaved in a similar manner under these conditions (not shown). Comparison of promastigote morphology in cultures containing whole or dialysed HIFCS identified differences in the proportions of each promastigote morphotype on each of the 4 days monitored. In contrast to the control culture where morphotype 1 promastigotes outnumbered all other promastigote forms on day 1, promastigotes in cultures with dialysed HIFCS were predominated on day 1 by forms characteristic of morphotype 2 promastigotes. By day 2 of the culture with dialysed serum, the other promastigote morphotypes were more numerous, especially morphotype 3 promastigotes. This was consistant with a drop in the proportion of morphotype 2 forms from approximately 90% to 55%. The proportion of this latter form continued to decrease, whereas the number of morphotype 3 forms increased. They were the major promastigote forms in cultures of *L. mexicana* containing dialysed HIFCS from 3 days post-inoculation.

Comparison of the effect on promastigote morphology 24 hours subsequent to sub-passage of supplementing culture medium with complete or dialysed HIFCS was also investigated for *L. major* and *L. panamensis*. The proportions of each promastigote morphotype for these species plus *L. mexicana* are shown in Figure 3.33. The results show greatly reduced numbers of morphotype 1 promastigotes and substantially elevated numbers of promastigotes with morphology characteristic of morphotype 2 promastigotes for all three species in cultures which had been supplemented with dialysed HIFCS.

Several materials were tested to see if they were involved in triggering cell differentiation. Supplementing HOMEM medium containing 10% dialysed HIFCS with

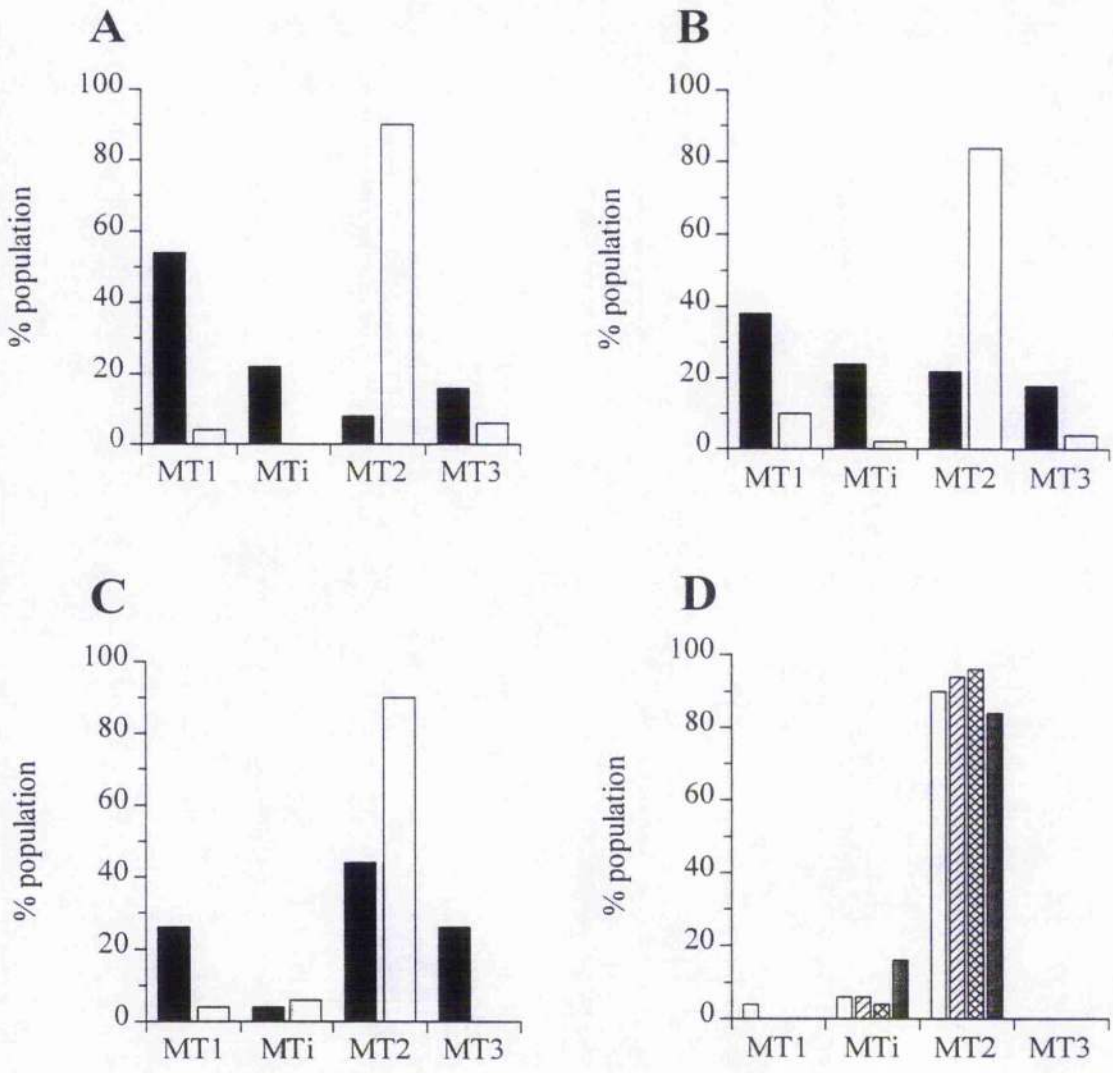


Figure 3.33: The proportions of each promastigote morphotype of *L. mexicana* (A), *L. major* (B) and *L. panamensis* (C) in single cultures supplemented with whole (closed bars) or dialysed (open bars) HIFCS. (D), the effect on cultures of *L. panamensis* supplemented with dialysed HIFCS of adding 0·01 mg ml^{-1} hemin, 0·01 mg ml^{-1} haemoglobin or 10 μM acetylcholine. Key: no additions, open bars; hemin, thinly striped bars; haemoglobin, hatched bars; acetylcholine, grey closed bars. Cell samples were removed and analysed 24 hours post-inoculation. The size criteria for each promastigote morphotype are defined in Table 3.1. Estimates are based on length and breadth measurements of 50 Giemsa-stained cells which were examined for each culture.

various compounds, glucose (not shown), proline (not shown), acetylcholine, hemin or haemoglobin, did not reverse the effect on promastigotes of *L. panamensis* of using dialysed serum (Figure 3.33, graph D).

3.4.3.2 Changing the amount of whole HIFCS

The results of experiments involving inoculation of promastigotes into HOMEM medium supplemented with dialysed HIFCS suggested that one or more dialysable components present in whole HIFCS was required for promastigote differentiation to forms with morphotype 1 morphology and that the absence of components trigger differentiation to promastigote morphotypes 2 and 3. The correlation of these changes with lack of growth seemed likely to be relevant. Consequently cultures were set up with various quantities of HIFCS to test whether there was a correlation between the concentration of HIFCS and the rate of differentiation to and from morphotype 1 promastigotes. The proportions of the different promastigote forms of *L. mexicana* in cultures on days 3 and 4 following inoculation into HOMEM medium which was supplemented with 5%, 10%, 20%, and 50% HIFCS and into 100% HIFCS are presented in Figure 3.34. As expected, control cultures with 10% HIFCS were dominated on days 3 and 4 by intermediates and morphotype 2 promastigotes, respectively. Lowering the amount of HIFCS to 5% resulted in cultures rich in morphotype 2 promastigotes on both days examined. Increasing the amount of HIFCS to 20 or 50% acted to delay the progression of development, such that promastigotes characteristic of morphotype 1 and intermediates were the most prevalent forms on day 3. By day 4 the proportions of these forms had declined and cultures were dominated by morphotype 2 and morphotype 3 forms. Promastigotes cultured in 100% HIFCS behaved differently from all other cultures. On day 3, all four promastigote forms were

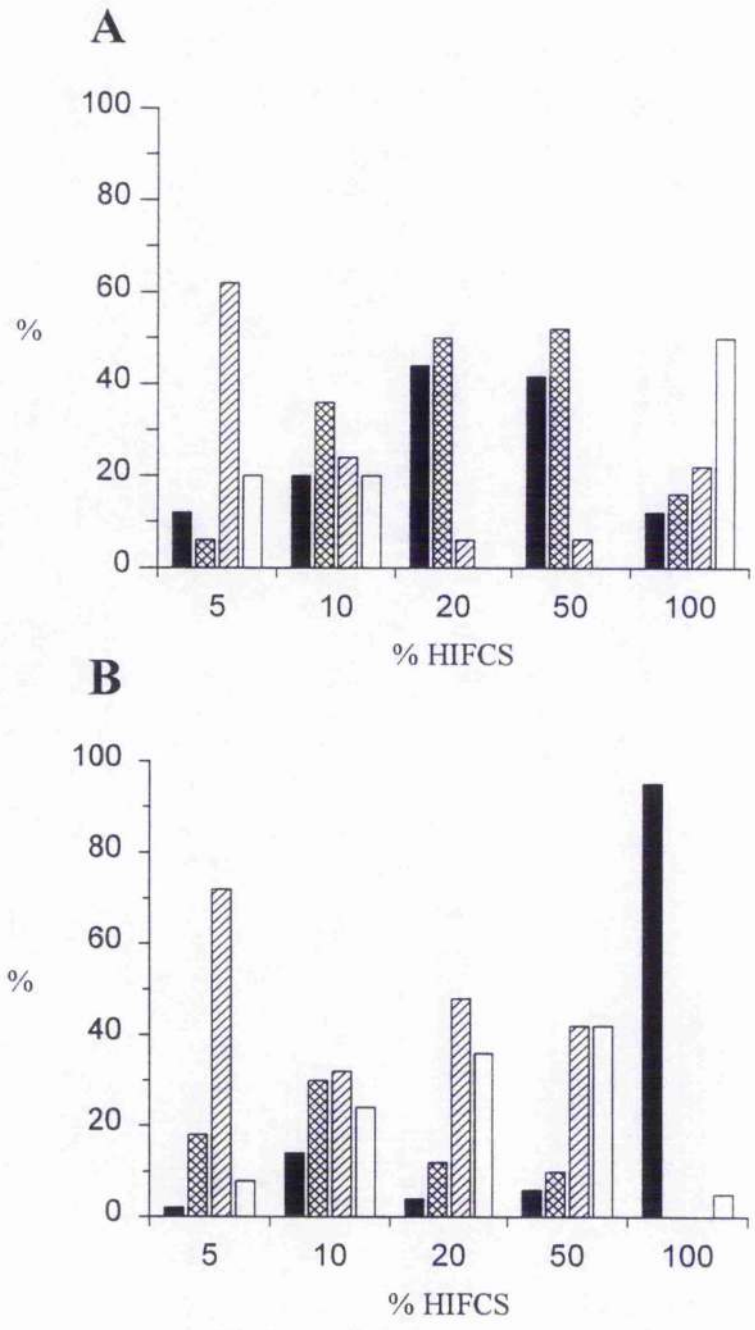


Figure 3.34: The proportions of each promastigote morphotype of *L. mexicana* on days 3 (A) and 4 (B) of culture in HOMEM medium supplemented with various quantities of HIFCS. Closed bars, MT1 promastigotes; hatched bars, MTi promastigotes; diagonally striped bars, MT2 promastigotes; open bars, MT3 promastigotes. The results presented are from one set of cultures at each concentration which was typical. The different promastigotes were characterised according to length and breadth measurements in Giemsa-stained smears (see Table 3.1 for definitions) and estimates are based on 50 cells from each culture on days 3 and 4 of growth.

present, with morphotype 3 promastigotes outnumbering all others, while on day 4 only promastigotes characteristic of morphotypes 1 and 3 remained. At this time morphotype 1 forms accounted for approximately 95% of the population.

3.4.4 The use of conditioned medium

The effect of conditioned medium on the differentiation of promastigotes was investigated. *L. mexicana* promastigote growth and morphology were assessed following initiation of late-log phase promastigotes into either complete HOMEM medium or spent medium harvested from cultures predominated by morphotype 2 promastigotes (Figure 3.35). Control cultures increased approximately 50-fold in cell density over the 4 days, at which time promastigotes were in the late-log phase/early stationary phase of growth. Promastigotes inoculated into spent medium also exhibited an initial increase in density, however this was substantially less than that in control cultures. An approximate doubling of promastigote numbers was observed over the first 2 days before there was cessation of growth. No further increase occurred. Promastigote numbers remained relatively consistant between days 2 and 3 but decreased subsequently. When promastigote morphology was assessed, it was clear that cells inoculated into spent medium behaved differently from control cultures. The proportions of the different promastigote morphotypes and their sequential development in control cultures was as expected, the cultures of promastigotes sub-passaged into morphotype 2 spent medium followed a different pattern. On day 1, promastigote morphotypes 2 and 3 comprised 50% and 30%, respectively, of promastigotes in the spent medium cultures, the remaining 20% exhibiting morphology typical of morphotype 1 and intermediate forms. Slightly reduced numbers of morphotypes 2 and 3 occurred on day 2, the numbers of intermediate promastigotes and morphotype 1

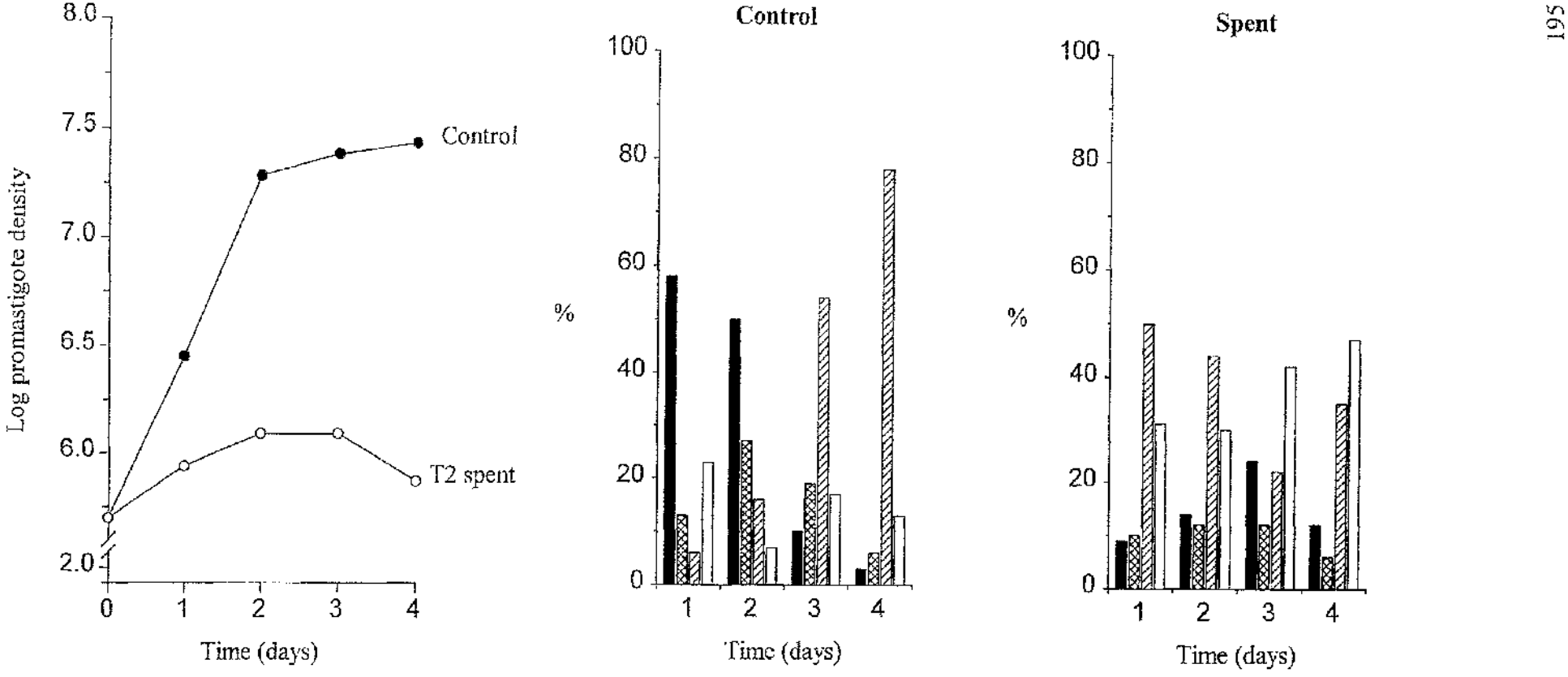


Figure 3.35: Changes in cell density (cells ml^{-1}) of *L. mexicana* promastigotes in complete HOMEM medium and in HOMEM medium harvested from single cultures in the late-log phase of growth (containing predominantely morphotype 2 promastigotes) [designated as spent medium] and the proportions of each promastigote morphotype in the resulting populations. Closed bars, MT1 promastigotes; hatched bars, MTi promastigotes; diagonally striped bars, MT2 promastigotes; open bars, MT3 promastigotes. Length and breadth measurements were made of 100 promastigotes which were characterised using the criteria defined in Table 3.1.

forms increasing. By day 3, morphotype 2 forms were substantially reduced in number, while promastigotes morphotypes 1 and 3 had increased. This decrease was only transitory since by day 4 the number of morphotype 2 forms had increased. At this time morphotype 3 promastigotes were dominant and comprised approximately 50% of all promastigote forms.

L. mexicana promastigote development in morphotype 3 spent medium (medium harvested from stationary phase cultures containing mainly morphotype 3 parasites) was also investigated (Figure 3.36). The proportions of each promastigote morphotype in control cultures and in cultures set up using medium harvested from cultures which had reached the stationary phase of growth were compared on day 2 post-inoculation. In addition, different starting densities were compared. In cultures initiated at $5x10^5ml^{-1}$, morphotype 3 promastigotes predominated when spent medium was applied. In contrast, the most numerous promastigote in control cultures were of morphotype 1 morphology. Increasing the starting density under both conditions acted to increase the number of morphotype 3 promastigotes which were present on day 2 of culture, making this the most prevalent promastigote form in all cultures set up at $5x10^6$ ml^{-1} and above. In contrast to this increase, the proportions of the other promastigote morphotypes decreased.

Comparison of the morphology of promastigotes sub-passaged at $5x10^5ml^{-1}$ into morphotype 2 and morphotype 3 spent media on day 2 post-inoculation revealed differences in the proportions of the various promastigote morphotypes which occurred. Although both populations contained significant numbers of promastigotes of all four morphotypes promastigotes in morphotype 2 spent medium were predominantely of morphotype 2 morphology at this time, while those in morphotype 3 medium were mainly morphotype 3 forms.

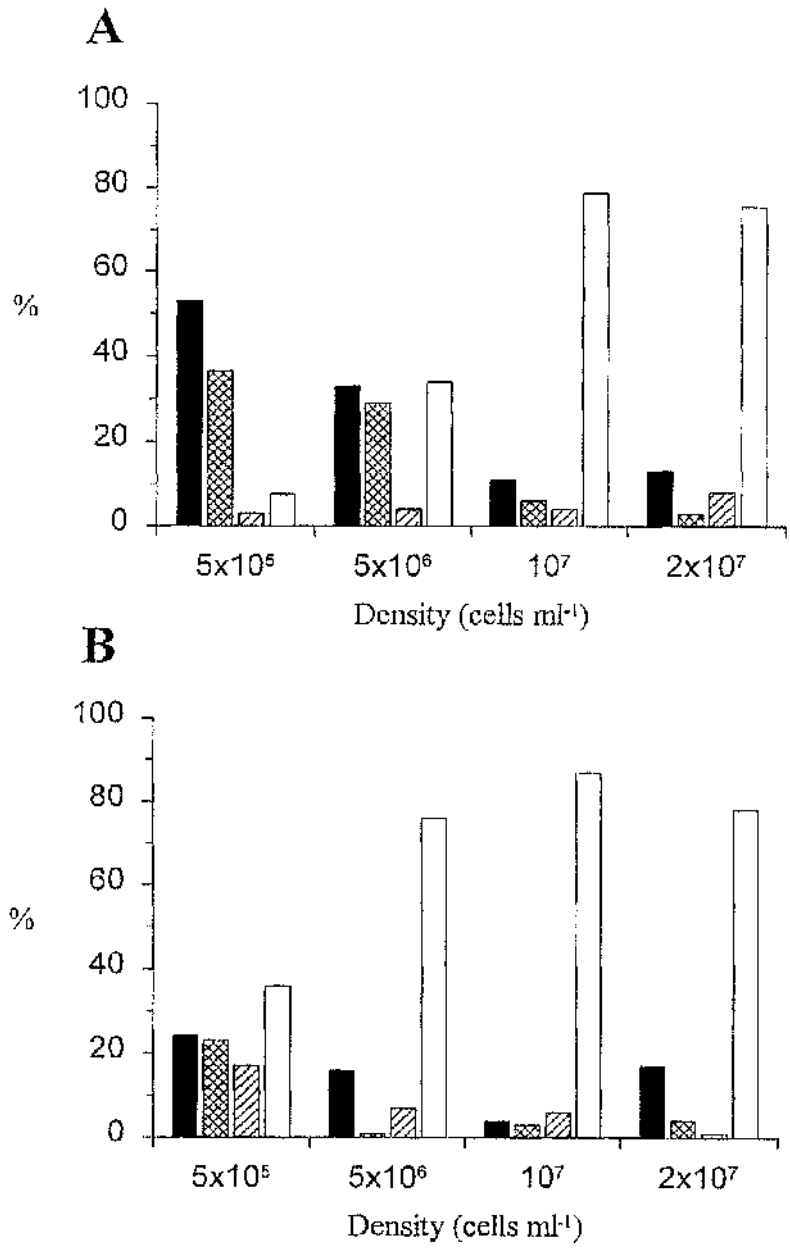


Figure 3.36: The mean proportions of each promastigote form in 2 day old cultures of *L. mexicana* set up in duplicate using: A, complete HOMEM medium; B, HOMEM medium supplemented with spent medium harvested from stationary phase cultures (MT3 spent medium). Closed bars, MT1 promastigotes; hatched bars, MTi promastigotes; diagonally striped bars, MT2 promastigotes; open bars, MT3 promastigotes. The size criteria for each promastigote morphotype are given in Table 3.1 and data are all from on measurements of 50 promastigotes.

3.4.5 Differentiation of morphotype 3 promastigotes

The growth and differentiation of morphotype 3 promastigotes of *L. major* was investigated. It was not possible to do this with *L. mexicana* or *L. panamensis* since methods to purify this parasite stage of these two species have yet to be developed. Isolation of different sub-populations of promastigotes using centrifugation through oil was attempted which although initially appeared promising was unsuccessful.

The procedure developed for purification of metacyclic promastigotes of *L. major* using peanut agglutinating lectin [Sacks, 1985] was applied to a stationary phase culture of *L. major* promastigotes and the resulting population of cells was sub-passaged under routine conditions for growth as promastigotes. Samples were taken on consecutive days of culture, days 0-4, and on day 6 and cell counts and Giemsa-stained smears were made. From these smears the proportions of each promastigote morphotype were estimated in each of the populations from measurements of 100 promastigotes. The data are shown in Figure 3.37. Day 0 cultures, which were representative of the purified population, contained promastigotes of each promastigote morphotype, but most had morphology characteristic of morphotype 3 forms. Although the increase in cell density was initially slow in the culture inoculated with the purified metacyclic population the maximum cell density and time taken to reach the stationary phase of growth were similar to the control cultures. Promastigote morphology also followed a similar trend to control cultures. Promastigotes of morphotype 1 morphology were most common in cultures in early-log phase but were gradually outnumbered by longer and more slender promastigotes as cultures progressed into mid-late-log phase of growth. Entry into stationary phase was coincident with an increase in the proportion of morphotype 3 promastigotes, although morphotype 2 promastigotes remained the most prevalent form.

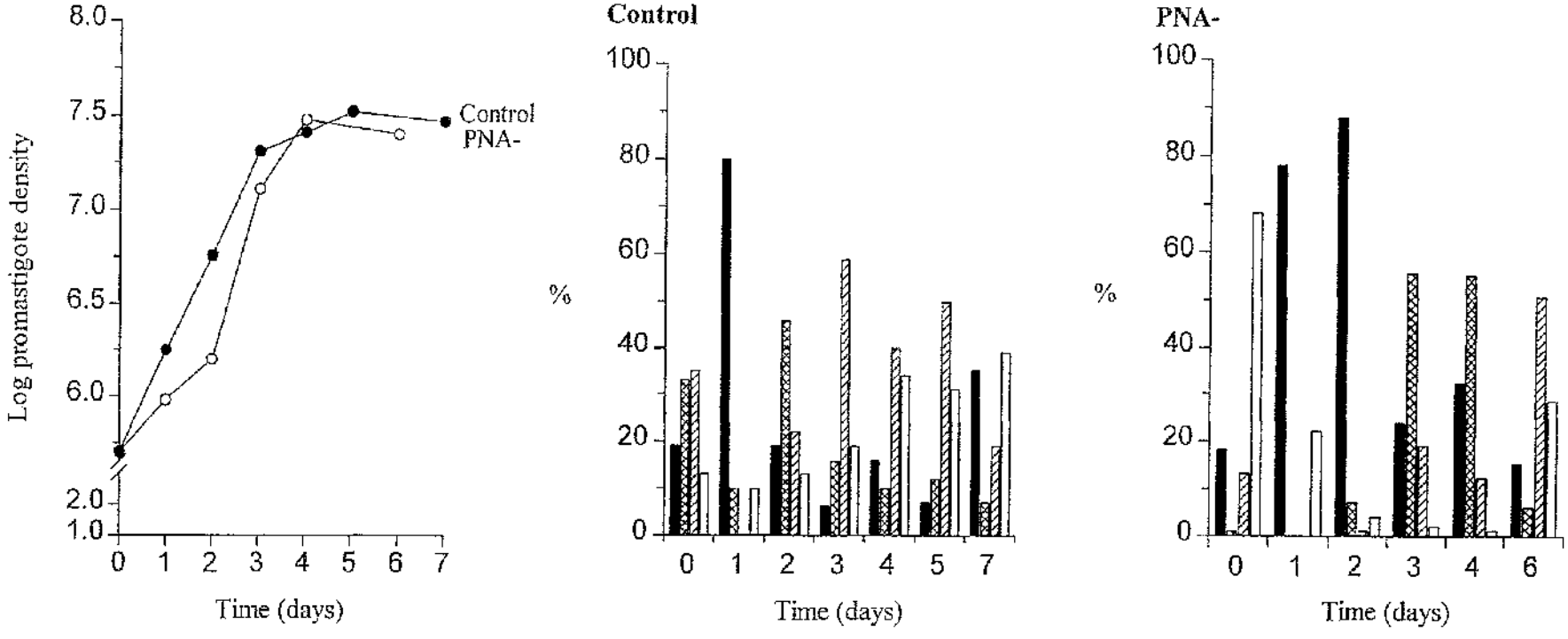


Figure 3.37: Changes in cell density (cells ml^{-1}) of *L. major* cultures initiated with mid-log phase (•) or peanut agglutinin lectin purified (o) promastigotes (PNA-) and the proportions of each promastigote morphotype in the resulting populations. Closed bars, MT1 promastigotes; hatched bars, MTi promastigotes; striped bars, MT2 promastigotes; open bars, MT3 promastigotes. The size criteria of each promastigote morphotype are given in Table 3.1 and estimates of the percentages of the different forms are based on measurements of 100 Giemsa-stained promastigotes (from single cultures) in each culture.

3.4.6 Induction of metacyclogenesis of *L. mexicana*

To investigate a potential relationship between inhibition of promastigote multiplication and metacyclogenesis, two compounds were assessed for their ability to inhibit promastigote multiplication and induce the production of promastigotes which morphologically resembled the metacyclic form (morphotype 3 promastigotes). Aphidicolin and DMSO were tested at a range of concentrations for their effect on promastigote growth and differentiation in complete HOMEM medium and complete SDM. Cultures were observed using an inverted microscope and assessed, by eye, relative to controls which contained no additions. In all experiments, promastigotes grew faster in SDM than in HOMEM. Aphidicolin notably reduced promastigote proliferation in both media at concentrations of between 0·75 $\mu g\ ml^{-1}$ and 2·5 $\mu g\ ml^{-1}$ but over 4 days its inclusion did not appear to enhance the production of metacyclics, when compared with control cultures. The effect of DMSO on promastigote multiplication differed depending on the culture medium applied. Concentrations of 0·25% - 2% DMSO substantially reduced promastigote growth in SDM but had no apparent effect on cell densities in HOMEM. When promastigote morphology was investigated in those cultures where growth was inhibited (those in SDM), no induction of metacyclogenesis was observed.

3.4.7 Transformation of promastigote morphotypes 1, 2 and 3 to axenic amastigotes

Differentiation of *Leishmania* promastigotes into forms which morphologically, biochemically and immunologically resemble the mammalian stage of the life-cycle (the amastigote) is possible through modification of culture conditions [reviewed by Bates, 1993a; Pan, 1993]. Such changes were applied to *L. mexicana* promastigote cultures

which were rich in each of the three promastigote morphotypes in order to compare the ability of each morphotype to differentiate into and to grow as axenic amastigotes. Growth curves for each morphotype are shown in Figure 3.38. Each grew well under conditions of acidic pH and elevated temperature, 32°C, although growth in cultures initiated with morphotype 3 promastigotes lagged behind the other two cultures. When cell morphology was investigated using Giemsa-stained smears, it was clear that all three promastigote morphotypes had begun to differentiate and had adopted amastigote-like morphology from as early as 2 days post-inoculation (Figure 3.39). By day 8 the aflagellates which originated from cultures containing each promastigote morphotype had reduced in size and were comparable with lesion amastigotes (Figure 3.40).

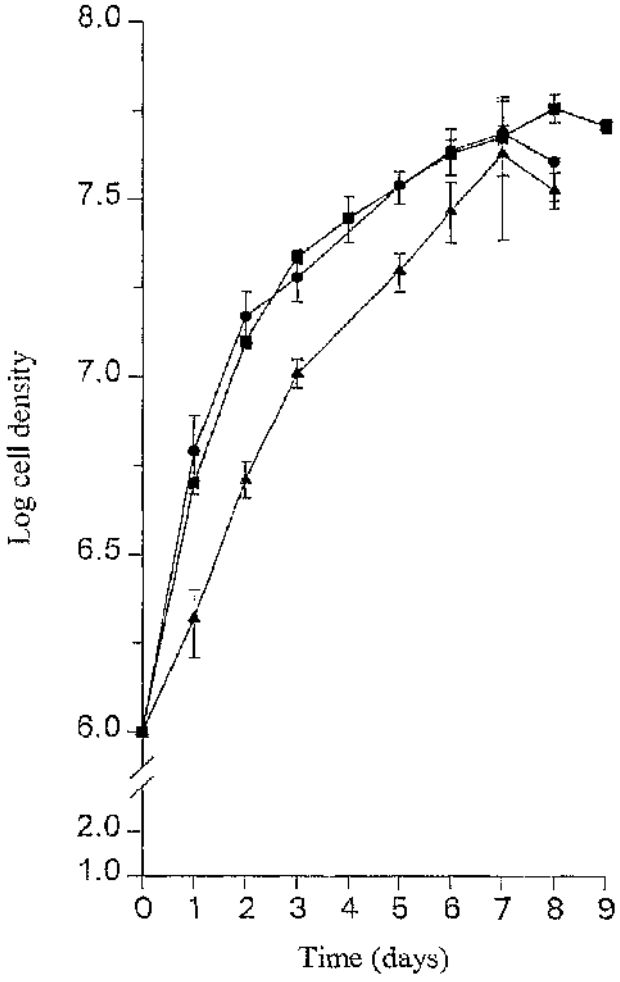


Figure 3.38: A comparison of the growth of *L. mexicana* in conditions developed for culture of axenic amastigotes when the cultures were initiated with morphotype 1 (circles), morphotype 2 (squares) and morphotype 3 (triangles) promastigotes. Promastigotes in the early-log phase, late-log phase and stationary phase of growth were sub-passaged at 10^6 ml^{-1} into complete SDM and incubated at 32°C. Samples were taken on consecutive days from triplicate cultures initiated with each of the promastigote morphotypes and cell counts were made. The bars represent one standard error from the mean cell density, n=3.

Figure 3.39: Comparison of length and breadth measurements of lesion amastigotes (A) with axenic amastigotes of *L. mexicana* produced from differentiation of morphotype 1 promastigotes (B), morphotype 2 promastigotes (C) and morphotype 3 promastigotes (D). Each set of measurements represents analyses of amastigotes from a typical lesion or culture forms harvested on the second day of growth from one of three individual cultures. Cells were measured at x1000 using a calibrated eye piece graticule. 1 unit = 1.25 μm.

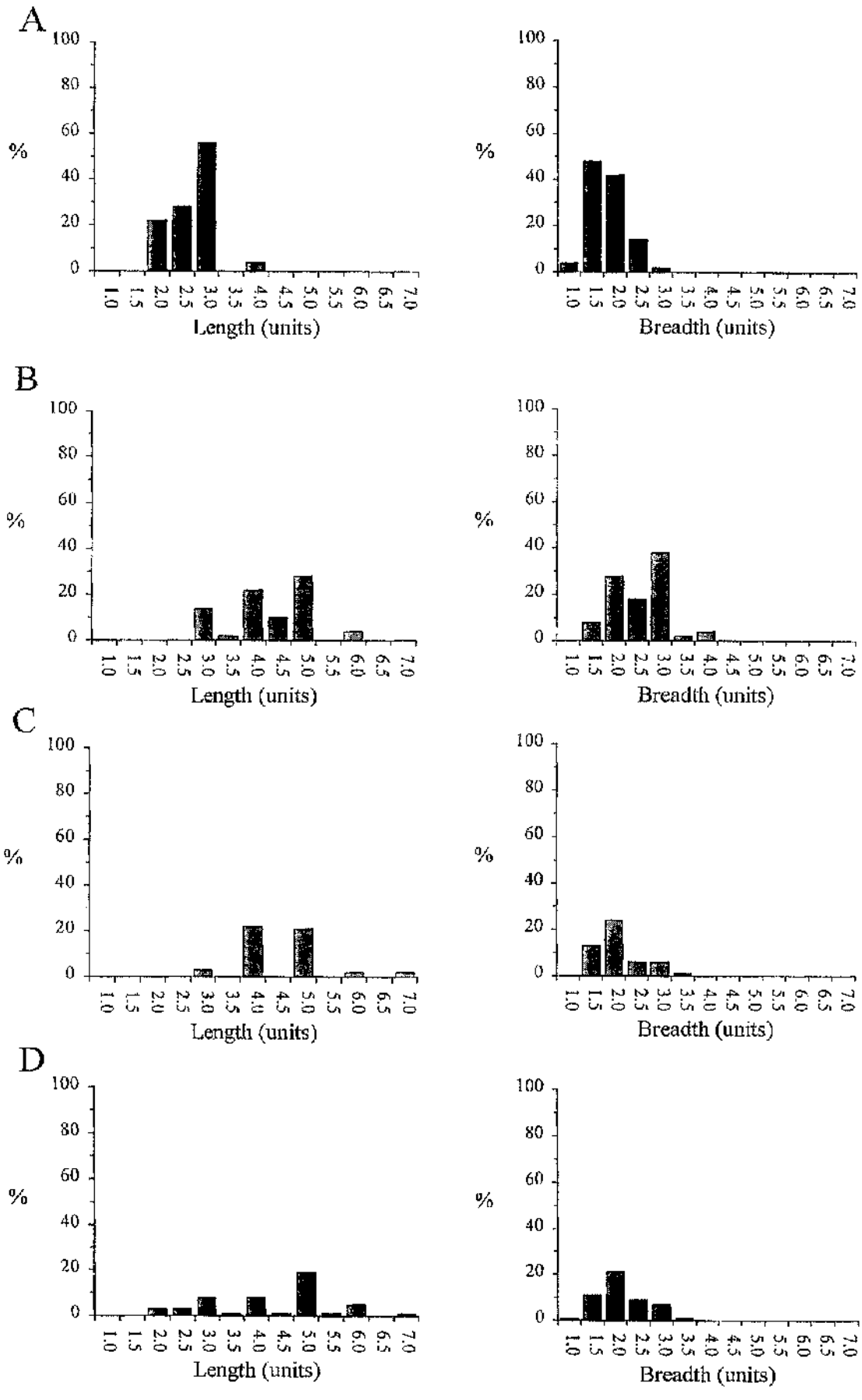
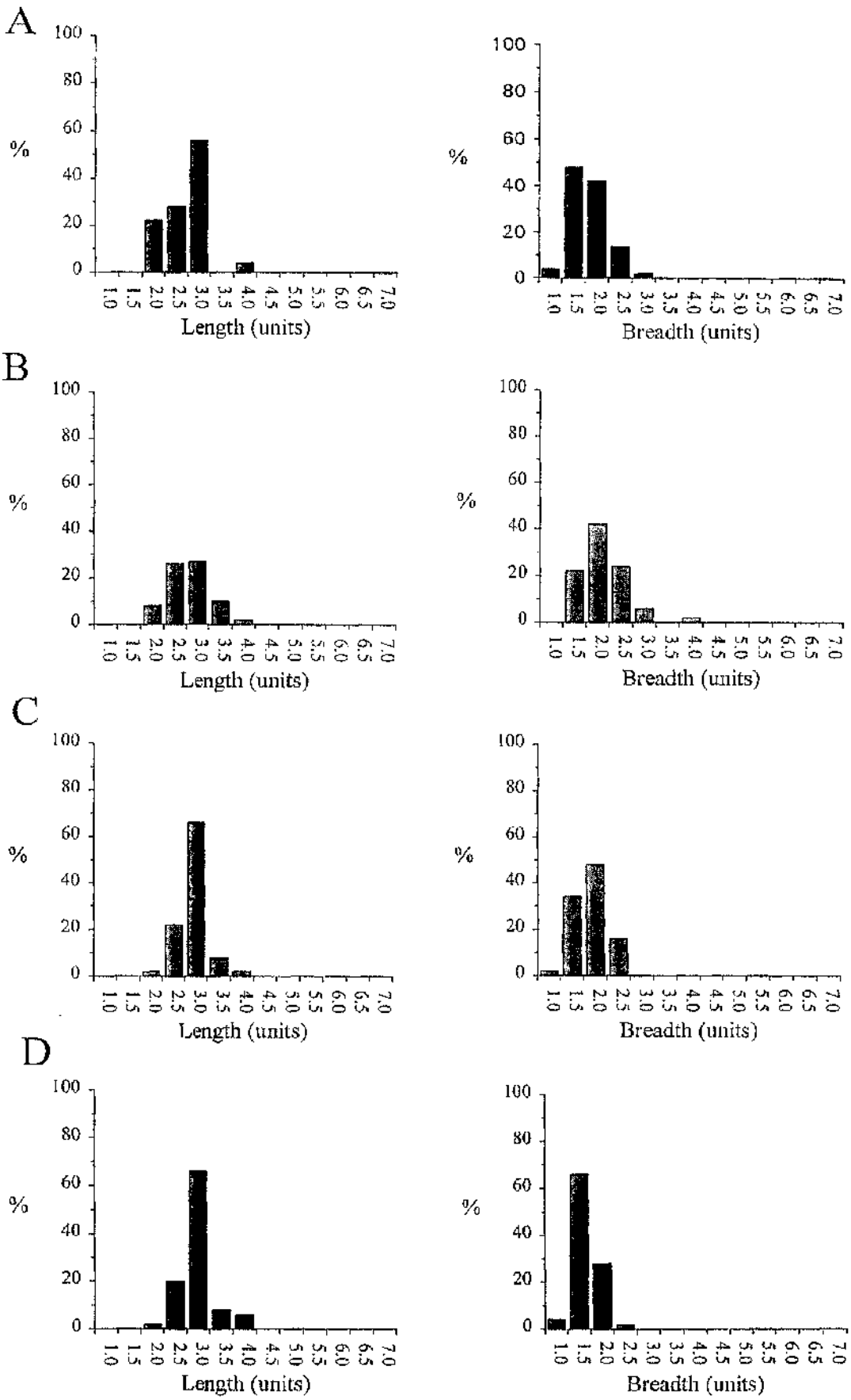

A
B
C
D
%
100
80
60
40
20
0
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
Length (units)
Breadth (units)

Figure 3.40: Comparison of length and breadth measurements of lesion amastigotes (A) with axenic amastigotes of *L. mexicana* produced from differentiation of morphotype 1 promastigotes (B), morphotype 2 promastigotes (C) and morphotype 3 promastigotes (D). Each set of measurements represents analyses of amastigotes from a typical lesion or culture forms harvested on the eighth day of growth from one of three individual cultures. Cells were measured at x1000 using a calibrated eye piece graticule. 1 unit = 1.25 μm.


A
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Length (units)
Breadth (units)

CHAPTER 4

DISCUSSION

Leishmania promastigote development in the sandfly is pleomorphic and involves congregation of specific morphological forms at different sites along the alimentary tract. At least six developmental forms have been identified, namely, stumpy procyclic promastigotes in the blood meal, slender midgut promastigotes, spatulate promastigotes in the hindgut, paramastigotes in the hindgut and foregut, and haptomonad promastigotes and metacyclic promastigotes in the foregut. Each of these morphotypes differs in appearance perhaps reflecting differing microenvironmental conditions in the different gut regions. Interestingly, an early study which characterised the morphological changes which occur in *in vitro* cultured promastigotes of *L. donovani* identified a developmental progression consisting of several morphologically distinct promastigote forms [Christophers *et al.*, 1926]. Whether a similar developmental progression is common to other *Leishmania* species and whether these different morphotypes represent distinct developmental stages was not investigated until recently. One major objective of this project was to determine whether the different promastigote morphotypes are also biochemically distinct, exhibiting adaptations for survival in their particular locality in the sandfly. Published data suggest that at least two biochemically, and morphologically, distinct promastigote stages of suprapylarian *Leishmania* species exist *in vivo*, namely, midgut promastigotes and metacyclic promastigotes. Both of these promastigotes also appear to occur *in vitro*. Promastigotes in the mid-late log phase of growth are presumed to correspond to midgut promastigotes while stationary phase cultures (of low sub-passage promastigotes) contain some

metacyclic promastigotes. Just how similar these culture forms are to those which occur in the sandfly has yet to be established, partly because retrieving large quantities of each from flies is unfeasable. Thus, biochemical studies have been mainly restricted to investigation of *in vitro* cultivated promastigotes. Both late-log phase and stationary phase promastigotes can be obtained in abundance with relatively little effort and consequently the majority of biochemical studies carried out on promastigotes have involved characterisation and comparison of these two forms while the other morphotypes which exist *in vivo* have been largely ignored. In addition, the ability to purify metacyclic promastigotes of *L. major* has provided a valuable source of this parasite stage for investigation. The purpose of this study was to investigate the biochemical characteristics of different developmental forms of leishmania promastigotes during growth *in vitro* and attempt to correlate the forms with those that occur in different localities in the gut of infected sandflies. The main findings of this study are discussed in three sections -morphology, biochemical evidence and differentiation.

4.1 Morphology

The results of this study confirm other reports, both old and relatively recent, of the sequential development of several morphologically distinct promastigote forms during *in vitro* culture [Christophers *et al.*, 1926; Bates, 1994; Charlab *et al.*, 1995]. Analysis of promastigote morphology of three species of *Leishmania* on consecutive days of culture allowed the morphological changes to be assessed. To ensure consistency the same batch of HIFCS and cells of similar sub-passage were used throughout. Furthermore, all experiments were carried out with promastigotes of relatively low passage number (< 15) as culture adapted parasites are likely to differ

significantly from those which occur *in vivo*. Evidence for this was demonstrated by Da Silva and Sacks [1987] who found that long-term culture of promastigotes resulted in reduced capacity to produce metacyclic forms. Three distinct *in vitro* promastigote morphotypes were defined for all three *Leishmania* species studied here; *L. mexicana*, *L. panamensis* and *L. major* (see Figures 3.4-3.6). These occurred sequentially and were consistantly observed in cultures set up under standard conditions. Each morphotype was defined based on promastigote cell body length and breadth measurements in Giemsa-stained smears (see Table 3.1), although two other considerations were also taken into account. It was critical for the purpose of this study that the forms which were defined could be consistantly obtained in large quantities for comparison. Each of the three morphotypes dominated cultures at different points in the growth cycle and therefore could be obtained relatively easily, although not as a homogeneous population, for investigation. Promastigote morphotypes 1, 2, and 3 corresponded to promastigote populations in early-log phase, late-log phase and stationary phase of culture, respectively each of which were judged by eye to contain a high proportion of a distinct promastigote form. The three categories which were defined illustrate the main developmental forms which occurred during promastigote growth *in vitro*, although clearly the life-cycle is a continuum and the presence of other forms cannot be ruled out. Indeed, it is likely that at least one other population of promastigotes occurs. This is suggested by the increase in number of rounded promastigotes (fitting the criteria used to describe morphotype 1 promastigotes) at the early stationary phase of growth (see Figures 3.4C, 3.5C and 3.6C). It is possible that this promastigote is equivalent to the stage which occurs in late infections attached to the foregut of the fly, which is also reported to be rounded in appearance. The progressive decrease in the number of this

morphotype in parallel with an increase in promastigotes of morphotype 3 morphology suggests that this stage could perhaps be the metacylic precursor.

Clearly body size is only one phenotypic marker and others if taken into account could identify additional populations of promastigotes. Flagellum length is one potential variable which may allow further promastigote morphotypes to be recognised. This was omitted here because in my opinion accurate quantitation without the aid of a computerised system for measurement of curved objects it was impractical. Since completion of this work, a more sophisticated method for measuring promastigote cell body shape has been reported. Blum and Balber [1996] applied flow cytometry which allows quick and accurate quantitation of large numbers of cells with relative ease. The implications of this technique for assigning more accurate size criteria and perhaps identifying additional promastigote morphotypes are clear. Moreover, cell sorting applications could potentially be applied to isolate and purify individual populations which could then be biochemically characterised. Success in achieving such a method would be highly beneficial for further biochemical characterisation.

Another consideration when defining the different morphotypes was their morphological resemblance to promastigotes which have been recognised *in vivo* since it was my intention to correlate each of the *in vitro* forms to a morphologically similar promastigote which had been identified in the fly. Promastigotes in the morphotype 1 category were short and rounded in appearance, resembling stumpy promastigotes (P1 and P2) which occur in the blood meal. In comparison, morphotype 2s were longer and more slender and were morphologically similar to the multiplicative midgut promastigotes (N1) of suprapylarian *Leishmania* species. Short slender cells comprised promastigotes in the morphotype 3 grouping which resembled the infective metacyclics (N5) of suprapylarian *Leishmania* species.

L. panamensis is a peripylarian *Leishmania* species and therefore undergoes a different developmental route within the sandfly gut from the other suprapylarian *Leishmania* species which are considered here (see sections 1.1 and 1.2). Promastigote development in the sandfly differs in that promastigotes free and multiplying in the midgut have not been observed and indeed are thought not to be a part of the life-cycle of this species (or closely related *L. braziliensis*, *L. guyanensis* and *L peruviana*). In contrast, an equivalent developmental period has been described in the sandfly hindgut. The exact nature of these hindgut promastigotes has yet to be determined and reports of their morphology and behaviour *in situ* are conflicting. Early documentation described them as multiplicative rounded paramastigotes [Hertig and McConnell, 1963; Johnson *et al.*, 1963; Lainson *et al.*, 1973; Lainson and Shaw, 1987] while a more recent study by Walters *et al.* [1989a] found the hindgut to be populated predominately by apparently non-dividing spatulate-shaped cells (promastigotes with a slender cell body and flattened posterior end, N2 and H2). The reason for these differences is unknown but could be related to the host-parasite combinations investigated and/or the differences in sandfly living conditions subsequent to infection. My observations of promastigotes of *L. panamensis* Boynton in *in vitro* cultures failed to identify significant quantities of either rounded paramastigotes or promastigotes which I would described as spatulate-shaped. It is possible that these forms were present but in such low numbers as to remain undetected or that the culture conditions applied simply did not favour their production. It was originally planned to attempt to obtain hindgut promastigotes of *L. panamensis* through modification of culture conditions to mimic the sandfly hindgut. However, insufficient information on the exact nature of the conditions prevailing in this region and of the morphology of the promastigotes which occur together with the lack of another form being observed in preliminary studies in which medium

composition was varied necessitated the adoption of an alternative approach for the investigation of this species. This approach took as a working hypothesis that the culture forms of this strain of *L. panamensis* which occurred in the late-log phase of growth were biochemically equivalent to hindgut promastigotes, although clearly they do not fit exactly with the morphological descriptions previously reported. It was hoped that biochemical comparison with promastigotes of *L. mexicana* in the corresponding phase of growth, comprising mainly morphotype 2 promastigotes, would reveal similarities and differences between the forms of each of these species and so at least provide some data on the extent that they resembled each other as well as possible adaptations for survival in two different gut regions.

Another promastigote stage of *L. panamensis* which has yet to be satisfactorily described is the infective metacyclic. This form of some other species (including *L. mexicana* and *L. major*), has been studied in-depth and a large body of evidence has been compiled confirming its morphological and biochemical distinction from log phase (multiplicative midgut) promastigotes. In addition, this form when compared using molecular approaches with multiplicative promastigotes and amastigotes has been shown to exhibit stage specific gene expression [Flinn and Smith, 1992]. I propose that the small, slender promastigotes which fall within the morphotype 3 category, defined here, correspond to metacyclics of *L. panamensis* Boynton. This hypothesis is based on observations made during this study and on descriptions of putative metacyclics of this species which have been reported in the literature. Firstly, promastigotes of this morphology increased in number with the advent of stationary phase producing a population of cells which was infective to mice and secondly, the gross morphology of these cells fits with putative metacyclics which have been identified *in vivo*. Of all the forms identified in the foregut of *Lu. gomezi*, Walters *et al.* [1989a] suspected that one

in particular corresponded to the infective metacyclic of *L. panamensis*. They chose a promastigote morphotype which was within the size criteria for metacyclics of other species [Killick-Kendrick, 1986; Warburg *et al.*, 1986; Lawyer *et al.*, 1987] but differed with respect to the flagellum length and cell body shape. The putative metacyclics, like those in this study, had a more rounded posterior end than the pointed metacyclics of suprapylarian species already described. Also in contrast to other species, for example *L. mexicana* [Bates and Tetley, 1993] entry into the stationary phase of growth did not appear to induce changes to the promastigote surface of *L. panamensis* which were visible by electron microscopy (not presented). Several sections of different promastigotes taken from a stationary phase culture were observed, including cells containing what appeared to be numerous lipid droplets (also found in metacyclics of *L. mexicana* [Mallinson and Coombs, 1989]), however no dense grainy coat was detected. The changes which occur in *L. panamensis* LPG with metacyclogenesis have yet to be fully elucidated and it is possible that this species differs from those which have been studied in detail.

The reason why *Leishmania* promastigotes undergo morphological alteration *in vivo* and *in vitro* has yet to be determined. It is unlikely that the variation is simply explained by the existence of promastigotes at various points in the cell cycle. Since only promastigotes possessing a single flagellum were included in the analysis of morphology I carried out, cell cycle variation is unlikely to account for the changes observed *in vitro*. In addition, analysis of the DNA content of promastigotes of *L. mexicana* during *in vitro* growth (Figure 3.29) showed only a small proportion of promastigotes were in the process of division at any one time and so this could not account for the predominance of different forms in the various phases of growth.

It has been proposed that the *in vivo* shape changes occur in response to changing environmental conditions within the sandfly gut. It is likely that promastigotes are subjected to highly variable conditions consistant with their existence along the entire length of the digestive tract, from the proboscis to the hindgut or midgut, of the sandfly. However, the exact environmental conditions prevailing in each of the gut regions are largely unknown and the speculations require confirmation.

One property of promastigote surroundings which is likely to vary during their development *in vivo* is the osmolarity of their surroundings, reflecting the fluctuating dietry habits of the fly from blood to plant juices (see section 1.3). Furthermore, plant juices, which are fed on frequently beween blood meals, themselves vary in tonicity [Salisbury and Ross, 1985]. Interestingly, studies on *in vitro* grown promastigotes confirmed that they are able to withstand quite dramatic changes in osmolarity, surviving both hypo- and hyper-osmotic stress by mechanisms which manifest visually as changes in cell body shape [reviewed by Blum, 1996]. Hypo-osmotic stress (achieved by diluting the medium with water) caused slender promastigotes to become shorter and rounder while hyper-osmotic stress (achieved by addition of mannitol) induced promastigotes to become slightly longer and thinner. The rounded promastigotes which were induced by hypo-osmotic stress closely resemble the morphotype 1 promastigotes which occurred in the early-log phase of growth. However, these forms of *L. panamensis*, or either of the other two morphotypes which occurred consistantly in *in vitro* culture, did not appear to result from the direct application of osmotic stress. The osmolarity of the culture medium harvested and measured at various points during promastigote growth was very similar to that of the iso-osmotic buffer used by Darling and Blum [1990] and remained relatively constant throughout the growth cycle (Table 3.2). Moreover, an additional response to hypo-

osmotic stress, alanine release, was not obvious when the amino acid contents of rounded (morphotype 1) and slender (morphotype 2) promastigotes of *L. mexicana* were compared (Table 3.13). If this shape change had been caused by changing osmolarity, the morphotype 1 promastigotes would be expected to contain substantially reduced quantities of alanine when compared with morphotype 2 promastigotes. Although based on only one determination of individual cell pellets of *L. mexicana*, the quantity of alanine in both of these morphotypes was comparable providing circumstantial evidence to support the proposal that the shape changes which occur *in vitro* are not simply due to direct osmotic stress. However, an indirect osmotic effect on promastigote shape has also been reported [Darling and Blum, 1990] and this cannot be ruled out in this study. Rounding of promastigotes in a manner identical to, but slower than, that resulting from hypo-osmotic stress was observed when promastigotes were incubated with certain potential energy substrates (glucose, mannose, fructose or proline) under iso-osmotic conditions. It is suspected that this shape change resulted from an osmotically-induced influx of water coincident with substrate uptake. This could correlate with the larger quantities of amino acids consumed by morphotype 1 promastigotes compared with morphotype 2s (Tables 3.5 and 3.6). In contrast to the shape change induced by direct application of hypo-osmotic stress, alanine release was not detected with promastigotes under iso-osmotic conditions in the presence of glucose. Consequently, it remains a possibility that the different *in vitro* morphotypes which I observed in the early-log phase and late-log phase of growth could be attributed simply to variations in the amounts of consumed substrates, reflected by differential water influx. This seems unlikely, however, since investigation of various properties of the different morphotypes provided biochemical evidence in support of the hypothesis that all three promastigote morphotypes represent distinct developmental stages.

The prime purpose of this study was to address the question of whether distinct developmental forms of promastigotes occur and in order to do this it was essential to define criteria to allow an experimental approach. In this context, the lack of clear distinctions between the different populations at the outset was unavoidable but necessitated a practical and working definition of the forms. The one adopted certainly allowed the hypothesis to be tested. This study concentrated on morphological criteria distinguishable at the level of the light microscope. The few electron microscopy studies included concerned mainly the surface structure of putative metacyclic forms. A fuller E.M. study could reveal important structural differences between the morphotypes, but was beyond the scope of my project.

4.2 Biochemical analyses of morphotypes

The biochemical parameters which were investigated were chosen with two main aims in mind. Firstly, some techniques were employed with the aim of identifying morphotype-specific features which could potentially become phenotypic markers of each stage. Secondly, other properties were analysed because they could potentially provide evidence of the similarity of the *in vitro* forms to *in vivo* promastigotes. Thus a study of energy metabolism was carried out, and this specifically involved analysis of substrate utilisation and enzyme activities in the different promastigote morphotypes. Finally, it was of interest to investigate transformation between each of the three morphological forms in order to gain some insight into the mechanisms involved, the intention being to investigate whether the differentiation events were stimulated by some exogenous compound or whether they were intrinsically programmed. *L. major* promastigotes were included in some of these studies since morphotype 3 promastigotes of this species can be purified and phenotypic markers are available.

The three promastigote morphotypes of *L. mexicana* and *L. panamensis* were characterised biochemically in an attempt to identify differences between them. When available, amastigotes, lesion and axenic, of *L. mexicana* were also analysed. The studies on promastigotes were carried out upon heterogeneous populations of promastigotes harvested from the early-log, late-log and stationary phases of growth which were judged by eye to contain a large proportion of the appropriate promastigote morphotype. Only one brief attempt was made to purify individual morphotypes. This involved separation of promastigote morphotypes 2 and 3 based on size differences using centrifugation. This was unsuccessful and was not pursued. The use of the populations as harvested was justified in that methods were not available to obtain pure populations of morphotypes and as the results showed that the populations obtained for use in different experiments were indeed morphologically very similar. Analysis of populations heavily enriched in a parasite form should allow identification of characteristics of that form.

Development of a technique for purification of metacyclic promastigotes of *L. major* has allowed biochemical characterisation of morphotype 3 promastigotes of this species and comparison with multiplicative promastigotes [for example, Mallinson and Coombs, 1989; Hart and Coombs, 1982]. In contrast, biochemical studies on promastigotes of other species, including *L. mexicana* [for example, Mallinson and Coombs, 1989], have largely focussed on comparison of mixed promastigote populations in the mid-log phase and stationary phase of growth. Promastigotes of this former population contain predominately forms which, according to my observations on promastigote morphology *in vitro*, are intermediate in morphology between morphotype 1 and morphotype 2 promastigotes. One study has been carried out in which a number of biochemical properties of *L. mexicana* undergoing the complete *in vitro* life-cycle

were analysed [Bates, 1994]. This investigation was set up from lesion amastigotes and monitored several characteristics during differentiation to and growth as the promastigote stage followed by differentiation to metacyclics and finally induced transformation *in vitro* to axenic amastigotes. A variety of promastigote stages were analysed including promastigote populations which appeared to contain forms resembling all three morphotypes of *L. mexicana* which were defined in this study. This was carried out at the same time as my own and although some of the results are both complementary and confirmatory, others are contradictory. These are referred to at appropriate sections of my discussion. In contrast to *L. mexicana*, the different morphological forms of *L. panamensis* have been studied little prior to this investigation.

As an alternative and complementary approach to biochemical searches for stage-specific characteristics, some workers have used molecular applications to discover stage-specific gene expression [Flinn and Smith, 1992]. However, similarly to biochemical studies these have been limited to investigation of multiplicative promastigotes, metacyclics and amastigotes and consequently other promastigote morphotypes have not been assessed.

4.2.1 Protein content and SDS-PAGE analyses of protein profiles, protein synthesis and nuclease activity

My results showed that the three promastigote morphotypes of *L. mexicana* differed in terms of protein content (Table 3.3) in a manner which corresponded to the observed changes in cell body size. Morphotype 2 promastigotes were found to contain the highest quantity of protein while morphotype 3 promastigotes contained the lowest. When my determinations were compared with those carried out by Bates [1994] on

promastigote populations of similar morphology it was clear that, although the quantities determined were of the same order of magnitude, the absolute protein content per cell differed. In Bates' study, promastigotes resembling morphotype 1 promastigotes contained 16 mg of protein per 10^9 cells, morphotype 2 promastigotes ca. 11 mg per 10^9 cells and morphotype 3 promastigotes 3·5 mg per 10^9 cells compared with 6 mg per 10^9 cells, 6·5 mg per 10^9 cells and 5 mg per 10^9 cells for promastigotes, in the same order, determined in this study. The reason why the protein contents differ between the two studies is uncertain but may be related to the fact that the promastigotes used in this study had been sub-passaged several times compared to those of Bates which were transformed directly from amastigotes. Another possible explanation could be that because morphotype 1 promastigotes are actively dividing, differences in determining the cell densities may account for the observed discrepency. In this study any promastigotes with two flagella were counted as two individual cells. If considered as only one promastigote by Bates the protein content per cell would be higher, as it indeed was. However, the same explanation could not account for the difference in protein content of late-log phase promastigotes (morphotype 2s) since the majority of these promastigotes were in the G1 phase of the mitotic cycle, having recently divided (Figure 3.29). A third possible explanation is that the differences could result form analyses of different mixes of cells in each population which seems probable as they were not homogeneous. My comparative results of protein profiles of the different morphotypes (section 3.3.2) provided circumstantial evidence that, in aggreement with Bates, morphotype 1 promastigotes contained more protein than the other two forms. It was necessary to reduce the quantity of morphotype 1 soluble and insoluble lysates to allow comparison of morphotypes 2 and 3. The lower protein content of stationary phase promastigotes than either of the other two forms is consistant with the presence of

metacyclics in this population and corresponds to their apparently smaller cell bodies. This finding correlates with other studies, including Mallinson and Coombs [1989] and Bates [1994], that the metacyclic promastigotes are intermediate in protein content between multiplicative promastigotes and amastigotes. The lower protein content of stationary phase promastigotes cultured under conditions to encourage metacyclic production is likely to reflect a higher proportion of metacyclics in Bates stationary phase population compared with the stationary phase promastigotes cultured in neutral HOMEM medium which were analysed here.

The protein content of *L. mexicana* axenic amastigotes was also determined. As expected these cells contained a lower quantity of protein than any of the promastigotes examined (Table 3.3). However, similarly to promastigotes, the protein content determined in this study differed from that of Bates [1994]. My results gave 2 mg of protein per 10^9 cells compared with approximately 5 mg per 10^9 cells in the latter study. The reason for these differences is uncertain but may be related to difficulties in determining accurate cell densities for these aflagellates. However, comparison of the determinations in both studies with the protein content of late-log phase promastigotes from the same study showed that axenic amastigotes contained approximately 3-fold less protein per cell. In contrast, Hart *et al.* [1981c] reported that in relation to the promastigote stage, lesion amastigotes of *L. mexicana* contained approximately 5-fold less protein per cell. This lower result could have been caused by rapid proteolysis by amastigote proteinases which in my study were inhibited both prior to harvest and at the point of cell lysis. On characterisation of axenic amastigotes of *L. mexicana* Bates [1994] concluded that the aflagellates were not identical to lesion amastigotes and that the method was in need of refinement. In agreement with this, the results of my studies showed that axenic cultivation of amastigotes of *L. mexicana* was a very variable

technique and the aflagellates which were obtained were somtimes larger and would not always sub-passage. No explanation for the variability could be found.

The smaller size of *L. panamensis* promastigotes in general to *L. mexicana* was reflected in their lower protein content (Table 3.3). Surprisingly, comparison of protein content in the different promastigote morphotypes of *L. panamensis* detected no difference in amount, suggesting that with this species developmental change may involve less dramatic overall changes.

SDS-PAGE proved a useful technique for comparing proteins in the different promastigote morphotypes. In addition to standard Coomassie Blue stained protein profiles, protein synthesis, nuclease activity and proteinase activity were compared in the three forms through application of slightly modified versions of this technique. Cell homogenates were centrifuged to produce crudely separated fractions of soluble and membrane components which were analysed separately.

To compare individual protein bands which had been stained with Coomassie Blue or which contained radioactive methionine it was necessary to adjust the volumes of sample which were loaded correlating with the differences in protein contents discussed above. Once profiles were obtained where the staining was relatively equivalent it was clear that although, as expected, most proteins were present in all three morphotypes, several varied quantitatively between them (Figure 3.9). The most prominent differences in gels stained with Coomassie Blue reflected differences in the soluble and insoluble protein content of the three stages of *L. mexicana*. However, some soluble proteins did appear to be more abundant, relative to others, in morphotype 2 promastigotes than in promastigote morphotypes 1 or 3. In contrast, comparison of Coomassie Blue stained profiles of the *L. panamensis* morphotypes did not detect any clear cut differences between the different morphological forms.

Incubations with radioactively labelled methionine identified both quantitative and qualitative differences in protein synthesis between the different promastigote morphotypes and between promastigotes and axenic amastigotes of *L. mexicana* (see Figures 3.10 and 3.11). Fortunately the rate of protein synthesis was high enough in the three morphotypes of both *Leishmania* species and in axenic amastigotes of *L. mexicana* to require only short (1-5 hours) incubations with the radioactive amino acid ^{35}S-methionine. Differentiation of the morphotypes would have prevented investigation of stage-specific protein synthesis over longer incubation periods. The most prominant difference between the morphotypes of *L. mexicana* was that protein synthesis was greater in morphotype 1 promastigotes than in either of the other two promastigotes. This is consistant with the highly multiplicative nature of this form. Membrane protein synthesis by morphotype 3 promastigotes was also notably higher than synthesis of soluble proteins of the same form and of soluble and insoluble proteins of morphotype 2 promastigotes. This perhaps relates to changes in surface membranes which accompany metacyclogenesis (discussed in section 1.6.4.2).

Although differences in protein synthesis were detected between the three morphotypes of *L. panamensis*, the changes were more subtle than those of *L. mexicana*. Generally the amount of synthesis was relatively consistant in all three forms although morphotype 2 promastigotes appeared to produce larger quantities of membrane proteins than the other two forms. This differs from protein synthesis in *L. mexicana* which was higher in morphotype 3 promastigotes. The reason for this difference is unknown but may be linked to differences in the life-cycle of these two *Leishmania* species.

Thus analysis of protein content and protein synthesis in the three promastigote morphotypes of *L. mexicana* has identified differences between them which although

difficult to quantify is consistant with the distinct morphotypes being different. In contrast, the same analyses on *L. panamensis* are suggestive that the three morphotypes are less different than those of *L. mexicana*, and indeed appear to be quite similar.

The results of my study confirm the results of Bates [1994] that nuclease activity is developmentally regulated in *L. mexicana* and provide evidence of an additional enzyme activity in morphotype 1 promastigotes which had not been reported previously (Figure 3.22, panel A). Since proteinase inhibitors were included in the incubation buffer and the cell samples were lysed on ice and boiled immediately afterwards it is unlikely that this activity results from proteolysis of the larger enzyme and potentially represents a stage-specific nuclease activity. The activity of this same enzyme was also detected in morphotype 2 promastigotes but was much reduced and probably correlates with a small proportion of morphotype 1 promastigotes in this population.

Substrate SDS-PAGE detected three nuclease activities in promastigotes of *L. panamensis* which were of approximately the same molecular mass as three of the enzymes detected in *L. mexicana*. No studies of substrate use or inhibitor sensitivity were carried out, thus any resemblance other than in size between these enzymes of the different *Leishmania* species were not identified. In contrast to the *L. mexicana* nucleases no enzymes appeared to be distinct to one morphotype. The nuclease profiles which were detected were similar for all three promastigote morphotypes. However, a difference in enzyme activity between the three morphotypes was apparent. The 40 kDa nuclease was of higher activity in morphotype 1 promastigotes than in the other two promastigote forms which contained similar amounts. So this represents a phenotypic difference between the *L. panamensis* forms.

A putative role in purine salvage from the sandfly for one 3`nucleotidase/nuclease activity of *L. donovani* promastigotes has been suggested, and

this is also thought to be relevant to promastigotes of *L. mexicana* (discussed in section 1.6.3). An enzyme of similar molecular weight was also detected in promastigotes of *L. panamensis*, however further experiments are required before any further similarity in structure or function to the enzyme of these other species can be deduced. It would be interesting to investigate whether other *Leishmania* species also possess this activity. Perhaps, like the promastigote surface proteinase (see section 1.6.4.1), it represents an enzyme which is conserved among *Leishmania* species.

The developmental changes in nuclease activites and the differences between *Leishmania* species potentially reflect the differing environments experienced in the sandfly host. It is interesting that the 40 kDa enzyme is of higher activity in the morphotype 1 promastigotes of *L. panamensis* than in the other two promastigote forms. This suggests that if this enzyme is involved in purine salvage from the sandfly that promastigotes resembling forms in the blood meal perhaps require a larger amount of purines than the other promastigote morphotypes. This is consistant with the highly multiplicative nature of the blood meal stages which is apparently not shared by the other morphotypes of this species and would be consistent with morphotypes 2 and 3 representing the reportedly non-dividing hindgut and metacyclic promastigotes, respectively (see sections 1.2.2.3.1 and 1.2.2.5). A similar change in activity of the 40 kDa nuclease of *L. mexicana* during differentiation from morphotype 1 promastigotes to morphotype 2 promastigotes was not observed. Studies on morphologically similar promastigotes in the sandfly gut have detected that both of these promastigotes are multiplicative in nature. It is possible that both stages are therefore in requirement of a source of purines for DNA synthesis which is not required by metacyclic promastigotes and is reflected in the fact that this enzyme was not detected in morphotype 3 promastigotes. The data gleaned from this study are suggestive that the nucleases of *L.*

mexicana potentially represent useful biochemical markers to distinguish not only between amastigote and promastigote stages but also between the different promastigote morphotypes. Further studies on these enzymes are merited to elucidate their precise roles in parasites survival in the fly .

4.2.2 Morphotype-specific antisera

Immunisation of rabbits with whole cell homogenates of the different promastigote morphotypes of *L. mexicana* produced highly sensitive antisera which recognised multiple proteins in soluble lysates of all three promastigote forms. The results indicate that many of the immunogenic molecules are common to all three morphotypes although quantitative differences for some of the proteins were detected when each morphotype soluble lysate was probed with the same antisera (Figure 3.12). The most prominant changes occurred in proteins of approximately 45-66 kDa and were most obvious when probed with antisera raised against morphotype 2 promastigotes. All three antisera detected greater quantities of one or more proteins in this size range in morphotype 3 promastigotes than in either of the other two forms. This correlates with the results of Western blotting analyses using partially stage-specific antisera. These were generated by complexing each of the different antisera with a different morphotype lysate the aim being to remove antibodies against molecules which were present in that lysate. When these sera were used to probe lysates of all three morphotypes only proteins of 45-66 kDa were detected in morphotype 3 promastigotes. It is therefore likely that rather than representing stage-specific proteins these molecules are produced in such excess by morphotype 3 promastigotes that they are incompletely removed by complexing with the other two morphotypes. Although the identity of these proteins was not investigated the Western blotting results suggest that they do not correspond to

the promastigote surface protein since the content in supernatant and pellet lysates of all three promastigote morphotypes of *L. mexicana* was relatively similar (Figure 3.17). Interestingly, on comparison of the results of the Western blots using polyclonal antisera (Figure 3.13) with Coomassie stained protein profiles (Figure 3.9, panel A) and autoradiographs of newly synthesised proteins (Figure 3.11), no proteins of 45-66 kDa were obviously expressed at a higher level in morphotype 3 promastigotes. It is therefore likely that the increased abundance of these proteins in morphotype 3 promastigotes detected in Western blots using polyclonal antisera is exaggerating by their highly immunogenic nature. This together with the fact that it is these stages of the parasite which are infective to the vertebrate host make these molecules worthy of further investigation. Analyses of membrane fractions of each parasite morphotype with the immune serum should elucidate whether these proteins are components of the cell surface and could provide potential markers of the different promastigote morphotypes.

These data, although difficult to quantify, are consistant with the results on protein content and synthesis suggesting that the different morphotypes of *L. mexicana* are indeed distinct. The results show that the three morphotypes contain a similar array of proteins but that these differ in quantity between the three forms.

4.2.3 Proteinases

The proteinase activities of *L. mexicana* log phase and stationary phase promastigotes and amastigotes have been studied in depth. In addition, proteinase activities during the axenic life-cycle of *L. mexicana* which includes populations of promastigotes which morphologically resemble the three promastigote morphotypes defined and investigated in my study have also been investigated [Bates, 1994]. My results generally confirm those already published, but also some additional activities

were detected. The most prominant difference relates to the apparent molecular masses of the enzymes, particularly those above 25 kDa. The discrepancy in size is probably due to the use of rainbow markers in this study which are of limited accuracy. Another difference was that, in general, greater enzymes activities were detected here, as judged by the larger quantity of gelatin hydrolysed than in other reported studies. Stimulation of activity by DTT in addition to a longer incubation period probably accounts for the observed increase in gelatin hydrolysis. These changes to the method were deliberately included in order to maximise proteinase activity in an attempt to identify differences in activities between the three promastigote morphotypes. This proved successful and allowed for differences in proteinase activity between the three promastigote morphotypes to be observed which had not been reported previously. Most of the enzymes which were present in morphotype 3 promastigotes were also detected in morphotype 2 promastigotes, although at lower activity, including the fastest moving enzyme which it was suggested may be metacyclic specific [Robertson and Coombs, 1992]. The occurrence of this proteinase in the latter population is probably an indication of the presence of some metacyclic promastigotes. This would be consistant with the identification of some metacyclics of *L. mexicana* and *L. major* in log phase populations of promastigotes by Mallinson and Coombs [1989] and in log phase populations of *L. mexicana* in this study (Figure 3.4C). In contrast to the other promastigote morphotypes of *L. mexicana* and to amastigotes, the proteinase activities of morphotype 1 promastigotes were virtually undetectable; the exceptions were a group of low mobility enzymes of approximately 70-100 kDa. Enzymes of equivalent mobility were also detected in *L. panamensis* promastigotes (see below).

There has been no detailed analysis of the proteinases of the different promastigote forms or of amastigotes of *L. panamensis*. My studies detected multiple

proteinase activities of apparent molecular mass 60-90 kDa in all three promastigote morphotypes (Figure 3.20). The highest mobility enzyme was also detected in amastigotes (see Figure 3.21) which had been obtained through *in vitro* infection of a macrophage-like cell line with stationary phase promastigotes. The activities of the promastigote enzymes were variable and attempts to enhance proteinase activity by increasing the amount of sample loaded were futile since gelatin hydrolysis appeared to be obstructed when the amount of protein was increased. Thus long incubation periods were required to maximise the amount of gelatin hydrolysed and even then the banding array obtained varied between experiments. At most 6 individual enzyme activities of this size range were detected.

Preliminary studies carried out to optimize the conditions for detection of activity looked promising for identifying differences in proteinase activity between the three morphotypes (see Figure 3.19). Three of the enzymes of stationary phase promastigotes which had been cultured under acidic conditions to maximise the number of metacyclic forms of *L. mexicana* [Bates and Tetley, 1993] were considerably more active than those of stationary phase promastigotes cultured in neutral HOMEM medium. A surface enzyme of approximately this size has been reported to be developmentally regulated in *Leishmania* promastigotes, undergoing increased expression in a manner which correlates with increasing infectivity [Kweider *et al.*, 1987; Davies *et al.*, 1990]. This suggested that metacyclic production of *L. panamensis* was also encouraged by acidic pH and that these forms either expressed higher quantities of these enzymes or contained proteinases of higher activity than populations of promastigotes which perhaps contained fewer morphotype 3 promastigotes in relation to morphotype 2s. However when the morphology of two stationary phase cultures of *L. panamensis* grown under these different culture conditions were compared the

proportions of morphotype 3 promastigotes were equivalent for each medium although the mean cell body statistics of promastigotes grown in the acidic medium was reduced. Stationary phase promastigotes grown in acidic SDM had an average length of 4·4 μm and width of 1·8 μm while a similar population grown in neutral HOMEM medium had an average length of 4·9 μm and width of 2·5 μm. Bates and Tetley [1993] reported a similar difference in promastigote body length between promastigotes of *L. mexicana* when growth and morphology of promastigotes in these two media were compared. It was concluded that the reduction in mean body length resulted from a more homogeneous population of metacyclics induced by the acid culture conditions. The approximate body length of the putative metacyclics of *L. panamensis* identified by Walters *et al.* [1989a] in the foregut of *Lu. gomezi* was 7-9 μm and is therefore longer than both populations of stationary phase promastigotes which were assessed here. It is possible that this difference in size between *in vitro* and *in vivo* metacyclics could be due to the use of different parasite strains. However, my results also suggest that the size criteria defined for the metacyclic stage of *L. panamensis* may be incorrect and that metacyclics are in fact smaller than reported.

Interestingly, increasing expression of a proteinase of similar molecular mass to those detected in *L. panamensis* promastigotes and *L. mexicana* promastigotes and amastigotes has been correlated with increased infectivity in a number of *Leishmania* species [Kweider *et al.*, 1987; Davies *et al.*, 1990]. Moreover, this enzyme, the promastigote surface proteinase (PSP or also known as gp63), has several characteristics in common with the *L. panamensis* enzymes. No characterisation of the *L. mexicana* enzymes were carried out. Whether any or all three of the enzymes which hydrolysed more gelatin in promastigotes grown in acidic conditions correspond to the PSP was not fully confirmed. However, all three behaved similarly under the conditions tested and

were approximately of the correct molecular mass. The molecular mass of the PSP has been variously reported to be in the range 63-72 kDa, although its mobility has also been reported to vary within a single study and appears to depend on the conditions employed for electrophoresis (especially the reducing condition). This together with my use of inaccurate markers of molecular mass may account for the apparently slightly higher molecular mass of the enzymes in my studies. The fact that all three enzymes exhibited activity over a broad range of pH, although the optimum differed slightly, and were inhibited by phenanthroline also correlates with their similarity to the PSP. None of these proteins, however, were recognized by a monoclonal antibody which was raised against the gp63 of *L. major* [Wallace and McMasters, 1987]. Rather than an indication of the absence of the PSP on *L. panamensis* promastigotes it is more likely that this lack of recognition reflects differences in glycosylation between the two species.

The presence of gp63 in the amastigote stage has been a matter for debate. It is now thought that amastigotes do contain the protein but in smaller amounts than promastigotes. Further differences relate to its location in the cell. In contrast to promastigotes the gp63 of amastigotes is soluble with an acidic pH optimum, and is located in lysosomes [see review on proteinases by Coombs and Mottram, 1997]. My results are in good accord with some of this data since proteins of slightly higher molecular mass to those of promastigotes were detected in purified lesion amastigotes of *L. mexicana* when lysates were probed with a monoclonal antibody raised against gp63 [Wallace and McMasters, 1987]. However, the quantities detected in amastigotes and promastigotes appeared relatively comparable. Furthermore, the results suggest that larger quantities of both the amastigote and promastigote protein are present in crude pellet fractions containing mostly membrane fractions than in the soluble supernatant fractions which contrasts with the report that the amastigote enzyme is soluble.

Amastigotes of *L. panamensis* which were obtained by infection and subsequent growth of parasites *in vitro* within a macrophage-like cell line contained one proteinase activity which was detected by gelatin SDS-PAGE. This proteinase was of slightly higher mobility than the promastigote enzymes which hydrolysed more gelatin when grown in acidic culture medium which could correlate with the smaller size of gp63 in the absence of the GPI-anchor. However, the mobility of this enzyme was very similar to that of another enzyme which was also present in promastigotes but did not change when promastigotes were subjected to acidic culture conditions. It is therefore likely that the amastigote enzyme does not correspond to gp63 and is also present in the promastigote stage.

The major surface proteinase of *Leishmania* promastigotes has had several functions attributed to it (see section 1.6.4.1). My results suggest that although it appears to be present in all promastigote forms of *L. panamensis* it is subject to developmetal regulation and is expressed in higher quantities or at higher activity in the metacyclic form than by early-log phase or late-log phase promastigotes. It thus seems likely that the activity is important for each of the different promastigote forms of this species during their development in the sandfly gut as well as subsequent to inoculaton into the vertebrate host.

Comparison of cysteine proteinases in promastigotes and amastigotes of *L. mexicana* using Western blotting techniques confirmed previous reports of stage regulation of these enzymes. Anti-type B and anti-type C antisera recognised different protein profiles in amastigotes and morphotype 3 promastigotes [Figures 3.14 and 3.15] confirming data published so far. In contrast, no proteins were recognised in promastigote morphotypes 1 and 2 which were not also detected with pre-immune serum. The result with morphotype 2 promastigotes was unexpected since proteinases

similar to, but of lower activity to those of metacyclics were detected with gelatin SDS-PAGE. It is therefore likely that the lower activity of these enzymes in morphotype 2 promastigotes results from decreased expression in this stage rather than the same quantity of enzyme which was of lower activity than the metacyclic enzymes. It has been suggested that cysteine proteinases of approximately 30 kDa and encoded by *lmcpb* genes have homologues in all leishmanias [Robertson and Coombs, 1994]. No proteins, however, were detected in soluble lysates of promastigotes of *L. panamensis* using anti-type B and anti-type C antisera raised against cysteine proteinases of *L. mexicana*. The amastigote stage of *L. panamensis* was not studied. Whether these enzymes are truly absent or are simply not detected by the method used was not pursued. However, evidence against the presence of an activity of this size was obtained by gelatin SDS-PAGE. Only one potential cysteine proteinase activity was detected in the promastigote stages of *L. panamensis* (Figure 3.20, panel B) which was larger than 30 kDa. This enzyme was not detected by any of the CP antisera suggesting that they are structurally unrelated. Interestingly the antiserum raised against the LmCPa protein of *L. mexicana* positively recognised a protein in all three promastigote morphotypes of *L. panamensis*, as well as two different proteins in all three promastigote morphotypes and amastigotes of *L. mexicana*. However, the single band recognised in *L. panamensis* was of slightly lower molecular weight than the *lmcpa* gene product of *L. mexicana*. Since no differences were obvious in the quantity of this protein in the different promastigote morphotypes no further analysis of this protein were carried out.

Although none of the proteinases detailed showed absolute stage specificity, the differential expression which was observed supports the hypothesis that the three morphotypes of *L. mexicana* differ biochemically. Furthermore, the results suggest that

the metacyclic promastigotes of *L. panamensis* differ in proteinase activity from the other two morphotypes and that other biochemical differences between this stage and the other two promastigote forms were probably being masked because mixed promastigote populations were being analysed.

4.2.4 Energy metabolism

4.2.4.1 Promastigotes

High pressure liquid chromatography proved to be a useful and accurate method for determining the amino acid content of simple and complex media. The concentrations detected in fresh simple media compared well with expected concentrations. However, the procedure adopted did not permit all amino acids to be quantified. Unfortunately proline, which is thought to be particularly important for parasites developing in the invertebrate gut, could not be analysed. Other methods to quantify proline use were not pursued. Incubation of parasites for two hours in simple media allowed direct comparison of amino acid use by the three different promastigote morphotypes of both species and by lesion amastigotes and axenic amastigotes of *L. mexicana*. These substrates were supplied at concentrations comparable to those in incomplete HOMEM medium. Longer incubations of promastigotes with the aim of comparing the effects of glucose and amino acid supplements on survival and growth of the cells were not carried out since the parasites would not grow in the absence of HIFCS. This supplement contains a large number of defined and non-defined components including potential energy substrates and a non-dialysable factor which I found to be essential for promastigote motility. Comparison of the concentrations of amino acids in fresh samples of simple and complex media indicated that whole HIFCS contained substantial quantities of some amino acids, particularly threonine, arginine,

alanine, tyrosine, tryptophan, phenylalanine, isoleucine and leucine, which were removed on dialysis. Dialysed foetal calf serum (with molecules under 10 kDa removed) was therefore included in the incubation media to allow substrate utilisation by active promastigotes to be assessed. In addition to these short term experiments, amino acid and glucose utilisation by the parasites growing in complex medium was analysed in order to see if the data obtained from the short term experiments gave useful information relevant to growing parasites.

My results have given detailed information of the amino acid uptake and release by promastigotes of *L. mexicana* and *L. panamensis* and amastigotes of *L. mexicana* and so provide important baseline data for further studies on functional significance and routes of amino acid catabolism. The large quantities of amino acids used by promastigotes of the two species in the presence and absence of glucose support the contention that amino acids constitute major energy substrates of these parasite stages [Marr, 1980]. In the absence of studies to follow the fates of individual amino acids, however, this suggestion remains to be confirmed. Nevertheless, other studies have demonstrated succinate production from amino acids [Van Hellemond *et al.*, 1997] as well as enzymatic pathways of amino acid catabolism in promastigotes which occur in an energy generating manner. These include proline [Krassner, 1969; Zeledon and de Monge, 1967; Krassner and Flory, 1972], arginine [Bera, 1987; Blum, 1992] and leucine [Blum, 1991] oxidation. Furthermore, the developmental regulation of an enzyme in *L. mexicana* which plays a central role in the catabolism and biosynthesis of amino acids, NAD-dependent glutamate dehydrogenase, is consistant with a catabolic role in the promastigote stage [Mottram and Coombs, 1985]. In contrast to promastigotes, only a cytosolic NADP-dependant enzyme was detected in amastigotes. This enzyme is thought to serve a biosynthetic role through provision of reduced

NADP. This correlates with the data available on energy generation by amastigotes, since amino acids are thought not to be major energy substrates [see Glew *et al.*, 1988]. It is therefore likely that the amino acids which were consumed by amastigotes of *L. mexicana* (Tables 3.7-3.9) were involved in biosynthesis rather than in energy generation.

The general consumption of amino acids by promastigotes of *L. mexicana* and *L. panamensis* was not affected by changing the gas phase from air to N_2, argon or 95% air/5% CO_2. However, the gas compositions within the aerobic incubation chambers were not monitored and it is probable oxygen tension was reduced due to the high parasite densities and this may account for the lack of change observed when conditions were made fully anaerobic. On the other hand, small quantities of dissolved oxygen in the medium could be responsible for the amino acid consumption detected in the 'anaerobic' chambers . Nevertheless, it would appear that promastigotes are able to consume amino acids when oxygen is severely limited, this possibly reflects the adaptability of these parasite stages for the frequently changing conditions within the sandfly gut. Indeed while observing promastigotes by light microscopy it became evident that promastigotes towards the outer edge of the coverslip first became sluggishly motile and then stationary. It seems likely that this behaviour reflects a lethal effect of complete aerobiosis on these parasites and although this requires clarification it does suggest that conditions within the fly gut are not fully aerobic. To my knowledge no measurements have been made regarding the oxygen and carbon dioxide concentrations within the different gut regions, although such information would anyway be of limited value since sandfly behaviour will have a direct influence on the gas composition at any one time. Moreover, changing parasite numbers will also influence the composition of the gas phase. Consistant with my results and with

putative variable conditions in the sandfly gut, it seems likely that promastigotes are able to undergo energy metabolism over a range of different concentrations of oxygen. In agreement with this was the finding that promastigotes of *L. major* catabolised glucose over a wide range of oxygen concentrations provided CO_2 (5%) was available [Keegan and Blum, 1990]. Indeed the rate of glucose catabolism increased as the pO_2 decreased from 95% to 6% and was still evident at as low a pO_2 as 1%, although ceased when all O_2 was removed. Whether this ability is common to other *Leishmania* species or to other energy substrates such as amino acids has not yet been determined, however the results of my study suggest that amino acid catabolism occurs at the same rate, at least short term, under conditions of reduced oxygen. Previous studies involving analysis of amino acid metabolism under changing gas phases have focussed on alanine production from glucose and consumption from endogenous pools which were found to alter depending on the amount of oxygen available [reviewed by Blum, 1993a; b; Blum, 1994]. Unfortunately my analyses of spent culture medium from promastigote cultures in air, N_2, argon or 95% air/5% CO_2 did not allow quantitation of alanine since alanine and arginine co-eluted. Thus the effects of changing gaseous compositions on the consumption and release of exogenous alanine by promastigotes remain undetermined.

The short term incubations also provided an opportunity to investigate glucose consumption in the presence and absence of amino acids by promastigotes of *L. mexicana* and *L. panamensis* and amastigotes of *L. mexicana*. However, insufficient quantities of glucose were consumed to allow accurate quantitation by the methods employed. This was probably due to the combined effect of a high initial concentration of glucose and an insensitive detection method. Other studies have detected changes in glucose use in relation to the promastigotes growth cycle [Mukkada *et al.*, 1974; Blum, 1996] and it is likely that further studies to optimize experimental conditions could

provide information on differential use of glucose by the three promastigote morphotypes.

My findings (Tables 3.5 and 3.6) that the different promastigote morphotypes consume and produce amino acids at different rates confirm other reports of altered amino acid use during the *in vitro* growth cycle (see section 1.6.1). It is likely that these differences are related to the changing environments which are experienced by the different morphotypes in the sandfly gut. Early-log phase promastigotes of both species, populations containing high numbers of morphotype 1s and resembling cells found within the blood meal, were found to consume large quantities of amino acids from the simple media, which correlates well with putative nutrient availability *in vivo*. This is also consistant with the highly multiplicative nature of these promastigotes *in vivo* and *in vitro* and although the fate of the amino acids was not followed it is likely that a substantial quantity of those consumed were used in protein biosynthesis. Although the overall situation was similar for both species, there were some differences. *L. mexicana* morphotype 1 promastigotes consumed slightly larger quantities of the detectable amino acids than equivalent morphotypes of *L. panamensis* and consumption by *L. mexicana* involved almost complete exhaustion (> 95%) of five of the available amino acids including alanine. In contrast, large quantities of alanine were excreted by morphotype 1 promastigotes of *L. panamensis*. The source of this alanine was not investigated but is unlikely to simply reflect release from an endogenous pool as occurs due to osmotic stress [reviewed by Blum, 1996] since the simple media were iso-osmotic and release of other amino acids was not detected. It would be interesting to investigate this inter-species difference in greater detail as it may represent a metabolic difference related to adaptation to different life-cycles. The finding that the addition of glucose had little effect on amino acid utilisation suggests that the sugar is not the

preferred energy substrate. This adds further support to the contention that the forms are equivalent to those that occur within the blood meal and are adapted for an environment likely to contain large amounts of amino acids and little glucose.

The general reduction in amino acid consumption by morphotype 2 promastigotes of *L. mexicana* and *L. panamensis* when compared with their respective morphotype 1 promastigotes ties in well with these forms existing outwith the blood meal and, in the case of *L. mexicana*, being equivalent to the midgut promastigotes identified in the sandfly which would potentially have access to both amino acids and glucose from plant and aphid sources. Increased release of alanine by these forms when glucose and amino acids were available suggests that glucose catabolism is also occurring (but that it is not full oxidised) although the lack of a similar effect of glucose on consumption of the other amino acids is suggestive that these amino acids are used in preferance to glucose. The data on amino acid utilisation by morphotype 2 promastigotes of *L. panamensis* shed no light on whether these forms are representative of the promastigotes which inhabit the hindgut of the sandfly. Indeed the finding that they behaved similarly to morphotype 2 promastigotes of *L. mexicana* in consuming large quantities of several of the amino acids in the presence and absence of glucose would be consistant with their existence in a similar environment.

Morphotype 3 promastigotes of both species generally consumed lower quantities of amino acids than the other two morphotypes consistant with reports that metacyclic promastigotes mainly use different energy substrates from multiplicative promastigotes (see section 1.6.1). However, in contrast to results of studies on other species which implied that the parasite preferred glucose at this stage [Mukkada *et al.*, 1974] my results suggest that amino acids may represent an important energy substrate for this stage of *L. panamensis* and *L. mexicana*. It is possible that the amino acid

consumption was mainly due to the morphotype 2 component of this population, but this was not determined since pure populations of metacyclics of these species could not be obtained. It would be of value to repeat these analyses with *L. major* as my results clearly show that this species also undergoes similar morphological transitions to those of *L. mexicana* and *L. panamensis* but in addition techniques allowing purification of metacyclic promastigotes of this species are available. Even then, however, the results would need to be applied to *L. mexicana* and *L. panamensis* with caution, as there may well be interspecies differences.

Since the fates of individual amino acids were not followed only limited interpretation of these results is possible. Consumption of radioactively labelled substrates from simple media would allow their metabolism to be mapped, while analysis of enzyme activities and soluble end-products during growth in complex medium would permit elucidation of the relative importance of individual pathways to each of the promastigote morphotypes. The results of other studies emphasising the complexity of the metabolism of these parasite stages suggest that they have adapted to withstand a diverse array of conditions by channelling their metabolism according to the prevailing situation at any time.

The results of this study indicate that in addition to amino acids all three promastigote morphotypes consume glucose when it is available. This conclusion is solely based on the general switch from consumption or negligable use of alanine in the absence of glucose to release in its presence. It seems likely that promastigotes possess the cellular machinery for both glucose and amino acid catabolism and switch the balance of use in accordance with the available nutrients. This may also explain why no differences in amino acid consumption were detected between morphotype 2 promastigotes of the two species investigated. The nutrients available to promastigotes

in the hindgut of the sandfly are probably significantly lower in quantity than those available to promastigotes in the midgut. However, promastigotes in both localities are potentially subjected to periods when energy substrates are limited and consumption of large quantities of amino acids by these forms may reflect their ability to maximise utilisation when substrates are available. In this regard, it is interesting that the results of a recent study showed that promastigotes of several *Leishmania* species can withstand periods of metabolic arrest induced by either starvation or by inhibition of respiration [Van Hellemond *et al.*, 1997]. The morphotype 2 promastigotes of the two species were exposed to rather similar conditions in my experiments which probably determined the similar consumptions.

Interpretation of amino acid and glucose utilisation during promastigote growth in complete HOMEM medium and correlation with the results obtained for each stage during the two hour suspension in simple media is complicated by the changing cell numbers during growth and the problems in correlating the different morphotypes exactly to the different days of culture. Nevertheless, the results can be cautiously interpreted. Substrate concentrations present after one day will have corresponded to the amounts of each amino acid and glucose remaining after differentiation of transformation intermediates taken from the mid-log phase of growth to morphotype 1 promastigotes and their subsequent growth. It would appear that amino acids are consumed in small quantities during this transition and thereafter in large amounts during promastigote growth in complex medium. By the late-log phase of growth (corresponding in this experiment to day 2 for *L. panamensis* and day 5 for *L. mexicana*) the concentrations of many amino acids were substantially reduced. Comparison of the amino acids which were used most during suspension in simple media by promastigote morphotypes 1 and 2 with those used by promastigotes during growth in complex media

up to late-log phase indicate that in both cases the same amino acids were the main ones used. In contrast, metabolism of alanine by *L. mexicana* morphotype 1 promastigotes differed depending on the conditions. Alanine was released in large quantities by promastigotes in the early-log phase of growth in complex medium but was consumed by morphotype 1 promastigotes in simple media. Alanine is thought to serve as an osmolyte guarding promastigotes against hypo-osmotic stress, being released under such conditions [reviewed by Blum, 1996]. However, the determination of the osmolarity of fresh and spent culture medium detected no change during the *in vitro* growth cycle of *L. panamensis* promastigotes (Table 3.2). The carbon skeleton of the released alanine probably originates from glucose catabolised by promastigotes in the complex medium as has been reported previously for *L. panamensis* [Darling *et al.*, 1987]. If this is the case then alanine release throughout the complete *in vitro* promastigote growth cycle of both *Leishmania* species suggests that glucose catabolism occurs continuously in these two species of *Leishmania* and is not restricted to cultures approaching the stationary phase of growth as reported for promastigotes of *L. tropica* [Mukkada *et al.*, 1974]. Quantitation of the amount of glucose used during short term incubations of the different promastigote morphotypes would have been useful for clarification of this since alanine can also be produced as an end-product of proline catabolism. In addition, the presence of several aminotransferase activities provide potential routes of alanine formation from other amino acids.

Analysis of the glucose content of culture medium confirmed that glucose was consumed by promastigotes of both species at all stages of *in vitro* growth. Only small quantities were consumed during the first few days of growth by both parasite species. Subsequently, at around the time of entry into the late-log phase of growth, glucose utilisation increased. Whether this increase was due to an increased use by individual

cells or simply reflected the rise in cell numbers is difficult to determine. However, a corresponding increase in the activity of hexokinase, a key glycolytic enzyme, in stationary phase promastigotes of *L. mexicana* (Table 3.12) suggests that there may indeed be increased glucose catabolism during metacyclogenesis, as was also reported for *L. tropica* [Mukkada *et al.*, 1974]. Interestingly, a similar increase in hexokinase activity was not detected with promastigotes of *L. panamensis* demonstrating a species difference. It is also notable that the activity of another glycolytic enzyme, pyruvate kinase, decreased with transition of *L. mexicana* to stationary phase. In contrast, pyruvate kinase in *L. panamensis* increased as the growth cycle progressed. This enzyme is probably less good as an indicator of the flux through glycolysis, for it catalyses the final step and it is known that other pathways may be followed in preference to pyruvate production depending on the growth conditions - for instance phosphoenolpyruvate may enter the glycosome (see section 1.6.1.1).

Further analyses of metabolism will be required to elucidate fully the pathways of energy generation occurring in the different *in vitro* promastigote morphotypes. Relating these to promastigotes *in vivo* is a challenging project since the conditions within the different gut regions have yet to be clarified. Moreover, presumably the exact conditions at any one time will be variable being influenced by sandfly behaviour and by other promastigotes in the gut. It is essential that promastigotes can withstand this variability if infections in the vector are to become established and it is likely that they are capable of catabolising a variety of energy substrates at different degrees of aerobiosis and alter their metabolism accordingly. The results of this study are in agreement with this hypothesis since it appeared that all three morphotypes survived and utilised amino acids when these were the only available substrates but consumed glucose in addition to amino acids when both were available.

The activities of several metabolic enzymes (Table 3.12) were assayed in the three promastigote morphotypes in an attempt to identify metabolic differences between them that could be related to differing *in vivo* environments and which could explain the differential consumption of amino acids. The activity of two glycolytic enzymes, hexokinase and pyruvate kinase, appeared to undergo stage regulation which differed between the two *Leishmania* species. The activity of hexokinase was generally higher in *L. mexicana* promastigotes, potentially indicating a higher rate of glycolytic flux than in *L. panamensis*. This interpretation may be incorrect, however, since the product of the reaction catalysed by hexokinase, glucose-6-phosphate, is also a substrate for the pentose phosphate pathway. The importance of this pathway, which is responsible for provision of carbohydrate intermediates used in nucleotide and other biosynthetic pathways [reviewed by Barrett, 1997], to the *Leishmania* appears debatable. One report suggested that it accounted for up to 40% of glucose used [Marr, 1980] while another study concluded that it contributed little to the overall glucose metabolism [Ghosh and Datta, 1971]. Flux through this pathway in relation to glycolysis and to glucose consumption was not investigated in this study, therefore hexokinase activity is probably not an ideal indicator of glycolytic flux. Nevertheless, differential activity of hexokinase was detected in the three promastigote morphotypes indicating that either the combined or individual pathways differ in flux. The *L. mexicana* hexokinase was of highest acitivity in morphotype 3 promastigotes which is consistant with the report of increased glucose consumption by stationary phase promastigotes [Mukkada *et al.*, 1974]. However, the activity in all three morphotypes was considerably higher than that reported in the same parasite by Mottram and Coombs [1985]. The reason for this discrepency is unknown but probably relates to different methodology. In contrast to *L. mexicana*, the same enzyme in *L. panamensis* was of slightly higher activity in

morphotype 1 promastigotes than in either of the other two promastigote forms, which had similar activity. It is possible that increased activity of this enzyme in morphotype 1 promastigotes is related to their enhanced requirement for nucleotides due to their high rate of multiplication.

Pyruvate kinase, the final glycolytic enzyme which is responsible for pyruvate production, differed considerably in activity between the two *Leishmania* species. The much higher activity in *L. mexicana*, which incidently is also considerably higher than that previously detected by Mottram and Coombs [1985], correlates with the higher quantity of pyruvate released during growth in complex medium by this species (compare Figures 3.27 and 3.28). In contrast, the two species released similar and rather low amounts of acetate and succinate. Perhaps these metabolites in *L. panamensis* arise not from glucose, as in *L. mexicana*, but from other substrates, such as amino acids. The results of a recent study indicated that the succinate produced and excreted by *L. infantum* promastigotes only partially originated from glucose and a higher proportion, it was suggested, was produced from amino acids [Van Hellemond *et al.*, 1997]. My enzyme data therefore suggest variation in the relative importance of different pathways of energy metabolism in *L. mexicana* and *L. panamensis*, differences which were not detected during analysis of amino acid or glucose uptake. The relatively similar activities of glutamate dehydrogenase, an enzyme central to amino acid degradation, in the two species do not support this proposal and highlights the need for additional studies to clarify these results. Perhaps the *L. panamensis* pyruvate kinase is significantly different from that of *L. mexicana* such that the assay conditions were not optimum for its activity. This could represent an interspecies difference important for the different life-cycles. Pyruvate kinase activities were also found to undergo stage-regulation. In *L. mexicana*, morphotype 3 promastigotes contained the lowest activity

and morphotype 2s the highest. Comparison with hexokinase activities suggests that the pathways of glucose catabolism in the three promastigote morphotypes of *L. mexicana* differ. It would appear that promastigote morphotypes 1 and 2 channel more glucose through puruvate than morphotype 3s, which presumably produce greater quantities of D-lactate and/or glycerol or divert the flow of carbon along the pentose phosphate pathway or back into the glycosome at the level of phosphoenolpyruvate (see section 1.6.1.1). This was not investigated, although the higher activity of isocitrate dehydrogenase in morphotypes 1 and 2 also indicate a higher flux through the citric acid cycle than in morphotype 3s. In contrast to *L. mexicana*, pyruvate kinase activity in *L. panamensis* was highest in morphotype 3 promastigotes and lowest in morphotype 1 promastigotes. Again these activities were not parallelled by the gradation of hexokinase activities. Morphotype 1 promastigotes had the highest activity of this enzyme but the lowest of pyruvate kinase while in the other two forms the activities were relatively comparable. This would be consistant with glucose being consumed at a faster rate by morphotype 1 promastigotes but the pathways subsequent to glucose-6-phosphate production differing. This could be investigated through analyses of the soluble end-products of energy metabolism, possibly using NMR [for example, Rainey and MacKenzie, 1991].

4.2.4.2 Amastigotes

The data obtained on the amino acid consumption by purified lesion amastigotes of *L. mexicana* allows their similarity to axenic amastigotes to be assessed. Small quantities of many of the detectable amino acids were consumed by lesion amastigotes resuspended at $3x10^8$ ml^{-1} in simple medim including aspartate, asparagine, threonine, alanine and leucine. When glucose was also available the consumption of amino acids

was generally increased. This may simply reflect a higher rate of cellular biosynthesis supported by glucose, but could also be a consequence of different pathways operating when glucose was absent. However, the results do not suggest amino acid catabolism to be important in energy generation. In addition, alanine consumption switched to production when glucose was added, which suggests catabolism of glucose.

The use of amino acids by axenic amastigotes was rather different from that of lesion amastigotes. The axenic amastigotes consumed negligable amounts of all of the amino acids analysed in the absence of added glucose and released large quantities of almost all of the amino acids in the presence of glucose at neutral pH. This provides further evidence that axenic amastigotes are not identical to lesion amastigotes. Although the results of amino acid consumption by lesion amastigotes at neutral pH provide a useful comparison with axenic amastigotes and promastigotes they probably do not reflect *in vivo* use. Acidification of the culture medium in the absence of glucose had little effect on amino acid consumption by axenic amastigotes. In contrast, inclusion of glucose in simple medium which was titrated to pH 5·5 resulted in consumption of most of the amino acids. Since glucose catabolism and proline uptake by amastigotes is optimal at acidic pH [reviewed by Zilberstein and Shapira, 1994] it is likely that these conditions are optimal for amastigote survival.

4.2.5 Amino acid content

Examination of the free amino acid pools of the three promastigote morphotypes of *L. mexicana* (Table 3.13) identified differences between the morphotypes and the results are consistant with a previous report on the amino acid content of mid-log phase and stationary phase promastigotes of *L. mexicana* [Mallinson and Coombs, 1989]. Stationary phase promastigotes generally had lower levels of amino acids than log phase

promastigotes in both cases. Attempts to harvest promastigotes rapidly and thus decrease the likelihood of changes occurring, either through efflux or modification of metabolism induced by harvesting conditions, during harvesting were unsuccessful. Few promastigotes were pelleted through the layer of silicone oil, indicating that the oil was too dense. This method was therefore abandoned in favour of that used previously by Mallinson and Coombs [1989]. The fact that these authors also detected differences between log phase and stationary phase promastigotes and that all three promastigote morphotypes analysed in this study were harvested identically suggests that the observed differences may be of some physiological significance. In addition, my results also show that morphotype 1 promastigotes differ in amino acid content. The observed pattern of amino acid content parallels the gradation of amino acid consumption from simple media by these three morphotypes and could therefore simply be due to increased uptake of amino acids. Indeed, decreases in the intracellular concentrations of some amino acid of *L. tropica* promastigotes were shown to be a consequence of starvation of these cells [Simon *et al.*, 1983]. Interestingly, however, the concentrations of some amino acids increased which could be a result of their release from proteins but lack of catabolism in energy generation.

Large intracellular quantities of alanine appear to be a common feature of leishmania and have been detected in several species [Simon *et al.*, 1983; Mallinson and Coombs, 1989; Knodler *et al.*, 1994; Darling *et al.*, 1990]. Other protozoa including *Trypanosoma* species [Williamson and Desowitz, 1961; O'Daly *et al.*, 1983], *Crithidia luciliae*, *Giardia intestinalis* and *Trichomonas vaginalis* [Knodler *et al.*, 1994] have also been reported to contain a large pool of intracellular alanine. It has been postulated that this amino acid plays an important role in prevention of cell lysis under osmotic stress [reviewed by Blum, 1996] which is consistant with the variable habitats encountered by

these different parasites during their life-cycles. My results confirm that all three of the morphotypes of *L. mexicana* contain a large pool of alanine providing evidence in favour of the hypothesis that these promastigotes represent distinct developmental stages and do not simply arise as a result of osmotic stress. Sub-passage of promastigotes into hypo-osmotic conditions has been shown to induce release of amino acids, particularly alanine, consequently resulting in a change in shape of promastigotes of *L. major* and *L. donovani* from long and slender to short and rounded [reviewed by Blum, 1996]. Comparable quantities of alanine in promastigote morphotypes 1 and 2 indicate that these promastigote morphotypes are not the result of changes in osmotic stress, which is also consistant with measurements of medium osmolarity (Table 3.2). In contrast to other studies on *L. tropica* [Simon *et al.*, 1983], *L. mexicana* and *L. major* [Mallinson and Coombs, 1989], I detected arginine to be present at a higher concentration than alanine in both of the morphologically distinct promastigote populations from the early-log phase (morphotype 1) and late-log phase of growth (morphotype 2) which were analysed. This discrepency may be due to the harvesting procedures correlating with the report that starvation of *L. tropica* promastigotes led to an increase in arginine content in promastigotes of *L. tropica* [Simon *et al.*, 1983].

The biochemical data accrued on both *Leishmania* species have identified differences between the three morphotypes which are in support of the hypothesis that each represents a distinct developmental form. Moreover, the results also provide evidence in favour of the proposal that each stage is adapted for potentially differing environments in the sandfly gut. In general the alterations which occurred in *L. panamensis* were less dramatic than those of *L. mexicana* which suggests that the morphotypes of this species are indeed less different. This may simply be a reflection on the choice of parameters which were analysed and it seems likely that further

characterisation will provide additional information on how the morphotypes of both species differ. For example, isoenzyme profiles or analysis of the antisera raised in mice in this study for stage-specific differences, especially surface proteins, could provide other phenotypic markers of all three morphotypes. Although no phenotypic markers which were absolutely stage-specific were identified in this study which could be applied to all three morphotypes, application of a number of the techniques which identified differences between the forms provides a means of identifying each of the different morphotypes. This should prove useful in further studies directed towards obtaining substantially enriched populations of the three morphotypes by modification of culture conditions. Such techniques are clearly warrented and may reveal differences which were masked during characterisation of the heterogeneous promastigote populations.

4.3 Infectivity

Previous studies on infectivity of *L. major* promastigotes have demonstrated that promastigotes from the log phase of growth are less infective than stationary phase promastigotes consistant with an increase in the number of metacyclics in the stationary phase population [for example see Sacks *et al.*, 1984; 1985; Mallinson and Coombs, 1989].

My studies on *L. mexicana* infectivity are in good accord with this since stationary phase populations survived considerably better in peritoneal exudate cells than late-log phase promastigote populations (Table 3.14). The finding that some of the morphotype 2 population were able to infect and multiply in macrophages could be explained by the presence of a small number of morphotype 3 promastigotes. Surprisingly, morphotype 1 promastigotes, were more infective to peritoneal exudate

cells than morphotype 2 promastigotes and were similar in infectivity to morphotype 3 promastigotes. This was unexpected and does not fit well with current concepts that only metacyclic forms are infective to mammals. The population of promastigotes sub-passaged to provide the morphotype 1 population was in the mid-late-log phase of growth and although some morphotype 3 promastigotes would be present it is unlikely that their numbers would be sufficient to account for the infectivity observed. Moreover, studies on the growth of purified metacyclics of *L. major* (see section 3.4.5) are suggestive that de-differentiation of metacyclics occurs upon sub-passage into fresh culture medium thus reducing the numbers of morphotype 3 promastigotes in the morphotype 1 population further. It may be that infectivity to macrophages *in vitro* is determined by different factors from infectivity to mammals, and data on the latter are now required to allow further interpretation of the results.

In contrast to *L. mexicana*, no clear cut differences in infectivity were detected with the different promastigote morphotypes of *L. panamensis*. All three populations were capable of survival within the macrophages. Interestingly, the percentage of infected macrophages and average numbers of amastigotes per macrophage detected were similar to those observed with morphotype 2 promastigotes of *L. mexicana*. It was suspected that *L. panamensis* would survive less well than *L. mexicana* since *L. panamensis* was found to be more sensitive to culture conditions and infected mice less readily (see section 3.1). The significance of the infectivity results for *L. panamensis* are uncertain. The finding that stationary phase promastigotes were not more infective than log-phase promastigotes could be because the number of metacyclics in the two populations was similar which would in turn explain why in other respects these two populations have been so similar. However, it may also be a reflection on the fastidiousness of this parasite species and the sub-optimal conditions used in the study.

It may also reflect the lack of correlation between infectivity to macrophages *in vitro* and to mice, as also potentially explains the results with *L. mexicana*.

4.4 Differentiation and transformation studies

The results from studying the ability of *L. mexicana* promastigotes to differentiate to amastigotes indicate that all three promastigote morphotypes were equally capable of differentiating to and dividing as amastigote-like forms (Figures 3.38, 3.39 and 3.40). This was surprising but in some respects correlates with the infectivity results discussed in section 4.3. In contrast to the infectivity studies, however, morphotype 2 promastigotes survived and multiplied as well as the other two morphotypes under these conditions. Unfortunately the ability of the aflagellates to be sub-passaged and maintain amastigote-like morphology was not assessed. Similarly no biochemical characterisation of the amastigote-like cells was carried out. Thus, whether the aflagellates bear resemblance other than in morphology to lesion amastigotes was not determined. However, these results are not consistant with metacyclic promastigotes being the only ones capable of differentiating to amastigotes.

Another approach to determining whether or not the promastigote morphotypes are distinct developmental forms was to investigate their ability to transform back and also the trigger for transformation.

Various experiments involving modification to culture conditions were carried out in an attempt to investigate the stimuli for differentiation between the different morphotypes. Exhaustion of amino acids within the blood meal may be the trigger for transformation of morphotype 1 promastigotes into more elongate, motile forms (morphotype 2s) *in vitro* and *in vivo*. My results clearly show that the early-log phase promastigotes consume large quantities of amino acids, almost exhausting supplies

within two hours, while *in vivo* studies have shown that this differentiation step occurs shortly before breakdown of the peritrophic membrane when sandfly digestion of the blood meal is virtually completed (see section 1.2.2.2). Short incubations involving suspension of promastigotes in buffered saline supplemented with 10% dialysed foetal calf serum in the absence of either glucose or amino acids provided some evidence to suggest that amino acid depletion was not the stimulus for differentiation to morphotype 2s since promastigote morphology was not observed to change during the incubations. However, the period of time taken for the differentiation to occur is probably in excess of two hours, based on the time taken in cultures for differentiation of morphotype 1 promastigotes into morphotype 2s. Furthermore, this differentiation step *in vitro* occurs despite the presence of large quantities of most of the amino acids in the culture medium (Tables 3.10 and 3.11), although clearly some have been virtually exhausted by the late-log phase of growth when morphotype 2 promastigotes predominate. Lack of data on the utilisation of other amino acids, especially proline, limit interpretation of these results and the role played by amino acids in triggering promastigote transformation therefore remains unclear. The results of other experiments (discussed below), however, involving modification of promastigote culture medium indicate that depletion of these energy sources may not be the only factor responsible for transformation to a more elongate promastigote form.

Studies carried out to elucidate other potential triggers of this differentiation step indicated that one or more dialysable components present in HIFCS are important determinants of both differentiation to morphotype 1 promastigotes and promastigote division (Figures 3.32 and 3.33). Unlike morphotype 2 promastigotes sub-passaged into HOMEM medium which was supplemented with complete HIFCS, morphotype 2 promastigotes (of all three species) which were sub-passaged into HOMEM containing

dialysed HIFCS did not transform back to morphotype 1 promastigotes and appeared to cease dividing. In some experiments promastigote numbers doubled during the first two days subsequent to sub-passage and declined thereafter. Although small quantities of each promastigote morphotype comprised the resultant cell populations, more than 90% were promastigotes typical of morphotype 2 forms - the main type used to initiate the cultures. Hemin, haemoglobin and acetylcholine (compounds found in blood), did not restore multiplication or this differentiation step for promastigotes of *L. panamensis* (Figure 3.33D). Interestingly, haemoglobin-derived peptides have been reported to be involved in differentiation of *T. cruzi* epimastigotes to metacyclics in the hindgut of the insect host [reviewed by Charlab and Garcia, 1997]. Restoring haemoglobin to dialysed HIFCS had no apparent effect on transformation to the morphotype 1 promastigote suggesting that similar molecules are insufficient to promote this transition. Extra quantities of glucose or proline, which would have been lost during dialysis, also had no effect on restoring differentiation (data not shown). If morphotype 1 promastigotes were solely the result of water uptake accompanying substrate entry then it would be expected that these potential energy substrates would have induced morphotype 2 promastigotes to differentiate into morphotype 1s. Further studies to elucidate the effects of HIFCS involved growth of promastigotes of *L. mexicana* in HOMEM medium which was supplemented with various concentrations of HIFCS. Clearly, the concentration of HIFCS present in the growth medium influenced the progression of appearance of the different forms (Figure 3.34). The results of this experiment, like those involving dialysed HIFCS, also suggest that one or more components of HIFCS determine the morphology of the promastigotes which occur, particularly influencing promastigotes of morphotype 1 morphology. It is likely that a higher quantity of these factors caused promastigotes to retain morphotype 1 morphology for longer before

differentiation to morphotype 2 promastigotes. Whether this differentiation step results because of consumption of the soluble component by the promastigotes was not clarified. However, experiments involving sub-passage of promastigotes into spent medium harvested from morphotype 2 or morphotype 3 cultures favour this hypothesis (see section 3.4.4). Moreover, increasing the starting density resulted in more rapid differentiation of morphotype 1 promastigotes (section 3.4.1) which also ties in with this theory.

One important feature which was overlooked in determining the above conclusions from these experiments was whether the slender promastigotes which were produced bore any resemblance, other than in morphology, to the morphotype 2 promastigotes of late-log phase cultures. Unfortunately, I had not discovered any phenotypic markers of this promastigote morphotype which could be used to confirm this beyond doubt. However, application of a number of these techniques would give some idea as to how similar they were to the morphotype 2s. Indeed, if this does represent a pure population of these forms then study of them may reveal differences which were masked during characterisation of the heterogeneous promastigote populations. In addition it would be interesting to analyse the DNA content of the cell population produced by sub-passage into HOMEM medium supplemented with dialysed HIFCS since they appear to be synchronous as regards morphology and division status. The mechanism of obtaining synchronicity of *Leishmania* promastigotes has been pursued on several occasions and has important implications for studies on the timing of cell cycle events. If these forms are indeed synchronous, then this procedure would provide a valuable tool to further advance our understanding of the development of these parasites.

One differentiation event which has been studied in some detail is the generation of metacyclic promastigotes (morphotype 3 promastigotes). These studies have been largely restricted to *in vitro* promastigotes, and the results considered to be biologically relevant extrapolated to highlight their potential importance *in vivo*. Although the mechanism involved has yet to be fully elucidated, there is evidence to suggest that generation of metacyclics is both induced and intrinsically determined [Milligan, 1996]. Nutrient depletion and acidic pH have been implicated as biologically relevant stimuli of this induction, they are thought to trigger differentiation through inhibition of promastigote multiplication. The results of my experiments investigating the effect of drug-induced inhibition of multiplication on metacyclogenesis are in contrast to this hypothesis. Both aphidicolin, which has been used to induce differentiation in *Theileria annulata* [Shiels *et al.*, 1994], and DMSO reduced division without concurrent metacyclogenesis. Whether these drugs also had non-specific effects damaging the cells in other ways which prevented metacyclogenesis is a possibility which was not investigated. Other modifications to the culture conditions, however, which also inhibited promastigote multiplication did appear to influence the timing of metacyclogenesis (section 3.4.4). Similarly to Mallinson and Coombs [1989] and Sacks and Perkins [1984;1985], incubation of promastigotes in spent culture medium resulted in generation of metacyclics earlier than in control cultures. Interestingly, the effect on promastigote morphology of sub-passage into spent medium appeared to differ depending on when the medium was harvested; morphotype 2 spent medium contained large numbers of morphotype 2 promastigotes on the second day of growth while morphotype 3 spent medium was predominated by morphotype 3 promastigotes at this time (Figures 3.35 and 3.36). These results suggest that the culture media are conditioned in some way by the different promastigote morphotypes which is in good

accord with the hypothesis proposed earlier that a soluble mediator is involved in determining promastigote morphology. Whether the same factor(s) is responsible for the different differentiation step requires clarification but if so then my results suggest that the progression of different morphotypes occurs as a result of consumption of this factor during growth and that promastigote morphology is probably controlled by a threshold level.

Investigation of morphotype 3 promastigotes of *L. major* gave similar results to Mallinson and Coombs [1989] who reported that these forms do not divide (Figure 3.28) and upon sub-passage into fresh medium de-differentiate into morphotype 1 forms in approximately 24 hours (Figure 3.37). Also similarly to this other study, these results are unlikely to be explained by the growth of contaminating morphotype 2 and intermediate promastigotes which were also present since culturing experiments showed that both of these promastigote morphotypes differentiate into morphotype 1 promastigotes on sub-passage into fresh medium. Although a large proportion of the *L. major* metacyclics differentiated into morphotype 1 promastigotes over 24 hours approximately one third of those present in the initiating population remained after this time. After a subsequent 24 hours this number decreased. Whether these forms also transformed back or died was not investigated here but perhaps this population of cells represents a sub-population of metacyclics which have progressed beyond the point of commitment to this differentiation event and are unable to de-differentiate. This appears unlikely, however, since death of these cells in the subsequent 24 hours, consistant with a decrease in number, is in contrast with the evidence that this promastigote stage is more tolerable to adverse conditions than other promastigotes.

Modification of culture conditions proved a useful means of investigating potential triggers of differentiation between the three promastigote morphotypes and

provided evidence that they can be induced by changing environmental conditions. For example, component(s) of HIFCS appeared to be key factors in influencing promastigote morphotypes 1 and 2 of all three *Leishmania* species which correlates with the probable conditions experienced by these forms *in vivo*. Intrinsic programming was not studied in detail here and therefore its occurrence cannot be ruled out, although the finding that the cycle does not proceed solely in one direction (promastigote morphotypes 2 and 3 were capable of de-differentiation) and that all three morphotypes transformed *in vitro* to amastigote-like forms suggests that the morphological changes are induced by changing environmental conditions. Clearly, more studies are required to elucidate the factors involved and their mechanism of triggering differentiation as well as to confirm whether the distinct morphotypes are indeed different developmental forms.

While these studies have provided new information on the *in vitro* promastigote developmental sequence and on some of the characteristics of three morphologically distinct promastigote stages, the overall picture of whether promastigote morphotypes 1 and 2 represent distinct developmental stages and how the *in vitro* developmental cycle relates to the promastigote life-cycle in the sandfly is still far from clear. Further biochemical analysis of the different morphotypes should determine other differences which can be related to differing environmental conditions in the sandfly gut, however, without obtaining the different morphotypes directly from sandflies for comparison with the *in vitro* morphotypes, the extent of the similarity cannot be assessed. Moreover, elucidation of the precise characteristics of the environments in which the parasites reside would be a great aid in our attempts to understand how the different morphotypes are adapted to their infection sites, and may also advance the development of satisfactory culture techniques.

Despite the limitations of determining how similar the *in vitro* forms are to morphologically similar *in vivo* promastigotes, the data presented in this thesis suggest that the three promastigote morphotypes which occur in *in vitro* culture represent distinct biochemical stages which exhibit adaptations supporting the contention that each form is adapted to the microenvironment in which it occurs *in vivo*. In addition, the data also suggest that all three promastigote stages can withstand changes to their immediate environment. This is exemplified by their apparant ability to maximise the use of potential energy substrates (amino acids and glucose) available at any time. Clearly, the *in vitro* developmental cycle and the different culture forms are worthy of further studies since the majority of biochemical investigations have been and will remain restricted to promastigotes obtained by this means and it is important for interpretation of results of such studies that the forms under investigation are representative of promastigotes in the sandfly.

References

Adler, S. (1964). Leishmania. In: *Advances in* Parasitology, Vol 2, pp 35-96 (Dawes, B., ed.). New York: Academic Press.

Adler, S. and Ber (1941). The transmission of *L. tropica* by the bite of *Phlebotomus papatasii*. *Ind. J. Med. Res.*, **29**: 803-809.

Adler, S. and Theodor, O. (1926a). The mouthparts, alimentary tract and salivary apparatus of the female in *Phlebotomus papatasii*. *Ann. Trop. Med. Parasitol.*, **20**: 109-142.

Adler, S. and Theodor, O. (1926b). Further observations on the transmission of cutaneous leishmaniasis to man from *Phlebotomus papatasii*. *Ann. Trop. Med. Parasitol.*, **20**: 175-194.

Adler, S. and Theodor, O. (1929). Attempts to transmit *Leishmania tropica* by bite: The transmission of *L. tropica* by *Phlebotomus sergenti*. *Ann. Trop. Med. Parasitol*, **xxiii**, 1-19.

Adler, S. and Theodor, O. (1931). Investigations on Mediterranean kala azar. I-V. *Proc. R. Soc. Lond, B.*, **108**: 447-502.

Akov, S. (1972). Protein digestion in hematophagous insects. In: *Insect and mite nutrition*, pp. 531-540 (J. G. Rodriguez, ed.). North-Holland/ American Elsevier, New York.

Albritton, A. (1952). *Standard values in blood.* Saunders, Philidelphia.

Almeida, M.C., Cuba Cuba, C.A., de Sa, C.M., Pharoah, M.P., Howard, K.M. and Miles, M.A. (1993). Metacyclogensis of *Leishmania* (*Viannia*) *braziliensis in vitro*: evidence that lentil lectin is a marker of complement resistance and enhanced infectivity. *Trans. R. Soc. Trop. Med. Hyg.*, 87:325-329.

Anez, N., Nieves, E. and Cazorla, D. (1989). The validity of the developmental pattern in the sandfly gut for classification of *Leishmania*. *Trans. R. Soc. Trop. Med. Hyg.*, **83**: 634-635.

Auclair, J.L. (1963). Aphid feeding and nutrition. *Annu. Rev. Entomol.*, **8**: 439-491.

Baker, J.R. (1965). The evolution of parasitic protozoa. *Symp. Br. Soc. Parasitol.*, **3**, 1-27.

Barrett, M.P. (1997). The pentose phosphate pathway and parasitic protozoa. *Parasitol. Today*, **13**: 11-16.

Bates, P.A. (1993a). Axenic culture of *Leishmania* amastigotes. *Parasitol. Today*, **9**: 143-146.

Bates, P.A. (1993b). Characterisation of developmentally-regulated nucleases in promastigotes and amastigotes of *Leishmania mexicana*. *FEMS Microbiol. Letts.*, **107**: 53-58.

Bates, P.A. (1994). Complete developmental cycle of *Leishmania mexicana* in axenic culture. *Parasitol.*, **108**: 1-9.

Bates, P.A., Robertson, C.D. and Coombs, G.H. (1994). Expression of cysteine proteinases by metacyclic promastigotes of *Leishmania mexicana*. *J. Euk. Microbiol.*, **41**: 199-203.

Bates, P.A., Robertson, C.D., Tetley, L. and Coombs, G.H. (1992). Axenic cultivation and characterisation of *Leishmania mexicana* amastigote-like forms. *Parasitol.*, **105**: 193-202.

Bates, P.A. and Tetley, L. (1993). *Leishmania mexicana*: induction of metacyclogenesis by cultivation of promastigotes at acidic pH. *Exp. Parasitol.*, **76**: 412-423.

Beach, D.H., Holz, G.G. and Anekwe, G.E. (1979). Lipids of *Leishmania* promastigotes. *J. Parasitol.*, **65**: 203-216.

Bera, T. (1987). The γ-guanidinobutyramide pathway of L-arginine catabolism in *Leishmania donovani* promastigotes. *Mol. Biochem. Parasitol.*, **23**: 183-192.

Berens, R.L., Brun, R. and Krassner, S. (1976). A simple monophasic medium for axenic culture of hemoflagellates. *J. Parasitol.*, **62**: 360-5.

Berens, R.L., Krug, E.C. and Marr, J.J. (1995). Purine and pyrimidine metabolism. In *Biochemistry and molecular biology of parasites* (Marr, J.J. and Muller, M., eds.), pp 89-117. Academic Press: London.

Berens, R.L. and Marr, J.J. (1978). An easily prepared defined medium for cultivation of *Leishmania donovani* promastigotes. *J. Parasitol.*, **64**: 160.

Bergmeyer, H.U., Bergmeyer, J. and Grassl, M. (eds.) (1983). Methods of Enzymatic Analysis Volume II: Samples, Reagents, Assessment of results. Verlag Chemie, Weineim.

Blackwell, J.M., Ezekowitz, R.A.B., Roberts, M.B., Channon, J.Y., Sim, R.B. and Gordon, S. (1985). Macrophage complement and lectin-like receptors bind *Leishmania* in the absence of serum. *J. Exp. Med.*, **162**: 324-331.

Blum, J.J. (1987). Oxidation of fatty acids by *Leishmania braziliensis panamensis*. *J. Protozool.*, **14**: 169-174.

Blum, J.J. (1991). Oxidation of leucine by *Leishmania donovani*. *J. Protozool.* 38: 527-531.

Blum, J.J., (1992). Arginine catabolism by *Leishmania donovani* promastigotes. *J. Protozool.*, **39**: 613-618.

Blum, J.J. (1993a). Intermediary metabolism of *Leishmania*. *Parasitol. Today*, **9**: 118-122.

Blum, J.J. (1993b). Energy metabolism in *Leishmania*. *J. Bioenerg. Biomemb.*, **26**: 147-155.

Blum, J.J. (1996). Effects of osmotic stress on metabolism, shape, and amino acid content of *Leishmania*. *Biol. Cell*, **87**: 6-16.

Blum, J.J. and Balber, A.E. (1996). Osmotic and metabolic-induced changes in light scattering of *Leishmania donovani* as measured by flow cytometry. *J. Euk. Microbiol.*, **43**: 213-217.

Bouvier, J., Etges, R. and Bordier, C. (1987). Identification of the promastigote surface protease in seven species of *Leishmania*. *Mol. Biochem. Parasitol.*, **24**: 73-79.

Brazil, R.P. (1978). Isolation of the intracellular stages of *Leishmania mexicana amazonensis* using cellulose column. *Annals Trop. Med. Parasitol.*, **72**: 579-580.

Bursell, E. (1963). Aspects of the metabolism of amino acids in the tsetse fly, *Glossina* (*Diptera*). *J. Insect Physiol.*, **9**: 439-452.

Button, L.L., Russell, D.G., Klein, H.L., Medina-Acoata, E., Karess, R.E. and McMaster, W.R. (1989). Genes encoding the major surface glycoprotein in *Leishmania* are tandomly linked at a single chromosomal locus and are constitutively transcribed. *Mol. Biochem. Parasitol.*, **32**: 271-284.

Camargo, E.P. (1964). Growth and differentiation in *Trypanosoma cruzi*. Origin of metacyclic trypaonosomes in liquid media. *Rev. do Inst. de Med. Trop. Sao Paulo*, **6**: 1120-1121.

Campbell, T.A., Mackow, M.C. and Gottlieb, M. (1990). Alterations in *Leishmania donovani* 3'-nucleotidase/nuclease activity in banding pattern in polyacrylamide gels. *Comp. Biochem. Physiol.*, **95B**: 711-720.

Cazzulo, (1992). Aerobic fermentation of glucose by trypanosomatids. *FASEB J.*, **6**: 3153-3161.

Chang, K.-P. and Hendricks, L.D. (1985). Laboratory cultivation and maintenance of *Leishmania*. In: *Leishmaniasis, Human Parasitic Diseases* Vol. 1 (Chang, K.-P. and Bray, R.S., eds.), pp. 213-244. Elsevier: Amsterdam.

Chang, C.S., Inserra, T.L., Kink, J.A., Fong, D. and Chang, K.-P. (1986). Expression and size heterogeneity of a 63 kilodalton membrane glycoprotein during the growth and transformation of *Leishmania mexicana amazonensis*. *Mol. Biochem. Parasitol.*, **18**: 197-210.

Charlab, R. and Garcia, E.S. (1997). Hemoglobin: Food for thought in vectors and parasites. *Parasitol. Today*, **13**: 351-353.

Charlab, R. and Ribeiro, J.M.C. (1993). Cytostatic effect of *Lutzomyia longipalpis* salivary gland homogenates on *Leishmania* parasites. *Am. J. Trop. Med. Hyg.*, **48**: 831-838.

Charlab, R., Tesh, R.B., Rowton, E.D. and Ribeiro, J.M.C. (1995). *Leishmania amazonensis*: Sensitivity of different promastigote morphotypes to salivary gland homogenates of the sandfly *Lutzomyia longipalpis*. *Exp. Parasitol.*, **80**: 167-175.

Chatterjee, T. and Ghosh, J.J. (1957). Transaminases of *Leishmania donovani*, the causative agent of kala-azar. *Nature*, **180**: 1425.

Childs, G.E, M^{c}Roberts, M.J. and Foster, K.A (1976). Partial purification of amastigotes from cutaneous lesions of American leishmaniasis. *J. Parasitol.*, **62**: 676-679.

Childs, G.E., Foster, K.A. and M^{c}Roberts, M.J. (1978). Insect cell culture media for the cultivation of New World *Leishmania*. *Int. J. Parasitol.*, **8**: 255-258

Christophers, S. R., Shortt, H. E. and Barraud, P. J. (1926). The morphology and life cycle of the parasite of Indian kala-azar in culture. *Ind. Med. Res. Mem.*, No. 4: 19-53.

Coombs, G.H., Craft, J.A. and Hart, D.T. (1982). A comparative study of *Leishmania mexicana* amastigotes and promastigotes, enzyme activities and subcellular locations. *Mol. Biochem. Parasitol.*, **5**: 199-211.

Coombs, G.H. and Mottram, J.C. (1997). Proteinases of Trypanosomes and *Leishmania* In: *Trypanosomiasis and Leishmaniasis* , pp 177-197. (Hide, G., Mottram, J.C., Coombs G.H. and Holmes, P.H., eds.). CAB INTERNATIONAL: U.K..

Darling, T.N. and Blum, J.J. (1987). *In vitro* reversible transformation of *Leishmania braziliensis panamensis* between promastigote and ellipsoidal forms. *J. Protozool.*, **34**: 166-168.

Darling, T.N. and Blum, J.J. (1989). Effects of anaerobiosis on adenine nucleotide levels and the release of ATP by *Leishmania major* promastigotes. *Comp. Biochem. Physiol.*, **94B**: 453-460.

Darling, T.N. and Blum, J.J. (1990). Changes in the shape of *Leishmania major* promastigotes in response to hexoses, proline and hypo-osmotic stress. *J. Protozool.*, **37**: 267-272.

Darling, T.N., Burrows, C.M. and Blum, J.J. (1990). Rapid shape change and release of ninhydrin-positive substances by *Leishmania major* promastigotes. *J. Protozool.*, **37**: 505-510.

Darling, T.N., Davies, D.G., London, R.E. and Blum, J.J. (1989a). Metabolic interactions between glucose, glycerol, alanine and acetate in *Leishmania braziliensis panamensis* promastigotes. *J. Protozool.*, **36**: 217-225.

Darling, T.N., Davies, D.G., London, R.E. and Blum, J.J. (1989b). Carbon dioxide abolishes the reverse Pasteur effect in *Leishmania major*. *Mol. Biochem. Parasitol.*, **33**: 191-202.

Da Silva, R. and Sacks, D.L. (1987). Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Inf. Immun.*, **55**: 2802-2806.

Davies, C.R., Cooper, A.M., Peacock, C., Lane, R.P. and Blackwell, J.M. (1990). Expression of LPG and GP63 by different developmental stages of *Leishmania major* in the sandfly *Phlebotomus papatasi*. *Parasitol.*, **101**: 337-343.

Dillon, R.J. and Lane, R.P. (1993). Digestion in the midgut of *Phlebotomus papatasi* and *Phlebotomus langeroni*. *Med. Vet. Entomol.*, **7**: 225-232.

Doyle, P.S., Engel, J.C., Pimenta, P.F., Da Silva, P.P. and Dwyer, D.M. (1991). *Leishmania donovani*: long-term culture of axenic amastigotes at 37°C. *Exp. Parasitol.*, **73**: 326-334.

Englund, P.T., Hajduk, S.L. and Marini, J.C. (1982). The molecular biology of trypanosomes. *Annu. Rev. Biochem.*, **51**: 695-726.

Eperon, S. and McMahon-Pratt, D. (1989). Extracellular cultivation and morphological characterisation of amastigote-like forms of *Leishmania panamensis* and *L. braziliensis*. *J. Protozool.*, **36**: 502-510.

Etges, R. and Bouvier, J. (1991). The promastigote surface proteinase of *Leishmania*, In: *Biochemical Protozoology*, pp 221-233 (Coombs, G.H. and North, M.J., eds). Taylor and Francis, London, Washington DC.

Evans, D. A., Lanham, S. M., Baldwin, C. I. and Peters, W. (1984). The isolation and isoenzyme characterization of *Leishmania braziliensis* subsp. from patients with cutaneous leishmaniasis acquired in Belize. *Trans. R. Soc. Trop. Med. Hyg.*, **78**: 35-42.

Evans, D. A. (1987). *Leishmania*. In: In vitro *methods for parasite cultivation*, pp52-75 (Taylor, A.E.R. and Baker, J.R., eds.). Academic press, New York.

Ewart, W.H. and Metcalf, R.L. (1956). Preliminary studies of sugars and amino acids in the honeydews of five species of coccids feeding on citrus in California. *Ann. Ent. Soc. Am.*, **49**: 441-447.

Flinn, H.M. and Smith, D.F. (1992). Genomic organisation and expression of a developmentally-regulated gene family from *Leishmania major*. *Nucleic Acid Res.*, **20**: 755-762.

Fong, D. and Chang, K.-P. (1982). Surface antigenic change during differentiation of a parasitic protozoan, *Leishmania mexicana*: identification by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, **79**: 7366-7370.

Frommel, T.O., Button, L.L., Fujikura,Y. and McMaster, W.R. (1990). The major surface glycoprotein (GP63) is pesent in both life cycle stages of *Leishmania*. *Mol. Biochem. Parasitol.*, **38**: 25-32.

Gemetchu, T. (1974). The morphology and fine structure of the midgut and peritrophic membrane of the adult female *Phlebotomus longipes* Parrot and Martin (Diptera: Psychodidae). *Ann. Trop. Med. Parasitol.*, **68**: 111-124.

Ghosh, D.K. and Datta., A.G. (1971). *Leishmania donovani*: Assay for a functional pentose phosphate pathway. *Exp. Parasitol.*, **39**: 235-246.

Ginger, M., Chance, M. and Goad, J. (1995). The incorporation of leucine into sterols by promastigotes of *Leishmania mexicana*. *BSP Trypanosomiasis and Leishmaniasis Seminar*, pp. 42.

Glaser, T.A., Wells, S.J., Spithill, T.W., Pettitt, J.M., Humphris, D.C. and Mukkada, A.J. (1990). *Leishmania major* and *L. donovani*: a method for rapid purification of amastigotes. *Exp. Parasitol.*, **71**: 343-345.

Glew, R.H., Saha, A.K., Siddhartha, D. and Remaley, A.T. (1988). Biochemistry of the *Leishmania* species. *Microbiological Reviews*, **52**: 412-432.

Gooding, R.H. (1975). Digestive enzymes and their control in haematophagous arthropods. *Acta Tropica*, **32**: 96-111.

Guerrant, G.O., Lambert, M.A. and Moss, C.W. (1982). Analysis of short-chain acids from anaerobic bacteria by high-performance liquid chromatography. *J. Clin. Microbiol.*, **16**: 355-360.

Gurdon, J.B. (1992). The generation of diversity and pattern in animal development. *Cell*, **68**: 185-199.

Hames, B.D. (1990). One dimensional polyacrylamide gel electrophoresis. In: *Gel Electrophoresis of Proteins: a practical Approach*, second edition, (Hames, B. D. and Rickwood, D., eds.) pp 1-139. Oxford University Press.

Handman, E., Greenblatt, C.L. and Goding, J.W. (1984). An amphipathic sulphated glycoconjugate of *Leishmania*: characterisation with monoclonal antibodies. *E.M.B.O. J.*, **3**: 2301-2306.

Hardy, I.L., Houk, E.J., Kramer, L.D. and Reeves, W.C. (1983). Intrinsic factor affecting vector competence of mosquitoes for arboviruses. *Annu. Rev. Entomol.*, **28**: 229-262.

Hart, D.T. and Coombs, G.H. (1982). *Leishmania mexicana*: energy metabolism of amastigotes and promastigotes. *Exp. Parasitol.*, **54**: 397-409.

Hart, D.T. and Opperdoes, F.R. (1984). The occurrence of glycosomes (microbodies) in the promastigote stage of four *Leishmania* species. *Mol. Biochem. Parasitol.*, **13**: 159-172.

Hart, D.T., Vickerman, K. and Coombs, G.H. (1981a). A quick, simple method for purifying *Leishmania mexicana* amastigotes in large numbers. *Parasitol.*, **82**, 345-355.

Hart, D.T., Vickerman, K. and Coombs, G.H. (1981b). Transformation *in vitro* of *Leishmania mexicana* amastigotes to promastigotes: nutritional requirements and the effect of drugs. *Parasitol.*, **83**: 529-541.

Hart, D.T., Vickerman, K. and Coombs, G.H. (1981c). Respiration of *Leishmania mexicana* amastigotes and promastigotes. *Mol. Biochem. Parasitol.*, **4**: 39-51.

Hendricks, L.D., Wood, D.E. and Hajduk, M.E. (1978). Haemoflagellates: Commercially available liquid media for rapid cultivation. *Parasitol.*, **76**: 309-316.

Hertig, M. and McConnell, E. (1963). Experimental infection of Panamanian *Phlebotomus* sandflies with *Leishmania*. *Exp. Parasitol.*, **14**: 92-106.

Hoare, C.A. (1948). The relationship of the hemoflagellates. *Proc. IVth. Int. Congr. Trop. Med. Malaria, Washington, D. C.*, **2**: 1110-1116.

Hoare, C.A. and Wallace, F.G. (1966). Developmental stages of trypanosomatid flagellates: a new terminology. *Nature*, **212**: 1385-1386.

Horvitz, H.R. and Herskovitz, I. (1992). Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. *Cell*, **68**: 237-255.

House, H.L. (1965). Digestion. In: *Physiology of Insecta*, Vol II, pp. 815-853 (Rockstein, M., ed.). Academic Press: New York and London.

Howard, K.M., Pharoah, M.M., Ashall, F. and Miles, M.A. (1991). Human urine stimulates growth of *Leishmania in vitro*. *Trans. R. Soc. Trop. Med. Hyg.*, **85**: 477-479.

Howard, M.K., Sayers, G. and Miles, M.A. (1987). *Leishmania donovani* metacyclic promastigotes: Transformation *in vitro*, lectin agglutination, complement resistance and infectivity. *Exp. Parasitol.*, **64**: 147-56.

Ilg, T., Harbecke, D. and Overath, P. (1993). The lysosomal gp63-related protein in *Leishmania mexicana* amastigotes is a soluble metalloproteinase with an acidic pH optimum. *FEBS Letts.*, **327**: 103-107.

Infante, R.B., Hernandez, A.G., Riggione, F. and Dawidowicz, K. (1980). A new method for the partial purification of *Leishmania* amastigotes from cutaneous lesions. *Parasitol.*, **80**: 105-112.

Iovannisci, D.M. and Ullman, B. (1983). High efficiency plating method for *Leishmania* promastigotes in semi-defined or completely-defined media. *J. Parasitol.*, **69**: 633-636.

Jacobson, R.L. (1995). *Leishmania*, LPG and the sandfly connection. *Parasitol. Today*, **11**: 203-204.

Jacobson, R.L. and Doyle, R.J. (1996). Lectin-parasite interactions. *Parasitol. Today*, **12**: 55-61.

Janovy, J., Lee, K.W. and Brumbaugh, J.A., (1974). The differentiation of *Herpetomonas megalseliae*: ultrastructural observations. *J. Protozool.*, **21**: 53-59.

Jenkins, W.T. and Thompson, C.C. (1989). Two kinetic methods to study the regulation of mammalian hexokinases. *Analytical Biochem.*, **177**: 396-401.

Johnson, P.T. and Hertig, M. (1970). Behaviour of *Leishmania* in Panamanian phlebotomine sandflies fed on infected animals. *Exp. Parasitol.*, **27**: 281-300.

Johnson, P.T., McConnell, E. and Hertig, M. (1963). Natural infections of leptomonad flagellates in Panamanian *Phlebotomus* sandflies. *Exp. Parasitol.*, **14**: 107-122.

Joseph, M.H. and Marsden, C.A. (1986) Amino acids and small peptides. In: *HPLC of Small molecules, A Practical Approach* (Rickwood, D. and Hames, B. D., Eds), pp 13-23. IRL Press Limited: Oxford.

Karp, C.L., Turco, S.J. and Sacks, D.L. (1991). Lipophosphoglycan masks recognition of *Leishmania donovani* promastigote surface by human kala-azar serum. *J. Immunol.*, **147**: 680-684.

Keegan, F.P. and Blum, J.J. (1990). Effects of oxygen concentration on the intermediary metabolism of *Leishmania major* promastigotes. *Mol. Biochem. Parasitol.*, **39**: 235-246.

Keegan, F. and Blum, J.J. (1992). Utilisation of a carbohydrate reserve composed primarily of mannose by *Leishmania donovani*, *Mol. Biochem. Parasitol.*, **53**: 193-200.

Keegan, F.P., Sansone, L. and Blum, J.J. (1987). Oxidation of glucose, ribose, alanine and glutamate by *Leishmania braziliensis*, *J. Protozool.*, **34**: 174-9.

Killick-Kendrick, R. (1979). Biology of *Leishmania* in phlebotomine sandflies. In: *Biology of the Kinetoplastida*, Vol 2. (Lumsden, W.H.R. and Evans, D.A., eds.), pp 395-460. Academic press: New York.

Killick-Kendrick, R. (1986). Part I.IV. The transmission of leishmaniasis by the bite of the sandfly. *J. Roy. Army Med. Corps.*, **132**: 134-140.

Killick-Kendrick, R. and Rioux, I.A. (1981). In: *Parasitological Topics* (Canning, E.U., ed.) pp 136-145, Society of Protozoology Special Publications.

Killick-Kendrick, R., Lainson, R., Leaney, A.J., Ward, R.D. and Shaw, J.J. (1977). Promastigotes of *Leishmania braziliensis braziliensis* in the gut wall of a natural vector, *Psychodopygus wellcomei*. *Trans. R. Soc. Trop. Med. Hyg.*, **71**: 381.

Killick-Kendrick, R., Leaney, A.J., Ready, P.D. and Molyneux, D.H. (1977). *Leishmania* in phlebotomid sandflies. IV. The transmission of *Leishmania mexicana amazonensis* to hamsters by the bite of experimentally infected *Lutzomyia longipalpis*. *Proc. R. Soc. Lond., B*, **196**: 105-115.

Killick-Kendrick, R., Molyneux, D.H. and Ashford, R.W. (1974). *Leishmania* in phlebotomid sandflies. I. Modifications of the flagellum associated with attachment to the midgut and oesophageal valve of the sandfly. *Proc. Roy. Soc. Lond. B*, **187**: 409-419.

Killick-Kendrick, R., Molyneux, D.H., Hommel, M., Leaney, A.J. and Robertson, E.S. (1977). *Leishmania* in phlebotomid sandflies. V. The nature and significance of infections of the pylorus and ileum of the sandfly by leishmaniae of the *braziliensis* complex. *Proc. R. Soc. Lond., B*, **198**: 191-199.

Killick-Kendrick, R., Molyneux, D.H., Leaney, A.J. and Rioux, J.A. (1979). Aspects of the life-cycle of *Leishmania* in the sandfly. In: *Second European Multicolloquy of Parasitol., Trogir, 1975.* (Petrovic, Z., ed.), *Association of Yugoslav. Parasitologists, Beograd*, 89-95.

Killick-Kendrick, R., Wallbanks, K.R., Molyneux, D.H. and Lavin, D.R. (1988). The ultrastructure of *Leishmania major* in the foregut and proboscis of *Phlebotomus papatasi*. *Parasitol. Res.*, **74**: 586-590.

Knodler, L.A., Edwards, M.R. and Schofield, P.J. (1994). The intracellular amino acid pools of *Giardia intestinalis*, *Trichomonas vaginalis*, and *Crithidia luciliae*. *Exp. Parasitol.*, **79**: 117-125.

Krassner, S.M. (1969). Proline metabolism in *Leishmania tarentolae*. *Exp. Parasitol.*, **24**: 348-363.

Krassner, S.M. and Flory, B.(1971). Essential amino acids in the culture of *Leishmania tarentolae*. *J. Parasitol.*, **57**, 917-920.

Krassner, S.M. and Flory, B. (1972). Proline metabolism in *Leishmania donovani* promastigotes. *J. Protozool.*, **19**: 682-685.

Kweider, M., Lemesre, J.-L., Darcy, F., Kusnierz, J.-P., Capron, A. and Santoro, F. (1987). Infectivity of *Leishmania braziliensis* promastigotes is dependent on the increasing expression of a 65,000-dalton surface antigen. *J. Immunol.*, **138**: 299-305.

Lainson, R., Ready, P. D. and Shaw, J. J. (1979). *Leishmania* in phlebotomid sandflies. VII. On the taxanomic status of *Leishmania peruviana*, causative agent of Peruvian 'uta', as indicated by its development in the sandfly, *Lutzomyia longipalpis*. *Proc. R. Soc. Lond. B*, **206**: 307-318.

Lainson, R. and Shaw, J. J. (1979). The role of animals in the epidemiology of South American leishmaniasis. In: *Biology of the Kinetoplastida*, Vol 2 (Lumsden, W. H. R. and Evans, D. A., eds.) pp. 1-116. Academic Press: London and New York.

Lainson, R. and Shaw, J.J. (1987). Evolution, classification and geographical distribution. In: *The Leishmaniases in Biology and Medicine*, Vol 1 (Peters, W. and Killick-Kendrick, R., eds.) pp. 1-120. Academic Press: London and Orlando, Florida.

Lainson, R. and Shaw, J.J. (1988). Observations on the development of *Leishmania (L.) chagasi* Cunha and Chagas in the midgut of the sandfly vector *Lutzomyia longipalpis* (Lutz and Neiva). *Ann. Parasitol. Hum. Comp.*, **63**: 134-145.

Lainson, R., Shaw, J.J., Ward, R.D. and Fraiha, H. (1973). Leishmaniasis in Brazil: IX. Considerations of the *Leishmania braziliensis* complex:-importance of sandflies of the Genus *Psychodopygus* (Mangabeira) in the transmission of *L. b. braziliensis* in North Brazil. *Trans. R. Soc. Trop. Med. Hyg.*, **67**: 184-196.

Lainson, R., Ward, R.D. and Shaw, J.J. (1977). *Leishmania* in phlebotomid sandflies. VI. Importance of hindgut development in distinguishing between parasites of the *Leishmania mexicana* and *L. braziliensis* complexes. *Proc. R. Soc. Lond. B*, **199**: 309-320.

Lawyer, P.G., Ngumbi, P.M., Anjili, C.O., Odongo, S.O., Mebrahtu, Y.B., Githure, J.I., Koech, D.K. and Roberts, C.R. (1990). Development of *Leishmania major* in *Phlebotomus duboscqi* and *Sergentomyia schwetzi* (Diptera: Psychodidae). *Am. J. Trop. Med. Hyg.*, **43**: 31-43.

Lawyer, P.G., Young, D.G., Butler, J.F. and Akin, D.E. (1987). Development of *Leishmania mexicana* in *Lutzomyia diabolica* and *Lu. shannoni* (Diptera: Psychodidae). *J. Med. Entomol.*, **24**: 347-355.

Leatherbarrow, R.J. (1992). *Grafit* Version 3·0, Erithacus Software Ltd., Staines, U.K..

Le Blancq, S. M. and Lanham, S.M. (1984). Aspartate aminotransferases in *Leishmania* is a broad spectrum transaminase. *Trans. R. Soc. Trop. Med. Hyg.*, **78**: 373-75.

Lewis, D.J. and Domoney, C.R. (1966). Sugar meals in Phlebotominae and Simuliidae. *Proc. R. Ent. Soc. Lond.*, A, **41**: 175-179.

Lockwood, B.C., North, M.J., Mallinson, D.J. and Coombs, G.H. (1987). *FEMS Microbiol. Lett.*, **48**: 345-350.

Lockwood, B.C., North, M.J., Scott, K.I., Bremner, A.F. and Coombs, G.H. (1987). The use of a highly sensitive electrophoresis method to compare the proteinases of trichomonads. *Mol. Biochem. Parasitol.*, **24**: 89-95.

McConville, M.J. and Ferguson, M.A.J. (1993). The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.*, **294**: 305-324.

McConville, M.J., Thomas-Oates, T., Ferguson, M.A. and Homans, S.W. (1990). Structure of the lipophosphoglycan from *Leishmania major*. *J. Biol. Chem.*, **265**: 19611-19623.

McConville, M.J., Turco, S.J., Ferguson, M.A. and Sacks, D.L. (1992). Developmetal modification of lipophosphoglycan during the differentiation of *Leishmania major* promastigotes to an infectious stage. *EMBO J.*, **11**: 3593-3600.

Mallinson, D.J. and Coombs G.H. (1989). Biochemical characteristics of the metacyclic forms of *L. major* and *L. mexicana mexicana*. *Parasitol.*, **98**: 7-15.

Marr, J.J. (1980). Carbohydrate metabolism in *Leishmania*. In: *Biochemistry and physiology of protozoa*, 2nd ed., vol. 3., pp 313-338 (Levandowsky, M. and Hunter, S.H., eds). Academic Press: New York.

Marr, J.J. (1991). Purine metabolism in parasitic protozoa and its relationship to chemotherapy. In: *Biochemical Protozoology*, pp 524-536 (Coombs, G.H. and North, M.J., eds). Taylor and Francis. London: New York.

Marsden, P.D. (1984). Selective primary health care: Strategies for control of disease in the developing world XIV Leishmaniasis. *Review Infectious Diseases*, **6**: 736-744.

Meade, J.C., Glaser, Y.A., Bonventre, P.F. and Mukkada, A.J. (1984). Enzymes of carbohydrate metabolism in *Leishmania donovani* amastigotes. *J. Protozool.*, **31**: 156-161.

Melo, N.M., De Azevedo, P., Roitman, I. and Mayrink, W. (1985). A new defined medium for cultivating *Leishmania* promastigotes. *Acta Trop.*, **42**: 137-141.

Michels, P.A.M., Hannaert, V. and Bakker, B.M. (1997). Glycolysis of Kinetoplastida in Trypanosomiasis and Leishmaniasis Biology and Control (eds G. Hide, J.C. Mottram, G.H. Coombs and P.H. Holmes) p 133-148. CAB International.

Milligan, K. (1996). PhD. Thesis, University of Glasgow.

Molyneux, D. H., Killick-Kendrick, R. and Ashford, R. W. (1975). *Leishmania* in phelbotomid sandflies. III. The ultrastructure of *Leishmania mexicana amazonensis* in the mid-gut and pharynx of *Lutzomyia longipalpis*. *Proc. R. Soc. Lond, B*, **190**: 341-357.

Molyneux, D. H. and Killick-Kendrick, R. (1987). Morphology, ultrastructure and lifecycles. In: *The Leishmaniases in Biology and Medicine*, Vol 1 (Peters, W. and Killick-Kendrick, R., eds.) pp 121-176. Academic Press: London.

Mottram, J.C. and Coombs, G.H. (1985). *Leishmania mexicana*: Enzyme activities of amastigotes and promastigotes and their inhibition by antimonials and arsenicals. *Exp. Parasitol.*, **59**: 151-160.

Mottram, J.C., Robertson, C.D., Coombs, G.H. and Barry, J.D. (1992). A developmentally regulated cysteine proteinase gene of *Leishmania mexicana*. *Mol. Microbiol.*, **6**: 1925-1932.

Mukkuda, A.J., Schaefer, F.W., III, Simon, M.W. and Neu, C. (1974). Delayed *in vitro* utilisation of glucose by *Leishmania tropica* promastigotes. *J. Protozool.*, **21**: 393-397.

Newton, A. and Ohta, N. (1990). Regulation of the cell division cycle and differentiation in bacteria. *Ann. Rev. Microbiol.*, **44**: 689-719.

North, M.J. and Coombs, G.H. (1981). Proteinases of *L. mexicana* amastigotes and promastigotes: analysis by gel electrophoresis. *Mol. Biochem. Parasitol.*, **3**: 293-300.

O'Daly, J.A. and Rodriguez, M.B. (1988). Differential growth requirements of several *Leishmania* spp. in chemically defined culture media. *Acta Trop.*, **45**: 109-126.

O'Daly, J.A., Serrano, L.E. and Rodriguez, M.B. (1983). Free amino acid pool and proteolytic enzymes of *Trypanosoma cruzi* cultured *in vitro*. *Internat. J. Parasitol.*, **13**: 433-440.

Opperdoes, F.R. and Borst, P. (1977). Localization of nine glycolytic enzymes in a microbody-like organelle in *Trypanosoma brucei*: the glycosome. *FEBS Letters*, **80**: 360-364.

Opperdoes, F.R. (1991). Glycosomes. In: *Biochemical Protozoology* (Coombs, G.H. and North, M.J., eds.) pp 134-144. Taylor and Francis: London., Washington DC.

Pan, A.A. (1984). *Leishmania mexicana*: Serial cultivation of intracellular stages in a cell-free medium. *Exp. Parasitol.*, **58**: 72-80.

Pan, A.A., Duboise, S.M., Eperon, S., Rivas, L., Hodgkinson, V., Traub-Cseko, Y. and McMahon-Pratt, D. (1993). Developmental life cycle of *Leishmania*-Cultivation and characterisation of cultured extracellular amastigotes. *J. Euk. Microbiol.*, **40**: 213-223.

Park, J.H., Schofield, P.J. and Edwards, M.R. (1995). The role of alanine in the acute response of *Giardia intestinalis* to hypo-osmotic shock. *Microbiol.*, **141**: 2455-2462.

Pimenta, P.F.P., Turco, S.J., Mconville, M.J., Lawyer, P.G., Perkins, P.V. and Sacks, D.L. (1992). Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. *Science*, **256**: 1812-1815.

Pupkis, M.F. and Coombs, G.H. (1984). Purification and characterisation of proteolytic enzymes of *Leishmania mexicana mexicana* amastigotes and promastigotes *J. Gen. Microbiol.*, **130**: 2375-2385.

Raghupathi, Rami R.S. and Campbell, J.W. (1969). Arginine metabolism in insects. Role of arginase in proline formation during silk moth development. *Biochem. J.*, **115**: 495-503.

Rainey, P.M. and MacKenzie, N.E. (1991). A carbon-13 nuclear magnetic resonance analysis of the products of glucose metabolism in *Leishmania pifanoi* amastigotes and promastigotes. *Mol. Biochem. Parasitol.*, **45**: 307-316.

Ready, P.D. (1978). The feeding habits of laboratory-bred *Lutzomyia longipalpis* (Diptera: Psychodidae). *J. Med. Entomol.*, **14**: 545-552.

Robertson, C.D. and Coombs, G.H. (1992). Stage-specific proteinases of *Leishmania mexicana mexicana* promastigotes. *FEMS Microbiol. Letts*, **94**: 127-132.

Robertson, C.D. and Coombs, G.H. (1994). Multiple high activity cysteine proteases of *Leishmania mexicana* are encoded by the *lmcpb* gene array. *Microbiol.*, **140**: 417-424.

Russell, D.G. and Wilhelm, H. (1986). The involvement o the major surface glycoprotein (gp63) of *Leishmania* promastigotes in attachment to macrophages. *J. Immunol.*, **136**: 2613-2620.

Russell, D.G. and Wright, S.D. (1988). Complement receptor type 3 (CR3) binds to an Arg-Gly-Asp-containing region of the major surface glycoprotein gp63 of *Leishmania* promastigotes. *J. Exp. Med.*, **168**: 279-292.

Sacks, D.L. (1989). Metacyclogenesis in *Leishmania* promastigotes. *Exp. Parasitol.*, **69**: 100-103.

Sacks, D.L. (1992). The structure and function of the surface lipophosphoglycan on different developmental stages of *Leishmania* promastigotes. *Inf. Dis.*, **1**: 200-206.

Sacks, D.L., Hieny, S. and Sher, A. (1985). Identification of cell surface carbohydrate and antigenic changes between non-infective and infective developmental stages of *Leishmania major* promastigotes. *J. Immunol.*, **135**: 564-69.

Sacks, D.L. and Perkins, P.V. (1984). Identification of an infective stage of *Leishmania* promastigotes. *Science*, **223**: 1417-1419.

Sacks, D.L. and Perkins, P.V. (1985). Development of infective stage *Leishmania* promastigotes within phlebotomine sandflies. *Am. J. Trop. Med. Hyg.*, **34**: 456-459.

Sacks, D.L., Saraiva, E.M. Rowton, E., Turco, S.J. and Pimenta, P.F. (1994). The role of the lipophosphoglycan of *Leishmania* in vector competence. *Parasitol.*, **108**: S55-S62.

Salisbury, F.B. and Ross, C.W. (1985). Osmosis. In: *Plant Physiology*, pp 47. Wadsworth Publishing Company, Belmont, California.

Saraiva, E.M.B., Pimenta, P.F.P., Pereira, M.E.A. and De Souza, W. (1983). Isolation and purification of amastigotes of *Leishmania mexicana amazonensis* by a gradient of metrizamide. *J. Parasitol.*, **69**: 627-629.

Schlein, Y. (1986). Sandfly diet and *Leishmania*. *Parasitol. Today*, **2**: 175-177.

Schlein, Y. (1993). *Leishmania* and sandflies: interactions in the life cycle and transmission. *Parasitol. Today*, **9**: 255-258.

Schlein, Y. and Romano, H. (1986). *Leishmania major* and *L. donovani*: Effects on proteolytic enzymes of *Phlebetomus papatasi* (Diptera, Psychodidae). *Exp. Parasitol.*, **62**: 376-380.

Schnur, L.F., Zuckerman, A. and Greenblatt, C.L. (1972). Leishmanial serotypes as distinguished by the gel diffusion factor excreted *in vitro* and *in vivo*. *Isreali J.Med. Sci.*, **8**: 932-942.

Schmidt, G.D. and Roberts, L.S. (1989). Order Kinetoplastida: Trypanosomes and their kin. In: *Foundations of Parasitology* (4th edition) pp 55-80. Times Mirror/Mosby, St Louis.

Shahabuddin, M. and Kaslow, D.C. (1993). Chitinase: a novel target for blocking parasite transmission? *Parasitol. Today*, **9**: 252-255.

Shiels, B.R., Smyth, A., Dickson, J., McKellar, S., Tetley, L., Fujisaki, K., Hutchinson, B. and Kinnaird, J.H. (1994). A stoichiometric model of stage differentiation in the protozoan parasite *Theileria annulata*. *Mol. Cell. Diff.*, **2**: 101-125.

Shortt, H.E. (1928). The life-history of *Leishmania donovani* in its insect and mammalian host. *Transactions of the 7th Congress of the Far Eastern Association of Tropical Medicine, December 1927*, Vol III. Calcutta: Thacker's Press, pp 12-18.

Shortt, H.E., Barraud, P.J. and Craighead, A.C. (1926). The life-history and morphology of *Herpetomonas donovani* in the sandfly *Phlebotomus argentipes*. *Ind. J. Med. Res.*, **13**: 947-959.

Simon, M.W. and Mukkada, A.J. (1983). Intracellular protein degradation in *Leishmania tropica* promastigotes. *Mol. Biochem. Parasitol.*, **7**: 19-26.

Steiger, R.F. and Black, C.D.V. (1980). Simplified defined media for cultivating *Leishmania donovani* promastigote. *Acta Trop.*, **37**: 195-198.

Steiger, R.F. and Meshnick, S.R. (1977). Amino-acid and glucose utilization of *Leishmania donovani* and *L. braziliensis*. *Trans. R. Soc. Trop. Med. Hyg.*, **71**: 441-443.

Steiger, R. F. and Steiger, E. (1976). A defined medium for cultivating *Leishmania donovani* and *L. braziliensis*. *J. Parasitol.*, **62**: 1010-1011.

Steiger, R. F. and Steiger, E. (1977). Cultivation of *Leishmania donovani* and *Leishmania braziliensis* in defined media: Nutritional requirements. *J. Protozool.*, **24**: 437-441.

Stephenson, G., Stokes, R.W. and Ward, R.D. (1987). A method for purification of *Leishmania* promastigotes from infected phlebotomine sandflies. *Ann. Trop. Med. Parasitol.*, **81**: 15-23.

Stobbart, R.H. and Shaw, J.J. (1973). In: *Physiology of Insecta*, Edition 2, *Vol. V.*, pp 361-446 (Rockstein, M., ed.).

Strangeways-Dixon, J. and Lainson, R. (1966). The epidemiology of dermal leishmaniasis in British Honduras. Part III. The transmission of *Leishmania mexicana* to man by *Phlebotomus pessoanus*, with observations on the development of the parasite in different species of *Phlebotomus*. *Trans. R. Soc. Trop. Med. Hyg.*, **60**: 192-201.

Tarella, C., Ferrero, D, Gallo, E., Pagliaroli, G.L. and Ruscetti, F.W. (1982). Induction of differentiation of HL-60 cells by dimethyl sulfoxide: evidence of a stochastic model not linked to the cell division cycle. *Cancer Research*, **42**: 445-449.

Turner, C.M.R., Aslam, N. and Dye, C. (1995). Replication, differentiation, growth and virulence of *Trypanosoma brucei* infections. *Parasitology*, **111**: 289-300.

Turrens, J.F. (1989). The role of succinate in the respiratory chain of *Trypanosoma brucei* procyclic trypomastigotes. *Biochem. J.*, **259**: 363-368.

Turrens, J.F. (1991). Mitochondrial metabolism of African trypanosomes. In: *Biochemical Protozoology* (Coombs, G.H. and North, M.J., eds.) pp 145-153.

Van Hellemond, J.J. and Tielens, A.G.M. (1997). Inhibition of the respiratory chain results in a reversible metabolic arrest in *Leishmania* promastigotes. *Mol. Biochem. Parasitol.*, **85**: 135-138.

Van Hellemond, J.J., Van der Meer, P. and Tielens, A.G.M. (1997). *Leishmania infantum* promastigotes have a poor capacity for anaerobic functioning and depend mainly on respiration for their energy generation. *Parasitol.*, **114**: 351-360.

Wallace, F.G. (1977). Developmental stages of trypanosomatid flagellates: a new terminology revisited. *Protozool.*, **3**: 51-56.

Walters, L.L. (1993). *Leishmania* differentiation in natural and unnatural sandfly hosts. *J. Euk. Microbiol.*, **40**: 196-206.

Walters, L.L., Chaplin, G.L., Modi, G.B. and Tesh, R.B. (1989a). Ultrastructural biology of *Leishmania (Viannia) panamensis* in *Lutzomyia gomezi* (Diptera: Psychodidae): a natural host-parasite association. *Am. J. Trop. Med. Hyg.*, **40**: 19-39.

Walters, L.L., Irons, K.P., Chaplin, G. and Tesh, R.B. (1993). The lifecycle of *Leishmania major* (Kinetoplastida: Trypanosomatidae) in the neotropical sandfly *Lutzomyia longipalpis* (Diptera: Psychodidae). *J. Med. Entomol.*

Walters, L.L., Irons, K.P., Modi, G.B. and Tesh R.B. (1992). Refractory barriers in the sandfly *Phlebotomus papatasi* (Diptera: Psychodidae) to infection with *Leishmania panamensis*. *Am. J. Trop. Med. Hyg.*, **46**: 211-228.

Walters, L.L., Modi, G.B., Chaplin, G.L. and Tesh, R.B. (1989b). Ultrastructural development of *Leishmania chagasi* in its vector *Lutzomyia longipalis* (Diptera: Psychodidae). *Am. J. Trop. Med. Hyg.*, **41**: 295-317.

Walters, L.L., Modi, G.B., Tesh, R.B. and Burrage, T. (1987). Host-parasite relationship of *Leishmania mexicana mexicana* and *Lutzomyia abonnenci* (Diptera: Psychodidae). *Am. J. Trop. Med. Hyg.*, **36**: 294-314.

Walton, B.C. (1987). American cutaneous and mucocutaneous leishmaniasis. In: *The Leishmaniases in Biology and Medicine*, Vol 2 (Peters, W. and Killick-Kendrick, R., eds.) pp 637-663. Academic Press: London.

Warburg, A., Hamada, G.S., Schlein, Y. and Shire, D. (1986). Scanning electron microscopy of *Leishmania major* in *Phlebotomus papatasi*. *Z. Parasitenkd.*, **72**: 423-431.

Warburg A. and Schlein, Y. (1986). The effect of post-bloodmeal nutrition of *Phlebotomus papatasi* on the transmission of *Leishmania major*. *Am. J. Trop. Med. Hyg.*, **35** (5): 926-930.

Williamson, J. and Desowitz, R.S. (1961). The chemical composition of Trypanosomes. I. Protein, amino acid and sugar analysis. *Exp. Parasitol.*, **11**: 161-165.

Wilson, M.E. and Hardin, K.K. (1990). The major *Leishmania donovani chagasi* surface glycoprotein in tunicamycin-resistant promastigotes. *J. Immunol.*, **144**: 4825-4834.

Woodcock, H.M. (1914). 2. Further remarks on the flagellate parasites of *Culex*. Is there a generic type, *Crithidia*? *Zool. Anz*, **44**: 26-33.

Young, D.G., Morales, A., Kreutzer, R.D., Alexander, J.B. and others (1987). Isolation of *Leishmania braziliensis* (Kinetoplastida: Trypanosomatidae) from cryopreserved Colombian sandflies (Diptera: Psychodidae) [correction appears *in J. Med. Entomol.*, **24**: 703] *J. Med. Entomol.*, **24**: 587-589.

Young, C.J., Turner, D.P., Killick-Kendrick., R., Rioux, J.A. and Leaney, A.J. (1980). Fructose in wild-caught *Phlebotomus ariasi* and the possible relevance of sugar taken by sandflies to the transmission of leishmaniasis. *Trans. R. Soc. Trop. Med. Hyg.*, **74**: 363-366.

Zeledon, R. and de Monge, E. (1967). Physiological studies on the culture form of four strains of *Leishmania braziliensis*. I. Nitrogen content, substrate utilization, and effect of metabolic inhibitors on respiration and its relation to infectivity. *J. Parasitol.*, **53**: 937-945.

Zilberstein, D and Shapira, M. (1994). The role of pH and temperature in the development of *Leishmania* parasites. *Ann. Rev. Microbiol.*, **48**: 449-70.

Zuo, X. and Coombs, G.H. (1995). Amino acid consumption by the parasitic protists *Entamoeba histolytica* and *E. invadens*. *FEMS Microbiol. Letts.*, **130**: 253-258.

APPENDIX

Composition of media used

PBS

10 mM phosphate buffer, pH 7·4
2·7 mM KCl
137 mM NaCl

PSGEMKA [Hart *et al.*, 1981a]

Phosphate buffer was prepared as follows:
1080 mls of 200 mM $Na_2HPO_4.2H_2O$ (26·11 g/litre) titrated to pH 7·3 with approximately 345 mls of 200 mM $NaH_2PO_4.2H_2O$ (10·40 g/litre). The following compounds were subsequently added to 1 litre.

	g/litre
180 mM NaCl	10·50
55·5 mM Glucose	10·00
0·65 mM EDTA	0·19
9·97 mM $MgCl_2.6H_2O$	2·03
9·93 mM KCl	0·74
Bovine albumin (Sigma # A4503)	0·02 % (w/v)
Final pH 7·3	

HOMEM, modified from Berens *et al.* [1976]

	In 1 litre of water
Minimum Essential Medium (MEM, Gibco # 11400)	1 x 1 litre pack
11 mM Glucose	2000 mg
3·6 mM Sodium bicarbonate	300 mg
1·0 mM Sodium pyruvate	110 mg
7·2 µM p-Aminobenzoic acid	1·0 mg
0·4 µM Biotin	0·1 mg
25 mM HEPES	5960 mg
MEM Amino acids (Gibco # 11130-036, 50X)	10·0 ml
MEM Non Essential Amino acids (Gibco # 11140-035, 100X)	10·0 ml
Titrated to pH 7·5 with sodium hydroxide, pellets and 1 M	

SDM (Gibco # 21720-016 or # 21720-024)

	mg/litre
5·4 mM $CaCl_2.2H_2O$	794
21·5 mM KCl	1600
3·3 mM KH_2PO_4	450
15·0 mM $MgSO_4.7H_2O$	3700
35·9 mM NaCl	2100
4·8 mM $NaHCO_3$	400
4·9 mM $Na_2HPO_4.7H_2O$	1321
0·9 mM Fumaric acid	100
11·0 mM D-Glucose	2000
1·4 mM α-Ketoglutarate	200
0·7 mM Malic acid	100
0·8 mM Succinic acid	100
5·8 mM Trehalose	2000
5·6 mM β-Alanine	500
2·3 mM L-Arginine	400
3·0 mM L-Aspartic acid	400
0·05 mM L-Cysteine	60
0·04 mM L-Cystine	100
5·4 mM L-Glutamate	800
12·3 mM L-Glutamine	1800
3·3 mM Glycine	250
2·6 mM L-Histidine	400
1·1 mM L-Isoleucine	150
1·1 mM L-Leucine	150
9·0 mM L-Lysine.HCl	1650
5·4 mM L-Methionine	800
0·9 mM L-Phenylalanine	150
14·8 mM L-Proline	1700
2·4 mM L-Serine	250
2·9 mM L-Threonine	350
2·0 mM L-Tryptophan	100
2·8 mM L-Tyrosine	500
2·6 mM L-Valine	300
Yeastolate	2000