STUDIES ON THE METACYCLIC FORMS OF *LEISHMANIA MAJOR* AND OTHER SPECIES

by

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ADPRT	Adenosine diphosphate ribosyltransferase
AGE	Advanced glycosylation endoproducts
ALAT	Alanine aminotransferase
ASAT	Aspartate aminotransferase
CatF	Cationised ferritin
CL	Chemiluminescence
ConA	Concanavalin A
DBA	Dolichos biflorus agglutinin
DMFO	α-Difluromethylornithine
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetracetic acid
EF	Excreted factor
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetracetic acid
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FMet-Leu-Phe	N-formyl-methionyl-leucyl-phenylalanine
GDH	Glutamate dehydrogenase
G-6-PDH	Glucose-6-phosphate dehydrogenase
GPI	Glucose phosphate isomerase
GPO	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
HBSS	Hanks balanced salt solution
Hepes	N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid

HI	Heat-inactivated
IEF	Isoelectric focussing
KDa	Kilodalton
LCA	Lens culinaris agglutinin
LDH	Lactate dehydrogenase
LIT	Liver infusion tryptose
LPO	Lactoperoxidase
MDH	Malate dehydrogenase
MPO	Myeloperoxidase
MTT	3-(4,5-dimethylthiazol-2-yl) -2,5,-diphenyl tetrazolium bromide:Thiazolylblue
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NBT	Nitroblue tetrazolium
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEP	Phosphenolpyruvate
PEP carboxykinase	Phosphenolpyruvate carboxykinase
6-PGDH	6-phosphogluconate dehydrogenase
PGM	Phosphoglucomutase
PK	Pyruvate kinase
PMN	Polymorphonuclear leucocytes
PMS	Phenazine methosulphate
PNA	Peanut agglutinin
RB	Respiratory burst
RCA	Ricinus agglutinin
RNA	Ribonucleic acid

SBA	Soybean agglutinin
SDS	Sodium dodecyl sulphate
SJA	*Sophora japonica* agglutinin
SOD	Superoxide dismutase
SSH	Sodium salicyl hydroxamate
Tris	2-amino 2(hydroxymethyl) 1,3-propandiol
UEA	*Ulex europaeus* agglutinin
WA	*Vicia villosa* agglutinin
WGA	Wheat germ agglutinin
XO	Xanthine oxidase

SUMMARY

Populations of stationary-phase promastigotes and purified metacyclic promastigotes of Leishmania major, and stationary-phase populations of L.mexicana mexicana (which contain putative metacyclics) were compared with their respective mid-log phase populations and isolated amastigotes using a variety of techniques.

Peanut agglutinin (PNA) was used successfully to separate out the metacyclic promastigotes from stationary-phase populations of L.major. These cells were found to possess a characteristic morphology, and assessment of numbers of metacyclics in populations by PNA treatment and from morphological criteria correlated well and showed that mid-log phase populations contained low numbers of metacyclics, whereas about half the stationary-phase populations were metacyclics. Studies using similar morphological criteria suggested that the situation was similar with L.mexicana mexicana, low numbers of putative metacyclics being present in mid-log phase populations with greater numbers in stationary-phase populations. However, attempts to separate out the two populations of promastigotes of this species using various agglutinins and density gradient centrifugation were unsuccessful.

Infectivity studies using mice showed that L.major metacyclic and stationary-phase promastigotes were more infective than mid-log phase cells, although this later form produced lesions. Metacyclic promastigotes were also found to be more infective than stationary-phase populations of cells. Similarly, L.mexicana mexicana stationary-phase populations were more infective to mice than mid-log phase population, although the difference was variable. Leishmania major metacyclic promastigotes and L.mexicana mexicana stationary-phase promastigotes survived and multiplied in murine resident peritoneal macrophages in vitro, whereas mid-log phase cells were either killed,

in the case of L.major, or produced non-multiplicative infections, as in the case of L.mexicana mexicana. Infectivity of L.mexicana mexicana stationary-phase populations also varied when tested in vitro. The greater infectivity to mice, and the ability to multiply in macrophages of L.mexicana mexicana correlated with an absence or presence in variable amounts of a series of low molecular weight proteinases. These were not detected in mid-log phase promastigotes, sometimes found in stationary-phase cells and present at high activity in amastigotes, as detected by Gelatin discontinuous polyacrylamide gel electrophoresis (Gel Disc-PAGE).

When exposed to macrophages, more L.major mid-log phase promastigotes than metacyclics became attached and they resulted in higher initial infection rates. This difference was also seen when attachment/uptake of the two promastigote forms was examined using rabbit peritoneal neutrophils. In contrast, with macrophages mid-log phase and stationary-phase promastigotes of L.mexicana mexicana gave rise to similar infection rates, although differences in attachment between the two forms, similar to those seen with L.major, were observed. With both species, amastigotes were more efficiently phagocytosed by macrophages than any promastigote form. Interestingly, however, L.mexicana mexicana amastigotes and mid-log phase promastigotes were attached and taken up by rabbit peritoneal neutrophils to the same extents, and no more efficiently than by macrophages.

The respiratory burst (RB) elicited by different forms of the two species was assessed using a variety of phagocytic cell types. Amastigotes of both species were found to be poor stimulators of the RB. In contrast, L.major metacyclics and L.mexicana mexicana stationary-phase promastigotes stimulated a similar RB to their

respective mid-log phase promastigotes. With L.major, the enhanced survival of the metacyclics compared to mid-log phase cells, despite stimulating a similar RB, correlated with their ability to withstand greater concentrations of hydrogen peroxide (H_2O_2). This resistance may possibly be due to the larger numbers of lipid inclusions present in these cells.

An interesting finding was L.mexicana mexicana and L.donovani mid-log phase promastigotes and some insect trypanosomatids, but neither promastigote form of L.major, could apparently "inhibit" the RB of neutrophils when added in large numbers. This ability approximately correlated with the presence of surface acid phosphatase on the cells. Neither promastigote form of L.major were found to possess acid phosphatase activity, nor did they secrete this enzyme. This is in contrast to all other Leishmania species investigated. The overall acid phosphatase activities of metacyclic and mid-log phase promastigotes of L.major were found to be similar with respect to specific activities, location, pH profile and isoenzyme profile, suggesting that this enzyme is not crucial in mediating the initial survival of this species in macrophages.

The effect of human serum on leishmanias was tested, and was found to be lytic for all promastigote forms of L.major and L.mexicana mexicana: none survived in whole serum. Heat-inactivating the serum removed the majority of this lytic activity, suggesting complement was involved. Both stationary-phase and metacyclic promastigotes of L.major were more resistant to the effects of serum than mid-log phase cells. This was possibly due to the presence of an irregular, finely grained "surface coat" which was present on all metacyclics, some stationary-phase cells and apparently absent in mid-log phase cells. In contrast, although both mid-log phase and stationary-phase promastigotes of L.mexicana mexicana were more resistant than any

promastigote form of L.major, they were both similarly susceptible to serum.

Examination of the isoenzyme profiles of proteinases by Gel Disc-PAGE, protein content, isoenzyme profiles of other enzymes by isoelectric focussing (IEF), enzyme content and free amino acid pool content of different promastigote forms and amastigotes of L.major and L.mexicana mexicana, suggested that the metacyclics of L.major and the stationary-phase promastigotes of L.mexicana mexicana are preadapted to life within the macrophage.

Studies examining the change of L.major mid-log phase promastigotes to metacyclics and vice versa showed that metacyclic production is induced by "spent" medium (from stationary-phase cultures) suggesting that nutrient depletion may be an important trigger. They also showed that metacyclics were capable of changing back to "mid-log" phase cells in fresh medium, and that this was apparently required before division occurred.

Overall, comparison of L.major mid-log phase and metacyclic promastigotes with amastigotes suggested that metacyclics possess preadaptations which allow them to resist host humoral microbicidal factors such as complement, and to survive and multiply in the host macrophage. Similar comparison of the different forms of L.mexicana mexicana showed that stationary-phase promastigotes are also apparently preadapted in some respects but not others, to multiply within the macrophage. The results also suggested that mid-log populations of this species may contain a proportion of forms which are similar to metacyclics in some respects but lack the crucial preadaptations that allow multiplication in macrophages. It is envisaged that these forms are an intermediate between multiplicative promastigotes and the metacyclics.

1.0 INTRODUCTION

1.1 *LEISHMANIA* AND THE LEISHMANIASES

The Leishmaniases comprise a group of diseases that have a major impact on the health of the population in large parts of the world. The importance of the leishamanias was reaffirmed by their inclusion as one of the six major diseases for study in the World Health Organisation Special Programme for Research and Training in Tropical Diseases (Alexander & Russell, 1985; Pearson, Wheeler *et al.*, 1983).

The leishmaniases encompass what has been referred to as a "spectrum of disease" (Molyneux & Ashford, 1983) which ranges from simple cutaneous lesions through grossly disfiguring mucocutaneous lesions to a visceral disease which has a high mortality rate if untreated (Molyneux & Ashford, 1983).

The causative agents of these diseases all belong to the genus *Leishmania* and are transmitted by blood sucking phlebotomine sandflies. They initially occur as extracellular flagellated promastigotes in the midgut of the vector. From there they migrate anteriorly, taking different morphological flagellated forms, until they reach the anterior foregut and proboscis where they occur as metacyclic promastigotes, which have characteristic morphology and are the end point of the cycle in the fly. When the fly takes a blood meal, the metacyclic forms are inoculated into the vertebrate host where they are taken up by mononuclear phagocytes and transform into amastigotes. These intracellular forms are non-motile, they divide by binary fission and eventually rupture the cell. The free amastigotes released are taken up by other mononuclear phagocytes and the infection is maintained in this way until parasites are taken up by another sandfly during a blood meal, whereupon they transform back to promastigotes in the fly mid-gut.

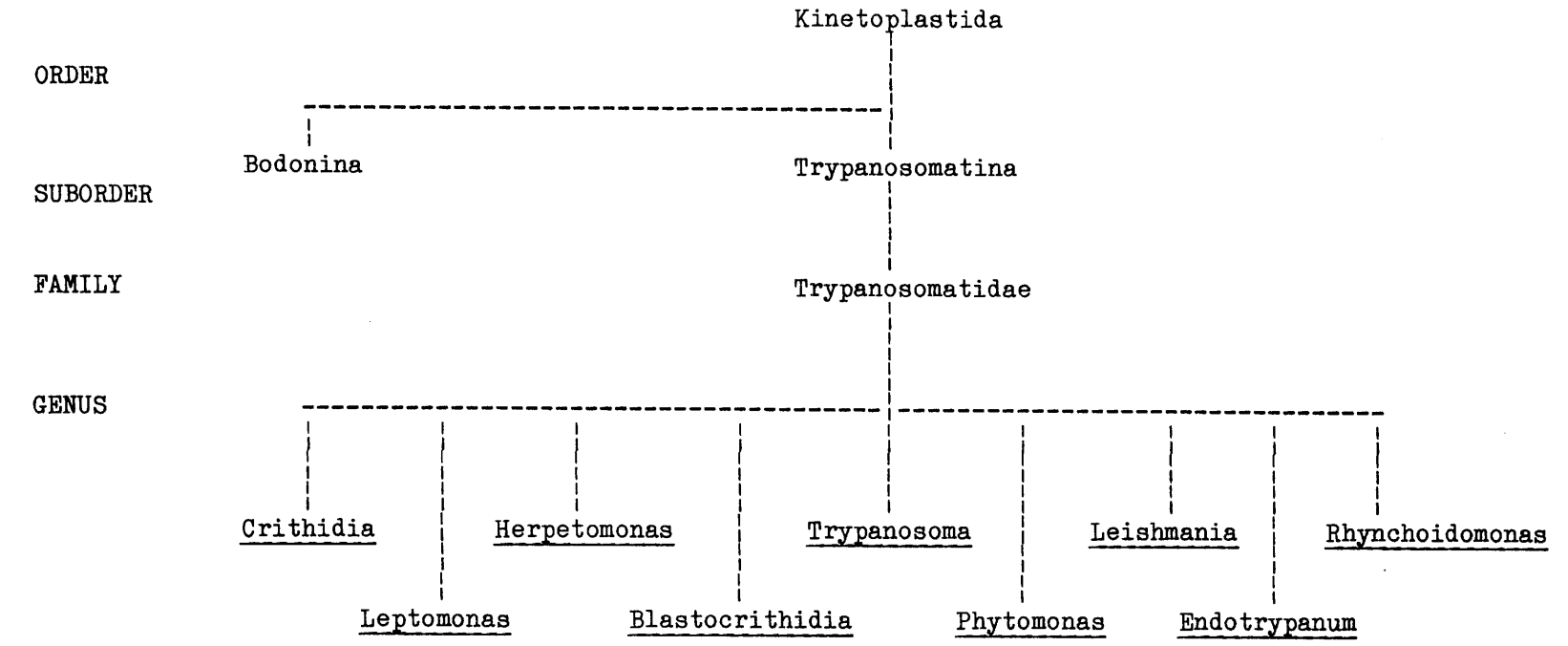
1.2 LEISHMANIA

1.2.1 Taxonomy

Leishmania are parasitic protozoa belonging to the family Trypanosomatidae and order Kinetoplastida (Table 1). The main distinguishing feature of the members of the Kinetoplastida is the presence of a Feulgen positive (DNA-containing) densely staining region in the mitochondrion; this is termed the Kinetoplast and is composed of catenated circles of DNA. It is located adjacent to the basal body of the single flagellum (Vickerman, 1976). There are two suborders belonging to this order, the Bodonina which are largely free living, and the Trypanosomatina which are exclusively parasitic (Molyneux & Ashford, 1983). There is only one family in the latter suborder and this contains members which are parasitic in either invertebrate or vertebrate hosts. Some genera, such as Leptomonas, Herpetomonas, Crithidia, Blastocrithidia and Rhynchoidomonas, are monoxenous (having a single host) and are almost exclusively parasites of insects, although some are parasitic in nematodes, acanthocephalan worms and ticks (Molyneux & Ashford, 1983; Vickerman, 1976). Others, however, such as Endotrypanum, Leishmania and Trypanosoma (with the exception of T.equiperdum), are digenetic having both vertebrate and blood-sucking invertebrate hosts. The genus Phytomonas are also digenetic, but are parasites of plants and are transmitted by plant sucking insects (Molyneux & Ashford, 1983).

Members of the Trypanosomatidae are of interest for a variety of reasons. Species belonging to the genus Crithidia, notably C.fasciculata, are easily cultivated in vitro and have been used as a model eukaryotic cells and subjected to detailed biochemical and physiological studies (McGhee & Cosgrove, 1980). Several Phytomonas species are pathogenic in plants and have been reported to severely damage plants of great economic importance such as oil (Elacis

Table 1: Taxonomic position of Leishmania.



From Molyneux and Ashford (1983).

guineensis) and coconut palms (Cocos nucifera), and coffee plants (Coffea liberaca) (McGhee & Cosgrove, 1980). Species in the genus Trypanosoma are important pathogens of humans and domestic animals, and are the causative agents of human trypanosomiasis in Africa (sleeping sickness caused by T.brucei rhodesiense and T.b.gambiense) and in South America (Chagas' disease caused by T.cruzi), Nagana in cattle (caused by T.b.brucei, T.congolense, T.vivax), surra in camels and horses (caused by T.evansi) and dourine (caused by T.equiperdum) in equines (Molyneux & Ashford, 1983). Further details are beyond the scope of this work and can be found in a number of reviews including those of McGhee and Cosgrove (1980) and Molyneux and Ashford (1983).

The species of Leishmania that are known to infect humans together with the diseases they cause and their distribution are given in Table 2. There is, however, much controversy surrounding the classification of leishmanias and as yet no universally accepted system has been produced (WHO, 1984; Chance, 1985). The one shown in Table 2 is based on that in WHO Technical Report Series 701 (1984). Other species that do not infect man but parasitise other mammals are L.hertigi (Tree porcupines-Coendou species),L.gerbilli (Gerbil-Rhombomys opimus) and L.enrietti (Guinea-pig - Cavia porcellus). The latter species produces a cutaneous infection in guinea pigs which is very similar to human cutaneous leishmaniasis and has been used extensively as a laboratory model for this disease (Molyneux and Ashford, 1983; Howard, 1985). Furthermore, various trypanosomatids from lizards have been included in the genus, notably L.tarentolae which infects the moorish gecko (Tarentola mauritanica). This species can be cultured very easily and has been used extensively in the past as a model for the leishamnias which infect humans. Recently however, it has been shown to be identical to Trypanosoma platydactyli from

Table 2: Leishmania species known to infect humans, the diseases they cause and their distribution.

SPECIES	TYPE OF DISEASE	PRIMARY GEOGRAPHICAL LOCATION
L.bra ziliensis braziliensis	Cutaneous and mucocutaneous (Espundia)	Found typically in Brazil
L.braziliensis guyanensis[a]	Cutaneous (Pian bois)	North Amazon basin and the Guyanas
L.braziliensis panamensis[a]	Cutaneous	Panama and Costa Rica
L.peruviana	Cutaneous	Western slopes of the Andes
L.mexicana mexicana	Cutaneous (Chideros ulcer)	S.Mexico, Belize and N.Guatemala
L.mexicana amazonensis[a]	Cutaneous, diffuse cutaneous	Amazon basin and Brazil
L.mexicana pifanoi	Diffuse cutaneous	Venezuela
L.mexicana garnhami	Cutaneous	Venezuelan Andes
L.mexicana venezuelensis	Cutaneous	Venezuela
L.aethiopica	Cutaneous (Chronic dry) Diffuse cutaneous	Ethiopian highlands and Mt Elgon, Kenya
L.tropica	Cutaneous (urban, dry) Leishmaniasis recidivans Visceral (rare)	Urban areas of Middle East and India
L.major	Cutaneous (rural wet)	Widespread in Africa, Middle East, Soviet Asia
L.donovani donovani	Visceral, mucocutaneous (Africa) Post kala-azar dermal leishmaniasis	NE Coastal China; Bihar, Bengal, Assam (India); Bangladesh; Kenya; Sudan
L.donovani infantum[a]	Infantile visceral	North Central Asia & N.W. China; Uzbekistan, Middle East, Mediterranean Europe and N.W. Africa
L.donovani chagasi[a]	Visceral also infantile Visceral	South and Central America

[a] Some workers consider these to be individual species. Adapted from Molyneux & Ashford (1983) WHO (1984), and Chang et al (1985).

geckos and this finding has brought into question the validity of saurian leishmanias as a group and their relationship to mammalian leishmanias (Chance, 1985; Wallbanks et al., 1985). This highlights the problems of identifying Leishmania species. The identification of parasites is necessary for a variety of reasons. For example, in the analysis of the ecology and epidemiology of the parasite; for identifying potential reservoirs and vectors; for assessing control programmes and for deciding appropriate treatment regimens (Chance, 1979; Evans et al., 1984; Marsden and Jones, 1985). Apart from minor differences (Gardner et al., 1977) all Leishmania species are morphologically and ultrastructurally identical (Chang et al, 1985). Early taxonomy was therefore based on clinical features in the host combined with epidemiology and geographic distribution. However, since there is no absolute distinction between parasites causing visceral and cutaneous infections (Beach and Mebrahth, 1985; Schuur et al., 1981), these parameters are of limited validity and more precise methods of identification are now employed. At present, the most widely used method is the analysis of isoenzymes by electrophoresis (WHO, 1984; eg. Evans et al., 1984). Other methods that can be used alone or in conjunction with isoenzyme analysis include the determination of the bouyant density of DNA (G+C analysis), radiorespirometry, excreted factor (EF) seroptyping (Chance, 1979; 1985) and lectin-binding studies (Jacobson et al., 1982; Schottelius, 1982). More recently, with the advent of hybridoma and recombinant DNA technology, the use of monoclonal antibodies (McMahon-Pratt and David, 1981) and DNA species-specific probes (Barker et al, 1986; Barker, 1987) and has become increasingly important in the identification of Leishmania (Wirth et al., 1986).

Because of the problems of identifying Leishmania, in the past some workers have used the wrong specific name when referring to

parasites in publications. This is especially the case with L.tropica/L.major. Where this occurs and the true identity of the parasite is known the species name used by the author is given first and the true species name is given in parentheses.

1.2.2 Cell biology

1.2.2.1 Cultivation of leishmanias

Sandflies can now be reared in the laboratory relatively easily (Young et al., 1981; Modi and Tesh, 1983) and readily infected with Leishmania (Killick-Kendrick, 1979; Sacks and Perkins 1984, 1985; Walters et al., 1987). A method has been developed for purifying parasites from sandflies (Stephenson et al., 1987), but yields are low making it impractical as a source of parasites for biochemical and other studies.

Fortunately, cell types morphologically similar to those in sandflies can be easily cultured in a range of media at 25°C-28°C (Chang and Hendricks, 1985). The commonest media are complex, either biphasic, eg. N.N.N., or monophasic, eg. LIT (Carmargo et al., 1964). In addition, a number of semi-defined media, frequently based on insect cell culture media such as Scheider's and Grace's, have been shown to support the growth of Leishmania (Childs et al., 1978; Hendricks et al., 1978). Several defined medium have also been developed. The first defined medium for Leishmania species infective to humans was medium REI of Steiger and Steiger (1976). Other media subsequently developed are RElll (Steiger and Steiger, 1977), HOSMEM (Berens and Marr, 1978), REIX and REX (Steiger and Black, 1980). DME2 (Iovannisci and Ullman, 1983) and, most recently, MD-29 (Melo et al., 1985). The last medium is of interest because it was used to grow a representative range of species and strains, whereas the others were initially used for the growth of only one or two species.

Just how similar promastigotes grown in culture are to those in the insect has been and still is an open question (Molyneux et al., 1975; Killick-Kendrick, 1979), although it is now well established that in a wide variety of undefined biphasic (Keithly & Bienen, 1981) and monophasic (Giannini, 1974; Rizvi et al., 1984) media and in semi-defined media (Sacks & Perkins, 1984; Wozencroft & Blackwell, 1987) Leishmania promastigotes undergo sequential development similar to that which occurs in the sandfly. They transform from the essentially non-infective, actively dividing log-phase cells, which are analagous to the multiplicative stage in the sandfly midgut, to the infective, stationary phase population which contains metacyclics thought to be analagous to the proboscis forms in sandflies. It should be noted, however, that with successive sub-passaging in vitro promastigotes can lose their virulence or infectivity to laboratory animals (Giannini, 1974; Dawidowicz et al., 1975; Giannini et al., 1981; Hart et al., 1981a; Semprevivo et al., 1981; Grimaldi et al., 1982; Pimenta & de Souza, 1983; Ayesta et al., 1985; Saraiva et al., 1986) and their ability to survive in macrophages in vitro (Ebert et al., 1979; Nolan & Herman, 1985; Katakura, 1986). This apparently can be modulated to some degree by the use of different media (Neal, 1984). Concomitant with such changes of virulence are other biochemical/physiological changes (see Sections 1.2.2.3 and 1.2.2.4) although the significance of those in relation to virulence has yet to be elucidated (Chang & Hendricks, 1985).

Amastigotes can be cultivated in a variety of ways. For large scale harvesting, they are usually grown in experimental animals; several methods for purifying amastigotes from host material have been developed (Infante et al., 1980; Hart et al., 1981b; Saraiva et al., 1983; Meade et al., 1984). These are, however, quite time consuming,

and although some methods (e.g. Hart et al., 1981b) can guarantee very pure preparations, some contamination with host material is inevitable. Chang (1980a) reported a method for growing L.mexicana amazonensis in a mouse macrophage-like cell line and more recently Pan (1984) described a similar system for L.mexicana pifanoi. They were able to obtain sufficient numbers of purified amastigotes to allow biochemical studies to be carried out. But these two are the only Leishmania-host cell combinations tested that permit continuous in vitro cultivation and result in reasonable multiplication in the number of amastigotes. In general, amastigotes are slow growing, fastidious organisms (Chang & Hendricks, 1985). Nevertheless, intracellular growth, although usually of limited duration, has been demonstrated with many host cell-parasite combinations. The most commonly used host cells are peritoneal macrophages from mice. They are usually separated from other cells by their adhesion to glass or plastic, on which they remain fixed as a monolayer (Chang & Hendricks, 1985). Interestingly, Nacy and Diggs (1981) found that amastigotes multiplied much better in non-adherent, suspended macrophages and related this to differences between the two populations of macrophages. Leishmania amastigotes have also been grown in human blood monocytes (Pearson, Harcus et al., 1983), macrophages derived from them (Berman et al., 1979), hamster peritoneal macrophages (Chang & Dwyer, 1976) and in a variety of macrophage-like murine cell lines such as J774 (Chang, 1980), P388D (Berens & Marr, 1979), and IC-21 (Scott et al., 1985). Amastigotes have been reported to survive and multiply in non-macrophage-like mammalian cell lives such as Sticker Dog Sarcoma cells series 503 (Lewis, 1974) and hamster peritoneal exudate line (Mattock & Peters, 1975). Cutaneous strains of Leishmania grow best at 32-35^{o}C (Berman & Neva, 1981; Biegel et al., 1983) although some will grow at temperatures above this; in general, New

World cutaneous strains are more sensitive to higher temperatures than Old World strains (Sacks et al., 1983). Leishmania donovani and subspecies are grown at 37°C.

Recently, Pan (1984) developed a rich semi-defined medium which was reported to substain the growth of Leishmania amastigotes axenically in vitro. Subsequent studies comparing the infectivity (Pan & Honigberg, 1985), ultrastructure (Pan & Pan, 1986) antigenic relationships (Pan, 1986) and biochemistry (McMahon-Pratt et al., 1987) of these amastigotes with ones from lesions or J774 cells and also promastigotes suggest that the in vitro amastigotes are very similar to true amastigotes and quite different in a variety of ways from cultured promastigotes. It is not yet clear whether other species or subspecies will also grow in this medium, and more biochemical studies are required to establish to what extent the in vitro amastigotes differ from amastigotes growing in animals.

1.2.2.2 Cell structure

The ultrastructure of typical amastigotes and promastigotes of Leishmania are illustrated in Figs. 1 and 2. Both forms have features characteristic of other eukaryotic cells and also several unusual features. The kinetoplast contains DNA in the form of maxicircles and minicircles which are arranged into regular arrays (Chang et al., 1985). The kinetoplast lies just beside the base of the flagellum within the single mitochondrion which exists in all Leishmania species and also all other kinetoplastid flagellates (Vickerman & Preston, 1976). This organelle generally lies just below the plasma and is reticulated. A three-dimensional reconstruction of L.mexicana mexicana amastigotes showed it to have a complex shape taking up approximately 10% of the total cell volume (Coombs et al., 1986). It is cristate in both amastigotes and promastigotes. From ultrastructural studies with

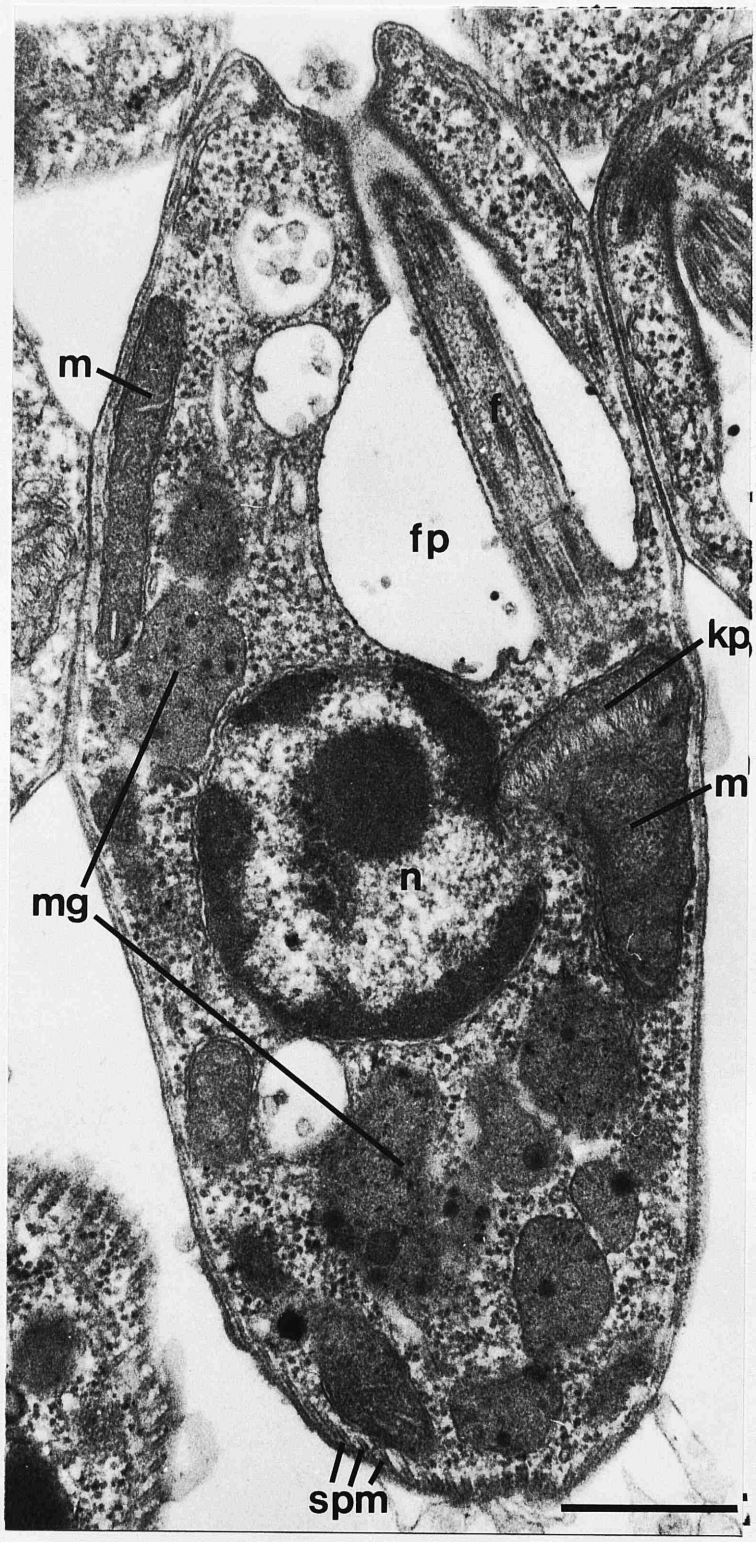


Fig. 1. A transmission electron micrograph of a Leishmania mexicana mexicana amastigote.

Scale bar = 0.5 μm. Micrograph courtesy of L.Tetley.

Key to subcellular structure: Flagellum, (f); flagellar pocket, (fp); kinetoplast, (kp); mitochondria, (m); "megasome", (mg); nucleus, (n); subpellicular microtubules, (spm).

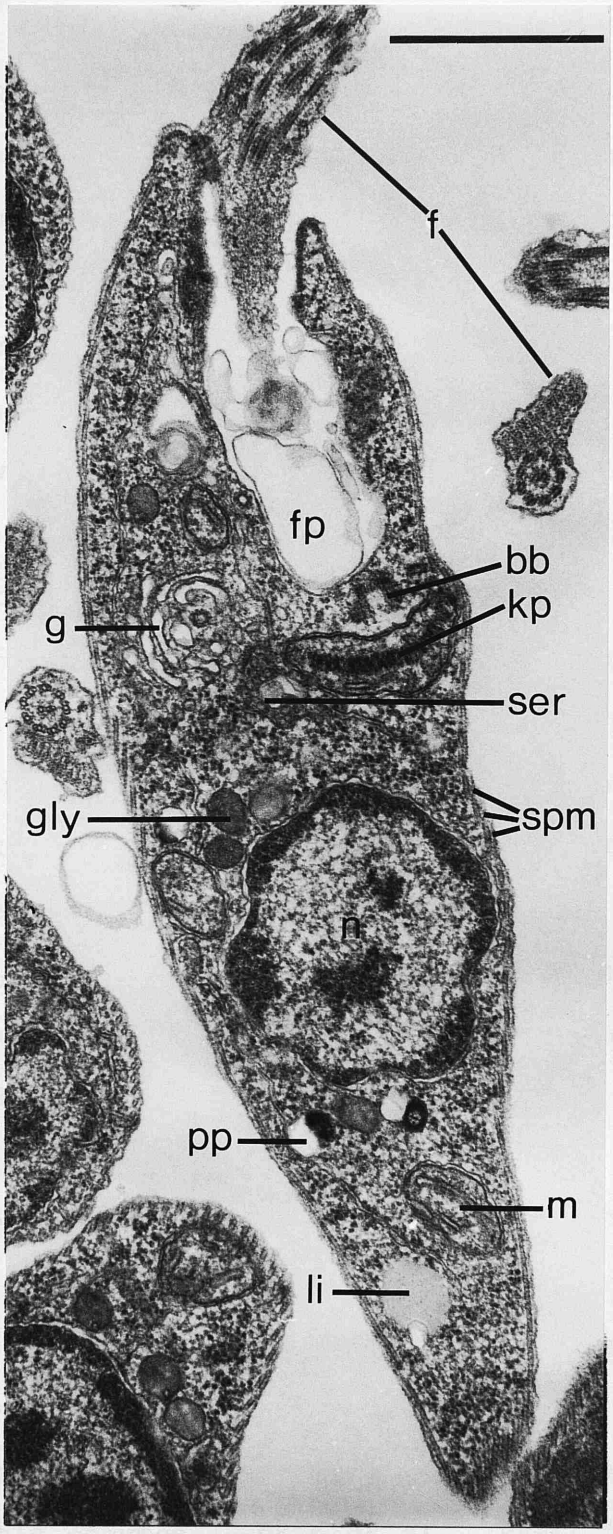


Fig. 2. A transmission electron micrograph of a Leishmania major promastigote.

Scale bar = 0.5 μm. Micrograph courtesy of L.Tetley.

Key to subcellular structures: Basal body, (bb); flagellum, (f); flagellar pocket, (fp); golgi apparatus, (g); glycosome, (gly); kinetoplast, (kp); lipid inclusion, (li); mitochondria, (m); nucleus, (n); paraxial rod, (par); polyphosphate granule, (pp); subpellicular microtubules, (spm).

L.donovani, Rudzinska et al. (1964) concluded that relative mitochondrial volume increased in transformation from amastigotes to promastigotes whereas from morphometric studies with the same species, Brun and Krassner (1976) reported a slight but significant decrease in volume. Respiration studies, however, with L.mexicana mexicana demonstrated that qualitatively and quantitatively there was little difference between the two main forms (Hart et al., 1981a).

Glycosomes are small membrane bound organelles unique to trypanosomatids (Opperdoes & Borst, 1977; Opperdoes, 1982; Hart & Opperdoes, 1984) and are associated with important biochemical pathways such as glycolysis, carbon dioxide fixation (Hart & Opperdoes, 1984; Mottram & Coombs, 1985a), B-oxidation of fatty acids, ether lipid biosynthesis (Hart & Opperdoes, 1984), and de novo purine and pyrimidine biosynthesis (Hammond & Gutteridge, 1982; Hassan et al., 1985). They appear to be variable in size in leishmanias and are apparently equally abundant in the two main forms of the parasite (Hart & Opperdoes, 1984; Mottram & Coombs, 1985a; Coombs et al., 1986).

"Megasomes" (Alexander & Vickerman, 1976) are thick membrane (10nm) bound, lysosomal-like organelles spherical to elongate or dumbell shaped and very variable in size (Coombs et al., 1986). There is a correlation between high proteinase activity and the presence of megasomes and recently they have been shown to contain proteinases and other hydrolytic enzymes (Pupkis et al., 1986). "Megasomes" are apparently present only in species belonging to the L.mexicana complex. Moreover, they are apparently only present in the amastigote stage (Coombs, 1982; Pupkis et al., 1986). In L.mexicana mexicana amastigotes they are the most abundant organelle, comprising up to 15% of the total volume of the cell (Coombs et al., 1986). A role in

amastigote survival within macrophages has been proposed (Coombs, 1982; Coombs & Baxter, 1984).

Trypanosomatids are more richly endowed than any other flagallate group with subpellicular microtubules. These microtubules lie underneath the cell body section of the plasma membrane and run in parallel to one another, in a spiral course along the long axis of the cell. They are inter-connected and are intimately associated with the plasmalemma. The spatial organisation is seen maintained in isolated plasma membranes (Dwyer, 1980) and the microtubules thus form the cytoskeleton of Leishmania species and collectively are responsible for rigidity and cell shape (Dwyer, 1980).

1.2.2.3 Surface membrane

The surface membrane of Leishmania is important as all physiological and biochemical interactions between the parasite and its host occur, at least temporally, across this membrane (Dwyer & Gottlieb, 1983, 1985). Consequently, they have been studied intensively using a variety of techniques and probes (Dwyer & Gottlieb, 1985). Unfortunately, however, most of the work has been carried out using only promastigotes. Promastigotes of L.donovani (Dwyer et al., 1974; Dwyer, 1977; Muhlpfordt, 1975), L.tropica, L.braziliensis and L.mexicana amazonensis (Muhlpfordt, 1975; Hernandez et al., 1980; Pimenta & De Souza, 1983; Ayesta et al., 1985) have been shown to possess an evenly distributed net negative surface surface. This has been demonstrated by agglutination of living cells with cationised ferritin (CatF) (Dwyer, 1977; Herman et al., 1980; Ayesta et al., 1985). Similarly, a net negative surface charge, but lower than that of the respective promastigotes, has been demonstrated on the surface of amastigotes of L.braziliensis (Hernandez et al., 1980) L.mexicana amazonensis (Pimenta & De Souza, 1983) and L.donovani

(Dwyer et al., 1974). The more detailed investigations of Pimenta and De Souza (1983) using cytospherometry showed that amastigotes of L.mexicana amazonensis had a net negative but heterogenous surface charge. This charge increased as the amastigotes transformed to promastigotes and after five sub-passages established a value which did not change even after extensive subpassaging. Interestingly, there was no difference in surface charge between pathogenic (5 sub-passages) and non-pathogenic (176 sub-passages) promastigotes or at any phase during growth in vitro. In contrast, Ayesta et al (1985) demonstrated that whereas a pathogenic strain of L.braziliensis bound cationised ferritin, a non-pathogenic strain of L.braziliensis (LBY-L.braziliensis yaracuyensis) did not. The reasons for these differences are not yet clear. It is not known what functional groups are responsible for the negative surface charge of Leishmania, although sialic acid has been implicated in the cases of L.mexicana amazonesis and L.donovani (Dwyer, 1977; Pimenta & De Souza, 1983) promastigotes. It has also been suggested that the net negative charge may be due, in part at least, to the phosphate groups of the membrane phospholipids and also to negatively charged membrane carbohydrate components (Dwyer & Gottlieb, 1983).

The surface carbohydrates of Leishmania have been characterised by lectin-binding studies using approaches similar to those described above for surface charge. Lectins are proteins that can bind non-cordently to specific carbohydrate groups, without modifying them chemically. Binding is reversible and all lectins have more than one specific carbohydrate combining site (Brown & Hunt, 1978; Lis & Sharon, 1973). The majority of Leishmania species infective to humans have been studied and a number of uniformly distributed sugar residues have been demonstrated. Promastigotes (and amastigotes where studied) of a variety of strains and species have been shown to possess on

their surface the following sugars or very similar residues α-mannose/glucose, D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine and α-L-fucose (Dwyer, 1974, 1977; Dawidowicz et al., 1975; Petavy et al., 1978; Doran & Herman, 1981; De Souza & Brasil, 1976; Jacobson et al., 1982; Schottelius, 1982; Ebrahimzadeh & Jones, 1984; Handman et al., 1983; Zehavi et al., 1983; Stokes, 1984; Wilson & Pearson, 1984; Gueugnot et al., 1984; Sacks et al., 1985; Grogl et al., 1987). These residues, however, appear to be present only on some species or strains of species. The only membrane ligands, however, which are apparently common to all species (regardless of strain, stage, or phase of growth in vitro) so far studied are those similar to or containing α-D-mannose/glucose residues. Lectin-induced agglutination is concentration dependent, and, for L.braziliensis promastigotes at least, is temperature and energy dependent, and inhibited by cytochalasin B (Hernandez et al., 1980).

Where amastigotes and promastigotes have been compared, both quantitative and qualitative differences in lectin binding have been demonstrated using electron microscopy. Wilson and Pearson (1984) showed that a transformation of L.donovani from amastigotes to promastigotes, a wheat germ agglutinin (WGA) binding site (inhibited by N-acetylglucosamine) was lost and a peanut agglutinin (PNA) binding site (inhibited by D-galactose) was gained. The latter, however, was thought to be internal. Hernandez et al. (1980) demonstrated that L.braziliensis promastigotes possess a greater number of Concanavalin A (ConA), binding sizes (inhibited by D-glucose and α-methyl-mannopyranoside) than amastigotes as measured by specific binding of ^{125}I-ConA. Saraiva et al (1986) tested 31 lectins against L.mexicana amazonensis stationary-phase, low sub-passage (2, infective) promastigotes and amastigotes, and found that some were bound more (as

judged by the minimum concentration required to agglutinate) by one or other of the forms. Notably, PNA was bound more by promastigotes and WGA were by amastigotes. Similarly, Stokes (1984) reported that L.mexicana mexicana promastigotes possess greater amounts of mannose and galactose on their surface compared to amastigotes. Studies with low sub-passage, pathogenic promastigotes and high sub-passage non-pathogenic promastigotes have also revealed quantitative and qualitative differences between them. For example, L.braziliensis pathogenic NR strain promastigotes (2-10 sub-passages) were found to possess both ConA (inhibited by α-methylmannopyranoside) and Ricinus agglutinin (RCA, inhibited by D-galactose) binding sites whereas promastigotes from the non-pathogenic LBY strain (500 sub-passages) apparently do not (Dawidowicz et al., 1975; Ayesta et al., 1985). Similarly, Saraiva et al (1986) examined both infective (2 sub-passages) and non-infective (130 sub-passages) stationary phase promastigotes of the same strain of L.mexicana amazonensis with 31 lectins. They found 6 of the lectins (D-galactose, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine binding) detected differences in cell surface carbohydrates; in all cases the lectins reacted more strongly or exclusively with the infective promastigotes. Ebrahimzadeh and Jones (1984) screened 5 isolates of L.tropica and found that the isolate which was not infective to Balb/c mice showed poor agglutination with the following lectins: ConA (inhibited by D(+)-mannose), RCA (inhibited by D(+)-galactose) and soybean agglutinin (SBA, inhibited by N-acetyl-D-galactosamine). In contrast, Handman et al (1983) found that virulent and avirulent clones as well as the wild-type population of a L.tropica isolate bound these lectins equally well.

Other workers have concentrated on the interesting differences in lectin binding between promastigotes at different phases of their

growth cycle *in vitro* and how these relate to differences in infectivity. Doran and Herman (1981) showed that *L.donovani* promastigotes from 3-day old cultures (log-phase) were less infective to hamsters than promastigotes from 10-day-old cultures (stationary-phase). The log-phase cells also agglutinated RCA_{60} (inhibited by α-D(+)-galactose and N-acetyl-D(+)-galactosamine). Subsequently, Sacks *et al* (1985) demonstrated with *L.major* that 100% of the non-infective log-phase promastigotes were agglutinated by PNA (inhibited by D-galactose) whereas only approximately 50% of the infective, stationary phase promastigotes were. They showed that it was the population that did not bind PNA (designated PNA^-) which were the infective promastigotes and this difference in binding of PNA was the basis of the first method developed for separating metacyclics from a mixed population. Similar differential binding was found to be common to the 5 strains of *L.major* they tested, and the proportion of PNA^- promastigotes varies in clones of varying virulence in some (Blackwell *et al.*, 1986), but not all cases (Marchand *et al.*, 1987). Similarly, Grogl *et al* (1987), using FITC-conjugated lectins, found that the infective stationary phase promastigotes of *L.braziliensis panamensis* (Franke *et al.*, 1985) bound ConA less intensely than the essentially non-infective log-phase cells. In addition, the stationary phase promastigotes also bound *Lens culinaris* agglutinin (LCA), whereas binding of this lectin to log-phase promastigotes could not be detected. The binding of both these lectins is inhibited by mannose and α-methylmannoside.

The surface membrane constituents of various *Leishmania* species have been identified using external radiolabelling techniques in conjunction with sodium dodecylsulphate-polyacrylamide gel elctrophoresis (SDS-PAGE) and autoradiography or Western blotting

followed by immunoprecipitation. Early studies using these techniques produced an array of surface orientated molecules of considerable variability and complexity (Dwyer & Gottieb, 1983, 1985; Alexander & Russell, 1985) probably due to variation in the way the parasite and sample were handled. For example, with respect to incubation temperature, the presence of protease inhibitors and amount of reduction occurring (Colomer-Gould et al., 1985; Heath et al., 1987). It now appears that the number of surface orientated molecules in promastigotes of Leishmania is fairly limited and the profile between species is remarkably similar (Gardiner et al., 1984; Colomer-Gould et al., 1985; Etges et al., 1985) where amastigotes have been studied, the pattern generated has generally been found to be similar to, but rather more complex than that for promastigotes and some stage specific molecules have been detected (Handman & Curtis, 1982; Handman et al., 1984; Colomer-Gould et al., 1985; Dwyer & Gottlieb, 1985; Heath et al., 1987). Some workers, however, have reported extensive differences between promastigotes and amastigotes (Sadick & Raff, 1985). Species-specific and strain-specific surface molecules of promastigotes have also been reported (Handman & Hocking, 1982; Heath et al., 1987; Kahl & McMahon-Pratt, 1987) and studies examining surface molecules of log-phase and metacyclic or stationary phase promastigotes have demonstrated that with L.major there is at least one surface antigen specific to the metacyclic forms (Sacks et al., 1985). Similarly, surface molecules specific to L.chagasi stationary-phase promastigotes have also been described (Rizvi et al., 1985).

One of the most abundant surface antigens present on Leishmania promastigotes is a glycoprotein with an apparent molecular weight of 63-68KDa which is uniformly distributed over the promastigote surface (Bordier, 1987). It is apparently present on the promastigotes of all Leishmania species so far studied (Fong & Chang, 1982; Gardiner et

al., 1984; Colomer-Gould et al., 1985; Etges et al., 1985; Misle et al., 1985) although with one Sudanese isolate it was not detected by immunoprecipitation by pooled Kala-azar serum (Heath et al., 1987). For one strain of L.major, as much as 70% of the radiolabel incorporated was found to be associated with this protein, and it has been calculated that it makes up 0.5-1% of the total cell protein in L.mexicana amazonensis and L.major promastigotes (Chang & Chang, 1987; Bouvier et al., 1985). There are conflicting reports as to the presence of this protein on the surface of amastigotes. Colomer-Gould et al (1985) readily demonstrated a 65KDa surface molecule on the surface of amastigotes of 6 species of Leishmania by surface iodination and also by immunoprecipitation by sera from infected patients. In contrast, other workers could detect the presence of this protein on L.mexicana amazonensis (Fong & Chang, 1982; Chang et al., 1986) or L.donovani amastigotes (Heath et al., 1987). However, the latter workers detected a 57KDa protein amastigotes by immunoprecipitation using human kala-azar sera whilst other experiments indicated that this protein may have common determinants with or be the same protein as the 63KDa protein. In addition, in vitro translation of the mRNA from both promastigotes and amastigotes demonstrated that both forms possessed mRNA that directs the synthesis of a 63KDa protein. Several interesting features have been demonstrated for a major surface protein around this molecular weight. Recently it has been reported that the major surface protein of L.major promastigotes (molecular weight 63DKDa) has proteinase activity and a surface protein of similar molecular weight possessing proteinase activity has subsequently been reported for all other Leishmania species tested (Bouvier et al., 1987; Chaudhuri & Chang, 1987). A role for survival in both the insect and mammalian host has

been suggested (Etges et al., 1986; Bouvier et al., 1987; Chaudhuri & Chang, 1987). Kweider et al (1987) also recently reported that the major surface protein of L.braziliensis promastigotes, with an apparent molecular weight of 65KDa, is preferentially expressed on stationary-phase promastigotes and appears to be involved in attachment of this form to macrophages, and their subsequent survival within these cells. Similarly, the major surface protein of L.mexicana mexicana and L.mexicana amazonensis promastigotes, with an apparent molecular weight of 63KDa has been shown to be involved in attachment to, and phagocytosis by macrophages (Chang & Chang, 1986; Russell & Wilhelm, 1986). In addition, it has been demonstrated that this protein is the predominant receptor site for the C3 component of the alternative complement pathway in L.mexicana mexicana promastigotes (Russell, 1987). Thus, there is a consensus that a surface protein around 63KDa is important, currently, however, it is unclear whether all workers were studying the same molecule and precise role that it performs.

Another abundant surface molecule present on Leishmania promastigotes which is at least as abundant as the 63-68KDa surface protein is a glycolipid (Russell, 1987). Leishmania donovani has a cell surface glycolipid that migrates SDS-polyacrylamide gels as a broad band with an approximate molecular weight of 15-30KDa, which is secreted into the culture medium and is preferentially expressed on stationary-phase promastigotes (Turco et al., 1984; King et al., 1987). Similarly, L.major promastigotes have a surface glycolipid that also runs as a broad band with an apparent molecular weight of 20-67KDa. This is also secreted into the culture medium, and a role in attachment, and survival in macrophages has been demonstrated (Handman & Goding, 1985; Handman et al., 1986). Promastigotes of L.mexicana synthesise a similar but antigenically distinct glycolipid (Handman et

al., 1984). The secreted form of these molecules, present in culture supernatants, is a member of the polymorphic family of carbohydrate-rich molecules which make up EF (Handman et al., 1987).

The surface membrane of leishmanias are also involved in transporting nutrients. Carrier-mediated uptake showing typical saturation kinetics and resulting in the accumulation of substrates to concentrations much higher than that in the external milieu (indicating active transport) has been demonstrated for hexose sugars (Schaefer et al., 1974, Schaefer & Mukkada, 1976; Zilberstein & Dwyer, 1984a), for the amino acids proline (Law & Mukkada, 1979; Zilberstein & Dwyer, 1985), methionine (Mukkada & Simon, 1977; Simon & Mukkada, 1977), α-aminoisobutyric acid (Lepley & Mukkada, 1983) and alanine (Bonay & Cohen, 1983), and for purines and nucleosides (Hansen et al., 1982; Aronow et al., 1987) with promastigotes of several Leishmania species. Interestingly, the uptake rates of nucleosides were much higher than those of free bases. The probable occurrence of active transport of proline into amastigotes of L.major and L.donovani in macrophages in vitro has been suggested albeit indirectly, by the studies of Zilberstein and Dwyer (1984b). Recently, the glucose transporter of L.donovani mid log-phase promastigotes has been isolated and characterised as a glycoprotein (Zilberstein et al., 1986).

By the use of enzymatic and cytochemical methods, several enzyme activities associated with the surface membrane of Leishmania have been demonstrated (Dwyer & Gottlieb, 1983, 1985). In addition to the proteinase described above, ATPase and adenylate cyclase activities have been shown, by the fine structure cytochemical studies, to reside on the cytoplasmic face of isolated surface membranes of L.donovani promastigotes (Gottlieb & Dwyer, 1982a; Dwyer & Gottlieb, 1983, 1985),

and also 3 distinct phospholipase activities have been demonstrated in isolated promastigote surface membranes of this species (Dwyer & Gottlieb, 1983). Furthermore, a protein kinase activity associated with the suface of L.donovani promastigotes, and which is at higher activity in stationary-phase cells compared to log-phase promastigotes has recently been reported (Das, Saha, Mukhopadhyay et al., 1986). However, the physiological role of these activities remains speculative (Gottlieb & Dwyer, 1982a; Dwyer & Gottlieb, 1983, 1985; Das, Saha, Mukhopadhyay et al., 1986). 3'-nucleotidases and 5'-nucleotidases have been detected on the extracellular face of isolated surface membranes and the surface membranes of intact promastigotes of L.donovani (Gottlieb & Dwyer, 1981a; Dwyer & Gottlieb, 1984) and L.mexicana mexicana (Hassan & Coombs, 1987) by cytochemical methods. Enzyme studies using intact cells and cell homogenates indicated that virtually all the activity was associated with the plasma membranes, and in both species the 3'-activity was higher than the 5' activity (Gottlieb & Dwyer, 1981a; Dwyer & Gottlieb, 1984; Hassan & Coombs, 1987). L.mexicana amazonensis promastigotes also possess these activities, the majority of which was sedimentable by centrifugation (Hassan & Coombs, 1987), and Pereira and Konigk (1981) presented evidence for the presence of a 5'-nucleotidase on the surface of L.tropica promastigotes. Hassan and Coombs (1987) also examined these enzymes in L.mexicana mexicana amastigotes and found a similar situation to that for promastigotes. In addition, 3' activity in L.donovani amastigotes was also demonstrated in preliminary studies (Gottlieb & Dwyer, 1982b). These two enzymes may have a role in the acquisition of essential purines from exogenous nucleotides. There is evidence that the observed 3'-nucleotidase activity of L.donovani promastigotes may be due to an enzyme that in vitro function as a nuclease hydrolysing exogenous nucleic acids (Zlotnick et al., 1985).

It has been suggested that the resultant nucleotides could be further catabolised by the surface located 5'-nucleotidase to yield nucleosides, which would be taken into the cells more easily (Hassan & Coombs, 1987).

The most extensively characterised enzyme associated with the external face of Leishmania surface membranes is acid phosphatase. It has been demonstrated cytochemically on the surface of L.donovani (Gottlieb & Dwyer, 1981a,b,c) and L.mexicana mexicana (Hassan & Coombs, 1987) promastigotes, and both the infective (less than 5 sub-passages) and non-infective (320 sub-passages) promastigotes of L.mexicana amazonensis (Pimenta & De Souza, 1986). Although Pimenta and De Souza (1983) could not detect activity on the suface of amastigotes of this latter species, Hassan and Coombs (1987) demonstrated activity, albeit lower than promastigotes, on the surface of L.mexicana mexicana amastigotes and Dwyer and Gottlieb (1983) reported its presence on L.donovani amastigotes. Surface membrane activity in amastigotes and promastigotes of L.mexicana mexicana and L.donovani promastigotes accounts for approximately half the total activity present in cell homogenates (Gottlieb & Dwyer, 1981c; Hassan & Coombs, 1987), the rest being cytosolic. Moreover, an acid phosphatase activity, distinct from the surface membrane bound activity, is secreted into the medium during culture in vitro of promastigotes of all Leishmania species tested with the exception of most, but not all, L.major strains (Gottlieb & Dwyer, 1982c; Lovelace & Gottlieb, 1986; Hassan & Coombs, 1987). The amount of enzyme secreted appears to vary between species, and between different isolates of the same species, L.major is unusual in that only 1 isolate out of 7 studied secreted any activity, and this was at very low levels (Lovelace & Gottlieb, 1986). The surface membrane and

secreted acid phosphatase activities of L.donovani promastigotes have been purified and extensively characterised (Glew et al., 1982; Remaley, Das et al., 1985; Lovelace et al., 1986). The surface membrane activity consists of 3 activities, one of which is characteristically resistant to L-(+)-tartrate (Remaley, Das et al., 1985). This activity and the purified secreted activity have been shown to be reistant to inactivation by oxygen radicals (Saha et al., 1985); the secreted enzyme being the more resistant. Furthermore, it has been reported that the tartrate-resistant surface membrane activity inhibits the production of oxygen radicals by stimulated phagocytic cells (Remaley et al., 1984; Remaley, Glew et al., 1985). In addition, an avirulent line of L.donovani promastigotes was found to possess lower activity of surface acid phosphatase than a virulent line (Katakura, 1985). Consideration of these data led to suggestions that acid phosphatase has a key role in survival within the sandfly alimentary canal and within macrophages in the vertebrate host (Gottlieb & Dwyer, 1982c; Dwyer & Gottlieb, 1983; Remaley et al., 1984; Remaley, Glew et al., 1985; Hassan & Coombs, 1987).

1.2.2.4 Other aspects of the biochemistry of leishmanias

Several other aspects of the biochemistry of leishmanias have been investigated quite extensively, in most cases because they are unusual and therefore provide potential targets, at which to direct novel drugs. The most studied of these are reviewed very briefly in the following paragraphs.

Energy metabolism has been extensively studied although in the main, with promastigotes (Mukkada, 1985). Promastigotes of all Leishmania species studied use oxygen (Mukkada, 1985) and for L.mexicana mexicana there is a positive correlation between oxygen levels and growth rate and cell density (Hart and Coombs, 1981).

Promastigotes contain enzymes that would allow them to utilise either carbohydrates or amino acids as energy substrates (Chatterjee and Ghosh, 1957; Martin et al, 1976; Simon et al 1983; Meade et al, 1984; Cazzulo et al, 1985; Mottram and Coombs, 1985a,b). Early studies indicated that Leishmania utilized various carbohydrates as energy substrates (Mukkada, 1985) however, more recent studies with a variety of Leishmania species have shown that promastigotes utilized glucose only towards the end of the log-phase or during early stationary-phase of growth (Mukkada et al., 1974; Marr & Berens, 1977; Cazzulo et al., 1985). Proline has been shown to be as good as, or superior to, glucose as a respiratory substrate for L.major (Law & Mukkada, 1979) and L.donovani (Krassner & Flory, 1972), and it has been suggested that Leishmania utilize amino acids as energy substrates (Mukkada, 1985). However, Hart and coworkers (1981a, 1982) found that low sub-passage (less than 10 sub-passages) L.mexicana mexicana promastigotes preferentially utilized glucose rather than amino acids throughout growth in vitro. This situation was reversed in high subpassage (more than 100 sub-passages) promastigotes and there was also a general increase in protein content and rate of respiration with increasing subpassage. These authors suggested that the use of long established promastigote culture lines by previous workers could explain the differences seen in the results, in addition it seems likely there are species differences. Where amastigotes and promastigotes have been compared, quantitative rather than qualitative differences have been found. Meade et al (1984) examined some enzymes of carbohydrate and amino acid metabolism in L.donovani amastigotes and promastigotes and found very little difference between the two stages. In contrast, L.mexicana mexicana amastigotes were found to differ from promastigotes in several ways. Amastigotes utilised fatty acids in preference to glucose in energy metabolism (Hart et al., 1981a; Hart &

Coombs, 1982) and contained much higher levels of carnitine than promastigotes which correlates well with this finding (Cooper & Coombs, 1985). Amastigotes also differ from promastigotes in their requirements for carbon dioxide and oxygen, high concentrations of the former being optimal whereas low levels of the latter seem sufficient (Hart & Coombs, 1981). The carbon dioxide fixation is probably mediated through phosphoenolpyruvate carboxykinase (PEP carboxykinase), an enzyme that together with malate dehydrogenase (MDH) is of much higher activity in amastigotes than promastigotes, carbon dioxide fixation occurring to a greater extent in amastigotes than promastigotes (Mottram & Coombs, 1985b; Coombs, 1987). Oxygen consumption is mediated via a cytochrome containing respiratory chain present in the mitochondrion of both amastigotes and promastigotes (Hart et al., 1981a).

Leishmania, like most parasitic protozoa are unable to synthesise purines de novo (Marr & Berens, 1985; Coombs, 1987) whereas they are able to synthesise de novo a portion, at least, of their pyrimidiae requirements (Marr & Berens, 1985). There have been extensive investigations of purine metabolism and a new potential drug, allopurinol riboside, is a product of the investigations (Coombs, 1987). The majority of the work has been carried out using L.donovani promastigotes (Coombs, 1987). Purine metabolism in L.donovani promastigotes and amastigotes is similar apart from adenine and adenosine metabolising enzymes which appear to be stage-specific (Marr & Berens, 1985). This is in contrast to L.mexicana mexicana where similar differences do not exist, only quantitative differences being found (Hassan & Coombs, 1985).

Polyamines (spermine, spermidine, and putrescine) have been detected in a variety of Leishmania species promastigotes (Bachrach et

al, 1979; Coombs & Sanderson, 1985). Morrow et al (1980) found all three polyamines were present in L.donovani amastigotes but could only detect putrescine and spermidine in promastigotes, whereas Coombs and Sanderson (1985) found the reverse was true for L.mexicana mexicana. The polyamines detected were present at much lower levels in amstigotes of both species compared to promastigotes. The role of polyamines remains to be elucidated, although polyamine levels have been shown to fluctuate during the growth cycle in vitro. Maximal levels were attained during log-phase growth indicating they may be involved in cell proliferation (Bachrach et al., 1979). A lot of attention has been given to ornithine decarboxylase which is a key enzyme controlling putrescine sythesis (Coombs, 1987). This enzyme is highly susceptible to the suicide inhibitor difluoromethylornithine (DFMO) and DFMO inhibits the growth of some (Kaur et al., 1986) but not all (Coombs et al., 1983) promastigotes, and therefore is a potential drug against Leishmania. DFMO has recently been passed for the use in the treatment of sleeping sickness (Coombs, 1987).

One of the reactions with which the polyamines have been implicated in Leishmania and other trypanosomatids is that of glutathione metabolism. This aspect of metabolism in trypanosomatids, including amastigotes and promastigotes of Leishmania is unique in that it involves an unusual low molecular weight cofactor that has been identified as a novel glutathione-spermidine conjugate for which the trivial name trypanothione has been proposed (Fairlamb & Cerami, 1985; Fairlamb et al., 1985; Penketh & Kennedy, 1986)

Proteinases were reported to be present in promastigotes of L.mexicana amazonensis (Chang & Fong, 1981), L.donovani, L.braziliensis (Carmargo et al., 1978) and L.tropica (Simon & Mukkada, 1983) before the recent discovery of suface activity (Etges et al., 1986) rekindled interest. The activities in amastigotes have been

little studied, however, with the only detailed research being on L.mexicana mexicana (Pupkis & Coombs, 1984). Amastigotes of L.mexicana mexicana and L.mexicana amazonensis were found to have 40, and 10-fold higher activities on azocasein compared to promastigotes, respectively, (Coombs, 1982; Pupkis & Coombs, 1984; Pupkis et al., 1986). In contrast, the activities in amastigotes of L.major and L.donovani were similar to the activities in promastigotes of these species (Pupkis et al., 1986). Analysis of the activities in L.mexicana mexicana promastigotes and amastigotes showed that those in the former were mainly particulate in nature, were not affected by cysteine proteinase inhibitors and had a broad pH optimum of between 6.0 and 7.5. In contrast, the activity in the latter was mainly soluble, was reduced by cysteine proteinase inhibitors and had a pH optimum of 5.5 (Coombs, 1982; Pupkis & Coombs, 1984). Subsequent purification of soluble proteinases from both forms of L.mexicana mexicana revealed that the major proteinase activity in amastigotes was indeed due to a cysteine proteinase with an apparent molecular weight of 31KDa, whereas a second minor activity was due to an enzyme with a molecular weight of 67KDa that showed some characteristics of a metalloproteinase. A soluble proteinase purified from the promastigotes was shown to be similar to the amastigote minor activity (Pupkis & Coombs, 1984). The amastigote-specific cyteine proteinase has been shown to be located in the amastigote-specific organelle, the "megasome" (Pupkis et al., 1986), and inhibitors of cysteine proteinases (such as leupeptin and antipain) have been shown to have activity against amastigotes within macrophages in vitro (Coombs & Baxter, 1984) indicating the potential of the proteinase as a chemotherapeutic target.

1.3 THE LEISHMANIASES

Leishmania has a wide ranging distribution occuring on all continents except Antarctica and Australasia. Its distribution and the diseases caused by different species are shown in Table 2. There are apparently no reliable figures on the annual incidence of leishmaniasis (Chance, 1981; Chang et al., 1985). Estimated figures vary from 400,000 (Chance 1981) to 12 million cases (Walsh and Warren, 1979). There is a concensus however, that it is more widespread than was recognised ten years ago.

Leishmaniasis has the most complex epidemiology of any human protozoal disease (Marsden, 1984), although it can be generalised to some extent. Apart from classical Bengali vesceral leishmanias (endemic in Bihar, Bengal, Assam, Bangladesh, possibly Orissa), visceral leishmanias of the East Coast of China (both due to L.donovani), and possibly urban cutaneous leishmaniasis due to L.tropica, the disease is a zoonosis. This means that in the majority of cases it is a disease transmitted from reservoir animals to animals and from reservoir animals to humans, but usually not from human to human or humans to animals. Transmission occurs via phlebotomine sandflies (Chang et al., 1985). However, the types of vectors, animal reservoirs and dynamics of transmission vary greatly in different parts of the world (Marsden, 1984).

1.3.1 The disease in humans

1.3.1.1 Visceral leishmaniasis

This is sometimes known as kala-azar, a Hindu name for black fever that refers to the earth-grey pigmentation of the strain seen in some cases (Chance, 1981). A small leishmanioma or papule may develop at the site of initial infection, this usually regresses without ulcerating. The incubation period of both the initial papule and

visceral disease are variable and difficult to determine. It has been known to develop within 3 weeks of exposure to infection (Molyneux & Ashford, 1983) and in experimentally infected volunteers the incubation period was 3-6 months, but it may be much longer; 9 years is the maximum recorded (Marsden, 1984). The amastigotes multiply within mononuclear phagocytes throughout the recticuloendothelial system, including the spleen, liver, lymph nodes and bone marrow (Pearson, Wheeler et al., 1983). The disease is characterised by splenomegaly and hepatomegaly accompanied by anaemia and intermittent fever. Wasting has also been specifically attributed to the infection (Harrison et al., 1986). Splenomegaly appears first, the white pulp of the spleen may become almost replaced by red pulp. This is followed by hepatomegaly where the kupffer cells of the liver become infected and increase in number possibly by local division or by recruitment of blood monocytes. These in turn become infected until small organised granulaomas occur (Chang et al., 1985) and the liver may cirrhose with subsequent ascites. Enlargement of the lymph glands is rare, but parasites have been found at gland biopsy (Marsden, 1984). There is also a marked increase in the γ-globulin level (IgG), and levels up to 5 g 100 ml^{-1} have been recorded (Pearson, Wheeler et al., 1983). This is non-specific and ineffective and is accompanied by a marked depression of cellular immune responses to Leishmania antigens during the illness (Marsden & Jones, 1985). If let untreated, the infection is thought to be fatal in at least 70% (Chang, 1981) to 90% (Molyneux & Ashford, 1983) of cases, though it is difficult to obtain accurate figures. Death usually results from secondary pulmonary or intestinal infections by normally inoffensive organisms which become pathogenic due to the general immunosuppression caused by the disease (Molyneux & Ashord, 1983; Marsden, 1984).

Post kala-azar dermal leishmaniasis is a sequel to treated and

apparently cured L.donovani infection and is seen most frequently in India (Pearson, Wheeler et al., 1983; Molyneux & Ashford, 1983; Marsden & Jones, 1985). This appears within 2 years of recovery. At this stage the parasites are present in much of the skin, but may become concentrated in hypo- or hyper-pigmented papules which occur all over the body (Chance, 1981; Molyneux & Ashford, 1983). Parasites are numerous in the lesions and unaltered skin but cannot be found in the bone marrow or deep body organs and symptoms of visceral leishmaniasis are not apparent (Molyneux & Ashford, 1983).

1.3.1.3 Cutaneous leishmanias

Several species from the Old and New World are capable of causing cutaneous leishmaniasis, but the basic pathogenesis is the same (Marsden & Jones, 1985) and cutaneous leishmanias in South America is normally sufficiently similar to that in the Old World that the two were not distinguished clinically by early workers (Molyneux & Ashford, 1983). However, the lesions due to the former tend to be more florid than those of the latter and may be much more persistent (Molyneux & Ashford, 1983). The time between infection and first appearance of the pupule is usually a few weeks and under experimental conditions as little as 4 weeks are necessary to produce a visible lesion (Molyneux & Ashford, 1983; Marsden & Jones, 1985). The papule formed eventually ulcerates or heals without treatment usually within 3-18 months after infection leaving a characteristic scar (Chance, 1981). In its simplest form classic cutaneous leishmaniasis involves only one lesion, although multiple lesions can occur due to an individual being bitten several times by different sandflies or because of multiple probing by the same sandfly (Beach et al., 1984; Killick-Kendrick et al., 1985). In addition, rapid bloodstream dissemination can occasionally occur and dozens of lesions may appear

(Chance, 1981; Marsden & Jones, 1985). There are other clinical manifestations which do not conform to the pattern described above.

Diffuse cutaneous leishmaniasis (DCL) is usually caused by members of the L.mexicana complex, but also in L.aethiopica infections (Chance, 1981; Molyneux & Ashford, 1983). It is difficult to assess the proportion of infections that go on to develop DCL. In Ethiopia it is rare, whereas in South America the prevalence varies from place to place. For example, in northern Brazil about 25% of infections due to L.mexicana complex are thought to develop DCL, while in Belize and Mexico it is almost unknown (Molyneux & Ashford, 1983; Marsden & Jones, 1985). The disease starts as a localised papule lesion which does not ulcerate. Satellite lesions subsequently develop around the initial papule and organisms metastasize to distant areas of the skin, often to the face and extremities, producing nodules 1-2 cm in size over the surface of the body (Chance, 1981; Pearson, Wheeler et al., 1983). The nodules tend to be non-ulcerating and contain a mass of infected macrophages (Molyneux & Ashford, 1983; Marsden, 1984). The disease appears to be due to the failure of the host to mount a cell mediated response to leishmanial antigen, though they respond normally to other antigenic stimuli (Chance, 1981; Marsden & Jones, 1985).

Leishmaniasis recidivans (also known as tuberculoid or lupoid leishmaniasis) occurs occasionally with L.tropica infections and in some ways is similar to DCL and yet very different in other aspects. The initial lesion apparently heals, but small lesions develop on or near the margin of the scar and these remain active and extend over a wide area of the skin (Molyneux & Ashford, 1983; Marsden & Jones, 1985). Amastigotes are difficult to find or isolate from the lesions and there is a strong cell mediated response (Marsden & Jones, 1985). Both these infections (DCL and leishmaniasis recidivans) are chronic

in nature and do not respond well to chemotherapy (Molyneux & Ashford, 1983).

1.3.1.3 Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis or Espundia is found predominantly in South America where members of the L.braziliensis complex are usually implicated as the causative agents. It has, however, also been reported elsewhere. For example, in Sudan over 50 cases have been documented; these cases appear to be due to L.donovani (Marsden & Jones, 1985; Marsden, 1986). Individuals usually initially develop cutaneous lesions. For example, more than 90% of patients with mucosal diseases have scars of a previous cutaneous infection (Marsden, 1984), which then are apparently cured. A percentage of (see below) these individuals can then go on and develop metastatic lesions in the oral-nasopharyngeal tissues although the time of onset of mucosal disease is variable ranging from months to 20-30 years (Chance, 1981; Marsden, 1984). Over 80% of the mucosal lesions affect the nose but there may be spread to the buccal cavity, pharynx and larynx. These lesions do not self-heal but continue to invade the tissues which become blocked with granulomatous infiltration; eventually the tissues are eroded producing gross deformities of the face (Molyneux & Ashford, 1983). Amastigotes are very difficult to isolate from thee lesions (Pearson, Wheeler et al., 1983). If untreated, death frequently results due to suffocation because of blockage of air passages, or through bacterial infection (Molyneux & Ashford, 1983). The percentage of patients with cutaneous lesions who subsequently go on to develop mucocutaneous disease is difficult to estimate, although Pessoa's (1941) report that 80.9% of patients with skin lesions of more than one year's duration develop mucous metastasis is widely quoted (Marsden, 1986). Recently, Marsden (1986) has questioned these figures, suggesting they were

probably from a hospital series and as such cannot be relied upon since patients with severe disease will naturally seek hospital assistance. Marsden and his co-workers have studied a rural population in the Tres Bracos area of Brazil, which is endemic for L.braziliensis braziliensis, and found that under 5% of patients with cutaneous leishmaniasis developed mucosal disease (Marsden, 1986).

1.3.2 Control of leishmaniasis

Leishmaniasis can be potentially controlled at a variety of points in the transmission cycle. They are: **(1)** control and avoidance of sandflies; **(2)** destruction of animal reservoirs; **(3)** treatment of infected people; **(4)** vaccination (Marsden, 1984). Often it is only feasible to control the disease at one of these points, usually by treatment of infected people, although attempts to improve control often involve attack at several points in the transmission cycle.

1.3.2.1 Control of vectors and reservoir hosts

Although the vectors are very susceptible to DDT and other insecticides (there appears to have been only one report of insecticide resistance in India; Ward, 1985), there are many difficulties in their control. For example, the epidemiology of leishmaniasis in different parts of the world are not identical and so each requires its own special control strategy. In addition, with only one stage - the adult - is control feasible as the immature stages are widely dispersed, subterranean and inaccessible (Molyneux & Ashford, 1983; Marsden, 1984; Ward, 1985). Control of leishmaniasis by eradication of sandflies by insecticide spraying is only practical (and successful) where in the insects infest human dwellings and surrounding areas, that is they are peridomestic (Molyneux & Ashford, 1983; Marsden, 1984). When this is the situation it has been observed in many countries malaria control campaigns have often led

accidentally to reduction in sandfly densities and in leishmaniasis morbidity, while resurgence of leishmaniasis morbidity has been observed in numerous areas after cessation of these campaigns (Marinkelle, 1980). Thus, in Europe, China, India and Brazil there has been a reduction in the incidence of visceral leishmaniasis (L.donovani complex) following specific anti-Leishmania campaigns and also following control of malaria with DDT (Marsden, 1984; Ward, 1985). In Peru, cutaneous leishmaniasis (L.peruviana) was reduced after peridomestic application of DDT to eliminate Oroya Fever (bartonellosis) (Molyneux & Ashford, 1983; Ward, 1985). Urban cutaneous leishmaniasis (L.tropica) in the USSR has virtually been eradicated by a combination of planned DDT spraying in the homes of people with leishmaniasis and also as a result of a campaign against malaria (Marinkelle, 1980). Where the transmission of leishmaniasis is sylvatic, as is the case with L.major in the Old World and the majority of cutaneous and mucocutaneous infections in the New World, control by insecticides is impractical and where it has been attempted it has proved unsuccessful (Molyneux & Ashford, 1983; Ward, 1985).

An alternative to the use of insecticides is the destruction of sandfly habitats. This has often occurred accidentally, for example in China extensive land reclamation in certain parts of the desert in Xinjiang has caused a change in local environmental conditions making it unsuitable for sandflies (Chao-Tsung, 1985). Similarly in Malta socio-economic development has reduced suitable breeding sites and contributed to the increase in annual incidence of visceral leishmaniasis (Ward, 1985). Destruction of the breeding places of sandflies has been successful in controlling L.major in the USSR, although it also coincides with destruction of the breeding places of the reservoir host as this is where the sandfly lives (Molyneux &

Ashford, 1983; Ward, 1985).

In some it is possible to control leishmaniasis by treatment or elimination of the reservoir host. In situations where humans are the only known hosts, dissemination of the disease can be prevented by effective treatment of cases (see below) (Marinkelle, 1980). In the majority of other cases a variety of mammals are the reservoir hosts and in certain areas elimination of these at the same time as implementing other control measures has proved successful. For example, in China visceral leishmaniasis was nearly eradicated by mass elimination of dogs in some endemic areas. In other areas control was affected by killing of infected dogs from patients' homes as well as treatment of patients and insecticide spraying (Chao-Tsung, 1985). Similarly, antirabies campaigns involving the killing of dogs as well as malaria eradication programmes and specific measures against sandflies have greatly reduced the incidence of visceral leishmanasis in several localities and eradicated it from many areas in the Mediterranean and Central Asia (Molyneux & Ashord, 1983). Major campaigns to control the rodent reservoirs of L.major (Rhombomys opimus) in the USSR combined with insecticide spraying have been successful in localised areas (Molyneux & Ashford, 1983) The rodents burrows were mapped by aerial photography, and then the rodents systematically destroyed by poisoning or deep ploughing of burrows. Recolonisation was prevented by the construction of physical barriers such as irrigation canals (Chance, 1981; Molyneux & Ashford, 1983).

1.3.2.2 Immunization

Cutaneous leishmaniasis in the Middle East (caused by L.tropica and L.major) is usually followed by life long immunity to reinfections (Chance, 1981; Marsden, 1984; Greenblatt, 1985, but also see Killick-Kendrick et al., 1985). A crude form of vaccination or leishmanisation

has been carried out in this area for centuries. This has usually been carried out on infants and involves material from an active some being inoculated using a thorn into an area of skin which would have covered by clothing. This results in a lesion and subsequently a scar, and immunity (Molyneux & Ashford, 1983; Marsden, 1984; Greenblatt, 1985). The modern equivalents of this are the vaccination programmes, mainly involving military personnel, against L.major which were carried out in Israel and in the USSR, and the en masse programme that is underway in Iran (Blackwell et al., 1986). Tens of thousands of individuals in the USSR and 5000 in Israel (Greenblatt, 1985) have been successfully vaccinated using live, cultured promastigotes; it was found to be important to use a recently isolated virulent strain (Molyneux & Ashord, 1983). There were, however, problems with using a live promastigote vaccine, such as the development of large unsightly chronic lesions. These were sufficiently common in Israel that vaccination was not compulsory or carried out en masse (Molyneux & Ashford, 1983). In time the vaccination campaigns in Israel were discontinued because of the severity of the lesions that developed. In Iran, however, a massive programme of leishmanization is in progress involving nearly a million individuals (Blackwell et al., 1986). The use of live vaccines is not acceptable with organisms such as L.braziliensis braziliensis or L.mexicana amazonensis when there may be a dangerous sequel to infection (Chance, 1981). Moreover, although in murine laboratory models of cutaneous leishmaniasis L.major protects against members of the L.mexicana and L.braziliensis complexes (Greenblatt, 1985), this may not be the case in humans (Killick-Kendrick et al., 1985). Other problems associated with the live vaccine were secondary infection of lesions (this occurred in up to 25% of the cases), reactivation of skin diseases, and interference with the immune response to other vaccines (e.g. triple vaccine)

(Marsden, 1984; Greenblatt, 1985). Thus the immediate prospect of a multivalent vaccine of this type are remote (Chance, 1981). Furthermore, although killed and attenuated organisms have been used successfully in the protection against cutaneous leishmaniasis using experimental animal models, they have proved unsuccessful when tried in humans (Greenblatt, 1985); some more recent studies with the former, however, show a little more promise (Greenblatt, 1985; Blackwell et al., 1985).

1.3.2.3 Chemotherapy

The treatment of leishmaniasis is in an unsatisfactory state, not only are there few effective drugs available, but also they are expensive, have many side effects and are difficult to administer (McGreevy & Marsden, 1986). In addition, there are marked differences in sensitivity to the first-line drugs among strains and species of Leishmania; indeed at least one truly resistant isolate has been reported (Geary et al., 1986). The first-line drugs in the treatment of leishmaniasis are the pentavalent antimonial Pentostom (sodium stibogluconate; Burroughs Wellcome) and Glucantime (meglumine antimoniate; Rhodia, Specia), the latter is wide used in Francophone and Latin American countries (Marsden, 1984). These two drugs appear to be equally effective (Marsden, 1984). China and India have manufactured their own antimonials, Pentamidine and Amphotericin B are usually second-line drugs for treatment, and have severe toxicity problems (McGreevy & Marsden, 1986).

Usually for Old World cutaneous leishmaniasis and infections caused by the L.mexicana complex in Central America specific treatment is not indicated as the lesions resolve naturally. Although multiple lesions, on a cosmetically important site and of duration longer than 6 months, are all strong indications for antimonial therapy which

these forms respond to readily (Molyneux & Ashford, 1983; Marsden & Jones, 1985). Where the infection is due to L.braziliensis braziliensis, antimonial therapy is mandatory because of the danger of metastases. Treatment of mucucutaneous leishmaniasis is difficult and requires high doses of antimonials. If these fail Amphotericin B is the drug most commonly used, and can be very effective (Molyneux & Ashford, 1983; Marsden, 1986). Similarly, DCL does not respond well to chemotherapy (Molyneux & Ashford, 1983), pentavalent antimonials are the first line drugs and are effective in many cases. Where they fail Pentamidine or Amphotericin B are used (Molyneux & Ashford, 1983; Marsden, 1984; Marsden & Jones, 1985; McGreevy & Marsden, 1986). Visceral leishmaniasis is usually responsive to antimonial therapy with cure rates of 70-100% having been recorded (Marsden & Jones, 1985). The disease in Brazil, India and China appears more responsive to treatment than in the Mediterranean region and Africa. In these latter areas and in relapsed cases alternate therapy with Pentamidine or Amphotericin B may be necessary (Molyneux & Ashford, 1983; Marsden & Jones, 1985).

There have been no new clinically useful drugs for leishmaniasis for very many years. Currently, however, there are several possibilities undergoing trials. These include allopurinol riboside, WR 6026 (a primaquine analogue), Ketoconazole and Pentostam encapsulated in liposomes.

1.4 HOST-PARASITE INTERACTIONS IN LEISHMANIAS

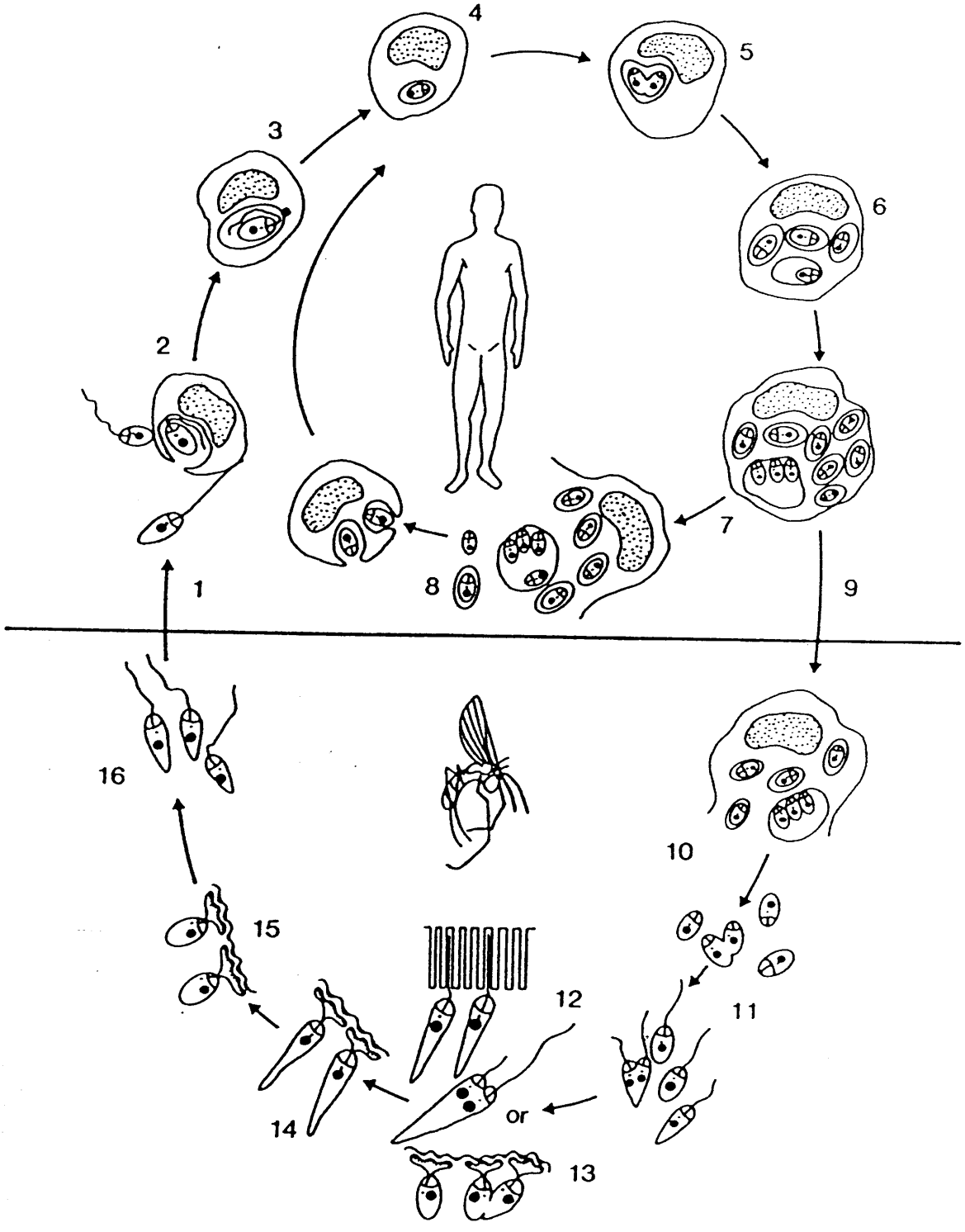
The digenetic life-cycle of Leishmania (see Fig. 3) means that it encounters very different environments - the digestive tract of the sandfly vector and a parasitophorous vacuole within a mononuclear phagocyte when in the vertebrate host.

Fig. 3. Leishmanial life-cycle in sandfly and in mammalian hosts

1. Delivery of metacyclic promastigotes into human skin by the bite of the sandfly vector; 2. attachment and engulfment by phagocytosis of promastigotes by a macrophage; 3. fusion of phagosome containing a promastigote with lysosome in a macrophage; 4. differentiation of promastigote into amastigote in the phagolysosome of the infected macrophage; 5. multiplication of an amastigote in a parasite-containing or parasitophorous vacuole; 6. formation of large parasitophorous vacuole and continuing replication of intravacuolar amastigotes; 7. rupture of heavily parasitised macrophage and release of amastigotes; 8. phagocytosis of released amastigotes by a macrophage; 9. ingestion of parasitised macrophage by sandfly after a blood meal taken from an infected person or reservoir animal; 10. rupture of the ingested macrophage and release of amastigotes in the abdominal mid-gut of the sandfly; 11. replication of amastigotes and their differentiation into promastigotes; 12. replication of promastigotes (termed nectomonads for L.mexicana mexicana, L.mexicana amazonensis and L.major) in the abdominal midgut and insertion of their flagella into the midgut epithelial cells; 13. replication of L.braziliensis complex in the pylorus and ileum of the sandfly hindgut as paramastigotes with broadened flagella attach to the chitinous cuticular epithelium via hemidesmosomes; 14. forward movement of promastigotes to thoracic midgut as haptomonads (L.mexicana amagonensis, L.braziliensis braziliensis and L.major) or "short promastigotes" (L.mexicana mexicana) which interdigitate between the microvilli or attach to the cuticular epithelium via hemidesmosomes at the standard valve; 15. sessile paramastigotes with broad flagella attached to the chitinous wall of oesophagus pharynx and cibarium; 16. metacyclic promastigotes found in the anterior thoracic midgut and proboscis of the sandfly.

Adapted from Chang et al (1985) with additional experimental information from Warburg et al (1986) and Walters et al (1987).

Fig. 3.



1.4.1 Leishmanias and the sandfly vector

1.4.1.1. The sandfly vector

As far as is known, the natural vectors of all Leishmania species belong to the subfamily Phlebotominae of the family Psychodidae. There are about 600 species distributed in all zoogeographical regions of the world (Molyneux & Ashford, 1983). Each vector of Old World Leishmania tends to transmit only one species of the parasite, in contrast it has been reported that New World vectors can support various Leishmania species (Schlein, 1986). Several well-known species of sandfly vectors for leishmaniasis are listed in Table 3. Sandflies have a holometabolous life-cycle with terrestrial larvae (4 instars) which are unlike the adults, a sessile pupa, and male and female adult flies. Fig. 4 shows the natural life-cycle and metamorphosis of a sandfly. Both male and female flies obtain sugar meals, either from plants or possibly honeydew (Killick-Kendrick, 1979; Young et al., 1980; Ward, 1985; Schlein, 1986). Only the females take blood and they are pool feeders using their mandibles and maxillae to cut a wound in the host's skin and sucking up the blood that accumulates (Ward, 1985). A blood meal is usually acquired before the eggs develop. Although in captivity female flies usually die after egg laying, it appears that under natural conditions 2-3 cycles of feeding and egg laying are normal but more than 5 would be exceptional (Molyneux & Ashford, 1983).

1.4.1.2 The life-cycle of Leishmania in the sandfly

The generalised gut structure of a sandfly is illustrated in Fig. 5. The life-cycle of leishmanias in the vector as described below have been elucidated mainly by studying experimentally infected sandflies kept in the laboratory and as such may not always represent what occurs in a natural infection (Killick-Kendrick, 1979). A sandfly

Table 3: Known sandfly vectors for *Leishmania* species infecting humans.

Sandfly species	*Leishmania* species	Location
	Old World	
Phlebotomus argentipes	*L.donovani donovani*	India
P.ariasi	*L.donovani infantum*	France
P.chinensis	*L.donivani*	China
P.dubosqi	*L.major*	Senegal
P.longipes	*L.aethiopica*	Ethiopia
P.longicuspis	*L.donovani infantum*	Algeria, Morocco, Tunisia
P.major syriacus	*L.donovani infantum*	Israel
P.martini	*L.donovani*	Kenya
P.orientalis	*L.donovani*	Sudan
P.papatasi	*L.major*	Saudi Arabia, Israel, USSR
P.pedifer	*L.aethiopica*	Ethiopia, Kenya
P.perniciosus	*L.donovani infantum*	Malta, Tunisia, Algeria
P.salehi	*L.tropica*	India
P.sergenti	*L.tropica*	USSR, Iraq
P.tobbi	*L.donovani infantum*	Cyprus
	New World	
Lutzomyia flavscutellata	*L.mexicana amazonensis*	Brazil
Lu.longipalpis	*L.donovani chagasi*	S. & C. America
Lu.olmeca	*L.mexicana mexicana*	Belize, Mexico
Lu.trapidoi	*L.braziliensis panamensis*	Panama
Lu.umbratilis	*L.braziliensis guyanensis*	Brazil, Fr.Guiana
Psychodopygus wellcomei	*L.braziliensis braziliensis*	Brazil

From Chang *et al* (1985).

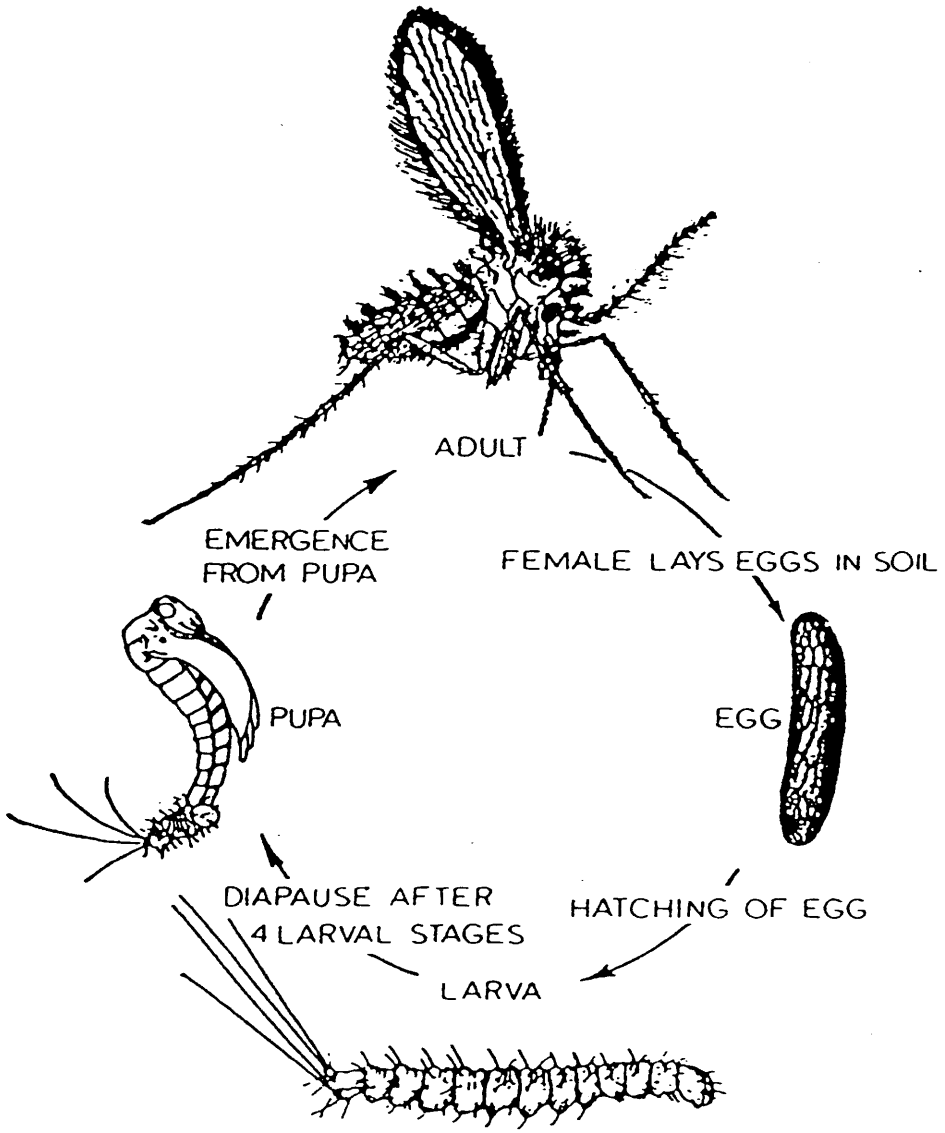


Fig. 4. Natural life-cycle and metamorphosis of a sandfly.

From Chang et al (1985).

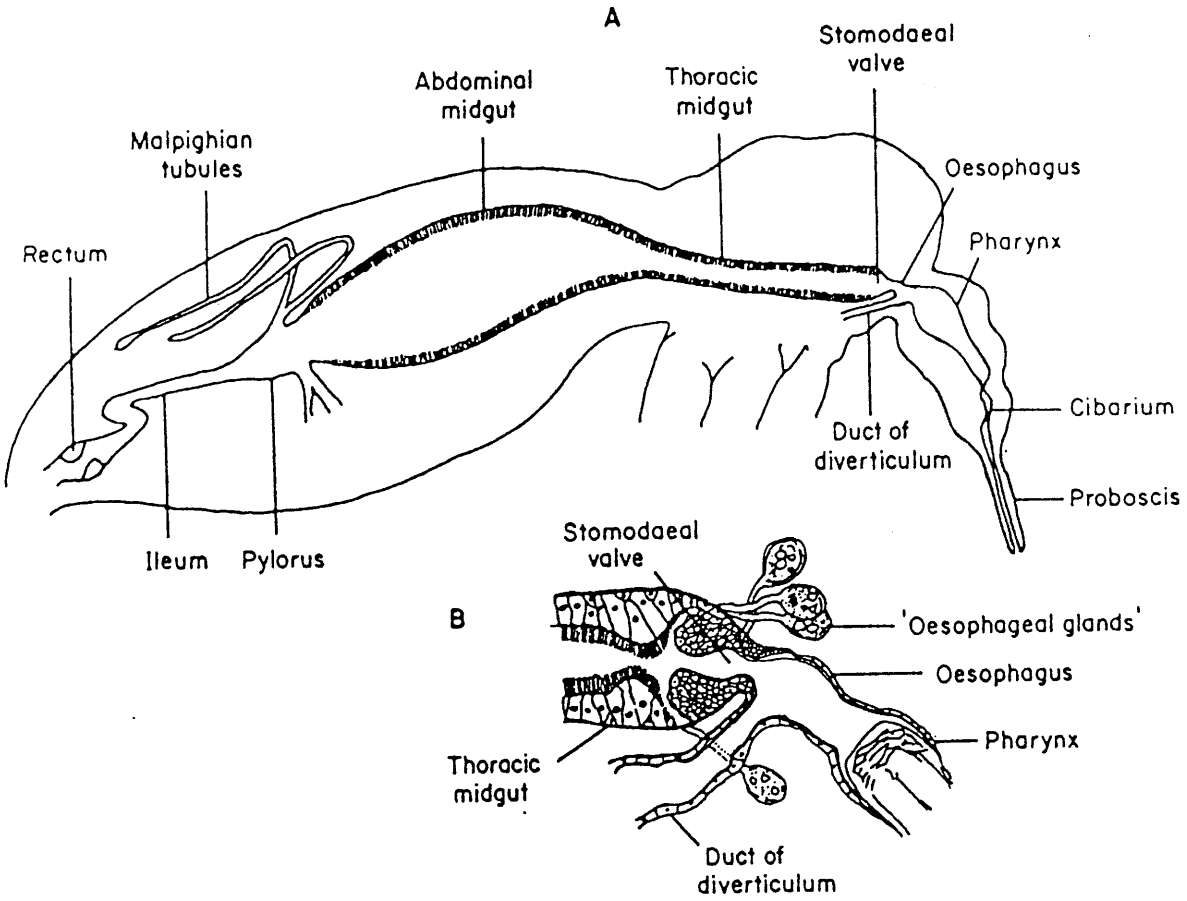


Fig. 5. Generalised gut structure of a sandfly.

(A) Sites of development of leishmanias.
(B) The stomodael valve and adjacent parts.

From Killick-Kendrick (1979).

becomes infected when it takes a blood meal containing macrophages infected with amastigotes or free amastigotes. In the first moments of infection, the blood and parasites are exposeed to the anticoagulant saliva, to considerable pressure variations in the proboscis, to the mechanical action of the cibarial and pharyngeal teeth, and usually to decreasing temperature (Killick-Kendrick, 1979; Molyneux & Ashford, 1983). The ingested blood passes immediately to the midgut without being stored in the crop (Schlein, 1986). Engorgement is quickly followed by production of the mucilaginous peritrophic membrane which is secreted by the abdominal midgut epithelium and which enclosed the blood meal (Killick-Kendrick, 1979; Molyneux & Ashford, 1983). The approximate numbers of amastigotes ingested by the fly is unknown but it must be very small because even when sandflies are experimentally fed on heavily infected cutaneous lesions it is very difficult to find parasites in stained preparations of blood meals (Strangways-Dixon & Laison, 1960; Killick-Kendrick, 1979; Walters et al., 1987). Those parasites present are often not found within intact macrophages (Killick-Kendrick, 1979; Strangways-Dixon & Laison, 1966; Walters et al., 1987). The early development of the amastigotes - before transformation into promastigotes - has only been examined in a few species, presumably because of the difficulty of finding parasites in fresh blood meals (Killick-Kendrick, 1979). Where it has been examined, workers have reported that the amastigotes of L.donovani in Phlebotomus argentipes (Shortt et al., 1926), L.mexicana mexicana in several sandflies (Warburg et al., 1986) undergo at least one cell division cycle before transformation into the promastigote. This appears to have been accepted as part of the cycle in the sandfly for all Leishmania species (Killick-Kendrick, 1979; Chang et al., 1985), although confirmation is required. Relevant to this is the finding from in vitro studies that the transformation of amastigotes to

promastigotes involves a division step (Hart et al., 1981c).

The transformation in the sandfly of amastigotes into the typical invertebrate form, the promastigote and the development of promastigotes of different morphologies which coincided with their migration to different part of the sandfly gut was described by early workers using light microscopy for several Old World (Alder & Theodor, 1926a; Shortt et al., 1926, Alder & Theodor, 1931; Alder et al., 1938) and New World (Hertig & McConnel, 1963; Strangways-Dixon & Laison, 1966) parasite-sandfly combinations. These observations have been confirmed by more recent studies (Laison et al., 1977; Killick-Kendrick, 1979; Walters et al., 1987) and extended using transmission (Killick-Kendrick et al., 1974; Molyneux et al., 1975; Killick-Kendrick, 1979; Walters et al., 1987) and scanning (Warburg et al., 1986) electron microscopy. For L.mexicana amazonensis it is thought that after the initial division of amastigotes, which are still within the bloodmeal which is encased in the peritrophic membrane, they transform to long slender promastigotes which have been termed nectomonads. This term was resurrected by Killick-Kendrick et al (1974) when that showed that the promastigotes in the abdominal midgut were morphologically distinct (on the basis of cell and mitochondrion morphology, microtubule number, size of cell and orientation of the kinetoplast with respect to the nucleus) from other promastigotes and cell forms that occurred later on and at different locations within the sandfly gut. Using similar techniques, other workers have subsequently confirmed the presence of nectomonads at this location in sandflies infected with L,major (Warburg et al., 1986) and L.mexicana mexicana (Walters et al., 1987). The presence of this morphologically distinct type of promastigotes was not been seen in preliminary studies with L.infantum and thus their occurrence cannot be said to be

typical for all species (Killick-Kendrick, 1979). The promastigotes undergo intense division within the bloodmeal and during this time some of the promastigotes themselves or their flagella become embedded in the peritrophic membrane and remain there as it is broken up and passed out (Killick-Kendrick et al., 1974; Molyneux, 1977; Walters et al., 1987). The majority of the promastigotes, however, move out of the bloodmeal through an opening at the anterior end of the membrane or escape as the membrane breaks up towards the end of digestion (Molyneux, 1977; Killick-Kendrick, 1979). For all leishmanias infecting humans with the exception of those of the braziliensis complex, the promastigotes now colonise the abdominal midgut, this is known as suprapylarian development. Members of the braziliensis complex, however, colonise the pylorus and ileum of the hindgut, this is known as peripylarian development. The latter is less well known than the former, but appears to be different and will therefore be considered separately.

1.4.1.2.1 Suprapylarian development

The promastigotes in the abdominal midgut are initially free in the lumen, and the nectomads of L.mexicana amazonensis have been reported to divide (Killick-Kendrick, 1979), although it it unclear of this occurs with other species (Killick-Kendrick, 1979). Some remain free in the lumen, and the nectomonads of L.mexicana mexicana (Walters et al., 1987) and L.mexicana amazonensis (Killick-Kendrick et al., 1974) appear to be orientated in "longitudinal masses". Other promastigotes became attached to the abdominal midgut wall (Shortt et al., 1926) and more detailed studies have shown that they interdigitate with the epithelial microvilli via their flagella (Killick-Kendrick et al., 1974; Warburg et al., 1986; Walters et al., 1987). The promastigotes then migrate anteriorly to such an extent

that the thoracic midgut, especially the anterior part, often becomes grossly swollen with parasites. Although this migration seems common to all mammalian suprapylarian leishmanias (Killick-Kendrick, 1979). There are differences between species and it is becoming apparent from electron microscopic studies that species and subspecies have their own characteristic development within the sandfly. Thus, with L.mexicana amazonensis as the nectomonads move forward they progressively transform, although with no clear cut transition (i.e. no intermediate forms), to another promastigote type, the shorter haptomonad (Killick-Kendrick et al., 1974), so that in the anterior thoracic midgut and at the stomodeal valve haptomonads are the only form present. In this region the haptomonads are closely packed together and do not appear to divide. Although the anterior thoracic midgut is lined with microvilli, none of the haptomonads attach to the epithelial lining (Killick-Kendrick et al., 1974). In contrast, L.tropica (Adler & Theodor, 1926a) and L.donovani (Shortt et al., 1926) promastigotes attach to the epithelium in the anterior thoracic midgut and divide at this site. Also, Laison et al (1977) reported dividing forms of L.chagasi at this site. With L.mexicana mexicana (Walters et al., 1987) as the nectomonads migrate anteriorly they appear to shorten and transform (but without any apparent intermediates) into "short promastigotes" rather than haptomonads. Also, both nectomonads and short promastigotes are present in the anterior thoracic midgut stomodael valve region, although the short promastigotes predominate as the infection matures. Both promastigote types occur free in the lumen of the anterior thoracic midgut and also interdigitate between the microvilli which are present at this site. Moreover, the "short" forms are commonly in division, and this characteristic together with the other differences described led Walters et al (1987) to consider them distinct from the haptomonads

described by Killick-Kendrick et al (1974) and to represent a new form. For L.major the situation appears to be different again (Warburg & Schlein, 1986; Warburg et al., 1986). The abdominal nectomonad forms as they migrate transform, via intermediate dividing forms which have their flagella inserted between the microvilli of the thoracic midgut, to short slender highly active unattached promastigotes with a long flagellum. These forms are present in large numbers in the anterior thoracic midgut and stomodael valve/oesophagus region and are identical in morphology to the promastigote forms seen subsequently in fluid egested during force feeding (Warburg & Schlein, 1986; Warburg et al., 1986) and in the proboscis of sandflies infected with other Leishmania species (Killick-Kendrick, 1986). A similar situation has been reported for L.infantum (Adler & Theodor, 1931). With L.major, haptomonads do occur attached at the stomodael valve, although it is unclear which form they arise from (Warburg et al., 1986). Thus, current data suggest that their is a large degree of variation between species with respect to the parasite forms that occur in the sandfly midgut.

As the promastigotes move anteriorly they form a "plug" at the anterior end of the thoracic midgut and stomodael valve (Adler & Theodor, 1926a; Shortt et al., 1926; Killick-Kendrick et al., 1974; Walters et al., 1987) and for L.donovani (Killick-Kendrick, 1979) and Leishmania mexicana mexicana (Walters et al., 1987) it has been reported that they appear to be embedded in a gel-like substance, the origin of which is unknown. Within the "plug" the parasites have a definite orientation, their flagella reaching forwards towards the head of the fly, and where they touch the cuticular epithelium of the stomodael valve they become attached (Adler & Theodor, 1926a; Killick-Kendrick et al., 1974; Warburg et al., 1986; Walters et al., 1987).

Killick-Kendrick et al (1974) found that the haptomonads of L.mexicana amazonensis attached to the cuticular epithelium via their flagella. The distal end of the flagellum expands to form a disc-like attachment organelle which these workers considered to be a hemidesmosome. Subsequently Walters et al (1987) reported that the short promastigotes of L.mexicana mexicana formed hemidesmosomes at the stomodael valve. Following colonisation of the stomadael valve, the parasites spread to the oesophagus and pharynx. This is accompanied by a further reduction in the size of the parasites which change to another morphological form - paramastigotes - distinguished by having the kinetoplast lying beside or slightly posterior to the nucleus (Killick-Kendrick, 1974). These forms occur in all leishmanias where the cycle in the fly has been studied in detail. They attach to the cuticular epithelium via hemidesmosomes and do not appear to divide (Molyneux et al., 1975; Killick-Kendrick, 1979; Warburg et al., 1986; Walters et al., 1987). In electronmicrographs of L.mexicana amazonensis (Killick-Kendrick et al., 1977) and L.mexicana mexicana (Walters et al., 1987) apparently healthy intact paramastigotes, as well as paramastigotes in various stages of degeneration, were seen in the pharynx and it has been suggested that this region represents a barren environment to the parasites and that colonisation of the pharynx by the apparently short lived paramastigotes is maintained by continual migration of parasites from the valve (Killick-Kendrick, 1979). In addition, for L.infantum, L.chagasi and L.major, forms similar or identical to those subsequently seen in the proboscis or in the fluid egested during force feeding have been reported in this region (Adler & Theodor, 1931; Laison et al., 1977; Warburg & Schlein, 1986). From the pharynx, the infection may spread forwards to the cibarium where paramastigotes morphologically identical to those in the pharynx attach to the cuticular lining (Killick-Kendrick, 1979).

Finally, promastigotes which have characteristic behaviour and morphology may occur in the proboscis. In fresh preparations of the mouthparts, the only parasites seen at this site are small unattached promastigotes with long flagella (up to twice the length of the cell body) which rapidly swim up and down the lumen and have never been seen in division (Killick-Kendrick, 1979; 1986). It is unclear, however, whether these forms develop from paramastigotes or migrate directly from the thoracic midgut where they also occur at least in the case of L.major and L.infantum.

1.4.1.2.2 Peripylarian development

The initial establishment of infection in the sandfly by members of the braziliensis complex is in the pylorus and ileum of the fly (Molyneux, 1977; Killick-Kendrick, 1979). This development was first described by Hertig and McConnell (1963) while observing the life-cycle of L.braziliensis panamensis in Panamanian sandflies. These workers described the presence of short, broad parasites which were attached to the cuticular epithelium of the hand triangle (pylorus and ileum). More recently, Killick-Kendrick, Molyneux et al (1977) confirmed this finding with L.braziliensis braziliensis and went onto describe the ultrastructure of the parasites in the pylorus and ileum. They found that the parasites in this region were in the form of paramastigotes which attach to the cuticular epithelium via hemidesmosomes. These forms multiply in this site and hind triangle infections tend to persist throughout the life of the sandfly (Hertig & McConnell, 1963; Killick-Kendrick, Molyneux et al., 1977). The sequence of development beyond this is imperfectly known, although the parasites do migrate anteriorly, and become attached in the anterior thoracic midgut, stomodael valve, and oesophagus (Hertig & McConnell, 1963). The parasites in these latter two sites again attach via

hemidesmosomes and conform to the haptomonad and paramastigote forms, respectively (Killick-Kendrick, Molyneux *et al.*, 1977). They can then go on to establish infections anterior to these sites and eventually in the proboscis; the only forms reported in this latter location have similar characteristics to leishmanias with suprapylarian development that occur in the same sites (Hertig & McConnell, 1963; Killick-Kendrick, 1979).

The time period from uptake of amastigotes in to the sandfly to the production of promastigotes in the proboscis is usually in the range 6-25 days, and is dependant upon the *Leishmania* sandfly system (Chang *et al.*, 1985).

1.4.1.3 Metacyclic promastigotes and their relation to transmission

One of the most controversial areas of sandfly-*Leishmania* relationships is the mode of transmission of the parasite to the mammal. The controversy has centred around two questions. Firstly, whether it is essential for the life-cycle to culminate in the production of infective metacyclic forms in the proboscis of the fly or whether transmission can be accomplished by the regurgitation of metacyclics or other forms of parasites from parts of the alimentary tract posterior to the mouthparts. Secondly, whether the multiple probing which is common in infected sandflies is caused by the presence of parasites in the cibrarium and beyond which interfere with the functioning of mechano- or chemo-sensilla which are present in these sites or whether it is simply caused by mechanical blockage of the oesophagus and stomodael valve by the large numbers of parasites which occur there. These two questions are interrelated and recent work *in vitro* with cultures of promastigotes, and *in vivo* with sandflies has shed some light on the situation.

1.4.1.3.1 Metacyclic promastigotes in culture

Early studies using promastigotes from cultures gave variable results as to their infectivity to humans or experimental animals. Some workers (Adler & Theodor, 1927) reported that they were unable to obtain any infection, whereas others produced infections with relative ease (Hindle, 1931; Berberian, 1939). The results of some of these latter workers also indicated that promastigotes taken early after inoculation of the cultures were less able to produce an infection than those taken later. Furthermore, Berberian (1939) observed with cultures of L.tropica in NNN medium that after 8 days the majority of parasites present were the "short metacyclic forms" and attributed the increased infectivity of older cultures to the production of these forms. More recently, Gianinni (1974) produced more convincing evidence that infectivity in vitro was adapted to growth phase, log-phase promastigotes of L.donovani being less infective to hamsters than stationary-phase promastigotes. Both populations of cells resulted in infections but the former gave rise to lower mean parasite numbers and greater mean times to death than the latter. There is now a large body of evidence that infections metacyclic promastigotes are produced during in vitro cultivation. Their generation is related to the growth cycle and is restricted to non-dividing organisms, thus the actively dividing cells in log-phase populations are in general less infective then the non-dividing stationary-phase cells. In animal studies, inoculation of log-phase promastigotes generally results in fewer animals becoming infected, and a much longer time for the infections to become apparent as well as lower parasite burden at a particular time, compared to infections resulting from the use of stationary-phase promastigotes. In vitro studies using macrophages have produced clear cut results. Log-phase cells give rise to intracellular infections which are either severely reduced by 72 hours

or remain static, whereas stationary-phase cells survive and multiply extensively so that not only the number of amastigotes per macrophage increases but the % infection also rises. Infectivity results such as these have been obtained for L.donovani and L.chagasi (Doran & Herman, 1981; Keithly & Bienen, 1981; Rizvi et al., 1985; Wozencraft & Blackwell, 1987) and it was calculated that 2% of log-phase promastigotes became established in vivo whereas 15% of stationary-phase cells did (Doran & Herman, 1981). There was, however, apparently no evidence that increased infectivity correlated with changes in morphology (Keithly & Bienen, 1981; Rizvi, pers. comm.). Similarly, stationary phase promastigotes were found to be more infective than log-phase cells with several other species including L.braziliensis panamensis, with approximately 30% of the former thought to be metacyclics (Franke et al., 1985), L.b.braziliensis (Kweider et al., 1987), L.major (Sacks & Perkins, 1984; Sacks et al., 1985), L.mexicana amazonensis (Scott & Sher, 1986) and L.mexicana mexicana, although no data were given in this latter case (Sacks & Perkins, 1984). Moreover, Sacks et al (1985) found that with L.major 50% of the stationary-phase promastigotes survived intracellular destruction in vitro whereas log-phase cells were all killed. Most interestingly, this correlated with the binding of PNA to the promastigotes with 100% of the log-phase cells being agglutinated whereas only 50% of the stationary-phase were. The unagglutinated promastigotes from stationary-phase cultures all survived in macrophages and were morphologically similar, if not identical to, the putative metacyclic promastigotes seen in the sandfly.

1.4.1.3.2 Metacyclic promastigotes in the sandfly

Until recently it was generally assumed that promastigotes in the gut of sandflies were invariably infective to a susceptible

vertebrate. Indeed many isolations of dermatropic speies of Leishmania have been made from wild-caught sandflies by inoculating parasites into the skin of hamsters (Killick-Kendrick, 1979). Early studies using material from sandfly guts had, however, given variable results which were not all consistent with this assumption. Adler and Theodor (1926b) inoculated material from two experimentally infected P.papatasi into human volunteers and produced two lesions, in contrast to Hindle (1931) who failed to infect six hamsters by inoculating them with material from P.mongolensis. Feng and Chung (1941) also reported difficulty in producing infections using parasites from sandflies. Of 20 hamsters inoculated with material from P.chinensis only 6 became infected. In addition, these workers could not find a correlation between infection of the animal and the state of the infection in the sandfly. Earlier, however, Parrot and Donatien (1927) produced evidence that not all forms in the sandfly are necessarily infective to normally susceptible vertebrates. They failed to infect four mice inoculated intradermally with promastigotes from the midguts of P.papatasi which had 34-48 hour old L.tropica infections. The flies were heavily infected and the methods used - inoculating the skin of the tails of mice - had been routinely successful with cultured promastigotes. In recent years Sacks and Perkins (1984, 1985) have confirmed this early observation, and showed that, similar to in vitro promastigote cultures, there is a sequential development in the sandfly from an essentially non-infective promastigote form to an infective form. In their studies sandflies were taken at various times after an infective feed, the parasites were released and inoculated into Balb/c mice footpads. Initial studies with L.major showed that promastigotes 3 days after the bloodmeal were essentially non-infective, whereas promastigotes 7-10 days after the bloodmeal were

highly infective. More detailed studies with this species similarly showed that promastigotes obtained 3 days after infection of the fly, when bloodmeals were still present, were relatively non-infective - only a small proportion of the mice developing lesions and even then grew very slowly. Promastigotes from flies 4 and 5 days after feeding, which still retained their bloodmeals, were progressively more infective - producing measurable lesions in all mice. Interestingly, day 5 promastigotes from flies that had passed their bloodmeals were significantly more infective than those that retained their bloodmeal. Comparison of days 6 and 7 promastigote populations indicated that they were more infective than those from prior time points, with the exception of day 5 promastigotes obtained from flies that had passed their bloodmeals. Results for L.mexicana amazonensis were similar, but flies retaining bloodmeals on day 5 were not observed and in this case promastigotes recovered immediately after bloodmeal passage were not optimally infective since day 6 promastigotes displayed even greater infectivity than day 5. Sacks and Perkins (1985) suggested that differentiation of promastigotes into an infective stage in vitro and in vivo occurred in response to nutrient depletion.

1.4.1.3.3 Metacyclic promastigotes and the mode of transmission

There are three observations which suggest that the only parasite forms likely to be deposited in the skin of the vertebrate are the metacyclic promastigotes. Promastigotes have been recovered from **(1)** the proboscis of infected sandflies by feeding them with a fine capillary (Adler & Theodor, 1931; Warburg & Schlein, 1986); **(2)** the fluid oozing from a bite of an infected sandfly (Adler & Ber, 1941); **(3)** the fluid on which infected sandflies had fed through a membrane (Adler & Theodor, 1929). In all these observations the only morphological form seen were promastigotes indistinguishable from the

highly characteristic small unattached promastigotes seen in the probosces and also other anterior parts of the alimentary canal. Therefore, it appears likely that these are the main and possibly only forms responsible for initiating the infection in the vertebrate host. However, it is doubtful as to whether it is essential for an sandfly to have metacyclic promastigotes in its proboscis before it can transmit an infection. Transmission has been achieved by the bite of naturally and experimentally infected sandflies which apparently do not have promastigotes in the proboscis (Laison et al., 1977; Killick-Kendrick et al., 1985; Stephenson & Ward, 1986). In addition, Adler and Theodor (1926b) and Sacks and Perkins (1984, 1985) produced infections using promastigotes released from the midguts of sandflies using methods which would have excluded the use of parasites present in the foregut. The latter workers clearly thought that the infective promastigotes came from the stomodael valve. Significantly, Warburg and Schlein (1986) could not detect any parasites beyond the pharynx of L.major-infected P.papatasi, although metacyclics were the only forms present in the liquid after feeding via capillary tubes. Furthermore, even though very few metacyclic promastigotes are ever seen in the proboscis of infected sandflies (Killick-Kendrick, 1979, 1986), under the right circumstances transmission can be very efficient. For example, Beach et al (1984) described the behaviour of P.dubosqi naturally infected with L.major which probed 11 times, took no bloodmeal, and produced 11 lesions each at the site of a probe. Similarly, Killick-Kendrick et al (1985) reported that a naturally infected P.papatasi probed 26 times within an area of 2 cm diameter; 11 lesions of L.major appeared 12 days later.

This general pattern in Leishmania-infected sandflies of repeated probing with only small or no bloodmeals taken has been reported previously using experimentally-infected flies (Adler & Ber, 1941;

Strangways-Dixon & Laison, 1966; Killick-Kendrick, Leaney et al., 1977) . As engorgment is presumed to be controlled by chemo- or mechano-receptors, these observations prompted Killick-Kendrick, Leaney et al (1977) to suggest that Leishmania infections in the foregut were interfering with the function of receptors in the cibrarium. Later, Killick-Kendrick and Molyneux (1981) described 2 such sensilla, one in the proboscis, the other in the roof of the cibarium (Lewis, 1984), and suggested that parasite interference with sensilla in the cibarium and proboscis monitoring engorgement could be the cause of the obstructed blood-feeding behaviour in Leishmania-infected sandflies (Killick-Kendrick, Leaney et al., 1977; Killick-Kendrick, 1979). Subsequently, Beach et al (1985) studying the behaviour of P.dubosqi experimentally infected with L.major found that only those flies with infections in the cibarium and beyond had altered feeding behaviour, those with infections up to the anterior midgut being unaffected, thus providing circumstantial support for the "sensilla" hypothesis. However, Warburg and Schlein (1986) working with P.papatasi experimentally infected with L.major showed that during feeding into fluid filled capillary tubes some flies were able to engorge whilst others could not, and may in the latter group regurgitated fluid containing only metacyclic promastigotes. Both groups of flies had a similar distribution of morphological forms up to the pharynx (but no attached parasites were present anterior to the oesophagus), and these workers suggested that the difference between the two groups was in the degree of restriction of the passage through the stomodael valve. They concluded that their observations supported the "blocked fly" hypothesis (Short & Swaminath, 1928) in which a fly with a blocked or partially blocked stomodael valve the rate of flow into the oesophagus is faster than the rate with which the blood can

flow posteriorly into the midgut. Consequently, the oesophagus expands until the pressure exerted by its walls exceeds that of the pharynged pump. This results in a backflow of fluid flushing with it unattached metacyclic promastigotes into the pharynx, cibarium and mouthparts and thereby passively depositing them in the skin. Indeed the idea that parasites interfere with flow through the foregut is supported by Jefferies et al (1986) who have shown clearly the theoretical limitation on fluid flow imposed on the infected sandfly by heavy infection in the paharynx.

Thus, although there is conflicting evidence, it appears that transmission can occur by the deposition in the skin of metacyclic promastigotes from the proboscis but a vertebrate may also acquire an infection from a fly which has no permanent infection of the cibarium or proboscis, via "flushing" or regurgitation of metacyclic promastigotes from the stomodael valve and oesophagus. Transmission is enhanced through the inability of the fly to engorge and this may be due to direct interference of receptor function, by physical blockage in the stomodael valve/oesophagus/pharynx or, as is most likely, by a combination of the two.

1.4.2 Leishmanias and the vertebrate host

Upon inoculation into the naive vertebrate host, the metayclic promastigotes will be subjected to the potentially adverse effects of serum components such as complement, as well as the microbicidal activities of mononuclear and polymorphonuclear phagocytes. The interaction of Leishmania with these humoral and cellular defence mechanisms will be dealt with below.

1.4.2.1 Complement

The complement proteins (C1-C9) are major serum components which form an enzyme cascade and are involved in two major pathways; the

classical pathway which is antibody mediated and the alternate pathway which is antibody independent. The two pathways differ in the way in which they produce the cleavage products of C3, that is C3a and C3b, after which they converge in a common pathway. The pathway will be explained in terms of its effect as an invading microorganism membrane in a naive host.

1.4.2.1.1 The classical pathway

C1 comprises 3 components, C1q, C1r and C1s, which are linked in a trimolecular complex. The activation of C1 and the classical pathways is initiated by binding of C1q to certain subclasses of IgG or IgM antibodies forming a complex with antigen. After binding, C1q acquires esterase activity and brings about activation and transfer to sites on the membrane of the microorganism of C4 and then C2. This complex has "C3-convertase" activity (C42) and splits C3 in solution to two molecules, C3a and C3b.

1.4.2.1.2 The alternative pathway

Normally, in the absence of antibody-antigen complexes or activating surfaces, there are low levels of C3b in the plasma due to proteolysis. The C3b can, in the fluid phase, form a complex with Factor B in the presence of magnesium ions. This in turn reacts with Factor D to form the alternate pathway convertase, C3bB, which itself can cleave C3. This creates a positive feedback loop in which the product of C3 breakdown helps to form more of the cleavage enzyme, but under normal conditions the lability of C3bB and the action of C3b inactivator (Factors H and I) prevent the system from getting out of hand and allow the feedback loop to just "tick over" (Roit, 1984). However, in the presence of activating surfaces (e.g. bacterial endotoxin and yeast zymosan or other microorganism membranes) onto

which C3b fixes, the C3b can recruit further Factor B and D in the presence of the stabilizer properdin (P) to generate more C3bB and, via the feedback loop, accelerate further C3 breakdown producing C3a and C3b which can produce more convertase or bind to other parts of the membrane.

The classical and alternative pathways converge at this point. C3a causes histamine release from mast cells and attracts polymorphonuclear leucocytes and monocytes, mainly by release of mast cell chemotactic factor. C3b becomes fixed to adjacent parts of the microorganism membrane. Monocytes, macrophages and polymorphonuclear leucocytes have specific receptors on their surface for C3b and this facilitates subsequent phagocytosis of particles onto which C3b is fixed. In addition, the membrane bound C3b becomes associated with C5, which is cleaved (by both pathway C3 convertases) to C5a and C5b. C5a decreases vascular permeability directly and through mast cell degranulation. It is the main macrophage and polymorph chemotactic agent in the complement system and also activates neutrophils to stimulate a respiratory burst and release lysosomal enzymes. The C5b produced binds with C6 and C7 to form a complex which binds to the membrane. This recruits the components C8 and C9, which generate the "membrane attack complex" forming a transmembrane channel fully permeable to electrolytes and water - this leads to cell lysis (Roit, 1984). As alluded to above, complement pathway inactivators are also present in serum, such as C1 inactivator and C4 binding protein which inhibit C1r/C1s and C42, respectively. Other inhibitors, Factors H and I, interact to clear C3b to its inactive form, C3bi, which is unable to combine with Factor B to form the alternative pathway C3 convertase. Macrophages, monocytes, and neutrophils have specific receptors on their surface for C3bi which enhance phagocytosis of particles on which it is present.

1.4.2.2 Leishmanias and complement

Many investigators have found that Leishmania promastigotes are susceptible to lysis by nonimmune serum from animals (Ulrich et al., 1968; Schmunis & Herman, 1970; Rezai et al., 1975; Mosser & Edelson, 1984) and from humans (Hindle et al., 1926; Laison & Strangways-Dixon, 1963; Pearson & Steigbigel, 1980; Mosser et al., 1986). Both the lytic ability of normal serum from animals of different species and the susceptibility of different species of Leishmania have been reported to vary. For example, Rezai et al (1975) when comparing dog, cat, sheep, guineapig, rabbit, rat and mouse serum found that, in general, L.enrietti was much more susceptible than L.tropica to lysis. In addition, they found that dog and cat serum had the greatest effect on both species whereas mouse serum had the least, being essentially non-lytic. In contrast, Schmunis and Herman (1970) tested guineapig, hamster, mouse, rat, gerbil and rabbit serum against L.braziliensis, L.donovani and L.tropica promastigotes and found only minor differences. All the sera were lytic, although rat serum was more effective against L.braziliensis and, interestingly, mouse serum had its greatest lytic effect upon L.braziliensis and L.donovani. Similarly, Mosser and Edelson (1984) also reported that L.enrietti and L.tropica (L.major) were both equally effected by guineapig serum. Furthermore, confirming Rezai et als results, these workers found that mouse serum was ineffective lytically against these species, and this phenomenon has also been reported with L.mexicana mexicana (Bray, 1983a). Similar variation appears to occur with normal human serum. Hindle et al (1926), working with L.donovani obtained variable amounts of lysis using normal human serum from different donors. More recently, Mosser et al (1986) examined the effect of normal human serum on L.donovani, L.mexicana mexicana, L.mexicana amazonensis,

L.braziliensis guyanensis and two strains of L.major, and found not only differences in susceptibility between species but also between strains. However, both groups reported these differences were overcome if higher serum concentrations were used. The variations as have been described for animal sera combined with the different methodologies used by workers to assess lysis makes it difficult to generalise as to the concentration of serum required to produce total lysis. However, with human serum, the majority of promastigotes are killed by concentrations over 20% and all are lysed by 100% (but see below) (Hindle et al., 1926; Pearson & Steigbigel, 1980; Mosser et al., 1986).

Complement has been shown to be the lytic agent in human serum as heat-inactivating at 56°C for 30 min completely abrogates lytic activity against all Leishmania spp. promastigotes (Hindle et al., 1926; Pearson & Steigbigel, 1980; Mosser et al., 1986). Similarly, with animal sera, heat-inactivated (HI) guineapig (Mosser & Edelson, 1984) and dog (Rezai et al., 1975) had no effect on L.enrietti, L.tropica (L.major), and L.enrietti promastigotes respectively. However, although Schmunis and Herman (1970) found that heat-inactivating guineapig, rat, gerbil and rabbit sera (as above) did remove all their lytic activity against L.braziliensis, rat and rabbit sera retained some lytic activity against L.donovani and L.tropica, as did rat serum against L.tropica. Heating to 65°C for 30 min, however, completely removed the lytic activity of all sera against all three species of Leishmania. It is difficult to explain these discrepancies although these authors suggested that serum lytic factors besides complement and antibodies may contribute to the lethal effect of some sera. Sera from animals and humans have also been shown to agglutinate Leishmania promastigotes (Schmunis & Herman, 1970; Bray, 1983a; Mosser et al., 1986) which has led workers to suggest the presence "natural"

antibodies to Leishmania in these non-immune donors. It seems more likely, however, that these antibodies result from exposure to other protozoa, mycobacteria, bacteria or other material encountered in the environment which have similar antigens to Leishmania (Pearson, Wheeler et al., 1983). The possible presence of such "natural" antibodies, however, prompted workers to investigate the mode of complement activation. Early studies with animal sera indicated that complement activation occurred via the classical pathway. Schmunis and Herman (1970) found that absorbing rabbit serum with L.donovani, L.braziliensis or L.tropica promastigotes removed the lytic and agglutinating activity against all three species, suggesting complement activation might occur via the classical pathway mediated by "natural" antibodies (possibly IgM). Similarly, Rezai et al (1975) reported that dog serum absorbed with L.enrietti lost its lytic activity and they attributed the activity to the classical pathway via such "natural" antibodies. In contrast, Mosser and Edelson (1984) showed that L.tropica (L.major) and L.enrietti were lysed by normal guineapig serum by activating the alternative pathway. They found that sera deficient in C2 and C4 components, and Mg-ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'(EGTA) chelated serum all retained their lytic activity, whereas treating the serum with ethylenediamine tetraacetic acid (EDTA) inhibited lysis. Furthermore, using a monoclonal antibody specific to the C3 component of complement, the presence of C3 on L.tropica (L.major) promastigotes was detected by immunofluorescence. Subsequently, these workers, using monoclonal specific to different forms of C3, showed that the form of the molecule on the surface of L.major promastigotes was C3b. This was rapidly inactivated to C3bi (in as little as 5 min), and the presence of both forms reached a plateau by 15 min (Mosser & Edelson, 1985).

With normal human serum, the pathway of complement activation appears to depend upon the Leishmania species. Using similar techniques to those described above, Pearson and Steigbigel (1980) showed that L.donovani promstigotes bind "natural" antibodies (IgG and IgM) and are killed by activation of complement by the classical pathway. Likewise Mosser et al (1986) found that L.donovani promastigotes weakly activate the alternative pathway and that lysis in this species occurs predominantly via the classical pathway. However, the lysis of L.braziliensis guyanensis L.mexicana amazonensis, L.mexicana mexicana and L.major promastigotes appears to be due to the alternative pathway, the classical pathway not being required for lysis (Bray, 1983a; Mosser et al., 1986; Russell, 1987). Absorbed sera were found to exhibit a slightly lower lytic titre for L.major, however, possibly indicating a very minor contribution by the classical pathway (Mosser et al., 1986). The binding of C3 from human serum has been visualised on the promastigote surface of all these species, and appeared to reach a plateau after about 15 min. The more detailed study of Russell (1987) showed that the most abundant form of C3 bound to L.mexicana mexicana promastigotes was C3b, and identified the predominant C3 acceptor site to be a glycoprotein with an apparent molecular weight of 63KDa. It is worth commenting that these studies did not distinguish between metacyclics and promastigotes which would account for some of the discrepancies.

There are very few studies regarding effect of serum on amastigotes and the majority of these have used human serum. Hoover et al (1986) reported that L.donovani amastigotes were 10-fold less susceptible to the cytotoxic effects of normal human serum compared to L.tropica (L.major) amastigotes. The majority of amastigotes of the latter species were killed in less than 1 min by 50% serum. Comparison of the susceptibility of a larger number of Leishmania species to

human and guineapig sera confirmed this difference and showed the variation depended on strain as well as species. At a serum concentration of 20%, the majority amastigotes of the L.major strains 1-S and 254 were inactivated, whereas L.mexicana mexicana, L.mexicana amazonensis and L.donovani (Sudan and Khartoum strains) remained viable. Leishmania major strain LRC-238 showed intermediate sensitivities between these two extremes. These differences in susceptibility remained even when higher serum concentrations were used (Mosser et al., 1985). Despite these differences, the amastigotes of all these species were found to consume complement and bind C3 (all by the alternative pathway only) to the same extent (Hoover et al., 1984; Mosser et al., 1985). Using human serum genetically deficient in complement components C5-C9, the interesting study of Hoover et al. (1985) showed that the killing of L.major amastigotes begins with the attachment of C5b and therefore occurs much earlier than has been demonstrated in other systems.

In general, amastigotes appear to be more resistant to the effects of serum compared to promastigotes from the same species or strain. The difference varies with species, however, and is least with L.major and greatest with L.mexicana mexicana (compare Mosser et al., 1985 with Mosser et al., 1986). There is evidence, for L.donovani at least, that the relative resistance of amastigotes may be because they are less efficient activators of the alternative pathway than promastigotes (Wozencraft & Blackwell, 1987).

The susceptibility of Leishmania promastigotes to complement-mediated lysis by sera from most sources led workers to speculate how promastigotes inoculated into the vertebrate host evade lysis and safely reach macrophages. Suggestions included: **(1)** rapid uptake by macrophages; **(2)** inactivation of complement by saliva from sandflies

and (3) differentiation of promastigotes into infective, metacyclic forms in the sandfly which are resistant to complement (Franke et al., 1985). There is no evidence to support or refute the first two theories, and both may play a role, but the confirmation of the occurrence of metacyclic promastigotes both in sandflies and in culture provides support for the suggestion that these forms may be complement-resistant. However, only a few studies to date have specifically examined differences between phase of growth in culture and resistance to serum lysis and those reported so far have produced conflicting but intriguing results. Franke et al (1985) using whole normal human serum found that all the promastigotes in log-phase cultures of L.major, L.mexicana amazonensis, L.donovani and L.braziliensis panamensis were killed. In contrast, when stationary-phase cultures were studied, although all L.major and L.mexicana amazonensis were killed, up to 30% of L.braziliensis panamensis and 10% of L.donovani survived. Stationary-phase L.braziliensis panamensis promastigotes were shown to be more infective than log-phase cells and serum-resistant promastigotes from stationary-phase populations were even more infective than the whole population, indicating that the serum-resistant promastigotes were the infective metacyclic population. The ability of a population of stationary-phase cells of a particular species of Leishmania to resist lysis by serum appeared to depend on the length of the stationary-phase. Those species where the population levels dropped off sharply after a short (L.major) or non-existent (L.mexicana amazonensis) stationary-phase did not survive, whereas where the stationary-phase was of several days duration (L.braziliensis panamensis and L.donovani) a proportion of the promastigotes were able to survive. Interestingly, L.braziliensis panamensis log- and stationary-phase promastigotes were killed predominantly by activation of the alternative complement pathway,

although the classical pathway possibly had a minor role, whereas survival of complement-resistant forms was not due to these forms not activating the complement cascade as C3 was visualised on their surface. This indicated that they may be able to prevent the cascade from proceeding to cell lysis. Similarly, Wozencraft and Blackwell (1987) using Mg-EGTA chelated human serum, and therefore only measuring alternative pathway activation, reported that stationary-phase L.donovani promastigotes were more efficient activators of this pathway and bound more C3 via this pathway than log-phase cells showing that stationary phase promastigotes of this species may also prevent the cascade from proceeding to cell lysis. In contrast, 40-60% of L.mexicana mexicana stationary phase promastigotes are resistant to lysis by 80% with human serum, and resistance in this case appears to be because the unlysed cells bind much less C3 (5 to 6-fold) and more slowly than the lysed cells. Intriguingly though, this does not appear to be due to reduced amounts of the predominant C3-receptor on the unlysed cells (see above) (Russell, 1987).

However, not all workers examining leishmanias in different growth-phases have detected differences in susceptibility to serum lysis. Mosser et al (1986) using human and guineapig serum were unable to detect any differences in susceptibility to lysis between mid-log phase and late stationary-phase promastigotes of L.major, L.mexicana amazonensis and L.donovani; the cells of all species and phases of growth were lysed in 20% serum and above. Similarly, Pearson and Steigbigel (1980) found that L.donovani promastigotes from day 3 through to day 7 were equally susceptible to lysis by human serum in this case a concentration of 10% serum producing 100% lysis. There are several possible explanations for these discrepancies but none have been confirmed as yet.

1.4.2.3 Phagocytic cells of the mammalian immune system

There are two main types of phagocytic cells; macrophages which arise from monocytes, which are actively phagocytic themselves, and polymorphonuclear granulocytes, more commonly known as polymorphonuclear leukocytes or PMNs. Monocytes and PMNs are similar to erthrocytes, lymphocytes and megakaryocytes in that they ultimately arise from haemopoietic stem cellsin the bone marrow. When committed, these are capable of differentiating into precursors of all the above named cell types (Roitt, 1984). In the case of the phagocytic cell types, the committed stem cell produces myeloblasts or monoblasts which then differentiate via a series of dividing intermediates into PMNs or monocytes respectively (Murphy, 1976; van Furth, 1980).

The PMNs comprise 3 cell types - basophils, eosinophils and neutrophils. Basophils are the least common PMN. Their function is not known in detail, although they do participate in allergic reactions and are not phagocytic. Eosinophils are the second most common PMN but during some parasitic helminth infections, against which they have a protective role, their numbers are dramatically increased. They are phagocytic, although this does not appear to be their major function. Neutrophils are the most common PMN in the bloodstream, are actively phagocytic and have a primary role in phagocytosis and destruction of microorganisms (Murphy, 1976; Wakelin, 1984) and therefore, for the purpose of this thesis, they will be the only phagocytic cell type referred to apart from monocytes and macrophages. Neutrophils occur both in the blood and body tissues (Wakelin, 1984) and have a short life. In culture they disintegrate after 1 or 2 days, and the limited data available indicate they may live for 3-4 days *in vivo* (Murphy, 1976).

Monocytes circulate in the bloodstream and are the immediate precursors of macrophages. They reside and are transported via the

appropriate tissue compartment or body cavity. There they finally differentiate via exudate macrophages and exudate resident macrophages into resident macrophages which can either be fixed or free. Unlike neutrophils, these macrophages are quite long-lived cells, the turnover time of macrophages in tissues being 1-5 weeks (van Furth, 1981). There appears to be a lot of heterogeneity in macrophages, the different populations having their own peculiar characteristics.

Neutrophils, monocytes and macrophages can be attracted and migrate to, and discouraged from leaving, sites of infection or injury by a variety of inflammatory mediators and chemotactic factors including complement components and lymphokines (LK's) (Roitt, 1984). These named factors also activate neutrophils, monocytes and macrophages, inducing many biochemical and physiological changes which ultimately result in increasing the microbicidal and tumoricidal activity of these cells (Cohn, 1979; Karnovsky & Lazdins, 1978).

1.4.2.3.1 Receptors

The majority of phagocytic cell functions are mediated by receptors, and for macrophages over 30 specific receptors have been documented (Adams & Hamilton, 1984). Those involved with binding and phagocytosis of particles (usually studied using zymosan or sheep red blood cells) and stimulation of the respiratory burst are amongst the most thoroughly studied (Adams & Hamilton, 1984). Currently, there are three categories of externally orientated receptors in the plasma membrane of phagocytic cells that have been implicated in these functions (Adams & Hamilton, 1984; Blackwell, 1985). These are described below, although undoubtedly other, as yet, undefined receptors are also involved.

1.4.2.3.1.1 Receptors for the Fc portion of antibodies

Neutrophils, monocytes and macrophages possess receptors which recognise the Fc portion of certain isotyes of IgG (Henson, 1977; Dorringhton, 1977; Adams & Hamilton, 1984). These receptors mediate attachment and phagocytosis of particles coated with IgG, and attachment to these receptors can also trigger the respiratory burst in the three cell types (Murphy, 1976; Wright & Silverstein, 1983; Yamamoto & Johnston, 1984).

1.4.2.3.1.2 Receptor for mannose-fucose (MFR)

It is now well established that a binding site that recognises glycoproteins having terminal glucose/mannose, N-acetyl-glucosamine, or fucose residues is present on macrophages from various species and sources (Adams & Hamilton, 1984) and also on human blood monocyte-derived macrophages, but not on human blood monocytes or the macrophage-like cell line J774 (Ezekowitz, Sim et al., 1983). This receptor is also known as a scavenge receptor as one of its main functions is the clearance of lysosomal enzymes (secreted by macrophage), bearing the appropriate recognitions structure from circulation (Stahl & Schlesinger, 1980). These receptors have also been shown to be involved in the serum-independent attachment and uptake of zymosan by human blood monocytes and macrophages (Ezekowtiz, Sim et al., 1983) and murine resident and inflammatory peritoneal macrophages (Ezekowitz, Sim et al., 1983; Berton & Gordon, 1983; Sung et al., 1983). It also mediates the triggering of the respiratory burst in inflammatory peritoneal macrophages by zymosan (Ezekowitz, Sim et al., 1983). Interestingly, the number of these receptors on inflammatory macrophages is the same as on resident murine macrophages, whereas activated macrophages possess many less (Adams & Hamilton, 1984; Ezekowitz, Hill et al., 1983).

1.4.2.3.1.3 Receptors for complement

Neutrophils, monocytes and most macrophages have at least two cell-surface receptors which recognise fragments of the third component of complement, these are CR1 which recognises C3b and CR3 which recognises C3bi (Adams and Hamilton, 1984; Berger et al., 1984; Sim & Walport, 1987). The role of these receptors in attachment, phagocytosis and triggering of the respiratory burst is at present unclear. Some workers have found that, for human blood monocytes and macrophages (Wright et al., 1983) and murine resident peritoneal macrophages (Yamamoto & Johnston, 1983), these receptors can mediate binding in the absence of serum but not phagocytosis of C3b or C3bi coated particles (sheep red blood cells). In contrast, with inflammatory or activated murine macrophages or human monocytes and macrophages in the presence of fibronectin it was found that the receptors did mediate phagocytosis. Moreover, it was reported that attachment to these receptors did not appear to trigger the respiratory burst even when they mediated phagocytosis (Wright & Silverstein, 1983; Yamamoto & Johnston, 1983). In contrast, other workers using similar serum-free conditions found that complement components derived from human blood monocytes and macrophages and murine resident, inflammatory and in vivo activated peritoneal macrophages mediate local oposonisation of zymosan particles. Subsequent attachment and uptake of the zymosan was shown to be mediated in part by the CR3. Further, it was also demonstrated, using murine inflammatory and in vivo activated peritoneal macrophages that attachment and uptake of zymosan proceeded via CR3 with triggering of the respiratory burst (Ezekowitz, Sim et al., 1983). The reason for these discrepancies in the role of these receptors is at present unclear.

1.4.2.3.2 Phagocytosis

Phagocytosis of particles can occur non-specifically by surface phagocytosis, or specifically by receptor-mediated phagocytosis (Edelson, 1982). In the latter case, phagocytosis is thought to occur by the so-called "zipper hypothesis". This involves sequential and circumferential interaction of receptors on the phagocyte with ligands on the particle. The motor mechanism for this process is provided by the actin and myosin microfilaments of the phagocyte which interact under the receptor-ligand area (Silverstein & Loike, 1980). The result is that the surface invaginates and cellular pseudopods move outward so that the cell surface completely surrounds the particle forming a phagocytic vesicle or phagosome, which is internalised within the cell.

1.4.2.3.3 Microbicidal mechanisms of phagocytic cells

Phagocytes have a considerable arsenal of mechanisms at their disposal for the destruction of other cells. These mechanisms are initiated as particles are bound and phagocytosed by these cells and can be conveniently split into those that are dependent on oxygen and those that are not (Nathan, 1983). The general microbicidal mechanisms available to phagocytes will be described below and their effect on leishmanias will be dealt with in Sections 1.4.2.5.2.1 and 1.4.2.5.2.2.

1.4.2.3.3.1 Oxygen-dependent mechanisms

Binding of particles to the membrane of phagocytic cells cannot only mediate ingestion (see above) but also trigger a chain of important metabolic events aimed at the destruction of invading microbes. This metabolic activity is characterised by chemiluminescence and an increased uptake of oxygen and hence is called the respiratory burst (RB). The RB results in the formation via

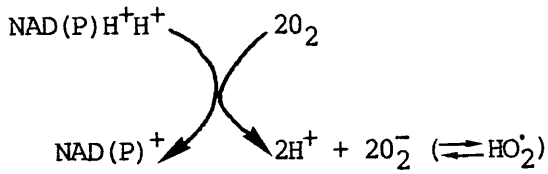
the partial reduction of oxygen of a series of highly reactive oxygen intermediates, all of which have been implicated in antimicrobial activity (Roos & Weening, 1979; Badwey & Karnovsky, 1980; Klebanoff, 1980; Klebanoff et al., 1983; Locksley & Klebanoff, 1983). The general importance of the RB in the microbicidal activity of phagocytes is shown by the occurrence of severe repetitive infections (bacterial and fungal) in patients with chronic granulomatous disease (CGD) or glucose-6-phosphate dehydrogenase (G-6-PDH) deficiency. The phagocytic cells of these patients are unable to produce a respiratory burst although they are normally phagocytic and retain the oxygen-independent microbicidal mechanisms (Roos & Weening, 1979; Badwey & Karnovsky, 1980).

The reaction sequence of the RB is normally initiated by perturbation of the membrane, for example the binding to an appropriate receptor, which in turn results in activation of a membrane-associated cyanide-insensitive flavoprotein oxidase. The events leading to the activation of the normally dormant oxidase are complex and poorly understood, although in a minimal simplified model the following are known to occur. Following ligand-receptor interaction, phosphatidylinositol-4,5-diphosphate (PIP_2) is hydrolyzed to inositoltriphosphate (IP_3) and diacylglycerol (DAG) by a guanine nucleotide regulatory protein (GNRP) dependent phospholipase C (PLC). DAG stimulates protein kinase C which when translocated to the plasma membrane leads to protein phosphorylation and the resulting phosphoproteins are necessary for activation of the oxidase. Macrophages can also activate the oxidase via another pathway which gives rise to a smaller independent, but coincident, RB upon ligand-receptor interaction. This requires activation of a phospholipase A_2 (PLA_2), which occurs due to elevation of calcium levels as a result of

IP_3 generation. The PLA_2 generates arachidonic acid, which is the start of a cascade leading to oxidase activation. Arachidonic acid itself is an important regulatory molecule and can modulate protein kinase stimulation. Undoubtedly, other signal transduction and regulatory demands are involved (Hamilton & Adams, 1987).

A) Superoxide anion and hydrogen peroxide production

The oxidase is linked via an incompletely understood transmembrane electron transport system to **(1)** the oxidation of an intracellular reduced pyridine nucleotide, NAD(P)H, which is supplied by the oxidation of glucose through the hexose monophosphate shunt (HMPS) pathway and **(2)** the reduction of molecular oxygen (Locksley & Klebanoff, 1983; Mauel, 1984). Oxygen initially accepts a single electron and is converted to the perhydroxyl radical ($HO_2^{\cdot}$) or its ionized form, the superoxide anion (O_2^-):



from Klebanoff (1980) and Thorne & Blackwell (1983)

The reduction of oxygen occurs initially at the site of membrane stimulation where the particle is attached and subsequently, after phagocytosis, within the plasma membrane of the phagososome so that the O_2^- is released into the phagosome (Karnovsky *et al.*, 1982; Locksley & Klebanoff, 1983; De Carvalho & De Souza, 1986). At neutral pH, the radical exists entirely as O_2^-, but at the acid pH which occurs in the phagosome (see below) a significant portion of the anion would exist as $HO_2^{\cdot}$ (Klebanoff *et al.*, 1983). The role of O_2^- as a primary toxic agent is somewhat controversial and although O_2^- can, under certain conditions, attack unsaturated lipids and breach membranes

(Fridovich, 1979) it is not thought to be reactive enough to be directly toxic (Badwey & Karnovsky, 1980; Klebanoff et al., 1983). Rather, the importance of O_2^- is in its ability through a series of other reactions to produce a series of highly reactive and toxic oxygen products (Klebanoff et al., 1983).

The superoxide anion can act as both a reductant and an oxidant (Locksley & Klebanoff, 1983; Klebanoff et al., 1983). As a reductant, as in the reduction of cytochrome C or nitroblue tetrazolium (NBT), it gives up an electron and is converted back to oxygen (Klebanoff, 1980). When it acts as an oxidant, it accepts a second electron and is immediately pronated to form hydrogen peroxide (H_2O_2). When two radicals interact, one is oxidised and the other reduced to form O_2 and H_2O_2. This dismutation can occur spontaneously or be catalysed by the enzyme superoxide dismutase (SOD). Spontaneous dismutation occurs best at pH 4.8 where $HO_2^{\cdot}$ and O_2^- are present in equal concentrations, for example in the acidic environment of the phagosome (Badwey & Karnovsky, 1980; Klebanoff et al., 1983; Locksley & Klebanoff, 1983). The reaction can best be written:

$$O_2^- + HO_2^{\cdot} + H^+ \longrightarrow H_2O_2 + O_2$$

from Klebanoff et al (1983)

The rate of spontaneous dismutation falls as the pH rises and O_2^- predominates. SOD has a wide catalaytic pH activity range but is most active at neutral or near neutral pHs (within the cytoplasmic range) where the rate of spontaneous dismutation is low (Badwey & Karnovsky, 1980; Klebanoff et al., 1983). There is no evidence that phagocyte, SOD is discharged into the phagosome, or that by catalysing the conversion of O_2^- to H_2O_2 it increases toxicity (Klebanoff et al., 1983). Thus, the role of phagocyte SOD is probably in converting any

O_2^- that diffuses from the phagosome into H_2O_2, which could then diffuse back into the phagososome or be inactivated by other enzymes inthe cytoplasm (see below) (Roos & Weening, 1979). Both O_2^- and H_2O_2 have been detected cytochemically in the phagosomes of stimulated phagocytes (Karnovsky et al., 1982; Badwey et al., 1983) and also chemically in the supernatants of phagocyte cell cultures (Nathan & Root, 1977; Johnston et al., 1978) and measurement of their release together with increased oxygen consumption are normally used for detecting triggering of the RB. However, the amounts of O_2^- and H_2O_2 released varies with cell type, state of cell activation, stimulating agent used and culture conditions, such as substratum, duration of cultivation and growth medium (Berton & Gordon, 1983). For example, in response to particulate (zymosan) or soluble (PMA-phorbol myristate acetate) stimuli neutrophils release more O_2^- and H_2O_2 than do monocytes (Johnson et al., 1976) and in turn monocytes release more of thee intermediates than macrophages derived from them, although there are conflicting reports as to how long the monocytes need to be cultured before this is seen (Locksley & Klebanoff, 1983; Murray & Cartelli, 1983). Moreoever, comparisons of resident macrophages, macrophages elicited by inflammatory agents and macrophages immunologically activated in vivo have shown differences between these populations with regard to O_2^- and H_2O_2 release (Johnson et al., 1980). It has been reported that all three types of macrophages release significant amounts of O_2^- upon stimulation, but that both inflammatory and activated macrophages release considerably more O_2^-, up to 15-fold, than do resident macrophages (Johnston et al., 1978). Both resident and inflammatory macrophages stimulated with PMA release only small amount of H_2O_2 (up to 26-fold less) in comparison to activated macrophages (Nathan & Root, 1977).

Unlike O_2^-, H_2O_2 is toxic and generally microbicidal in its own right (Klebanoff & Rosen, 1979; Badwey & Kanovsky, 1980; Klebanoff, 1980; Nathan, 1983), but thee are also two mechanisms known by which its toxicity may be amplified. The first is the metal-catalyzed interaction of H_2O_2 and O_2^- to form even more highly reactive radicals of oxygen such as hydroxyl radical ($OH^\bullet$) and possibly singlet molecular oxygen ($^1O_2$). These are stronger oxidants than H_2O_2. The second mchanism is the ability of peroxidase to catalyse the oxidation and halogenation of target substance by H_2O_2 and a halide the so called myeloperoxidase system (Badwey & Karnovsky, 1980; Klebanoff, 1980; Klebanoff et al., 1983).

B) Hydroxyl radical and singlet oxygen production

The hydroxyl radical ($OH^\bullet$) is formed by combination of two reactions and the resulting complete reaction is termed the metal-catalyzed Haber-Weiss reaction.

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^\bullet$$
$$O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$$
$$\text{----------}$$
$$H_2O_2 + O_2^- \longrightarrow O_2 + OH^- + OH^\bullet$$

The first part of the reaction involves the generation of OH^- though the oxidation of ferrous iron (Fe^{2+}) by H_2O_2. The limiting reagent, Fe^{2+}, is subsequently generated by the interaction of O_2^- with Fe^{3+}. The $OH^\bullet$ has been shown to be produced by a variety of stimulated phagocytes and by enzyme systems which generate both H_2O_2 and O_2^- for example xanthine-xanthine oxidase (XO) or acetaldehyde-XO. In general, the involvement of $OH^\bullet$ in the microbicidal (bactericidal) activity of these systems has been demonstrated by the use of $OH^\bullet$ scavengers such as mannitol, benzoate and ethonol (Klebanoff & Rosen, 1979; Badwey &

Karnovsky, 1980; Klebanoff, 1980). The OH• is thought to manifest its toxicity through lipid peroxidation and oxidation of nucleic acids (Badwey & Karnovsky, 1980).

There are a number of reactions that are capable of generating $^1O_2$ under conditions that may be relevant in vivo. These are: the spontaneous dismutation of O_2^-, although production of O_2^- by this reaction is controversial, interaction of O_2 with OH•; and production by the "modified Haber-Weiss reaction" (but see below - peroxidase section) (Badwey & Karnovsky, 1980). The reactions in details are:

$$HO_2 + O_2^- + H^+ \longrightarrow {}^1O_2 + H_2O_2$$

$$O_2^- + OH\cdot \longrightarrow {}^1O_2 + OH^-$$

$$O_2^- + H_2O_2 \longrightarrow {}^1O_2 + OH^- + OH\cdot$$

from Badwey & Karnovsky (1980)

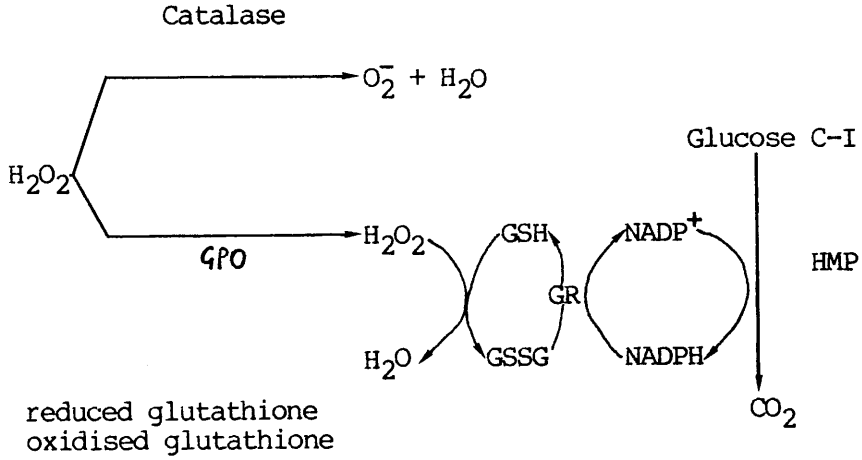
$^1O_2$ has been detected in the acetaldehyde-XO reaction by chemical methods, and its microbicidal activity implicated by the use of $^1O_2$ quenchers such as diazabicyclooctane (DABCO) and histidine. However, these studies should be interpreted with caution as both the detection method and quenching agents are relatively non-specific. This also makes it difficult to demonstrate the production of $^1O_2$ by stimulated phagocytes, so that at present the role of $^1O_2$ in phagocyte microbicidal activity is not firmly established (Klebanoff & Rosen, 1979; Badwey & Karnovsky, 1980; Klebanoff, 1980; Locksley & Klebanoff, 1983).

C) Peroxidase-catalysed systems

Neutrophils contain in their cytoplasm, primary, azurophilic granules which contain the hemeprotein myeloperoxidase (MPO). Monocytes also contain peroxidase positive granules, although they are fewer in number than in the neutrophil (Klebanoff, 1980; Klebanoff et

al., 1983; Locksley & Klebanoff, 1983). The monocyte peroxidase appears to be identical to that of the neutrophil, that is MPO, similarly macrophages freshly derived from monocytes, recently-derived bone marrow macrophages, and various elicited peritoneal macrophages contain granular peroxidase which resembles neutrophil MPO (Thorne & Blackwell, 1983). Cells containing a granule peroxidase can release this enzyme into the phagosome, or under some conditions into the extracellular fluid, where it reacts with H_2O_2 to form an enzyme-substrate complex which oxidases halide to a toxic agent or agents (Karnovsky et al., 1982; Klebanoff et al., 1983). MPO has a pH optimum of 5.5 and therefore acts most efficiently in the acid environment of the phagosome. It is thought that catalase can act as a peroxidase at low pH in cells which lack MPO (Thorne & Blackwell, 1983). Either chloride, iodide, or bromide can meet the halide requirement for MPO. Of these, chloride is present in phagocytes at concentrations greater than those required for microbicidal activity (Badwey & Karnovsky, 1980; Locksley & Klebanoff, 1983). The primary product formed, at least for some halides, appears to be the corresponding hypohalous acid. These are highly toxic, this is thought to result from halogenation and oxygenation of cell surface components (Badwey & Karnovsky, 1980; Klebanoff et al., 1983; Locksley & Klebanoff, 1983). The potency of this system is shown by the fact that the concentration of H_2O_2 necessary for microbicidal activity in vitro is typically reduced 100-fold by the addition of MPO and chloride to the antibacterial systems (Locksley & Klebanoff, 1983). The production of $^1O_2$ by in vitro MPO-H_2O_2- halide systems has also been described, but the criticisms applied to other $^1O_2$ producing systems similarly apply here (Badwey & Karnovsky, 1980).

Cellular protection from oxygen metabolites Protection from the oxygen metabolites described above in the cells that produce them, and also in the potential pathogens, is afforded by a number of enzymes. SOD binds O_2^- and converts then it to H_2O_2 and oxygen. This prevents direct damage by O_2^-, avoids the formation of possibly toxic $^1O_2$ and inhibits the reaction of O_2^- with H_2O_2 to give $^1O_2$ and $OH^\bullet$. The H_2O_2 produced by this reaction, or by spontaneous dismutation that normally occurs in the RB, can be inactivated by the enzyme systems shown below:



GSH reduced glutathione
GSSG oxidised glutathione
GPO glutathione peroxidase
GR glutathione reductase

from Klebanoff et al (1983)

1.4.2.3.3.2 Oxygen-independent mechanisms

Phagocytes possess a variety of oxygen-independent mechanisms which are potentially microbicidal, although these are not as well defined as the oxygen-dependent mechanisms and often act in conjunction with or provide a suitable environment for the latter mechanisms (Nathan, 1983; Thorne & Blackwell, 1983).

A) Acidification of the phagosome

Receptor binding not only leads to the events described above, but also to stimulation of an enzyme in the membrane that pumps protons into the lumen of the phagosome causing it to be acid (Hume,

1984). The pH within the phagosome can be as low as 4.5 (Jensen & Bainton, 1973; Ohkuma & Poole, 1978). This is detrimental to microorganisms by itself (Nathan, 1983) but also provides an optimal environment for the dismutation of O_2^- and the catalytic activity of MPO (see above) and for other lysosomal/granular hydrolytic enzymes (see below).

B) Hydrolytic enzymes

The hydrolytic enzymes of neutrophils are contained in two types of granule, the azurophil (primary) granule and the specific or secondary granules. There are large number of these within the cell, they are built up during cell maturation and cannot be replenished in natural cells (Parker, 1984). The azurophil granules contain a diverse array of hydrolases, most of which have an acidic pH optimum including acid proteases e.g. cathepsins, glycosidases e.g. β-glucuronidase, nucleases e.g. 5'-nucleotidase, neutral proteases e.g. elastase and collagenase, and also MPO (see above). Cationic proteins (see below) and lysozyme are present (Parker, 1984), and possibly acid phosphatase (Beaufay, 1972). The specific granules lack most of the conventional hydrolase enzymes, but do contain alkaline phosphatase, lysozyme, probably collagenase and also lactoferrin (see below) (Parker, 1984). Monocytes and macrophages have a similar spectrum of proteolytic enzymes to neutrophils, although the overall activity in macrophages is less. The enzymes are packaged in azurophilic peroxidase granules in monocytes. These are gradually lost as the monocytes differentiate into resident macrophages in which the hydrolases are contained in lysosomes (Parker, 1984). Further, macrophages unlike neutrophils have the synthetic apparatus which continually replenishes their enzyme stores. Synthesis can also be regulated, so that in activated macrophages the number of lysosomes is increased, and greater amounts

of lysosomal hydrolases are manufactured (Elsbach, 1980). Soon after they are formed phagosomes fuse granules or lysosomes, the contents of which are released into the phagosome (Parker, 1984). The spectrum of enzymes released are generally microbicidal as they are able to degrade many or all of the diverse components of microorganisms. Indeed they have been shown to have a variety of roles in the degradation or killing of different species of bacteria within phagocytes and in in vitro systems (Elsbach, 1980; Thorne & Blackwell, 1983).

C) Iron-binding proteins

Lactoferrin is synthesised by neutrophils and is a constituent of the specific granules so that it is also released into the phagosome during granule fusion. Although originally thought to be bacteriostatic, this has not been substantiated using purified preparations. Lactoferrin has, however, been shown to catalyse the formation of $OH^{\cdot}$ (see above) and is thought to provide the iron requirement for this reaction (Nathan, 1983; Parker, 1984). Macrophages possess receptors for lactoferrin, which they may ingest after its release by degranulating neutrophils (Nathan, 1983) and thus it may have a similar function in these cells. Macrophages also synthesise their own iron-binding protein, transferrin, and also have receptors for this molecule on their surface (Nathan, 1983; Adams & Hamilton, 1984).

D) Cationic proteins

Cationic proteins are present in the azurophilic granules of neutrophils, and have also been detected in macrophages from some but not all tissue sites (Nathan, 1983). Purified cationic proteins from both these sources have been shown to have antibacterial and antifungal activity (Nathan, 1983; Thorne & Blackwell, 1983).

1.4.2.4 Interactions of leishmanias and their mammalian host

Details of Leishmania-phagocyte interactions have almost exclusively been studied using in vitro systems which avoid the complications which may occur in animal infection experiments. Unfortunately relevant information on the early phase of the infection in mammals is sparse and usually has involved the use of promastigotes from culture as the parasite challenge. It is thought, however, that following inoculation of promastigotes in a mammal, some will be phagocytosed by skin macrophages (Marsden & Jones, 1985), whilst others are engulfed by PMNs and monocytes. The latter cell types appear to form the initial response to the infection - a poorly organised mixed PMN/monocyte infiltrate which is seen as early as 1 hour after inoculation of parasites (Wilson et al., 1987). At this time the majority of intracellular leishmania are found in PMN. Over the next 48 hours, however, the situation changes as the infiltrate becomes primarily mononuclear and by 48 hours intracellular parasites are effectively found only in mononuclear phagocytes, in which they divide (Wilson et al., 1987). With Leishmania species normally causing cutaneous disease, the PMN infiltrate is followed by a rapid invasion of lymphocytes (Marsden & Jones, 1985). Interestingly, this does not occur with those species normally causing visceral leishmaniasis (i.e. L.donovani complex), and it has been suggested that it is this that allows the parasites to disseminate to the viscera (Wilson et al., 1987). These infiltrates together result in a characteristic erythematous, puritic papule (Marsden & Jones, 1985). The subsequent events of infection depend on the species involved (see Table 3) and the clinical details of the diseases has already been given in Section 1.3.1.

1.4.2.5 *Leishmania*-phagocyte interactions *in vitro*

Although *Leishmania*-phagocyte interactions comprise a continuous sequence of events, they can conveniently be discussed as a series. It appears there are four components in the establishment of leishmanias as intracellular parasites: attachment, entry, survival and multiplication (Chang, 1983; Chang & Fong, 1983; Alexander & Russell, 1985; Chang *et al.*, 1985). The detailed changes that occur in the parasite and the host cell during thee events are still uncertain and the subject of much controversy. The majority of the studies aimed at an unravelling the situation have involved peritoneal macrophages (usually resident) from experimental animals. It is not known if the responses of these macrophages are representative of other macrophage populations, such as skin macrophages, although doubts have been expressed (Blackwell, 1985) and more recently some workers have started using the possibly more relevant human monocytes and macrophages derived from them. In some studies macrophage-like cell lines have also been used. Details are given below.

1.4.2.5.1 Attachment and phagocytosis

In studies examining attachment and phagocytosis, many workers have unfortunately not distinguished between the two. Attachment does appear to be an essential requisite to phagocytosis and so for convenience, in this thesis they will be considered together, except where they were specifically examined separately.

Leishmanias, like any other intracellular agent, must first come close and then attach themselves to the host cells before entry. The importance of these events can be readily envisioned both during primary infection by metacyclic promastigotes delivered into susceptible hosts by the sandfly vector as well as in the secondary infection by amastigotes released from heavily parasitised cells

during the course of leishmaniasis. Farah et al (1975) reported that L.tropica promastigotes actively sought mouse macrophages while the latter remained passive. In contrast, Aikiyama and Haight (1970) found that the contact between L.donovani and hamster peritoneal macrophages occurred randomly, although in some cases the macrophages appeared to migrate towards a promastigote. Similarly, the detailed studies of Bray (1983b) with L.mexicana mexicana showed that mouse macrophages were not attracted to any product of promastigotes. However, in the presence of normal serum the promastigotes (and to a lesser extent amastigotes) interacted with serum components to produce a factor that was chemtoxic to a variety of murine phagocytic cells including peritoneal macrophages, peritoneal and blood neutrophils, and to a lesser extent blood monocytes. The factor responsible appeared to have the characteristics of the C5a component of complement (although C3a was also thought to be produced) which was, in this case, produced via activation of the alternative pathway. In other species, however, the classical pathway would be involved. This appears to be the currently accepted view (Chang et al., 1985). Thus, it appears in vivo that the extracellular promastigotes and amastigotes will be confronted with multifarious cell types and this has been confirmed in vivo. Both these stages will attach avidly to phagocytes (neutrophils, monocytes and macrophages) (Chang, 1981a,b,c; Pearson & Steibigel, 1981; Bray, 1983a; Pearson, Sullivan et al., 1983; Saraiva et al., 1987) less well or poorly to lymphocytes (Benoliel et al., 1980; Chang, 1983) and not at all to erythrocytes (Klempner et al., 1983). Therefore, there is some prediction of leishmanias for attachment to their normal host cells.

The orientation of promastigotes attaching to phagocytes and their subsequent entry into them is a matter of some controversy. The orientation of attachment was described as predominently flagellar end

first in the following promastigote-macrophage systems: L.donovani-hamster (Miller & Twohy, 1967; Giannini et al., 1981), L.braziliensis-mouse (Merino et al., 1977), L.braziliensis panamensis-cell line P388D (Aikawa et al., 1982), L.mexicana mexicana-mouse (Alexander, 1975), L.enrietti-guineapig (Rahman & Sethi, 1978) and L.tropica-mouse (Zenian et al., 1979). These latter workers found that glutaraldehyde-fixed parasites attached randomly by almost any part of their surface and were phagocytosed. They suggested that promastigote motility is the deciding factor governing the orientation of parasite attachment and uptake. Contrary to this is the finding that promastigotes of L.donovani attach and are taken up predominantly posterior-end first (Aikiyama & Haight, 1971) or in no particular orientation (Ebert et al., 1979). The interaction of L.donovani promastigotes with hamster peritoneal macrophages and human blood phagocytes (neutrophils, monocytes and macrophages) have been quantified and have shown that with this species at least, the majority of promastigotes become attached and internalized either head first or tail first, both of which occur at the same frequency (Chang, 1979; Pearson et al., 1981; Pearson, Sullivan et al., 1983). It remains to be determined if this is true for other promastigote-macrophage interactions (Chang, 1983).

A problem related to this and which is also controversial is to what extent the parasite is actively involved in attachment to, and internalization into, the phagocyte. Studies with promastigotes using non-phagocytic cells or cells of low phagocytic ability have suggested that promastigotes can be actively involved in entry. Thus, L.mexicana mexicana promastigotes were reported to enter D.S. cells, a cell line of low phagocytic ability (Lewis, 1974). Similarly, L.tropica appear to be able to enter fibroblasts in culture (Avakijan, 1971), L.braziliensis panamensis has been observed within an unspecified non-

phagocytic cell line (Merino et al., 1977) and L.braziliensis sensu lato within skin fibroblasts (Chang, 1978). Interestingly, Chang (1978) was unable to demonstrate the entry of L.donovani promastigotes into the same skin fibroblasts whereas this was reported to occur in a recent study (Schwartzman & Pearson, 1985). Phagocytosis was not observed, however, and there was some doubt as to whether the parasites were truly within the cells (Schwartzman & Pearson, 1985). In only two of these studies (Lewis, 1974; Chang, 1978) was the mechanism of entry analysed. In both cases, the flagellum was found to lead entry in the cells by a form of "induced phagocytosis", although how leishmanias do this is unknown (Chang, 1978). Nevertheless, it has been suggested that this may be a general ability of all leishmanias.

This question has also been examined using normal macrophages as the host cell by examining promastigote or amastigote interactions at low temperature; and after treatment of the macrophages with metabolic inhibitors or inhibitors of microfilament action. Unfortunately, workers often have not differentiated between attachment and uptake. Also, the treatment was often applied in the presence of both parasite and macrophage, making it difficult to distinguish between its effects on the macrophage versus the parasite. There are conflicting results regarding the effect of temperature on attachment. For instance, the attachment of promastigotes of L.mexicana mexicana to murine peritoneal cells (Bray, 1983a), L.donovani to hamster peritoneal macrophages (Chang, 1981), L.braziliensis NR strain to J774 cells (Hernandez et al., 1986) and L.tropica to murine peritoneal macrophages (Zenin et al., 1979) has been reported to be temperature dependent, low temperatures inhibiting attachment. In contrast, attachment of L.mexicana amazonensis amastigotes to rat peritoneal macrophages (Benoliel et al., 1980), promastigotes of L.tropica (L.major) to human monocyte plasma membrane vesicles (PMV) (Klempner

et al., 1983) and promastigotes of L.braziliensis to murine peritoneal macrophages (Merino et al., 1977) was reported to be independent of temperature. There appears to be agreement, however, that ingestion (as opposed to attachment) of both amastigotes (Wyler, 1982) and promastigotes (Merino et al., 1977; Zenian et al., 1979) is dependent on temperature, although there is no clear evidence whether the effect is exerted on the parasite or the macrophage or both. Similarly, variable results have been reported using metabolic inhibitors. Simultaneous treatment of L.tropica promastigotes and murine peritoneal macrophages with iodoacetamide inhibited attachment (Zenian, 1981), whereas simultaneous treatment of L.mexicana amazonensis amastigotes and rat peritoneal macrophages with iodoacetate had no effect on attachment (Benoliel et al., 1980). However, pre-treatment of these latter macrophages or simultaneous treatment of murine peritoneal macrophages with iodoacetate drastically reduced uptake of L.mexicana amazonensis amastigotes and L.mexicana mexicana promastigotes, respectively (Benoliel et al., 1980; Bray, 1983a). Pre-treatment of the parasites alone, however, did not affect uptake (Benoliel et al., 1980; Bray, 1983a), suggesting that uptake requires only the phagocytic ability of the macrophage. Results from the use of cytochalasins (inhibitors of microfilament action) led to essentially the same conclusions but raise questions about the attachment phase. Thus, simultaneous treatment of promastigotes of L.donovani and hamster peritoneal macrophages (Chang, 1979), L.mexicana amazonensis amastigotes and rat peritoneal macrophages (Benoliel et al., 1980), and L.mexicana mexicana promastigotes and murine peritoneal macrophages (Bray, 1983a) with cytochalasin B inhibited uptake but not attachment. Zenian et al (1979), however, found that simultaneous treatment of parasite-

macrophage cultures with cytochalasin D reduced both the numbers of L.tropica promastigotes attached and ingested. These workers and also Alexander (1975) found that pre-treating macrophages with cytochalasins, but then leaving it out from the incubation mixture containing the parasites, had no effect on attachment and or uptake. In direct contrast, Bray (1983a), working with L.mexicana mexicana promastigotes and murine peritoneal macrophages, reported that pre-treating the macrophages with cytochalasin B even after extensive washing inhibited attachment but that pre-treatment of the promastigotes had no effect. Other workers reported quite different effects. Using monocyte-PMV Klempner et al. (1981) found that pre-treating L.tropica (L.major) amastigotes with cytochalasin B inhibited attachment to human blood monocytes, whereas pre-treatment of the monocytes had no effect on attachment but inhibited uptake. This indicated that the attachment phase depends on the active participation of the amastigote. This author also reported that the situation may be similar with promastigotes of this species and Aikiyama et al. (1982) reported the same for L.braziliensis panamensis promastigotes and the P388D cell line. Therefore these suggest that there may be a role for the parasite in attachment. There seems to be agreement, however, that participation of the macrophage is required for internalization of leishmanias.

In discussion of the details of attachment/uptake I will deal with mononuclear phagocytes and neutrophils separately.

1.4.2.5.1.1 Mononuclear phagocytes

It is clear that Leishmania-phagocyte interactions are very complex and it has been proposed that attachment and subsequent uptake are the result of multiple pathways of ligand-receptor binding mechanisms (Chang et al., 1985). Leishmania attachment/uptake with

macrophages/monocytes can be categorized phenomenologically into serum-dependent and serum-independent pathways.

A) Serum-independent

Chang (1981a) was the first worker to examine the interactions in detail using a radioisotopic method for assessing attachment of L.donovani promastigotes to hamster peritoneal macrophages. He found that attachment followed saturation kinetics, becoming saturated in his experiments after about 30 min. Inhibition experiments using antibodies, monosaccharides, glycosidases and proteases suggested the presence of antigenic ligands, probably glycoproteins or glycoconjugates of the surface of the parasite which attached to cell surface antigenic receptors, probably proteins, on the macrophage. Further studies around this time partially characterised both the interactions of both amastigote and promastigote attachment and uptake with macrophages and monocytes and produced similar general findings, although the details were different (Bray, 1983a; Klempner et al., 1983; Wyler & Suzuki, 1983; Zehavi et al., 1983). For example, attachment of L.donovani appeared to be mediated, in part at least, by the presence on promastigotes of glycoproteins with terminal sugar residues similar to N-acetylglucosamine, sialic acid, mannose or glucose (Chang, 1981a). Whereas, terminal mannose or galactose residues appeared to be more important in the attachment of L.tropica promastigotes to murine peritoneal macrophages (Zehavi et al., 1983). In direct contrast, Bray (1983a) found a wheatgerm agglutinin lectin-like receptor on the surface of L.mexicana mexicana promastigotes which bound to a terminal N-acetylglucosamine glycoprotein ligand on murine peritoneal macrophages. It is interesting to note that recently a very similar situation to that reported by Bray (1983a) has also been reported to be important in the attachment of L.braziliensis NR

strain to J774 macrophage-like cells (Hernandez et al., 1986). However, the majority of recent studies in this area have concentrated almost exclusively on ascertaining the role of specific receptors, MFR and CR3, present on macrophages in the attachment and uptake of leishmania promastigotes and amastigotes. Unlike earlier studies, Wilson and Pearson (1986) found, using a variety of monosaccharides, that they could not inhibit the uptake of L.donovani stationary phase promastigotes by human blood monocytes and derived macrophages. This is not surprising since the minimal terminal structure on glycoproteins recognized by the MFR consists of a small chain of saccharides with additional mannose or N-acetyl-D-glucosamine residues linked to the 6 position of the penultimate 1,6-linked mannose in the chain. Hence monosaccharides are poor antagonists of MFR activity, whereas polyvalent ligands are not (Blackwell, 1985). Thus, Wilson and Pearson (1986) found in a study using soluble polyvalent ligands, that promastigote attachment to macrophages and subsequent uptake was inhibited by approx. 50% by neoglycoproteins BSA-fucose or mannose, but not by BSA-galactose or N-acetyl-glucosamine, and also by mannan, a polysaccharide with high mannose linkages in the terminal sugars and which specifically binds to the MFR. Interestingly, interactions with monocytes were not affected similarly. Further, by specifically depleting the MFRs by allowing the macrophages to adhere to mannan plated coverslips promastigote ingestion was inhibited by 70%, although full inhibition could not be achieved. These studies indicated that the MFR had a major role in the attachment and uptake of L.donovani promastigotes by human monocyte derived macrophages but also that other receptors may also be involved. Additional support for this proposal has come from the work of Blackwell and her co-workers studying the attachment and uptake of L.donovani promastigotes and amastigotes by murine peritoneal macrophages. Hence, Channon et al

(1984) examining the role of the MFR found that they could inhibit attachment and uptake of promastigotes and amastigotes using monosaccharides, but the inhibition was not specific to monosaccharides rcognised by the MFR. However, with soluble mannan they found they could inhibit attachment and uptake of promastigotes by about 80%; intriguingly amastigote attachment and uptake appeared to be relatively unaffected being inhibited by only 20%. It was concluded therefore, that the MFR appeared to be of importance in mediating the attachment and uptake of promastigotes. On the other hand, amastigotes appeared to be less dependent on the MFR and probably attached to other macrophage receptors with at least equal affinity. Subsequently, Blackwell et al (1985) found that by depleting the MFR using mannan-plated coverslips there was a similar large inhibition of attachment and uptake promastigotes but in this case inhibition of amastigote attachment and uptake was increased to 60%.

These workers also examined the role of CR3 using M1/70, a monoclonal antibody that binds specifically to the binding site of CR3, anti-C3-Fab which binds to C3 and sodium salicyl hydroxamate (SSH) which inhibits the covalent binding of C3 to the activator surface. Using these reagents in solube form, they found similar inhibitions for both promastigotes and amastigotes to those obtained with soluble mannan. Specific depletion of CR3 using M1/70-plated coverslips again produced similar results for both promastigotes and amastigotes as those obtained with mannan-plated coverslips. Moreoever, use of mannan and M1/70 together in soluble form, or with one of them plated onto coverslips and the other soluble, did not enhance inhibition. Thus it appears that, under serum-free conditions, both CR3 and MFR are required to promote optimal attachment and ingestion of the promastigote. Neither receptor alone is capable of

even allowing parasite attachment. However, it should be noted that the parts played by receptor-mediated attachment and uptake of amastigotes are still not fully explained.

The indirect indication from these studies that macrophage-derived cleaved-C3bi present on promastigotes mediated binding by the CR3, was shown to be the case by the use of immunoelectron microscopy (Wozencraft et al., 1987). After 20 mins under serum-free conditions, C3 deposition was detected on both promastigotes and amastigotes. For promastigotes the degree of C3 deposition under serum-free conditions was comparable to that observed in the presence of exogenous (serum) C3. Addition of SSH prevented deposition on promastigotes, whereas for amastigotes deposition of C3 and subsequent attachment of the cells to macrophages was not eliminated by SSH and it was suggested that a proportion of C3 binding to the amastigote surface may be via non-covalent linkages and that the C3 bound may not be in the correct form to mediate attachment to CR3 (Wozencraft et al., 1987). This may explain the relative unimportance of CR3-mediated attachment and uptake found with amastigotes in the presence or absence of serum (see below and above).

The ability of macrophages to locally opsonise leishmania parasites by secretion of complement components under serum-free conditions does not appear to be restricted to L.donovani. Hence, Mosser and Edelson (1985) demonstrated that the numbers of L.tropica (L.major) promastigotes becoming attached and being ingested by murine peritoneal macrophages was inhibited using soluble M1/70. The inhibition was shown to be specifically due to inhibitor of attachment to CR3, although the extent of the inhibition (54%) was lower than that for L.donovani (see above) indicating differences in the requirements of different species. This appears to have been confirmed by a recent report from Mosser and his co-workers (1987). Again using

M1/70 which is specific to the CR3, they confirmed that attachment and uptake of L.major promastigotes was inhibited by about 50%. Further, incubation with an Advance Glycosylation Endproduct (AGE) bound to BSA also produced approximately 50% inhibition. AGEs apparently arise from a time-dependent non-enzymatic reaction of glucose with proteins, and a novel receptor distinct from other scavenge receptors that specifically binds to AGE moieties has been found on macrophages. Significantly, unlike the L.donovani system, the use of these reagents together produced an additive effect, inhibiting attachment and uptake by 90%. These workers concluded that these two receptors can account from the majority of L.major attachment and uptake. It remains to be determined whether this holds true for other Leishmania-phagocyte systems.

B) Serum-dependent

It has been reported that normal, fresh serum from various sources when at sub-lytic dilutions in the incubation medium enhances promastigote attachment and uptake in comparison to when there is either no serum or HI serum. This effect appears to be due in the main to complement. Thus, Mosser and Edelson (1984) reported that fresh serum increased the attachment and uptake by murine resident peritoneal macrophages of L.tropica (L.major) and L.enrietti promastigotes by 2- and 5-fold respectively. They subsequently demonstrated (Mosser & Edelson, 1985) that the CR3 specific antibody, M1/70, could inhibit attachment and internalization of the latter species by about 50% (a similar effect to that in the absence of serum). Likewise, Blackwell et al (1985) found that fresh serum increased (4-fold) the numbers of L.donovani promastigotes becoming attached and internalized by murine peritoneal macrophages, although there was no effect with amastigotes. This effect with promastigotes

could be inhibited by 80% with soluble or plated M1/70. In contrast to serum-independent studies, mannan (again either soluble or plated) had only a small inhibitory effect of about 35%, indicating tha for serum-dependent promastigote attachment CR3 acts at least partially independently of MFR. Also they showed that depletion of macrophage FC receptors using 2.4.52-Fab-coated coverslips caused a 30% reduction, presumably mediated by "natural" antibodies. Hence receptors other than MFR may wor k independently or in conjunction with CR3 in these conditions (Blackwell, 1985). In addition, it has been demonstrated that HI serum slightly increased the attachment and uptake of L.donovani promastigotes (but not amastigotes) compared to that occurring in the absence of serum (Blackwell et al., 1985). Other workers, however, studying L.enrietti or L.tropica (L.major) promastigotes found no difference (Mosser & Edelson, 1984). Nevertheless, these results suggest that serum factors other than complement such as "natural" antibodies, may play a role in macrophage-leishmania interactions.

Studies with immune serum (normally HI) have produced variable results. For example, the attachment and uptake of L.donovani amastigotes by human blood monocytes and L.tropica promastigotes by murine resident peritoneal macrophages (Farah et al., 1975; Chang, 1981b) were both inhibited by the presence of anti-leishmania antiserum from various sources. In contrast, L.mexicana mexicana promastigotes by murine resident peritoneal macrophages was enhanced in the presence of antiserum (Bray, 1983a). It should be noted that prior treatment of macrophages with immune serum, but excluding it from the incubation medium, can significantly enhance the attachment and uptake of promastigotes and amastigotes (Herman, 1980; Bray, 1983a) as can pre-treatment of promastigotes with antisera (Herman,

1980; Chang, 1981a; Channon et al., 1984). This apparently is mediated by antibodies binding to the Fc receptors on the macrophages and subsequently the parasite in the former case and by the binding of antibodies first to the parasite and then the Fc receptor of the macrophage in the latter case. Rather surprisingly, it was reported that pre-treating amastigotes with antisera inhibited attachment and upake (Chang, 1981b) whereas an increase albeit small in these parameters had been found earlier (Herman, 1980).

1.4.2.5.1.2 Neutrophils

In comparison to macrophages and monocytes, there have been relatively few studies with neutrophils, and all have involved human blood neutrophils. Pearson and Steigbigel (1981) showed that the uptake of L.donovani promastigotes in the presence of fresh serum had saturation kinetics and was saturated after about 15 mins. The uptake appeared to be due almost entirely to complement as heat-inactivating the serum reduced uptake to negligible levels. Similarly, it has been reported that in the absence of serum neutrophil-PMV do not adhere to L.tropica (L.major) amastigotes (Klempner et al., 1981). In contrast, Chang (1981c) reported that L.donovani amastigotes were taken up by neutrophils in the presence of HI serum, although the uptake was increased in the presence of fresh serum (Chang, 1981b). Uptake in both conditions showed saturation kinetics, becoming saturated between 30 and 60 min. Furthermore, neutrophils were found to be more efficient at phagocytosing amastigotes than were monocytes (Chang, 1981b,c).

1.4.2.5.1.3 Specific cell surface molecules on promastigotes

The roles of surface molecules of Leishmania in attachment to macrophages and their subsequent uptake has been considered in some recent studies. Only promastigotes have been studied in any detail so

far. There appear to be two main molecules involved, and both are major surface constituents of promastigotes.

A) Major glycoprotein

Using purified antibodies specific to the major surface glycoprotein (characterised by its apparent molecular weight of 63KDa) of Leishmania mexicana mexicana stationary-phase promastigotes, Russell and Wilhelm (1986) demonstrated, using serum-free conditions and a radioisotopic assay, that the antibodies added to the incubation medium could inhibit promastigote interaction with J774 cell line or murine resident peritoneal macrophages by 60-75%. By inserting the glycoprotein into proteoliposomes and following their interaction with the J774 cell-line, they were also able to show that the major glycoprotein on L.mexicana mexicana promastigotes is capable of attaching to macrophage surface receptor(s) and that the receptor(s) involved can also mediate phagocytosis. Similarly, using the same assay and conditions, pre-treating J774 cells with the purified major surface glycoprotein of L.mexicana amazonensis (apparent molecular weight 63KDa) was shown to inhibit the attachment of these parasites to the J774 cell line by 50%. (Chang & Chang, 1986). Likewise, Kweider et al (1986) reported that a monoclonal antibody specific to the apparently equivalent, major surface protein of L.braziliensis (apparent molecular weight 65KDa) could, when pre-incubated with stationary-phase promastigotes, inhibit the attachment and uptake of these promastigotes by murine peritoneal macrophages.

The mechanism by which the major glycoprotein mediates attachment is unknown although some suggestions have been made. The glycoprotein of L.mexicana mexicana promastigotes referred to above has been shown to be the major-C3 binding protein and could mediate interaction in this way, Further, a glycoprotein which is the major surface protein

of most Leishmania promastigotes, characteristically with an apparent molecular weight of 63-68KDa, has been shown proteolytic activity. It has been postulated that this activity is necessary for the cleavage of C3 to C3bi. This would inhibit further activation of the complement cascade and result in parasite-bound C3bi, the ligand for which the macrophage CR3 is specific (Bordier, 1987; Bouvier et al., 1987). Presently, the role of this molecule in the interaction of amastigotes with macrophages is uncertain, as it is yet to be confirmed that it is present on amastigotes. Its absence or lower levels on amastigotes, however, may explain in part at least, why amastigotes are poor activators of the alternative complement pathway compared to either log- or stationary-phase promastigotes and also why complement in the absence or presence of serum has apparently a minor role in attachment and uptake of amastigotes by macrophages.

B) Major glycolipid

Although virtually all leishmania promastigotes appear to possess similar major surface glycolipids, the role of this molecule in the interaction with macrophages has only been studied with L.major promastigotes. The intact cellular membrane form of the glycolipid can insert itself non-specifically into the membranes of many cell types including myeloma cells, J774 cell line and erythrocytes (Handman & Goding, 1985; Handman et al., 1987), whereas the water soluble form found in culture supernatants binds only to the J774 cell line by the carbohydrate moiety (Handman & Goding, 1985). Moreover, the attachment of L.major promastigotes (but not L.mexicana mexicana) to the J775 cell line, in the presence of HI serum, is inhibited 80% by a monoclonal antibody which specifically recognises the carbohydrate epitope of L.major glycolipid (tentatively the terminal galactose residues), and similarly by high concentrations of the purified

carbohydrate portions of this molecule (Handman & Goding, 1985; Handman et al., 1987). However, it is unclear whether the glycolipid is involved simply in attachment or whether its binding also mediates phagocytosis. Indeed, additional studies with an isolate of L.major that lacks the glycolipid molecule showed that these parasites were avidly phagocytosed by J774 cells, more so than an isolate with the glycolipid, but the intracellular parasites were subsequently killed - indicating a role for the glycolipid in intracellular survival (Handman et al., 1986).

Recently, there has been an attempt to bring these different ideas together. It has been suggested that the glycolipid may be involved in early events in promastigote-macrophage interaction by mediating "docking" of the promastigote onto the macrophage receptor and that this is followed by subsequent attachment of the major glycoprotein: **(1)** through a C3 bridge to CR3; **(2)** directly, via a sugar-lectin interaction to the mannose-fucose receptor, or **(3)** both of the mechanisms (Blackwell et al., 1986; Handman et al., 1987).

1.4.2.5.1.4 Interactions of macrophages with log- and stationary-phase promastigotes of Leishmania

With respect to attachment, as with most other areas of research on leishmanias, very little work has been carried out specifically comparing log-phase and stationary-phase promastigotes. When this has been done, the latter promastigote has also been shown to be more infective than the former. It was reported that in the presence of HI serum only 35-40% of murine resident peritoneal macrophages became attached to log-phase promastigotes of L.braziliensis or L.chagasi, whereas 70-80% of the macrophages had stationary-phase promastigotes attached. When longer incubation periods were used so that ingestion could be followed, similar results were obtained with stationary-phase

cells being more infective both in terms of the % macrophages infected and the number of parasites/100 macrophages (Rizvi et al., 1985; Kweider et al., 1987). In another study, L.donovani log-phase and stationary-phase promastigotes attached to a similar percentage of the macrophages although upon longer incubations it was found that about twice as many stationary-phase promastigotes were ingested compared to log-phase promastigotes (Rizvi et al., 1985). Interestingly, however, under serum-free conditions far greater numbers of L.donovani stationary-phase promastigotes attached and were ingested by murine resident peritoneal macrophages than log-phase promastigotes. The involvement of CR3 was confirmed by inhibition studies using M1/70 and it was concluded that stationary-phase promastigotes are more efficient activators of the alternative complement pathway than log-phase cells (Wozencroft & Blackwell, 1987). In direct contrast to these studies, it was reported that the uptake by murine resident peritoneal macrophages of L.major stationary-phase promastigotes in the presence of HI serum was the same (no data given) (Sacks et al., 1985) or less (Scott & Sher, 1986) than that of log-phase cells. Similarly Scott and Sher (1986) demonstrated no difference in the uptake of log-phase versus stationary-phase promastigotes of L.mexicana amazonensis.

Studies with avirulent/non-infective (high sub-passage) and virulent/infective (low sub-passage) promastigote populations have also produced contradictory results. Ebert et al (1979) reported that in the presence of HI serum less log-phase, avirulent L.donovani promastigotes attached and were subsequently ingested by hamster peritoneal macrophages compared to log-phase virulent cells. Similarly, Saraiva et al (1987) examining attachment and uptake of L.mexicana amazonensis promastigotes in the absence of serum reported

that stationary-phase infective (low sub-passage) promastigotes attached to and were ingested much better by resident murine peritoneal macrophages than stationary-phase non-infective (sub-passage 300) promastigotes and gave rise to a larger percentage of infected macrophages and mean number of parasites per infected macrophage. In contrast, Giannini et al (1981), examining only attachment, reported no difference in the numbers of L.donovani stationary-phase, infective (low sub-passage) or non-infective (high sub-passage) promastigotes attaching to hamster resident peritoneal macrophages (in presence of HI serum). Similar results have been reported for infective and non-infective strains of L.tropica promastigotes attaching to murine resident peritoneal macrophages (Ebrahimzabeh & Jones, 1985).

1.4.2.5.2 Microbicidal events during and following attachment and uptake of leishmanias

Unlike Toxoplasma gondii, upon attachment and during phagocytosis both promastigotes and amastigotes of Leishmania stimulate a RB (Blackwell & Alexander, 1983). Following phagocytosis of either form of Leishmania, the parasite is retained within a phagosome or parasitophorous vacuole. Using pre-labelled secondary lysosomes, it has been observed for many species of Leishmania, in several different parasite-macrophage systems, that fusion of the parasitophorous vacuole with secondary lysosomes occurs both in vitro (Alexander & Vickerman, 1975; Chang & Dwyer, 1976, 1978; Lewis & Peters, 1977; Alexander, 1981a,b) and in vivo (Berman et al., 1979). Fusion of primary lysosomes with Leishmania-containing parasitophorous vacuoles has also been demonstrated in human blood monocytes (Chang, 1981c). This is in contrast to the situation with other intracellular parasites such as T.gondii and Mycobacterium tuberculosis which

prevent phagosome lysosome fusion, and Trypanosoma cruzi which escapes from the phagosome (Blackwell & Alexander, 1983; Mauel, 1984; Moulder, 1985).

Thus leishmanias are apparently exposed to the microbicidal activities of the host macrophage. There have been many studies aimed at understanding how they survive. Our current understanding is summarised below.

1.4.2.5.2.1 Oxygen-dependent mechanisms

The role of oxygen-dependent microbicidal mechanisms in the outcome of infection by leishmanias has been investigated using several phagocyte and phagocyte-free in vitro systems.

A) Stimulation of the respiratory burst

Work from several laboratories, mainly using murine macrophages but also human monocytes and monocyte-derived macrophages, indicates that in general promastigote and amastigote forms of Leishmania species differ in their ability to trigger the RB. By using the qualitative NBT technique (see Materials and Methods Section 2.5.5.1) and by quantitatively measuring O_2^- or H_2O_2 production, it was shown that L.donovani and L.tropica promastigotes, in the absence of serum, trigger a large RB in murine resident peritoneal macrophages comparable to that elicited by the potent soluble stimulant PMA or the potent particulate stimulant zymosan (Murray, 1981a,b, 1982a). In contrast, amastigotes trigger a much reduced RB. Hence, under conditions in which L.donovani promastigotes stimulated 70-80% of the macrophages amastigotes stimulated only 40-50% of the macrophages even though amastigotes resulted in greater numbers of infected macrophages containing more parasites (Murray, 1982a; Channon et al., 1984). Further, by scoring the individual formazan-positive macrophage-associated parasites (=parasites triggering) it was shown that whereas

80% of promastigotes triggered only 25% of amastigotes did. These studies highlighted the large difference between the two forms in their triggering of the RB (Channon et al., 1984). Similarly, it was reported that 85-90% of L.mexicana mexicana promastigotes triggered, whereas only 10-22% of amastigotes did (Alexander & Russell, 1985). Using the ferricytochrome C technique for quantitatively measuring O_2^- production, Murray (1982a) found that L.donovani promastigotes in the absence of serum, stimulated the production of approximately 20 times as much O_2^- as did amastigotes. However, using the quantitative NBT technique (see Materials and Methods Section 2.5.5.1), in the presence of HI serum, which (unlike the above assay which only measures extracellular O_2^-) measures O_2^- generated on the surface membrane and within the parasitophorous vacuole, only a 2-fold difference between L.donovani promastigotes and amastigotes was reported (Scott et al., 1985). Other workers using the qualitative NBT assay and the ferricytochrome C assay and resident murine peritoneal macrophages have confirmed that promastigotes of L.mexicana mexicana stimulate the production of much greater quantities of O_2^- than amastigotes (Stokes, 1981, 1983).

The differences seen in triggering of the RB by L.donovani promatigotes and amastigotes when exposed to murine resident peritoneal macrophages were maintained when the macrophages were pre-activated in vivo (Murray, 1982a; Haidaris & Bonventre, 1982). However, when macrophages were pre-activated in vitro by exposure to LK's, both promastigotes and amastigotes of L.donovani stimulated 86-90% of macrophages to reduce NBT (Murray, 1982a). Nevertheless, the quantitative difference in stimulation of O_2^- production by the two forms of this species (Murray, 1982a; Scott et al., 1985) and L.mexicana mexicana (Stokes, 1983) were seen with resident macrophages

were maintained even when activated *in vitro*.

Work with human phagocytes has been more limited but has produced similar results. Murray and Cartelli (1983) found that in the absence of serum the same percentage of blood monocytes (about 90%) are stimulated to reduce NBT by *L.donovani* promastigotes or amastigotes, although promastigotes stimulated the production of 6-times more H_2O_2 than amastigotes. In contrast, Pearson, Harcus *et al* (1983) found that 70% of blood monocytes in the presence of HI serum were triggered by *L.donovani* promastigotes, whereas only 34% were stimulated by equal numbers of amastigotes. Further, measuring the RB quantitatively by luminol-enhanced chemiluminescence (CL) at various parasite-monocyte ratios, they found that although the peak response to amastigotes was lower than for promastigotes at all the ratios used, only at a ratio of 10 parasites to 1 monocyte was the RB response significantly lower with amastigotes.

Allowing the monocytes to mature into macrophages reduced by an order of magnitude, the amount of H_2O_2 secreted in the absence of serum in response to either form of *L.donovani* (Murray & Cartelli, 1983). In addition, Murray and Cartelli (1983) observed that promastigotes stimulated 90% of macrophages to reduce NBT whereas only about 50% were stimulated with amastigotes, and the latter form stimulated 6-times less H_2O_2 production than the former. Similarly, Pearson *et al* (1982) using luminol-enhanced CL in the presence of HI serum reported that the response of macrophages to *L.donovani* amastigotes was only 15% of that to promastigotes at the same ratio. Studies with human blood neutrophils have only been carried out using *L.donovani* promastigotes and have shown that promastigotes pre-oposonized in fresh serum stimulated a RB as measured by O_2^- production using ferricytochrome C (Pearson & Steigbigel, 1981; Remaley, Glew *et al*., 1985).

Where it has been examined, the size of the RB response of murine resident peritoneal macrophages, human blood monocytes and monocyte-derived macrophages appears to be dependent on the numbers of leishmanias (L.tropica promastigotes or L.donovani promastigotes and amastigotes have been studied) they are exposed to (Murray, 1981a, 1982a; Pearson et al., 1982; Murray & Cartelli, 1983; Pearson, Horcus et al., 1983). As with other stimuli, the RB response to leishmanias increases to a maximum and then declines to background levels (Meshnick & Eaton, 1981; Pearson et al., 1982; Pearson, Harcus et al., 1983), and it has been shown for L.donovani and human monocyte-derived macrophages that the luminol-enhanced CL response of macrophage monolayers did not differ 24 and 72 hours after infection indicating that intracellular parasites of this species at least, are not a stimulus for continuous oxidative activity (Pearson et al., 1982). Furthermore, by taking samples or measuring CL at various time intervals, the kinetics of the response has been examined. Hence, Murray (1981a) found that with a ratio of 5 L.tropica promastigotes to 1 murine resident peritoneal macrophage peak H_2O_2 production occurred after about 90 min, whereas at a ratioa of 10:1 Meshnick found that peak luminol-enhanced CL (in this case due mainly to O_2^- production) occurred after about 30 min. Pearson, Harcus et al (1983) observed with human blood monocytes and a ratio of 20 parasites to 1 phagocyte that the peak luminol-enhanced CL to both L.donovani promastigotes and amastigotes was at 30 min. In contrast, using similar methodology with a ratio of 10:1, but in this case with human monocyte-derived macrophages, the peak response to L.donovani promastigotes was at 60 min, whereas that for amastigotes was later at 135 min, and was much lower (as discussed above) (Pearson et al., 1982).

There are no published accounts concerning the stimulation of the

RB by infective, stationary-phase promastigotes, although there are two references to unpublished work concerning this matter. Preliminary observations with L.major proboscis promastigotes from the proboscis of P.papatasii suggested that unlike midgut forms, the former parasites failed to trigger a RB (as measured by the qualitative NBT technique) on contact with murine resident peritoneal macrophages (Alexander & Russell, 1985). A fuller account of this preliminary experiment has recently appeared (Killick-Kendrick, 1987). Along similar lines it has been reported that more macrophage (murine resident peritoneal)-derived O_2^- was produced in response to log-phase promastigotes of L.donovani than to stationary-phase promastigotes, although this was still greater than that elicited by amastigotes (Blackwell et al., 1985).

i) Mechanisms for the differential respiratory burst response to promastigotes and amastigotes

Several investigators have examined the effect of various treatments on promastigotes and amastigotes in attempts to explain the differential RB to the two forms. Stokes (1983) reported that heat-killed promastigotes of L.mexicana mexicana and L.mexicana amazonensis were poor stimulators of O_2^- production by murine resident peritoneal exudate cells in comparison to glutaraldehyde-fixed and living promastigotes. It was suggested that surface integrity of the promastigote (which would be destroyed by heat but not glutaraldehyde) was necessary for the macrophages to be stimulated to produce O_2^- and indicated the importance of surface components. In contrast, Channon et al (1984) demonstrated that, in the absence of serum, heat-killed, glytaraldehyde-fixed and sodium azide-(NaN_3) treated L.donovani promastigotes were as stimulatory (in terms of both the percentage of macrophages stimulated and the percentage of parasites triggering) as

live promastigotes. Interestingly, these two parameters were increased compared to the controls when amastigotes were subjected to the same treatments. In contrast, however, Haidaris and Bonventre (1982) found that heat-killed and live L.donovani amastigotes stimulated the same amount of O_2^- production by in vitro-activated macrophages. Nevertheless, Channon et al (1984) suggested that their results indicated that amastigotes possess an azide-sensitive mechanism (which is also fixative and heat-sensitive) that either competes for O_2^- or causes localised inhibition/inactivation of the RB. Clearly though, these mechanisms, if they exist, could only cause very localised effects, rather than a general desensitisation of the RB, as it has been shown for L.donovani, L.mexicana mexicana and L.mexicana amazonensis amastigotes with resident murine peritoneal macrophages (Stokes, 1983; Channon et al., 1984) and for L.donovani amastigotes with human blood monocyte-derived macrophages (Pearson et al., 1982) that concurrant stimulation with zymosan or promastigotes plus amastigotes produces a similar RB (as measured by O_2^- production, CL and % formazan-positive promastigotes) to that when zymoson or promastigotes were used alone. Moreover, Channon et al (1984) also examined the effect of various monosaccharides and mannan on triggering of the RB by L.donovani promastigotes and amastigotes, and concluded that the RB response to promastigotes is mediated by the MFR and that the differential RB elicited by promastigotes and amastigotes is most likely because of qualitative and quantitative differences in the distribution of surface ligands involved in binding to the macrophage membrane. Similarly, Stokes (1984) found that O_2^- production by murine resident peritoneal macrophages stimulated by L.mexicana mexicana promastigotes was greatly inhibited by mannose and complex carbohydrates rich in mannose, and concluded that mannose ligands on the surface of promastigotes are recognized by specific receptors

involved in mediating production of the RB and that the reduced RB elicited by amastigotes could be accounted for by the reduced numbers of these ligands on this form.

Other receptors have also been implicated in stimulation of the RB, but these have only been examined in the promastigote form. Mosser et al (1987) found that under serum-free conditions AGE-BSA not only inhibited binding and uptake of L.major promastigotes to murine resident peritoneal macrophages by about 50% but also reduced the amount of O_2^- produced by a similar amount. Furthermore, by using a monoclonal antibody specific to CR3 (M1/70) both binding and uptake, and O_2^- production were inhibited by 50%. In addition, using the AGE-BSA and M1/70 together O_2^- production was reduced by 80% indicating that under these conditions attachment and uptake via either the AGE receptor or, more interestingly via the CR3, can trigger the RB. Somewhat contradictory results have recently been reported by Mosser and Edelson (1987). These workers investigated the binding and ingestion under serum-free conditions of L.major promastigotes by resident murine peritoneal macrophages and found that promastigotes that had been briefly opsonized (5-7 min) in fresh human serum lacking late complement components (which leads to the deposition of C3b on the promastigote membrane) triggered the production of less than half the O_2^- produced by unopsonized promastigotes. The majority of promastigotes ingested by macrophages in the absence of serum or with HI serum were killed by 24-48 hours, whereas by the use of fresh serum or pre-opsonising the parasites in serum led to 5-10 fold enhancement in parasite survival. It has concluded that this was related to the reduced RB triggered under the latter conditions, and that this was mediated through attachment and ingestion via a complement receptor on the macrophage which did not lead to triggering of the RB.

ii) Possible mechanisms of inhibition of the respiratory burst

Acid phosphatase: The possible role of acid phosphatase in aiding survival in the macrophage by inhibiting the respiratory burst was first shown by Remaley et al (1984). These workers found that the purified major surface acid phosphatase activity of L.donovani promastigotes (characteristically resistant to L-(+)-tartrate) was able to inhibit the RB (as measured by the quantitative ferricytochrome C technique) of human blood neutrophils when stimulated by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe). By inactivating the enzyme preparation by boiling or by including a potent inhibitor of L.donovani acid phosphatase (complex E) in the incubation mixture, and by adding varying amounts of the active enzyme preparation, it was shown that the inhibition of the RB was due to the catalytic activity of the enzyme. The effect was time dependent. The enzyme had no effect if added with the stimulating agent whereas preincubation of the enzyme with the neutrophils produced inhibition, the maximum effect occurring after 30 min. A similar inhibitory effect on oxygen consumption and H_2O_2 production by neutrophils was also observed but interestingly there was always a residual phosphatase-resistant capacity for oxidative metabolite production which accounted for 15-30% of the total. This residual amount of oxidative metabolism could not be explained by the presence of an acid phosphatase-resistant subpopulation of neutrophils since a uniform, but incomplete decease of formazan deposits was observed using the qualitative NBT technique (Remaley, Glew et al., 1985). The inhibitory effect was manifested when either fMet-Leu-Phe or ConA were used as stimulating agents, but not when PMA was used (Remaley et al., 1984; Remaley, Glew et al., 1985). Moreover, the acid phosphatase had no effect on neutrophil

chemotactic peptide binding or other functions of the stimulated neutrophil such as degranulation and membrane depolarization (Remaley, Glew et al., 1985). Overall, these observations were taken to suggest that the enzyme must be exerting its effect by interacting with components on the external surface of the neutrophil (Remaley, Glew et al., 1985) (see below for details on mode of action). By testing for effects of the other two surface acid phosphatase activities (both tartrate-sensitive) and the secreted acid phosphatase tartrate sensitive) of L.donovani promastigotes, it was shown that the inhibition appeared to be specific to the tartrate-resistant surface enzyme. Further testing of acid phosphatases and alkaline phosphatases from a variety of sources generally confirmed the specificity of the inhibition, although interestingly prostatic acid was as effective at inhibiting O_2^- production as the tartrate-resistant acid phosphatase of L.donovani (Das, Saha, Remaley et al., 1986). More significantly, by stimulating human blood neutrophils with oposonized L.donovani promastigotes and opsonized zymosan (in the absence of serum) in the presence or absence of Complex E, Remaley, Glew et al (1985) were able to show that promastigotes inhibited 2-3 fold the rate of production and the total O_2^- concentration released by the neutrophils and that this was specifically due to surface acid phosphatase activity. It is surprising that other workers have not detected such inhibition when using increasing numbers of L.donovani promastigotes with human blood monocytes (see Pearson, Harcus et al., 1983). However, some confirmatory evidence for a role for acid phosphatase against the microbicidal defences of the macrophage has recently been supplied by Katakura (1986). He showed that a virulent line of L.donovani promastigotes had twice as much acid phosphatase activity as an avirulent line, and significantly, this was due to having twice as

much L-(+)-tartrate-resistant activity. Indeed, Remaley, Glew et al (1985) have suggested that a protective role for the enzyme during interaction with neutrophils or monocytes is unlikely due to the magnitude of the RB they release, but more likely it enhances survival in the relatively unreactive resident macrophage where the balance between the ability of macrophages to generate toxic oxygen metabolites and the ability of the parasite to neutralise these metabolites may be tipped in favour of the parasite by the acid phosphatase. The role of acid phosphatase in respect to L.donovani amastigote survival is now known, and similarly, the role of this enzyme in other Leishmania species has yet to be clarified.

The mechanism by which acid phosphatase inhibits the RB is not well understood. Purified enzyme was found dephosphorylate several phosphoproteins, which are phosphorylated at serine residues, including pyruvate kinase, phosphorylase kinase and histones. However, the specific activity of leishmanial phosphatase on these phosphoproteins was very low compared to other phosphoprotein phosphotases and, moreover, there was no correlation between the ability to dephosphorylate proteins and to inhibit O_2^- production. Also, neutrophil plasma membrane phosphoproteins were not dephosphorylated by leishmanial acid phosphatase, and these data indicated that the inhibitory mechanism of the enzyme was not mediated by its ability to dephosphorylate phagocyte phosphoproteins. Interestingly, though, both PIP_2 and IP_3 were hydrolysed. The enzyme was optimally active on PIP_2 at neutral pH and at this pH the activity of the enzyme on PIP_2 was relatively high, being 21% of that obtained with methylumbelliferyl phosphate. As both PIP_2 and IP_3 have been shown to be involved inthe series of events linking attachment to a phagocyte receptor to activation of the membrane bound oxidase and hence the RB, it was suggested that leishmanial acid phosphatase may

inhibit the production of an RB by breaking down either or both of these compounds (Das, Saha, Remaley et al., 1986).

Protein kinase and lipase: There are two other enzymes present on the surface membrane of L.donovani promastigotes that could possibly interfere with production of the RB by phagocytes and lower RB elicited by stationary-phase promastigotes compared to log-phase cells. Leishmania donovani promastigotes possess a surface-bound protein kinase activity and this is at 6-fold activity in stationary-phase cells compared to log-phase promastigotes (Das, Saha, Mukhpadhyay et al., 1986). This protein kinase could interfere with the protein phosphorylation events in the phagocyte that lead to activation of the membrane oxidase and production of the RB. Similarly, it has been suggested that L.donovani stationary-phase promastigotes could interfere with RB function via a surface bound lipase acting on DAG in the phagocyte membrane, as this latter component is also involved in linking ligand-receptor interaction to activation of the membrane bound oxidase (Blackwell et al., 1986). To date, there is no information available on the relative activity of these enzymes on amastigotes.

B) Susceptibility of leishmanias to products of the respiratory burst

Several groups have examined the susceptibility of leishmania promastigotes and a lesser extent amastigotes, to products of the RB using various cell-free systems. Mostly commonly this has involved using reagent H_2O_2 or employing enzyme systems which generate H_2O_2 but not O_2^- (for instance glucose plus glucose oxidase). In some cases, the susceptibility to other toxic intermediates has been examined using xanthine- or acetaldehyde -XO systems which produces O_2^-, H_2O_2, $OH^\bullet$ and possibly $^1O_2$. The MPO system is usually mimicked in vitro by addition

of lactoperoxidase (LPO) and potassium iodide (KI) (Murray, 1981a, 1982a; Haidaris & Bonventre, 1981; Pearson & Steigbigel, 1981; Reiner & Kazura, 1982; Locksley & Klebanoff, 1983; Pearson, Harcus et al., 1983; Thorne & Blackwell, 1983; Channon & Blackwell, 1985a; Katakura, 1986). There is considerable variation in LD_{50} (lethal dose 50% killing) values obtained for H_2O_2 (reagent or generated) even with the same species and strain. This is apparently due to variations in experimental conditions, such as, parasite density, sub-passage number, treatment time, activity of glucose oxidase, glucose concentration, degree of agitation of reaction mixture or method of measuring viability (Thorne & Blackwell, 1983). Nevertheless, the LD_{50}'s that can be ascertained from the data presented or were stated by the authors are given in Table 4 for reagent, H_2O_2 and Table 5 for generated H_2O_2. By examination of the results obtained from within the same laboratory, it appears that: **(1)** L.tropica promastigotes are more susceptible to H_2O_2 than L.donovani promastigotes; **(2)** within each species amastigotes are consistently, but variably (2 to 10-fold), more resistant to H_2O_2 than promastigotes; **(3)** addition of a peroxidase and a halide markedly, but variably (5 to 100-fold), enhances the sensitivity of Leishmania, although within the same system amastigotes are still more resistant than promastigotes. However, it should noted that there is one report for L.donovani promastigotes that addition of LPO and KI did not enhance the effect of reagent H_2O_2, although it did in a H_2O_2-generating system (Pearson & Steigbigel, 1981).

A few workers have also examined the susceptibility of Leishmania to toxic products other than H_2O_2, although these results are somewhat contradictory. Reiner and Kazura (1982) reported that XO and acetaldehyde in combination had no effect on L.donovani promastigotes. In contrast, Murray (1981a) using the XO-xanthine system, at similar

Table 4: Sensitivity, *in vitro*, of *Leishmania* species to catalase-inhibitable reagent hydrogen peroxide.

Species and stage	Parasite density (ml^{-1})	Incubation time (h)	Temperature (c)	Viability assay	LD_{50} H_2O_2 Concentration	LD_{50} H_2O_2 Conc. (with peroxidase + halide)	Reference
L.donovani							
promastigote (Sub 19)	5×10^6	1	37	FM; FDA/EB	0.1mM		Channon & Blackwell 1985a Thorne & Blackwell 1983
promastigote (Sub 1)	5×10^6	1	37	FM; FDA/EB	0.5mM		
amastigote	5×10^6	1	37	Trans; FDA/EB	0.75mM		
amastigote	5×10^6	1	37	Trans	0.1mM	0.01-0.001mM+MPO(260mU)+KI(0.1mM)	Hardaris & Bonventre 1982
promastigote (Sub 1 or 2)	3×10^6	1	37	Culture	0.5mM		Pearson, Harcus *et al.*, 1983
amastigote	3×10^6	1	37	Trans	1.0-5mM		
amastigote	3×10^6	1	37	Trans	0.1-1.0mM	0.01mM+LPO(100mU)+KI(0.1mM)	
L.mexicana mexicana							
promastigote	5×10^6	1		T.B.	0.1-0.2mM	0.01-0.02mM+LPO+KI	Thorne & Blackwell 1983
amastigote	5×10^6	1		TB	1-2mM	0.1-0.2mM+LPO+KI	
L.tropica							
promastigote					0.25mM		Locksley & Klebanoff 1983
amastigote					0.8mM		

Abbreviations: See Table 5.

Table 5: Sensitivity, *in vitro*, of *Leishmania* species to catalase-inhibitable glucose oxidase generated hydrogen peroxide.

Species and stage	Parasite density (ml^{-1})	Incubation time (h)	Temperature (^{o}C)	Glucose concentration (mM)	Viability assay	LD_{50} (nmol H_2O_2 min^{-1} ml^{-1})	LD_{50} (with peroxidase+ halide) (nmol H_2O_2 min^{-1} ml^{-1})	Reference
L.donovani								
promastigote (Sub 19)	5×10^6	1	37	5.5	FM;Culture:FDA/EB ErythB	3.1	0.09+LPO+KI	Channon & Blackwell 1985a Thorne & Blackwell 1983
promastigote (Sub 1)	5×10^6	1	37	5.5	FM;Culture;FDA/EB ErythB	4.7		
amastigote	5×10^6	1	37	5.5	Trans	8.7	0.9+LPO+KI	
promastigote	5×10^6	1	37	5.5	M	1.5	0.4+LPO(10mU)+KI(0.05mM)	Murray, 1982a; 1987a
amastigote	$5\text{-}10 \times 10^6$	1	37	5.5	Trans	8.1	0.9+LPO(10mU)+KI(0.05mM)	
promastigote	2×10^6	24	37	13.0	M;Culture	8.8		Pearson & Steigbigel 1981
L.mexicana mexicana								
promastigote	5×10^6	1		5.5	TB	1.7-3.1		Thorne & Blackwell 1983
amastigote	5×10^6	1		5.5	TB	8.7-16.2		
L.tropica								
promastigote	5×10^6	1		5.5	M;Culture	0.5		Murray 1981a

Abbreviations: Tables 4 & 5: Culture, subsequent culture for 1-3 days; ErythB; erythrosin B uptake by non-viable parasites; FDA/EB, combined fluorescence diacetate esterase staining for viable parasites and ethidium bromide staining for non-viable parasites, assessed after parasites had been in culture for 24hrs aftr treatment; FM, flagellar motility; LPO, Lactoperoxidase; M, motility; MPO, myeloperoxidase from human blood neutrophils; TB, trypan blue uptake by non-viable parasites; Trans, transformation of amastigotes to promastigotes in subsequent cultures over 42-96 hours; Sub, subpassage.

concentrations to those used in the above study found that both L.tropica and L.donovani promastigotes were susceptible to the products produced, and addition of LPO and KI enhanced the effect. Similarly, L.donovani amastigotes were found to be susceptible to the XO-acetaldehyde system, although the concentrations used were greater than those used by Reiner & Kazura (1982) (Murray, 1982a). By the use of inhibitors and scavengers such as SOD, catalase, mannitol, benzoate and histidine, Murray (1981a, 1982a) showed that only catalase effectively inhibited killing, suggesting that H_2O_2 alone was the reactive species involved. Other investigators using the XO-xanthine system and various inhibitors, have observed somewhat different effects. Haidaris and Boventre (1982) observed a 50% inhibition of the killing of L.donovani promastigotes by the addition of catalase, and smaller (15%) inhibition by SOD. This latter enzyme has been reported to have a greater effect (87% inhibition) on the killing of L.braziliensis promastigotes (Locksley & Klebanoff, 1983) and similarly the killing of L.mexicana amazonensis promastigotes was completely abrogated by SOD and to a lesser extent by catalase (Nabi & Rabinovitch,1984). These results indicate that other toxic intermediates besides H_2O_2 may be involved in the killing of Leishmania.

Although it might be expected that log-phase promastigotes would be more susceptible to toxic oxygen intermediates compared to stationary-phase or metacyclic promastigotes, there is no published work examining the in vitro susceptibility of the two forms. Nevertheless, work with promastigotes of differing virulence indicates that they have different susceptibilities. A virulent line of L.donovani promastigotes have been shown to be more resistant to reagent H_2O_2 than avirulent promastigotes of the same strain

indicating a role for H_2O_2 resistance in determining virulence. Interestingly, there was no difference in susceptibility with the H_2O_2/LPO/KI system (Katakura, 1986). Similarly, sub-passage 1 L.donovani promastigotes have been reported to be more resistant to reagent and genereated H_2O_2 than sub-passage 19 promastigotes, being intermediate in sensitivity between these high sub-passage promastigotes and amastigotes (Thorne & Blackwell, 1983; Channon & Blackwell, 1985a).

i) Mechanisms of protection against oxygen metabolites

To explain the differences in susceptibility of promastigotes and amastigotes to toxic oxygen products, several workers have examined the two forms for the presence and activity of enzymes likely to be involved in the protection of cells from toxic oxygen intermediates. SOD activity has been detected in L.tropica (Meshnick & Eaton, 1981), L.donovani (Murray, 1981a), and L.mexicana mexicana promastigotes (Ghafoor & Coombs, 1981) at levels similar to those in other aerobic cells such as Crithidia fasciculata and Toxoplasma gondii, although they were substantially higher than the level in bloodstream forms of Trypanosoma brucei brucei (Ghafoor & Coombs, 1981; Meshnich & Eaton, 1981; Murray, 1981a). Activity levels in amastigotes of L.mexicana mexicana were reported to be the same as those in promastigotes of this species (Ghafoor & Coombs, 1981) although L.donovani amastigotes have been reported to possess about twice the activity of promastigotes of this species (Murray, 1982a). The presence of other enzymes such as catalase, GR and GP appears to be more variable. Ghafoor and Coombs (1981) could not detect catalase activity in either L.mexicana mexicana promastigotes or amastigotes. Similarly, activity was not detected in L.tropica (Meshnick & Eaton, 1981) or L.donovani promastigotes (Channon & Blackwell, 1985b) although Murray (1981a)

found low amounts of activity in promastigotes of these latter two species. More interestingly, catalase activity was detected in the amastigotes of L.donovani (Murray, 1982a; Channon & Blackwell, 1985b). GP activity was not detected in promastigotes or amastigotes of L.mexicana mexicana (Ghafoor & Coombs, 1981), and likewise Channon and Blackwell (1985b) could not detect it in either form of L.donovani. In contrast, Murray (1981a, 1982a) detected low amounts in L.donovani promastigotes and about 14-fold higher activity in amastigotes. Very low levels of GP activity has also been reported in L.tropica promastigotes (Meshnick & Eaton, 1981; Murray, 1981a). GR activity has been reported in amastigotes and promasti gotes of L.donovani and L.mexicana mexicana, the activity being 3-4 fold higher in amastigotes (Ghafoor & Coombs, 1981; Channon & Backwell, 1985b). The role and contribution of these enzymes in neutralizing oxygen metabolites in leishmanias is unclear, although it appears to be generally accepted that leishmania are resistant to the affects of O_2^- because they possess SOD (Klebanoff et al., 1983; Locksley & Klebanoff, 1983). However, the low levels of other scavenger enzyme activity present in both forms of Leishmania, especially compared to Toxoplasma gondii, make it difficult to say whether they could play an important role in scavenging oxygen metabolites. Nevertheless, the generally higher activity of the enzymes in amastigotes compared to promastigotes, if confirmed, argues for same role in intracellular survival, possibly by neutralizing toxic oxygen metabolites. Indeed, Channon and Blackwell (1985a) working with L.donovani, found that the respective LD_{50} values obtained for each form of the parasite correlated with saturation of the ability to remove reagent H_2O_2 from a phagocyte-free system. The ability of promastigotes to react with H_2O_2 becoming saturated at lower concentrations compared to amastigotes. Further, Channon and Blackwell (1985b) reported that this process could be partially

inhibited in both forms (although more so in promastigotes) by reducing the temperature, indicating a role for enzymatic and non-enzymatic mechanisms in H_2O_2 removal. With amastigotes, aminotriazole (AT) and NaN_3, inhibitors of catalase and peroxidases, respectively, inhibited H_2O_2 removal to a large extent. This strongly suggests a role for these enzymes in protective H_2O_2 scavenging. Neither inhibitor had any effect on promastigotes, indicating the enzymatic mechanism in this form involved enzymes other than haem-containing enzymes. The non-enzymatic mechanism could not be identified by these authors although they suggested it was possibly due to the presence of an unsaturated lipid sink, either as a normal component of parasite membranes or incorporated in lipid inclusions. Interestingly, it has recently been reported that the unique co-factor trypanothione is involved in enzymatic and non-enzymatic H_2O_2 removal in L.donovani and other leishmanias (Penketh & Kennedy, 1986; Fairlamb et al., 1987). Cell lysates of amastigotes and promastigotes of this species have an NADH-dependent H_2O_2 consumption which has an absolute requirement for trypanothione. Further, most of the H_2O_2 removal by intact promastigotes and amastigotes apparently involved a non-enzymatic reaction with a large endogenous pool of reduced trypanothione (Penketh & Kennedy, 1986). Amastigotes also apparently have much higher intracellular concentrations of trypanothione on a cell to cell basis in comparison to promastigotes (Kennedy, pers. comm.) and could explain their increased resistance to H_2O_2.

c) In vitro studies with phagocytes

There are relatively few in vitro studies with phagocytes specifically examining the role of toxic oxygen metabolites in the killing of leishmanias; those reported are summarised here.

i) Promastigotes

Log-phase promastigotes of L.donovani (Murray, 1981a; Scott et al., 1985) and L.tropica (Murray, 1981a) are rapidly (by 18hrs) and efficiently killed by murine resident peritoneal macrophages. However, two macrophage-like cell lines, J774 and IC-21, which do not produce a RB after membrane perturbation (for instance by stimulation by PMA, zymosan, or promastigotes), allow log-phase L.donovani promastigotes to survive and multiply (Murray, 1981b; Scott et al., 1985). Similar results were obtained with log-phase L.tropica promastigotes and the J774 cell line (Murray, 1981b). If the J774 cells are exposed to LK's they become able to respond to perturbation of their membrane by producing a RB comparable to that of resident macrophages and are then able to kill L.donovani and L.tropica log-phase promastigotes (Murray, 1981b). Killing of these promastigotes by resident macrophages or LK-activated J774 cells was much reduced by loading the macrophages with catalase or depriving them of glucose. Moreover, killing was unaffected when these phagocytic cells were loaded with SOD, mannitol, benzoate, DABCO or histidine (Murray, 1981a,b). Pre-treatment of murine resident peritoneal macrophages with PMA (which totally exhausts the RB response) (Murray, 1982b), or with medium conditioned by tumor cells (which substantially diminished the RB) (Szuro-Sudol et al., 1983) totally abolishes the killing of log-phase L.donovani promastigotes or substantially reduces the killing, respectively.

Similarly, normal human blood monocytes and neutrophils kill L.donovani promastigotes. With cells from CGD patients, however, the killing does not occur in neutrophils (Pearson & Steigbigel, 1981) and is much reduced in monocytes (Murray & Cartelli, 1983; Pearson, Harcus et al., 1983). Also, the killing capacity of monocytes can be prevented (for the first 6 hours at least) by depriving them of

glucose or by treatment with catalase, but not SOD (Murray & Cartelli, 1983). In addition, as the monocytes mature into macrophages the magnitude of their RB response is reduced and L.donovani promastigotes are able to survive in them (Murray & Cartelli, 1983; Pearson, Harcus, et al., 1983). Finally, lymphokine-activated macrophages can kill L.donovani promastigotes but it can be prevented (to the same extent as in monocytes) by glucose deprivation, and by treatment with catalase, but not SOD (Murray & Cartelli, 1983).

Taken together, these observations strongly indicate a major role for oxygen-dependent mechanisms in the killing of promastigotes by a variety of phagocytes, and for H_2O_2 being the predominant promastigoticidal product. These are confirmed by studies on the stimulation of the RB and on the susceptibility of promastigotes to toxic oxygen metabolites in phagocyte-free systems (as already discussed above).

ii) Amastigotes

Amastigotes of all species of Leishmania tested are relatively resistant in vitro to toxic oxygen metabolites in comparison to promastigotes and also stimulate a smaller RB than promastigotes. It has been shown that L.donovani amastigotes are not killed by human blood monocytes (Murray & Cartelli, 1983; Pearson, Harcus et al., 1983). Amastigotes of this species, L.tropica, L.major, L.mexicana amazonensis, L.mexicana mexicana, and L.enrietti (see for example Behin et al., 1979; Buchmuller & Mauel, 1981; Haidaris & Bonventre, 1981, 1982; Murray et al., 1982; Murray, 1982b; Nacy et al., 1983; Scott & Sher, 1986) are also not killed by murine resident peritoneal macrophages, elicited inflammatory macrophages and macrophages activated in vivo or in vitro. In contrast, some L.braziliensis strains are (Scott & Sher, 1986). Amastigotes of most species (but not

L.mexicana amazonensis see Scott et al., 1983) are killed by all these murine cells (although this can depend on mouse strain, see Nacy et al., 1983) by the subsequent addition and continual replenishment of LK to the incubation medium (see for example, Behin et al., 1979; Buchmuller & Mauel, 1981; Hardaris & Bonventue, 1981, 1982; Murray, 1982b; Nacy et al., 1983; Hockmeyer et al., 1984; Scott & Sher, 1986). Although such activation may proceed by oxygen-independent pathways (see below), and it is unclear by which mechanism oxygen-dependent cidal activity might occur as there is no evidence that lymphokine induces an intraphagosomal RB in previously infected macrophages (Locksley & Klebanoff, 1983) and also amastigotes elicit a small RB response even from the activated cells. There are, however, several studies using L.donovani amastigotes, that have implicated oxygen-dependent mechanisms in the killing of these amastigotes. Murray (1982b) showed that killing could be prevented by withholding glucose or by adding catalase, similarly Haidaris and Bonventre (1982) found that addition of catalase, inhibited killing by LK-activated elicited macrophages, but in this case only about 50%. Murray (1982b) extended his studies and showed that addition of mannitol or benzoate did not prevent killing, but pre-treatment of the macrophages with PMA did. Moreover, Buchmuller and Mauel (1981) found that when elicited infected macrophages were incubated with LK and AT or trypan blue (another peroxidase inhibitor) killing of L.enrietti was strongly inhibited. In addition, L.donovani amastigotes were killed by normal human blood neutrophils and by CGD-neutrophils, although the leishmanicidal activity increased only with increasing numbers of normal neutrophils (Chang, 1981c). Thus, there is good evidence, for at least L.donovani and possibly L.enrietti that oxygen-dependent mechanisms can be involved in the killing of amastigotes. H_2O_2 appears to be the main amastigoticidal product implicated, although as both

Haidaris and Bonventre (1981) and Buchmuller and Mauel (1981) used elicited macrophages which may contain granule peroxidase by virtue of their recent migration from the blood it seems probably thatthis is also involved. Indeed the latter workers presented evidence for a role for peroxidase in amastigote killing.

1.4.2.5.2.2 Oxygen-independent mechanisms

There have been several observations implicating oxygen-independent mechanisms in the killing of leishmanias, although in most cases it is unclear which mechnism is involved. Thus, although CGD cells cannot undergo an RB to a variety of stimuli including leishmanias, their leishmanicidal defect is far from complete. For example, as described above, human blood CGD-neutrophils are capable of showing leishmanicidal activity against L.donovani amastigotes (Chang, 1981c) and a similar situation is seen with human blood CGD-monocytes and L.donovani promastigotes (Murray & Cartelli, 1983; Pearson, Harcus et al., 1983). Furthermore, even though glucose-deprivation and catalase treatment of normal human blood monocytes or LK-activated macrophages prevented their killing L.donovani promastigotes, this was only manifested in the first 6 hours. Thereafter, they achieved virtually normal levels of killing (Murray & Cartelli, 1983). In a similar way, although LK-activated human blood CGD-monocytes and macrophages still lacked the ability to produce a RB, they could prevent L.donovani amastigote multiplication and reduced promastigote infections by 80% (Murray & Cartelli, 1983). Likewise with the macrophage like cell line IC-21 which lacks RB and cannot kill log-phase L.donovani promastigotes. If they are activated in vitro by pre-treatment with LK, and if LK is kept in the subsequent incubation medium, they can kill promastigotes even though they still lack the ability to produce a RB (Scott et al., 1985).

The only evidence for a role for a specific oxygen-independent mechanism, involving lysosomal enzymes other than peroxidase, comes from the work of Alexander (1981a). This worker found that pre-treating murine resident peritoneal macrophages with the polyanion poly-D-glutamic acid (which inhibits phagosome-lysosome fusion) moderately enhanced the survival of L.mexicana mexicana amastigotes. With promastigotes, however, there was a dramatic increase in survival. Hence, it appears that in this respect as with other microbicidal mechanisms, amastigotes are more resistant than promastigotes.

A) Resistance of leishmanias to lysosomal enzyme degradation

In view of the results of Alexander (1981a) and others (see above Section) and the fact that fusion of the parasitophorous vacuole with lysosomes has been shown to occur with all Leishmania-phagocyte systems examined, it seems likely that all leishmanias will be exposed to the contents of lysosomes. How leishmanias resist degradation by the hydrolytic enzymees in the lysosomal compartment of monocytes and macrophages is still unknown. Several possibilities have been suggested (1) leishmanias promastigotes may shelter in non-hostile cells whilst they transform to the more resistant amastigote; (2) leishmanias may have a surface which is resistant to lysosomal enzyme degradation (3) leishmanias may release factors which affect overall macrophage digestive function or which inactivate lysosomal enzymes, either directly, by inactivating or inhibiting the enzymes, or indirectly, by changing the pH of the parasitophorous vacuole. It should be noted that results and evidence obtained regarding these mechanisms are contradictory and inconclusive. Further, it is possible that survival mechanisms may be working in concert but that the relative importance of each one may vary depending on the species and

form involved.

i) Shelter in non-hostile cells

Leishmania braziliensis (Chang, 1978), L.tropica (Avakjan, 1971) and possibly L.donovani promastigotes (Schwartzman & Pearson, 1986) have been reported to enter skin fibroblasts and transform into amastigotes. With L.braziliensis promastigotes, it was shown that they transform into amastigotes which somehow avoid phagosome-lysosome fusion. The amastigotes remain viable and survive within the fibroblasts for up to 20 days and can be liberated into the extracellular milieu by cytolysis and/or exocytosis (Chang, 1978). It has been suggested, therefore, that the ephemeral promastigotes may take temporary refuge in the more hospitable fibroblasts so that they can transform into the more resistant (to both oxygen-dependent and -independent microbicidal mechanisms) amastigotes before confrontation with the hostile phagocytes (Chang, 1978).

ii) Resistance to lysosomal enzyme degradation

The suggestion that leishmanias may have a surface resistant to lysosomal enzymes stems from observations that extraneous particles lodged in the parasitophorous vacuole are degraded and disappear whereas amastigotes do not. For instance, host vacuolar membrane tightly adhering to the amastigotes of L.donovani may be carried over during the isolation of the parasite from infected hamster spleen and remain bound to the parasite after ingestion by hamster resident peritoneal macrophages in vitro. It was reported that once this material and the parasite reach the parasitophorous vacuole, the host material was rapidly digested (within one day) suggesting that lysosomal enzymes remain active and therefore that the amastigotes must have a resistant surface (Chang & Dwyer, 1976, 1978). Chang and

Dwyer (1978) suggested that this effect may be mediated through the glycoproteins which cover the exposed surface of L.donovani parasites in parasitophorous vacuoles (Dwyer et al., 1974) being similar to those which bind lysosomal hydrolases (Avila & Convit, 1976) such that they bound to and inhibited the enzymes. This suggestion is supported by the finding that pre-treatment of L.mexicana amazonensis promastigotes with a sublethal concentration of tunicamycin (which inhibits protein glycosylation) results in their surface proteins being only partially glycosylated and at the same time reduces their infectivity for mice (Kink & Chang, 1987) and renders them susceptible to intracellular degradation by macrophages (Chang et al., 1985). A similar effect has been reported for L.donovani promastigotes in mice, and in macrophages in vitro. In the latter case it was also shown that tunicamycin-treated promastigotes were ingested as well as control promastigotes by hamster resident peritoneal macrophages, infection levels of the former dropped to 10% of the controls by 24 hours and to 2% by 48 hours (Nolan & Farrell, 1985). These data point to a role of protein glycosylation in the intracellular survival of leishmanias and are consistent with the idea that the carbohydrate moiety of a glycoprotein protects the protein against proteolytic degradation (Olden et al., 1982).

Direct evidence for the refractory nature of the surface of leishmanias has been presented by Chang (1983). Radiolabelled surface glycoproteins of L.mexicana amazonensis promastigotes were detergent solubilized and treated at pH5, 37^{o}C, with Triton X-100 solubilized lysosomes purified from rat liver. At least one surface glycoprotein at 68KDa and the other at 43KDa persisted throughout the entire 24 hour period of incubation, while other "irrelevant" molecules disappeared or faded, resulting apparently from the hydrolytic action of the lysosomal enzymes. When radioiodinated living promastigotes

were fed to macrophages, the labelled surface molecules also persisted for up to 36 hours without being degraded. The detergent-solubilized surface proteins of L.tropica (L.major) promastigotes and amastigotes have also been reported to be extremely resistant to proteolytic digestion by papain, chymotrypsin and staphococcal V8 protease. Interestingly, those of amastigotes were apparently more resistant than those of promastigotes (Handman et al., 1981; Handman & Curtis, 1982).

iii) Alteration of overall macrophage digestive function

This is difficult to distinguish from inhibition of lysosomal enzymes (covered in the next section), and there appears to be only one line of evidence for this mechanism. Thus, Kutish and Janovy (1981) found that if resident hamster peritoneal macrophages were initially infected with a virulent strain of Leishmania donovani promastigotes and then with Leptomonas costoris, they retained significantly more Leptomonas costoris after 16 hours than did non-infected control macrophages pre-infected with an avirulent strain of Leishmania donovani. This effect was abolished if heat-killed promastigotes of the virulent strain were used. Simultaneous infection gave similar results to pre-infection, but the effect was reduced. Also, if the cultures were incubated for longer periods of time (up to 48 hours) then the Leptomonas costoris were totally cleared even though the Leishmania donovani remained. This indicated that the alteration of the digestive function was either **(1)** transitory, possibly manifested whilst the promastigotes transformed to amastigotes or, **(2)** incomplete leaving an overall low level of residual enzyme activity or specific enzymes fully active (see section below) to which the Leishmania were resistant. The fact that the parasite was altering overall disgestive function is suggested by the

work of Dedet et al (1982) with Leishmania mexicana amazonensis-infected macrophages which showed that phagosomes containing particulates do not easily fuse with established parasitophorous vacuoles and therefore the Leptomonas costoris would have been retained in phagolysosomes by themselves.

iv) Inhibition of lysosomal enzyme activity within parisitophorous vacuoles

The are several lines of evidence that suggest that this occurs. For example, glutaraldehyde-heated amastigotes of Leishmania donovani (Chang & Dwyer, 1978) and L.mexicana mexicana (Alexander & Vickerman, 1975) are rapidly digested, within 24 hours of phagocytosis, by resident peritoneal macrophages. This indicated that the parasites need to be alive to survive rather than simply an enzyme-resistant suface. This is supported by the observation that the lysosomal enzyme, acid phosphatase, although readily detected at electron microscope level in lysoso mes of uninfected murine resident peritoneal macrophages and in lysosomes adjacent to parasitophorous vacuoles containing L.mexicana mexicana (Lewis and Peters, 1977) was hardly detectable in the parasitophorous vacuole itself, only small amounts were found at the periphery of the vacuole. (Alexander & Vickerman, 1975; Lewis & Peters, 1977). Further, in parasitophorous vacuoles containing glutaraldehyde-killed amastigotes there was intense acid phosphatase activity. Similarly, acid phosphatase activity was rarely detected at the light microscope and level in the parasitophorous vacuoles of 6-7 day old bone marrow derived macrophages containing L.mexicana amazonensis amastigotes (Ryter et al., 1983; Barbieri et al., 1985); Clearly the finding of acid phosphatase activity on the parasites themselves means that this enzyme is not an ideal marker, and so these results should be regarded

with some caution. However, Barbieri et al (1985) also reported that another lysosomal enzyme , arylsufatase, was also rarely detected in L.mexicana amazonensis-containing parasitophorous vacuoles. Moreover there is evidence to indicate that the inhibition may be selective and limited specific to certain enzymes as exogenous β-glucuronidase and horseradish peroxidase, which are taken up into the parasitophorous vacuoles of L.mexicana amazonensis-infected macrophages were not degraded at a different rate from that in the control cells (Shepherd et al., 1983). Peroxidase from granules has also been detected in the parasitophorous vacuoles of human blood monocytes containing L.donovani amastigotes (Chang, 1981c). Furthermore, acid phosphatase was apparently readily detected (much greater staining than in other studies) in the parasitophorous vacuoles of hamster resident peritoneal macrophages infected with virulent L.donovani promastigotes (Ebert et al., 1979) indicating that there may also be species differences.

B) Possible mechanisms of inhibiting lysosomal enzymes within parasitophorous vacuoles

i) Altering the pH of the parasitophorous vacuole

A role in altering the pH of the parasitophorous vacuole has been suggested for the high activity 31KDa cysteine proteinase which is located in unique organelles in amastigotes, but not promastigotes of members of the L.mexicana complex (L.mexicana mexicana, L.mexicana amazonensis) (Coombs, 1982; Pupkis & Coombs, 1984; Pupkis et al., 1986) Cysteine proteinase inhibitors leupeptin and antipain have been shown to be potent inhibitors of proteinase activity in L.mexicana mexicana amstigotes, but not that of promastigotes (Coombs, 1982; Coombs et al., 1983). They are also leishmanicidal against amastigotes in murine resident peritoneal macrophages and the J774 cell line

(Coombs & Baxter, 1984). This suggests a role for the cysteine proteinase in survival within macrophages and it has been envisaged that the proteinase activity in L.mexicana mexicana amastigotes could be responsible for the breakdown of proteins to peptides that they could subsequently be degraded to ammonia or to amines which was then secreted into the parasitophorous vacuole and therby raise the pH and render the majority of lysosomal enzymes, which have acidic pH optima, inactive (Coombs, 1982). This could also account for the the very large parasitophorous vacuoles which are characteristic of members of the L.mexicana complex growing in macrophages (Coombs, 1982; Pupkis et al., 1986). Proteinase secretion from L.mexicana mexicana amastigotes has not been detected (Coombs, 1982; Pupkis et al., 1986) and also uptake of gold-labelled BSA, low density lipoprotein and CatF into the magasomes was not observed (Pupkis et al., 1986); which makes it difficult to envisage how the mechanism as postulated could work. Further, although urea and ammonia are secreted by L.mexicana mexicana amastigotes during short term incubation in vitro, the levels were much lower than those secreted by promastigotes under the same conditions. Also, ammonia and urea production by, and levels within L.mexicana mexicana-infected and non-infected murine resident peritoneal macrophages, J774 and P388D cell lines, were not found to be different. Finally, although L.mexicana mexicana amastigotes contain amines (polyamines) they are at lower levels than in promastigotes (Coombs & Sanderson, 1985). This proposed mechanism is also not supported by published information concerning the pH within the parasitophorous vacuole; although there are conflicting results regarding this. Coombs and Alexander (1982, unpublished) reported that the pH within parasitophorous vacuoles of L.mexicana mexicana-infected macrophages was different from that in vacuoles of non-infected

macrophages, the former having a relatively higher pH. In direct contrast, the pH of parasitophorous vacuoles of the J774 cell line infected with L.mexicana amazonensis or L.donovani was reported to be the same as that in lysososmes in uninfected cells (Chang, 1980b; Rivas & Chang, 1983). Moreover, this observation with L.donovani coincides with the recent observation that amastigotes of this species (but not promastigotes) apparently carry out various metabolic activities optimally at pH 4.0-4.5 (Mukkada et al, 1985)

ii) Inhibition/inactivation of lysosomal enzymes by excreted factors

EF's are a diverse and polymorphic family of glycoconjugates which apparently are found with all leishmanias and which have previously been used as the basis for serotyping and classifying leishmanias. They are excreted into the medium during in vitro culture of promastigotes and are also apparently produced by amastigotes in macrophages in vivo and in vitro (Hernandez, 1983; Handman et al., 1987). They are thought to be released from the suface of the parasite and also from within the cell by exocytosis (Hernandez, 1983). It should be noted that because EF comprises a series of components the different excreted products may have different functions (Hernandez, 1983).

That EFs may protect parasites against intracellular degradation was first suggested by the observations of Handman and Greenblatt (1977). They reported that the addition of partially purified EF from cultures of the same species would allow L.enrietti and L.tropica (L.major) promastigotes to survive and multiply in otherwise non-permissive macrophages. Susequently EFs have been shown to inhibit lysosomal enzymes and lysosomal degradation, and several mechanisms by which they accomplish this have been proposed. Hence Rodriguez et al (1983) found that murine resident peritoneal macrophages or the J774

cell line which were treated with a purified EF from L.braziliensis (NR strain) promastigote cultures took about twice as long as control cells to digest [I^{125}] bovine albumin. Futhermore, partially purified EFs from L.tropica (L.major) promastigote cultures have been shown to partially inhibit Escherichia coli β-galctosidase activity (Zehavi et al., 1983) similarly, partially purified EFs from L.donovani promastigote culture were found to inhibit B-galactosidase activity in lysates of macrophages from both resistant and susceptible mouse strains. This inhibition was dependent on EF concentration and the duration of the incubation. The maximum inhibition was 70% and, interestingly, there was no effect on acid phosphatase, β-glucuronidase or N-acetyl-β-glucosaminidase activity (El-On et al., 1980). It was postulated that EF may inhibit enzyme activity via electrostatic interactions by virtue of its high negative charge, which is a characteristic of other inhibitors of lysosomal enzymes such as heparin or chondroitin-sulphate (Avila & Convit, 1976; El-On et al., 1980). More recently, Eilam et al (1985) found that human fixed or unfixed erythrocytes coated with purified EF from L.major promastigote cultures were degraded at a much slower rate by murine resident peritoneal macrophages than control erythrocytes. They also found that EF bound calcium, and that L.major-infected macrophages and those engulfing EF-coated erythrocytes had significantly less surface band calcium but more exchangable calcium than control macrophages. It was suggested that the EF could afford protection in a number of ways: **(1)** by simply inhibiting enzyme activity either whilst remaining in the membrane or released into the P.V. as explained above, **(2)** by binding calcium thus maintaining the parasite in a calcium-rich microenvironment which may increase the stability of the amastigote membrane and **(3)** by depleting the free calcium within the parasitophorous vacuole and so inhibiting the activity of enzymes

which are dependent on calcium.

A similar finding has recently been reported by Handman et al (1986) for the well characterised glycolipid of L.major which forms a component of the EF. They identified an isolate of L.major that lacked this major surface component, this was also non-infective for mice and killed rapidly in murine resident peritoneal macrophages or the J774 cell line. If promastigotes of this isolate were allowed to incorporate purified cellular form glycolipid into their membranes then they could survive and multiply in macrophages or J774 cells until the glycolipid was diluted out by multiplication. It was suggested that possession of the glycolipid allowed promastigotes to survive because they may be diverted to a different intracellular component or that once within the parasitophorous vacuole the highly negatively charged water soluble moiety released from it could inhibit lysosomal enzymes by electrostatic interactions.

iii) Inactivation of lysosomal enzymes by leishmanial enzymes

As well as the high cysteine proteinase activity in L.mexicana mexicana amastigotes, these cells also have much higher DNase, RNase and anylsufatase activity compared to promastigotes. These enzymes are also apparently located in megasomes (Pupkis et al., 1986). The higher activity suggests they may together with the proteinase also play an important part in the survival and growth of amastigotes in macrophages, possibly by simply degrading lysosomal enzymes directly (Coombs, 1982). However, as **(1)** nothing is known about the secretion of DNase, RNase and anylsulfatase by amastigotes **(2)** proteinases are apparently not secreted by amastigotes **(3)** protein uptake into the megasosomes has not been detected (Pupkis et al., 1986) this role for these enzymes must remain speculative.

Similarly, the recently discovered surface proteinase activity on

all Leishmania promastigotes (Bouvier et al., 1987) would seem a good candidate for degrading lysosomal proteins. However, the alkaline pH optimum reported for this enzyme on L.major (between pH 7-10) (Etges et al., 1986) would seem to preclude this role within an acidic parasitophorous vacuole. Currently available information indicates that the surface proteinase activity of L.mexicana amazonensis promastigotes is more likely to be involved. This enzyme is about 2.5 fold more abundant on virulent compared to avirulent promastigotes, and the purified enzyme has a pH optimum of 4.0. It is very active, being 5-fold more active than cathepsin D on lysosomal proteins at pH 4.5 (Chaudhuri & Chang, 1987). Because of uncertainties regarding its presence on amastigotes, its role in the survival of this form is unknown, although it may be involved in protecting the promastigote from degradation whilst is transforms to the more resistant amastigote. The major surface glycoprotein (with an apparent molecular weight of 65KDa) of L.braziliensis promastigotes may have a similar role as it is preferentially expressed as the more infective stationary-phase cells, and incubation of these cells with a monoclonal antibody specific to it severely inhibits their intracellular survival in macrophages (Kweider et al., 1987).

It has also been suggested that the surface protein kinase of L.donovani promastigotes may be involved in inactivating lysosomal enzymes by phosphorylating them (Das, Saha, Remaley et al., 1986) however, its neutral pH optimum and lack of knowledge as to whether it is present on amastigotes means that we must withhold judgement at present.

1.4.2.5.3 Other changes in macrophage function due to infection with Leishmania

Not surprisingly, other changes in macrophage activities, as a

result of infection with Leishmania have been reported. It has been shown that leishmanial antigens are present on the surface of murine resident peritoneal macrophages infected with L.tropica (L.major) (Farah et al., 1975; Handman et al., 1979) and human blood monocyte derived-macrophages infected with L.donovani (Berman & Dwyer, 1981). It has also been shown that L.mexicana mexicana infection of elicited murine peritoneal macrophages had no effect on antibody dependent cytotoxicity, phagocytosis, secretion of lysosomal enzymes, and lysozyme content but infected macrophages were found to decrease in their random and chemotoctic migration through filter and to increase in pinocytic activities (Bray et al., 1983). Increase in pinocytic activity has also been reported for L.mexicana amazonensis-infected J774 cells (Chang, 1980b).

Infection with L.donovani altered the arachidonic metabolism of murine macrophages, leading to infected macrophages producing increased amounts of these metabolites in vivo (Reiner & Malemud, 1984) and in vitro (Reiner & Malemud, 1985), Bray et al (1983) however found that prostaglandin secretion was the same in L.mexicana amazonensis macrophages compared to uninfected cells.

Results regarding alterations in oxidative metabolism are equivocal. Pearson et al (1982) found that 24 hours after infection with L.donovani, human blood monocyte-derived macrophages responded less well to opsonized zymosan than did uninfected macrophages; after 72 hours, however, the RB was greater than or equal to that of control macrophages. Interestingly, this depression was not parasite specific as macrophages "infected" with zymosan also showed depression of the RB after 24 hours. In contrast, elicited murine peritoneal macrophages pre-infected for 24 hours with L.mexicana mexicana showed enhanced bactericidal properties in a short term assay (Bray et al., 1983).

Nevertheless, a recent report has presented convincing evidence that at least some species of Leishmania can alter the oxidative metabolism of the macrophages they infect. Hence Buchmiller-Rouiller and Mauel (1987) reported that for periods up to 72 hours after infection L.enrietti-infected, in vivo-activated, murine peritoneal macrophages had a much reduced RB response to PMA compared to uninfected cells. This was found to be dependent on the number of parasites present and the inhibition was more pronounced when live rather than killed organisms were used. This inhibitory effect was not observed in macrophages "infected" with latex beads. Inhibition of the RB due to L.major infection was also reported. Such an effect would promote superinfection.

1.5 AIMS OF PROJECT

Although in the past it was clearly suspected by some authors that the production in the sandfly of a particular infective form of promastigote - the metacyclic - was required for initiation of the infection in the vertebrate host, it is only over recent years that accumulative evidence has unequivocally shown this to be the case. An important advance, however, was the recognition that similar forms also develop during in vitro culture of promastigotes. There being few if any in the essentially non-infective log-phase cultures but there are large numbers in the infective stationary-phase cultures. This provides an ideal laboratory model to study such forms, although unfortunately at present only L.major metacyclic promastigotes can be purified from the mixed stationary-phase populations.

A major aim of this project was to characterise the metacyclics that were produced and could be purified from the strain of L.major used in this laboratory. In particular I wished to compare them to the other forms that occur in the life-cycle. As such studies were likely

to be hampered by the difficulty in obtaining large numbers of pure amastigotes of L.major, and also to broaden the base of my research, L.mexicana mexicana were also included as large numbers of pure amastigotes of this species can be readily obtained, although the occurrence of metacyclics was uncertain.

At the outset very little or nothing was known about the cell biology/biochemistry of metacyclic promastigotes and their interaction with macrophages. Thus, the broad aim of my project, using the models listed above using and a variety of techniques, was to characterise the metacyclic forms of Leishmania in order to find out how they are adapted to survive in the two divergent environments they occupy: (1) the nutritionally barren foregut of the invertebrate vector; and (2) in the hostile macrophage, the host cell in the vertebrate host.

2.0 MATERIALS AND METHODS

2.1 THE PARASITES

2.1.1 Maintenance and cultivation of parasites

The parasite used in this study had not been cloned. Leishmania mexicana mexicana amastigotes (M379; MNYC/BZ/62/M379) were grown in female CBA mice (Zoology Department, University of Glasgow). Infections were initiated by subcutaneous inoculation of amstigotes, harvested from a mouse and washed twice in PSGEMKA buffer, pH7.3 (see section 2.1.1), into the shaven rump of mice (3×10^7 amastigotes per animal). The area surrounding the point of inoculation was shaved at regular intervals and large subcutaneous lesions developed within 3-5 months after infection. These lesions were used for the production of promastigotes by transformation of amastigotes (see below) and harvesting of amastigotes (see section 2.1.2). Leishmania donovani amastigotes (L82; LV9; MHOM/ET/67/L82) (Bentin and Kingman Ltd., Grimsden, Hull, England). The hamsters were infected by intra-peritoneal inoculation of a crude, twice-washed (in PSGEMKA buffer, pH 7.3) preparation of amastigotes ($2-4 \times 10^8$ per animal). Amastigotes were harvested from spleens 2-5 months after the hamsters had been infected (see section 2.1.2). Promastigotes were produced by transformation of amastigotes (see below). The L.major strain (HMOM/SA/83/RKK2) used in this study was routinely maintained as the amastigote form by the subcutaneous inoculation of twice washed (in HBSS, pH7.3, see section 2.1.2) stationary - phase promastigotes into the footpads of male or female Balb/c mice (1×10^6 cells per footpad) (Department of Zoology, University of Glasgow). Amastigotes were harvested from these lesions after 4-6 weeks and were used for the production of promastigotes (see below). When amastigotes were required for study twice-washed (in HBSS, pH 7.3), stationary - phase promastigotes were inoculated subcutaneously into the footpads of

female Golden Syrian hamsters (1×10^7 per footpad). This caused the production of large, unruptured footpad lesions which were used three weeks after infection as a source of amastigotes (see section 2.1.2).

Promastigotes of each of these species were derived by the *in vitro* transformation of amastigotes from an infected animal in the following manner. Part of a lesion was aseptically excised and added to 5 ml HOMEM medium (Berens *et al.*, 1976) Supplemented with 20% (v/v) heat-inactivated foetal calf serum (HIFCS), and 25 ug gentamycin sulphate ml^{-1}, 1 mg streptomycin sulphate ml^{-1} and 1000 benzylpenicillin ml^{-1} to prevent bacterial growth (transformation medium). This was then incubated at 25°C for 48-72 hours. The promastigotes produced were then routinely grown in 5 ml HOMEM medium supplemented with 10% (v/v) HIFCS and 25 µg gentamycin sulphate ml^{-1} (standard medium) in sealed 30 ml universals using air as the gas phase with incubation at 25°C. Cultures were sub-passaged when at mid-logarithmic (mid-log) phase of growth (approximately every 2-3 days) by transfer of approximately 1×10^5 cells into fresh stndard medium. For experimental work promastigotes were grown in bulk in 500 ml medical flats containing 100-200 ml standard medium. The cultures were inoculated with mid-log phase promastigotes to give a starting density of 1×10^5 cells ml^{-1}, and reached mid-log phase of growth (density 2-5×10^6 cells ml^{-1}) two days later. At this stage they were then either harvested (see section 2.1.2) or allowed to grow to stationary-phase. In the latter-case, the cell numbers were monitored every 24 hours using an Improved Neubauer Haemocytometer. Cultures were taken as having reached stationary-phase when the cell density had dropped by approximately 10% over the preceeding 24 hours but with the majority of promastigotes still being motile. This stage was usually reached after 7-8 days in culture the final density being $1\text{-}2 \times 10^7$ cells ml^{-1}. The cells were then harvested or used for the purification

of metacyclics (see section 2.1.2). Only promastigotes that had been sub-passaged less than 11 times were used in this study.

Herpetomonas muscarum muscarum (ATCC 30260) was routinely grown in 5 ml LIT medium (Carmargo et al., 1964) supplemented with 10% HIFCS and 25 μg gentamycin sulphate ml^{-1} with incubation at 25°C in sealed 30 ml universals using air as the gas phase. Herpetomonas muscarum ingenoplastis (ATCC 30269) was routinely grown in 25 ml LIT medium and incubated at 25°C in sealed 30 ml universals with a minimum gas (air) phase. Both cultures were sub-passaged every 2-3 days. For experimental work H.muscarum muscarum and H.muscarum ingenoplastis were grown in 100 ml bottles containing 80 ml or 100 ml LIT medium, respectively. The cultures were inoculated with actively dividing cells to give a starting density of 1×10^6 cells ml^{-1}. The cells were harvested (see Section 2.1.2) 2 days later when H.muscarum muscarum had reached a density of $0.6\text{-}1 \times 10^7$ cells ml^{-1} and H.muscarum ingenoplastis approximately 2.5×10^6 cells ml^{-1}.

2.1.2 Harvesting and purification of parasites

Parasites were harvested and washed by centrifugation at 2000 x g for 10 min at 4°C unless otherwise stated. Parasites were always washed before use. The number of washes and medium used depended upon the study undertaken and these details will be stated in the appropriate sections below.

Amastigotes of L.mexicana mexicana and L. donovani were harvested from cutaneous lesions and infected spleens, respectively. They were purified using the method of Hart et al (1981b), as modified by Mottram and Coombs (1985b). In brief, the infected tissue was aseptically removed, placed in 20 ml PSGEMKA buffer, pH 7.3 (20 mM sodium phosphate buffer, 104 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM EDTA, 0.5 mM D-glucose, 0.02% [w/v] bovine serum albumin) and cut into

small pieces. The chopped lesion was then homogenised between two gauze meshes to release the parasites and the crude suspension filtered through No1 Whatman filter paper to remove large pieces of debris. Saponin, 0.5% (w/v) was added to lyse red blood cells, and the amastigotes were gently dispersed by two strokes of a Potter homogeniser working at low speed. The cells were harvested from the saponin solution by centrifugation, and washed three times in PSGEMKA buffer. They were resuspended to give a parasite density of approximately 10^8 ml^{-1}, and passed down an ion-exchange column containing 0.1 g of both α-cellulose and Sigmacell (type 50) respectively, layered on top of 2 g CM-Sephadex C25. This removed contaminating host white cells, the purified amastigotes were then washed appropriately, checked microscopically for purity and counted. Leishmania major amastigotes were purified as above. For each purification, four infected hamster footpads were used. The crude suspension contained 1.6-3.3 x 10^9 in total and following the purification proceedure resulted in 3 x 10^8 - 1.4 x 10^9 pure amastigotes. The final preparation of amastigotes contained fewer than 0.1% (cell/cell) contamination, similar to the preparations of amastigotes of other species used.

Mid-log phase and stationary-phase promastigotes of Leishmania species and cells of H.muscarum muscarum and H.muscarum ingenoplastis were harvested by centrifugation and washed appropriately. Metacyclic promastigotes of L.major were purified from the stationary-phase cultures using the method of Sacks et al (1985). This was carried out as follows: stationary-phase promastigotes were harvested and washed three times in Hanks balanced salt solution with phenol red, pH 7.3, with phenol red but without sodium bicarbonate (HBSS). They were then resuspended to 4-5 x 10^8 cells ml^{-1} in with 100 µg PNA ml^{-1}, and

incubated at room temperature for 30 min, to allow agglutination of non-metacyclics. After this the suspension was carefully layered on top of HBSS containing 50% (v/v) HIFCS and left for a further 30 min, to allow the large aggregates of agglutinated promastigotes to sediment out. Unagglutinated metacyclic promastigotes remaining above the interface were then carefully removed. A small layer of the supernatant was always left just above the interface as it was found, in some cases, the smaller aggregates of agglutinated promastigotes remained here rather than entering the HBSS/50% HIFCS layer. The purified metacyclics were then counted and washed appropriately. Usually 2-6 x 10^8 metacyclics were isolated from 200 ml stationary-phase cultures, this being equivalent to 21$\pm$ 9% (n=23) of the total numbers of promastigotes initially present.

2.2 ASSESSMENT OF DIFFERENT MORPHOLOGICAL FORMS IN PROMASTIGOTE CULTURES

"Smears" of L.major and L.mexicana mexicana promastigote cultures were taken at mid-log and stationary-phase growth. These were air-dried, fixed in methanol for 2 min, stained in Giemsa's stain for 20 min, washed, allowed to dry and coverslips added. The stained "smears" were examined at x 1000 magnification, and the number of metacyclics (L.major) or putative metacyclics (L.mexicana mexicana) as a percentage of the cells present was assessed (from morphological criteria, mainly by the length of the flagellum in relation to the cell body) by counting at least 100 promastigotes in each "smear".

2.3 DETERMINATION OF THE BINDING OF AGGLUTININS TO PROMASTIGOTES

2.3.1 Preparation of cells for agglutinin-mediated agglutination assay

Leishmania major and L.mexicana mexicana promastigotes were washed three times in phosphate buffered saline, pH 7.3 (PBS, 20 mM sodium phosphate buffer, 150 mM NaCl) and resuspended in this buffer

to density of 4 x 10^8 cells ml^{-1}.

2.3.2 Agglutinin-mediated agglutination assay

The binding of agglutinins to the cells were assessed by quantifying their ability to agglutinate promastigotes using a modification of the agglutination assay of Sacks et al (1985). Equal volumes (0.1 ml) of the promastigote suspension and the agglutinin dilutions in PBS (at twice the final concentration) were gently mixed and incubated for 30 min at room temperature to allow agglutination to occur. After gentle mixing the suspension was carefully layered onto 1 ml of PBS containing 50% (v/v) HIFCS and the agglutinated promastigotes left to sediment out for 30 min at room temperature. The overlaying layer (0.2 ml) containing the unagglutinated promastigotes was removed, diluted where necessay and the number of promastigotes counted in a haemocytometer. This count was compared to that of the control (using PBS only, no lectin) and the % agglutination determined from the formula:

[1-(number of free promastigotes in sample / number of free promastigotes in control)] x 100.

In some cases with L.major cultures, after agglutination with 100 µg PNA ml^{-1}, the % unagglutinated promastigotes was determined from the formula: (number of free promastigotes in sample/number of free promastigotes in control) x 100. This gave the % metacyclics presnt in these cultures.

2.4 DENSITY GRADIENT CENTRIFUGATION OF PROMASTIGOTES

2.4.1 Preparation of cells for density gradient centrifugation

Leishmania mexicana mexicana stationary-phase promastigotes were washed once in 150 mM NaCl, and resuspended to a density of 1 x 10^8 cells ml^{-1} in 150 mM NaCl.

2.4.2 Density gradient centrifugation

A solution of stock iso-osmotic Percoll (SIP) was made by adding 9 parts Percoll to 1 part 1.5 M NaCl. A series of dilutions from SIP were made using 150 mM NaCl, 6 ml of each dilution was added to 10 ml tubes and the gradient pre-formed by centrifuging with the brake off at 30,000 x g for 15 min, at 4°C using a 35° fixed angle rotor on a MSE Europa 1ml aliquots of the promastigote suspension were loaded onto each of the gradients and they were centrifuged with the brake off at 400 x g, at 4°C for various lengths of time using swing out buckets on a MSE Chilspin. The resulting band pattern was recorded by placing the centrifuge tubes against graph paper and the band position,size and opacity marked directly onto the graph paper. The bands were then removed via the top of the tube and examined in wet preparations at x 400 using phase contrast microscopy.

2.5 MURINE RESIDENT PERITONEAL MACROPHAGE-PARASITE INTERACTION STUDIES

2.5.1 Medium

The medium used for all macrophage studies except where indicated otherwise was RPMI 1640 pH 7.2-7.3 supplemented with 10% (v/v) HIFCS, 50 U benzylpenicillin ml^{-1}, 50 µg streptomycin sulphate ml^{-1}, 25 µg gentamycin sulphate ml^{-1}, 2 mM L-glutamine, 25 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (Hepes) and 20 mM sodium hydrogen carbonate. This will be known hereafter as RPMI 1640 medium.

2.5.2 Macrophages

Groups of 3-6 female Balb/c or CBA mice were used for each experiment, and resident peritoneal exudate cells were obtained by lavage with 5 ml RPMI 1640 medium per mouse. The cellular exudates

from the mice were pooled, the number of cells counted and 1 x 10^5 peritoneal exudate cells (in 0.4 ml) were plated into each chamber on 8-chamber Lab-Tek tissue culture slides. the cells were allowed to adhere overnight at 37°C with a gas phase of 95% air 5% CO_2, and non-adhered cells were removed by washing the next day. the adherent cells remaining were assumed to be and will be referred to hereafter as macrophages. For the purpose of calculating the numbers of parasite cells required for the various parasite to macrophage ratios used in the studies below, the number of macrophages present was assumed to be the same as the number of peritoneal exudate cells initially added. All experiments were performed in duplicate (except for those in section 2.5.5.2) and were carried out at 34°C with a gas phase of 5% CO_2, 95% air.

2.5.3 Uptake experiments

Parasites were washed twice in HBSS pH 7.3, resuspended in RPMI 1640 medium and dilutions made to give the required densities. In experiments where uptake was examined in the absence of serum, washed parasites were resuspended in RPMI 1640 medium without HIFCS, and immediately prior to exposure the macrophage cultures to be used were washed twice in RPMI 1640 medium without HICFS. Macrophage cultures were then exposed to parasites in a final volume of 0.4 ml per chamber at various macrophage:parasite ratios. At appropriate time points the parasites were removed and the macrophages washed four times with warm (37°C) HBSS, wet-fixed for 2 min in methanol and stained with 10% Giemsa's stain, for 10 min.

2.5.4 Infection experiments

Macrophages were exposed to washed promastigotes in RPMI 1640 medium as described above for 2 hours. The promastigotes were then removed by repeated washings, those cultures to be fixed at this time

were washed four times in warm HBSS, fixed, and stained as described above. Other cultures which were re-incubated were washed four to five times with warm RPMI 1640 medium before subsequent incubation. After 24 and 72 hours the medium was either replaced and the cultures re-incubated for longer periods, or the cultures were fixed and stained as above.

The slides were examined at x 1000 magnification and over 250 macrophages were counted for each culture to determine the % macrophages infected, the number of parasites ingested per 100 macrophages and the number of parasites attached per 100 macrophages (for uptake experiments and 2 hour infection time points); or simply the % macrophages infected and the number of amstigotes per 100 macrophages. (for the other infection time points).

2.5.5. Measurement of the respiratory burst of resident peritoneal macrophages

The RB was assessed using the reduction of NBT by O_2^- which forms a blue black formazan precipitate (Baehner et al., 1976). This was determined qualitatively by counting the % macrophages stimulated to reduce NBT, i.e. with formazan deposits therefore formazan positive, and quantitatively by measuring the absolute amount of formazan produced.

2.5.5.1 Qualitative nitroblue tetrazolium assay

Washed parasites (see section 2.5.3) were resuspended in RPMI 1640 medium containing 0.5 mg NBT ml^{-1}, and macrophages were exposed to parasites in a volume of 0.4 ml per chamber at various macrophage parasite ratios. At appropriate time points the parasites were removed, the macrophages washed twice in warm HBSS, fixed for 1-2 min, in methanol and counterstained in 0.2% (w/v) safranin (dissolved in

70% [v/v] ethanol) for 1 min. Controls run in parallel were macrophages exposed to medium containing 0.5 mg NBT ml^{-1} without parasites, and macrophages exposed to 0.4 ml zymosan suspension per chamber (10 mg ml^{-1} [w/v] suspension in RPMI 1640 medium containing 0.5 mg NBT ml^{-1}). The slides were examined at x 1000 magnification and at least 250 macrophages were counted for each culture to determine the % macrophages stimulated. The figures for macrophages stimulated with parasites or zymosan were corrected for non-specific stimulation of the RB by subtraction of the figure for the control treated with medium only.

2.5.5.2 Quantitative nitroblue tetrazolium assay

Quantitative reduction of NBT was quantified by a modification of the method of Haidaris and Bonventre (1982). Resident peritoneal exudate cells (2×10^6 per tube) were plated in Leighton tubes, left overnight and non-adherent cells washed off the following day as described above. Washed (see section 2.5.3) promastigotes were resuspended in RPMI 1640 medium containing 0.5 mg NBT ml^{-1} and mixed with the macrophages for 1 hour at a parasite:macrophage ratio of 20:1. The promastigotes were removed and the monolayers were rinsed twice in warm PBS pH 7.3, and allowed to dry overnight. A control consisting of medium containing 0.5 mg NBT ml^{-1} without parasites was also included. Precipitated formazan was extracted with pyridine (1 ml per tube) by placing the tubes containing pyridine in a boiling waterbath for 15 min. The absorbance of the resultant solution was measured at 515 nm against a pyridine blank using a MSE Spectroplus spectrophometer and the results for experimental tubes corrected for non-specific stimulation of the RB by subtraction of the absorbance for the control treated with medium only. The amount of reduced NBT present was calculated using an extinction coefficient of 2.8 mM^{-1} cm^{-1}

(Haidaris & Bonventre, 1982) and was expressed per the number of macrophages present assuming that half the peritoneal exudate cells were macrophages. As the promastigotes themselves also reduced the NBT, tubes containing promastigotes only were run in parallel with the above experiments and the amount of NBT reduced by the promastigotes determined as described above (see section 2.16 for details). Using the 2 hour uptake figures (ie. % macrophages infected, number of parasites ingested per 100 macrophages and number of parasites attached per 100 macrophages) for the parasite:macrophage ratio of 20:1 and the assumption that half the peritoneal exudate cells were macrophages, the number of promastigote attached and ingested per macrophage monolayer and hence their contribution to the reduction of NBT was calculated.

2.6 ANIMAL INFECTION EXPERIMENTS

Parasites were washed once in HBSS pH 7.3, resuspended in this buffer to the correct density and dilutions made where necessary. Experiments investigating the relative infectivity of different forms of L.mexicana mexicana were performed by injecting parasites into the rumps of female CBA mice, and the left-hind footpads of female CBA and female or male Balb/c mice. Experiments with L. major promastigotes were performed by injecting cells into the left-hind footpads of female Balb/c mice. Five to ten mice were used in each group of an experiment. the infections were followed weekly or bi-weekly. Footpad width was measured using a direct reading Mitutoyo micrometer. Results were expressed as mean footpad width $\pm$ standard deviation; cases where a footpad did not produce a lesion over the whole length of the experiment this was excluded from the figures contributing to the mean. Rump lesions were assessed by measuring the length, width and height using a micrometer and the volumes estimated using the method

of Honigberg (1961) which assumes the lesion to be a hemisphere. The rump lesions results were expressed as an infectivity index (Franke et al., 1985) which was calculated by the following formula:

$$I = \% L/100 \times V$$

where %L was the percent of animals in each group with a lesion, and V was the mean volume of the lesions produced within each group.

2.7 RABBIT PERITONEAL NEUTROPHIL - PARASITE INTERACTION STUDIES

2.7.1 Neutrophils

Neutrophils were obtained using a modification of the method of Lackie (1977). New Zealand White 1/2 Lop rabbits were inoculated intraperitoneally with 500 ml 0.9% (w/v) sterile saline containing 50 mg oyster glycogen and the fluid drained off after 4 hours, at which time the exudate contains almost pure polymorphonuclear leukocytes (Cohn & Morse, 1959), 98% of which are neutrophils (J. Lackie, pers. comm.). The cells were counted, pelleted by centrifugation at 500 x g for 10 min, at 4°C, resuspended in exudate solution to a density of 1 x 10^7 cells ml^{-1} and kept at 4°C until use. Neutrophils were stored in the exudate solution for up to 72 hours; this did not appear to significantly alter, either the cells ability to phagocytose particles, or the cells ability respond with a RB upon exposure to appropriate stimulating agents. Neutrophils were used directly from exudate solutions in all experiments, and in all cases this was within, 4 hours to up to 72 hours after preparation.

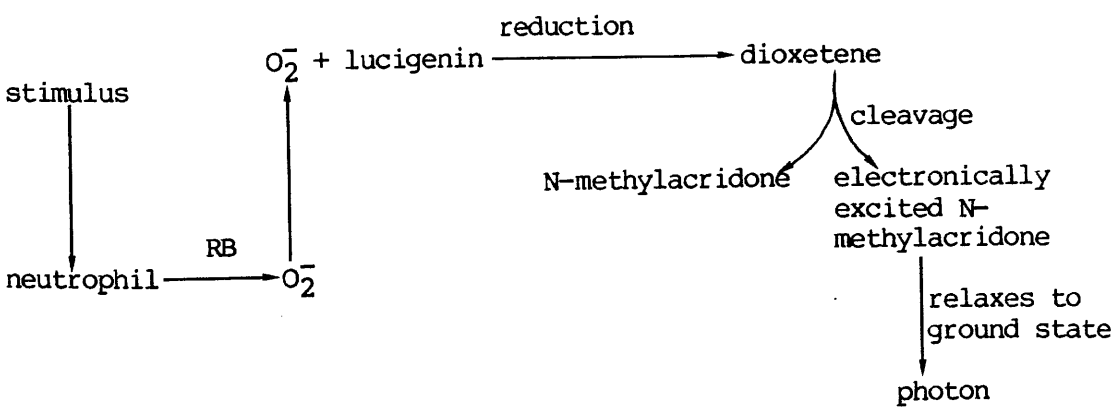
2.7.2 Attachment/Uptake Assay

Parasites were washed twice in HBSS pH 7.4, without sodium bicarbonate and phenol red, buffered with 10 mM Hepes (HBSS-HEPES), and resuspended in this buffer to the required density. The assay was performed in a total volume of 5 ml, HBSS-HEPES with 10% (v/v) exudate containing 1 x 10^6 neutrophils ml^{-1} and with either 1 x 10^6 or 5 x 10^6

parasites ml^{-1}, and incubated at 37°C in a shaking waterbath (80 strokes min^{-1}, 10 mm stroke length). At 15, 30 and 60 min. intervals the tubes were gently vortexed and 0.4 ml aliquots were taken and the cells sedimented onto glass slides by cytocentrifugation at 230 x g for 15 min. using an IEC Centra-48 cytocentrifuge. The slides were wet-fixed in methanol for 5 min, air dried, and stained as described in section 2.2. At least 100 neutrophils were counted at each time point sample and the number of parasites associated with them (= attached + ingested) determined. At the same time, using the ratio of 5 parasites : 1 neutrophil the density of extracellular parasites in the suspension at 0, 15, 30 and 60 min was also determined using a haemocytometer, and compared to that in a control which lacked neutrophils. The results are expressed as a percentage of the cell density in the control.

2.7.3 Measurement of the respiratory burst of neutrophils exposed to trypanosomatids

The RB of the neutrophils was assessed by measuring O_2^- production using lucigenin (9, 9'-bis-N-methylacridium nitrate) - enhanced CL (Baxter et al., 1983; Buchmuller-Rouiller & Mauel, 1986; Donaldson & Cullen, 1984; Williams & Cole, 1981a,b). Lucigenin is thought to be reduced by O_2^- in a reaction which results in the release of light and the following reaction sequence has been postulated (Allen, 1981):



The light produced was measured in a LKB 1251 Luminometer coupled to a BBC computer. I am indebted to Dr J. Lackie, Department of Cell Biology, University of Glasgow, for allowing me the use of this apparatus. All experiments were performed in the Department of Cell Biology with the kind permission of Prof A. Curtis. The luminometer was programmed to have a time constant of 0.5 sec a delay time of 1.0 sec, and was in continuous mixing and continuous counting mode; results were given as millivolts (mV). The continuous counting mode entailed counting each sample for 0.5 sec with a 4.5 sec gap before counting the next sample. The samples were counted continuously ten times and then, after a delay of 216 sec, counted another ten times with a delay of 210 sec between each set of counts. The continuous mixing mode meant that each sample was mixed before counting by rotating the cuvette and changing direction every 0.5 sec. Usually 24 samples were run in each experiment and this number of samples took 120 sec to read.

2.7.3.1 Chemiluminescence assay

Parasite cells were washed twice in HBSS-HEPES pH 7.4, resuspended in this buffer and diluted to the required densities. In other experiments, glutaraldehyde-fixed and sodium fluoride (NaF)-treated parasites were used. These were prepared by centrifuging cells from growth medium/balanced salt solution and resuspending to a density of 2.5×10^8 cells ml^{-1} in 2.5% (v/v) glutaraldehyde or 5 mM sodium fluoride (both in HBSS-HEPES) and incubating for 15 min at 18°C. They were then treated as above, NaF-treated cells were all motile after the treatment, glutaraldehyde-fixed cells were non-motile but appeared to be intact as observed by light microscopy.

All assays were performed at 37°C (maintained by water circulation through the sample holder) in 4 ml Sterilin polystyrene

cuvettes, the neutrophil suspension was maintained on ice before use, other solutions and parasite cell suspensions were kept at room temperature before use. Each sample had a final volume of 1 ml, and experimental samples contained 0.2 ml of the appropriate parasite suspension, 0.1 ml lucigenin (1×10^{-3} M), 0.1 ml neutrophil suspension in exudate (1×10^{6} cells) and 0.6 ml HBSS-HEPES. Control samples run in parallel were: neutrophils and lucigenin without any external stimulus (= neutrophils alone control); neutrophils and 1 µg PMA ml^{-1}; and parasite cells with lucigenin and without neutrophils. As both PMA and lucigenin were initially disolved in dimethyl sulphoxide (DMSO) and then diluted to the desired concentration with HBSS-HEPES, a DMSO control consisting of neutrophils, lucigenin and DMSO to the same concentration as it would be if PMA were included were also run in order to see if DMSO stimulated or inhibited the control value CL. All experiments were performed in triplicate, readings were started after the samples had been incubated in the machine for 2 min, to allow for temperature equilibration. The CL of an experimental mixture was determined by subtraction of that of the neutrophils alone control. On one occasion the effect of inhibitors on neutrophil CL was determined. This was done using a parasite (L.major mid-log phase promastigotes):neutrophil ratio of 10:1, with either 600 U of SOD (bovine erythrocyte), or 600 U of catalase (bovine liver) being added immediately prior to the start of the assay. CL was determined as summed mV counts over 40 min, and results are expressed as % inhibition compared to an untreated control.

2.8 HUMAN MONONUCLEAR PHAGOCYTE - PARASITE INTERACTION STUDIES

Mononuclear cell preparations were prepared form blood obtained from healthy adult donors. Blood was taken in 10 U heparin ml^{-1} (bovine lung), mixed with 1% (v/v) Dextran 110 and incubated at 37°C

for 1 hour. The leukocyte-rich supernatant was removed, layered onto an equal volume of Ficol-Hypaque and centrifuged at 400 x g for 30 min, at 4°C to obtain the mononuclear cell fraction. This was carefully removed, and the number of cells counted. A ("smear" was then made, air dried, fixed and stained as described in section 2.2, and the number of monocytes judged by morphological criteria, counted. The cells were then washed twice in HBSS-HEPES, pH 7.2 (by centrifugation at 400 x g for 10min, at 4°C), resuspended to a density of 1 x 10^9 monocytes ml^{-1} in RPMI 1640 medium, and kept on ice in a glass universal until use. In all cases the monocytes were used within 3-4 hours after preparation. When required the monocytes were washed twice in HBSS-HEPES, pH 7.2 as described above, and resuspended in this buffer (at room temperature) to give a density of 1 x 10^7 monocytes ml^{-1}. The RB elicited on contact with parasite cells was measured by lucigenin-enhanced CL as already described for rabbit peritoneal neutrophils. In this case, however, 15 samples were run in each experiment and this number of samples took 75 sec to read.

2.9 DETERMINATION OF THE SUSCEPTIBILITY OF PROMASTIGOTES TO HYDROGEN PEROXIDE and HYDROGEN PEROXIDE/PEROXIDASE/HALIDE COMBINATIONS

2.9.1 Preparation of cells and the microbicidal assay

Leishmania major promastigotes were washed twice in PBS, pH 7.3 with 5mM glucose (PBSG, pH 7.3) and resuspended in this buffer to a density of 1 x 10^7 cells ml^{-1}.

A modification of the microbicidal assay of Channon and Blackwell (1985a) was used. Before each experiment the concentration of the H_2O_2 reagent stock solution was determined spectrophotometrically at 230 nm using an extinction coefficient of 81 $M^{-1}cm^{-1}$ (Penketh and Klein, 1986) and dilutions were made in PBSG. The assays were carried out in a total volume of 1 ml in eppendorf tubes. 5 x 10^6 promastigotes were

added to H_2O_2 solutions alone, or with 100 mU LPO (bovine milk) and 0.1 mM KI added. Controls run in parallel were the above mixtures without H_2O_2 and a catalase control (at the highest H_2O_2 concentration used) with 3000 U of catalase (bovine liver) added immediately prior to the start of the assay. After addition of the promastigote suspension, the tubes were vortexed and incubated at 34°C in a non-shaking water bath for 1 hour. After this period the assay was terminated by the addition of 3000 U of catalase which hydrolysed the H_2O_2, with susequent vortexing of each tube. 30 µl aliquots were then placed on glass slides, overlayed with 18 x 18 mm coverslips and the promastigotes examined by phase-contrast microscopy at x 400 magnification. Promastigote viability was assessed by flagellar motility. Promastigotes were scored as "fast", slow or non-motile; a promastigote was scored "fast" if the flagellum was a blur (Channon & Blackwell, 1985a). At least 250 organisms were assessed for each dilution and the percentage of the cells that were (1) "fast" and (2) motile (= "fast" + slow)calculated. The effects of H_2O_2 were assessed by comparison of the results obtained with those for the controls (without H_2O_2), and were expressed as a % "fast" and % motile (= "fast" + slow) with the controls being 100%.

2.10 DETERMINATION OF THE SUSCEPTIBILITY OF PROMASTIGOTES TO SERUM LYSIS

2.10.1 Human serum

Three independent samples of normal human serum were tested and all were obtained from the Scottish Blood Transfusion Service, Glasgow. They were stored at -70°C until required and had been freeze-thawed three times at the time of use. Aliquots of each serum were heat-inactivated at 56°C for 30 min before storing at -70°C. None of the sera were tested for antibodies against Leishmania.

2.10.2 Preparation of cells for the lytic assay

The lytic assay used was a modification of that described by Franke et al (1985). Leishmania major and L.mexicana mexicana promastigotes were washed twice in HBSS, pH 7.3 and resuspended in this buffer to a density of 1 x 10^7 cells ml^{-1}. Equal volumes (0.1 ml) of the cell suspension and the serum dilutions in HBSS (at twice the desired concentration) were mixed together. Promastigotes to be exposed to undiluted serum were washed twice as described above and resuspended in 0.2 ml whole serum. Controls run in parallel were promastigotes exposed to 10% (v/v) (L.major) or 25% (v/v) (L.mexicana mexicana) HI serum in HBSS and HBSS alone. Incubation was at 37°C for 45 min in 5% carbon dioxide in air. The cells were then mixed, placed on a haemocytometer, fixed in formalin vapour for 15 min, and the survival of the promastigotes (as a percentage of survival in HBBS alone) determined by counting the number of morphologically intact, unlysed promastigotes.

2.11 ANALYSIS OF PROTEINASE ACTIVITY USING GELATIN DISCONTINUOUS POLACRYLAMIDE GEL ELECTROPHORESIS (GELATIN DISC-PAGE)

2.11.1 Preparation of parasite homogenates and soluble and particulate fractions for Gelatin Disc-PAGE

Parasite cells were washed three times (2000 x g, 15 min, at 4°C) in 250 mM sucrose, pelleted, and stored at -70°C until required. In most studies, the rest of the procedures for Gelatin Disc-PAGE were kindly performed by Dr B. Lockwood (Department of Biological Sciences, University of Stirling). I carried out the studies using particulate and soluble fractions and L.major amastigotes (see results section 3.6.4). Parasite homogenates were prepared by resuspending the cells in 250 mM sucrose containing 0.25% (v/v) Triton X-100. Particulate and soluble fractions were prepared by centrifuging parasite homogenates

at 100,000 x g for 1 hour at 4°C. The supernatant fraction was removed, and the pellet resuspended in 250 mM sucrose to the same volume as the supernatant. For testing the binding proteinases o to Con-A, parasite homogenates were prepared in 0.5% (v/v) Nonidet P40 in PBS, pH 7.3. Bound and non-bound fractions were prepared using the method of Lepay et al (1983) except that Con A-agarose which was used in place of Con A-sepharose.

2.11.2 Running the gel and staining for proteinase activity

Gels were prepared and run as described by Hames and Rikwood (1981) and Lockwood et al (1987). Samples were prepared by 1:1 dilution of parasite homogenates (50 µg protein), and particulate and soluble fractions (both 100 µg protein) with sample buffer (125 mM Tris-HCl pH 6.8, 4% [w/v] SDS. 5% [v/v] 2-mercaptoethanol, 20% [v/v] glycerol). The samples were loaded onto a polyacrylamide discontinuous gel system comprising a 2.5% (w/v) stacking gel and a 7.5% (w/v) running gel containing 0.2% (w/v) gelatin (from porcine skin), and subject to electrophoresis.

After electrophoresis the gels were immersed for 1 hour in 1 litre of 2.5% (N/V) Triton X-100 to remove the SDS and restore proteolytic activity. The proteinase bands were usually developed by immersing the gels in incubation buffer (100 mM sodium acetate-acetic acid buffer, pH 5.5, containing 1 mM dithiothreitol (DTT) for 4 hours at 37°C; this standard procedure was used where otherwise indicated. To study activities at a range of pH the following buffers (100 mM) were used: sodium acetate-acetic acid, pH 4.0, 6.0; Tris-HCl, pH 8.0. The effect of inhibitors was tested by adding these to the pH 5.5 incubation buffer. The bands were visuallised by staining in 0.05% (w/v) PAGE blue 83, 25% (v/v) isopropanol, 10% (v/v) acetic acid for at least 3 hours before destaining in 10% (v/v) acetic acid.

Densitometric scans of the gels were made at 550 nm using a Gelman DCD-16 scanner. Molecular weights of individual proteinases were determined from their mobility compared to those of protein standards.

2.12 ELECTRON MICROSCOPY

Cells were purified or harvested as described in section 2.1.2 and washed once in HBSS pH 7.3 and pelleted by centrifugation at 1000 x g for 15 min at 25°C. Cells to be stained for ultrastructural features were processed and stained according to Coombs et al (1986). Some cells were stained for acid phosphatase activity using the method described by Hassan and Coombs (1987) using disodium glycerol-2-phosphate as substrate and lead as the capture agent, and the sections examined unstained or after staining by the method above.

2.13 ENZYME ANALYSIS

2.13.1 Preparation of parasite homogenates and soluble and particulate fractions for enzyme assays

Cells used in enzyme assays were washed three times (see Section 2.11.1) in 250 mM sucrose, pelleted and used immediately, or stored as pellets at -70°C until required. Parasite homogenates were prepared by resuspending the cells in 250 mM sucrose and sonicating, whilst cooled on ice, using an MSE Soniprep 150 fitted with an exponential microprobe programmed to deliver three 4-second bursts of 6 μm amplitude sonication with a 10-second cooling period between bursts. In some cases, as specified in the text, cells were lysed in 250 mM sucrose containg 100 μg ml^{-1} of the proteinase inhibitor leupeptin to protect enzymes from dedradation by proteinases. Particulate and soluble fractions were prepared, after lysis by sonication as described in section 2.11.1.

2.13.2 Enzyme assays

Parasite homogenates were used in all enzyme assays unless otherwise stated. All enzyme assays were carried out in a total volume of 1.5 ml at 25°C using a Pye Unicam SP8000 incorporating a temperature controlled cell holder, unless otherwise indicated. Enzyme activities were calculated using an extinction coefficient of for NAD(P)H 6.2 x 10^3 $M^{-1}cm^{-1}$ where appropriate.

The conditions used for all enzyme assays were confirmed to be optimal with respect to substrate concentration for the amount of parasite extract added and gave linear rates of reaction over the period of incubation used.

Alanine aminotransferase (ALAT) (EC 2.6.1.2) was assayed at 340 nm using a modification of the method of Bergmeyer and Bernt (1974). The rection mixture contained 80 mM potassium phosphate buffer (pH 7.6 for L.mexicana mexicana promastigote homogenates, pH 6.0 for L.mexicana mexicana amastigote homogenates; and pH 8.0 for L.major homogenates), 0.56 M L-alanine, 0.1 mM NADH, 2 U of rabbit skeletal muscle lactate dehydrogenase (LDH), and parasite homogenate. The reaction was initiated by the addition of α-ketoglutegate to 9.0 mM.

Aspartate aminotransferase (ASAT) (EC 2.6.1.2) was assayed at 340 nm using a modification of the method of Bermeyer and Bernt (1974). The reaction mixture contained 80 mM Tris HCl (pH 8.4 for L.mexicana mexicana promastigote and amastigote homogenates; pH 8.2 for L.major extracts), 92 mM L-aspartate, 0.094 mM NADH, 11 U of porcine heart MDH (in glycerol) and parasite homogenate. The reaction was initiated by the addition of α-ketoglutarate to 11.5 mM.

Glutamate dehydrogenase (GDH) (EC 1.4.1.3) was assayed at 340 nm as described by Mottram and Coombs (1985b) in a reaction mixture containing 100 mM Tris-HCl pH 8.5, 320 mM $(NH_4)_2SO_4$, 0.2 mM NADH and parasite homogenate. The reaction was initiated by the addition of

α-ketoglutarate to 6.6 mM.

MDH (EC 1.1.1.37) was assayed at 340 nm (Mottram & Coombs, 1985b) in a reaction mixture containing 1M glycine, 0.4 M hydrazine, 1 M NaOH buffer, pH 9.5, 133 mM malate and 2 mM NAD^+. The reaction was started by the addition of parasite homogenate.

PEP carboxykinase (EC 4.1.1.49) was assayed at 340 nm Mottram & Coombs, 1985b) in a reaction mixture containing 100 mM imidazole-HCL pH 6.6, 4 mM $MnSO_4$, 20 mM $NaHCO_3$, 0.1 mM NADH, 4 mM phosphoenol pyruvate (PEP), 15 U of bovine heart MDH (in glycerol) and parasite homogenate. The reaction was started by the addition of 1 mM ADP. In order to avoid interference from high promastigote NADH oxidase activity, that is stimulated by Mn^{2+} and HCO_3^-, promastigote PEP carboxykinase was assayed anaerobically with the same reaction mixture as above, but in a 0.5 ml total volume. Amastigote Mn^{2+} stimulated NADH oxidase was low in comparison to the PEP carboxykinase activity and was simply subtracted to obtain the PEP carboxykinase activity.

Pyruvate kinase (PK) (EC 2.7.1.40) was assayed at 340 nm (Mottram & Coombs 1985b) in a reaction mixture comtaining 80 mM Tris-HCl pH 7.3, 8 mM $MgSO_4$, 75 mM KCl, 0.1 mM NADH, 4 mM PEP, 15 U of pig heart LDH, and parasite homogenate. The reaction was initiated by the addition of 1 mM ADP.

Proteinase was assayed using azocasein as substrate according to the method of Pupkis and Coombs (1984). The reaction mixture contained 0.4 M sodium phosphate buffer, pH 6.0 1 mM DTT and 10 mg azocasein in a total volume of 0.75 ml. The reaction was started by the addition of parasite homogenate. After 1 hour incubation at 32°C the reaction was stopped by the addition of 0.75 ml 5% (w/v) trichloroacetic acid (TCA), centrifuged at 10,000 x g for 1 min at 18°C and the supernatant absorbance measured at 366 nm using a MSE Spectroplus

spectrophotometer. One unit (U) of azocasein activity was defined as that which caused the hydrolysis of 1 mg azocasein min^{-1} under specified assay conditions. The A 1%/366 of azocasein was taken as 32 (Pupkis & Coombs, 1984).

Phosphoric monoester hydrolase (Acid phosphatase) (EC3 1.3.2) was assayed using a modification of the method of Gottlieb and Dwyer (1981c). The reaction mixture contained 50 mM succinic acid-NaOH pH 6.0, 5 mM p-nitrophenylphosphate (PNPP) in a total volume of 0.5 ml. The reaction was started by the addition of parasite homogenate. After 1 hour incubation the reaction was stopped by the addition of 0.1 ml 100 mM NaOH and the released p-nitrophenyl was measured at 410 nm on a Perkin-Elmer Lamda 5 spectrophotometer. Enzyme activities were calculated using an extinction coefficient of 14.3×10^3 $M^{-1}cm^{-1}$ (Glew et al., 1982). The effect of pH on enzyme activity in parasite homogenates, and particulate and soluble fractions was determined by using the following buffers (50 mM): sodium acetate-acetic acid pH 4.5-5.5; succinic acid-NaOH pH 5.0-6.0; and Tris-maleate pH 5.5-8.0. The effects of inhibitors was assessed by adding the inhibitor to the reaction mixture immediately prior to adding the parasite homogenate to start the assay. The results are expressed as % inhibition related to the activity in the absence of inhibitors.

In preliminary experiments the acid phosphatase activities of live parasite cells and that released into medium during growth in vitro were also examined. Experiments examining the AP activities of living L.major mid-log phase promastigotes were carried out using the method of Hassan and Coombs (1987). Parasite cells were washed twice in Tris-maleate saline, pH 7.0 (TMS, 50 mM Tris-maleate buffer, 0.9% [w/v] NaCl), resuspended in TMS with 5 mM PNPP to a density of 5×10^7 cells ml^{-1} and incubated at 25°C. At 5, 15, 30 and 45 min time points 0.2 ml aliquots of the incubation mixture were centrifuged at 10,000 x

g for 1 min and the supernatant analysed for released p-nitrophenol as described above.

The acid phosphatase released by L.major promastigotes into HOMEM standard medium (see section 2.1.1) during growth was studied using the following proceedue. 10 ml aliquots were taken at 24 hour intervals (up to 168 hours) from a 200 ml culture seeded with mid-log phase promastigotes to give a starting density of 1 x 10^5 promastigotes ml^{-1}. Each aliquot was centrifuged at 2000 x g for 20 min at 4°C to remove the promastigotes, and the resulting supernatant filtered (0.22 um milipore filter) and frozen at -70°C. When all the supernatants were collected they were defrosted and concentrated by freeze-drying for 24 hours. The concentrated aliquots were then each resuspended in 1 ml deionised, distilled water and assayed for acid phosphatase as described above.

2.14 ANALYSIS OF ISOENZYMES

2.14.1 Preparation of parasite homogenates for isoelectric focussing

Cells used for isoelectric focussing were washed three times (see Section 2.11.1) in 250 mM sucrose, pelleted and used immediately or stored as pellets at -70°C until required. Parasite homogenates were prepared by resuspending the cells in 250 mM sucrose and sonication as described in section 2.12.1. In some cases, as specified in the text, the cells were lysed in the presence of 100 µg ml^{-1} leupeptin.

2.14.2 Isoelectric focussing

Flatbed isoelectric focussing (IEF) was performed using carrier ampholytes as outlined in the Pharmacia guide to IEF. The agarose IEF, 3.3 g sorbitol, 25 ml deionised distilled water and 1.9 ml PHARMALYTE PH 3 - 10. This was cast onto a hydrophilic polyester sheet (Gelbond). Once the gel was set, it was left overnight at room temperature in a

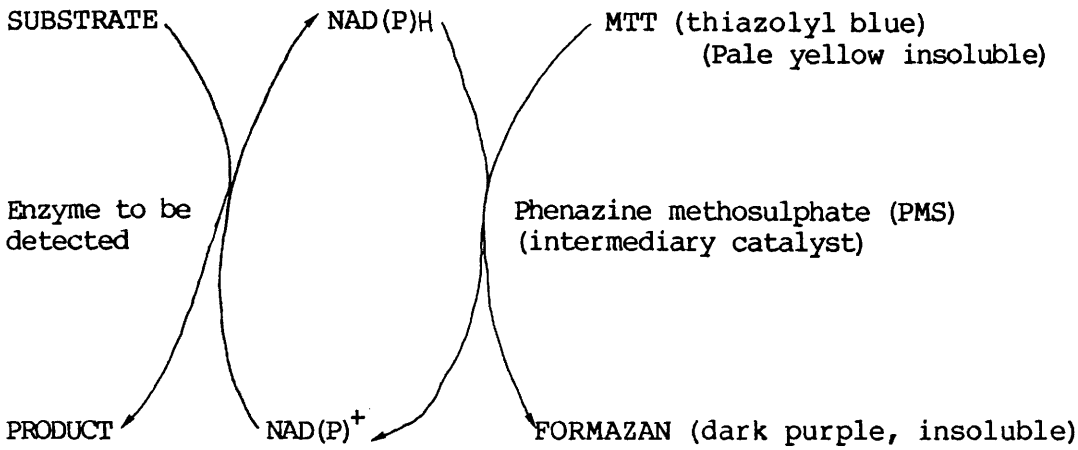
moist chamber. the following day the set gel was placed on the cooling plate of a flatbed electrophoresis apparatus (Pharmacia FBE 3000). The anode and cathode electrode strips were soaked in 0.05 M H_2SO_4 and 1 M NaOH, respectively. 10 - 20 µl of the sample homogenates containing equal amounts of protein 300 - 800 µg protein), unless indicated otherwise, were applied to strips of filter paper (applicators) which was placed at the centre of the gel approximately 1 cm apart. A constant power supply (LKB Bromina 2197 was set to deliver a maximum of 1500 V, 50 mA and 20 W was applied. The sample applicators were removed after 30 min and electrophoresis was continued for a total of 2.5 hours by which time the human haemoglobin markers used had focussed.

2.14.3 Staining for protein and enzymes in gels

Protein The focussed Gel was fixed in 5% (w/v) sulphosalicylic acid, 10% (w/v) TCA for one hour and then washed (2 x 15 min) in destain solution (ethanol/acetic acid/water, 35/10/55,v/v). The gel was then immersed in 0.2% (w/v) PAGE blue 53 dye in destain solution all filtered (using a N^O2 Whatman filter) and constantly agitated until protein bands were fully stained. Finally, the stained gel was washed well in destain solution to remove background staining, dried and photographed.

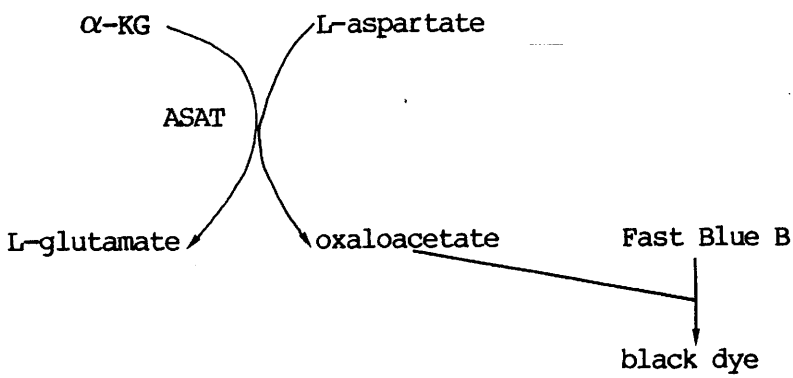
Enzymes All enzymes were stained using modifications of the methods layed out by Harris and Hopkinson (1976). All enzymes were stained by incubation at 37^{O}C in the dark, and the reaction stopped by submerging the gel in 10% (w/v) acetic acid for 10 min. The gels were then washed in the reaction mixture buffer, dried and photographed. Approximate pIs of resulting isoenzymes were determined by comparison to pI standards. Unless otherwise stated, enzymes were stained using a zymogram technique of overlaying the agarose gel with a reaction

mixture (that contained the appropriate substrates and buffer) which had been mixed with an equal volume of 2% (w/v) agar. The basis of the visualisation proceedure for the reactions involving reduction of $NAD(P)^+$ was:



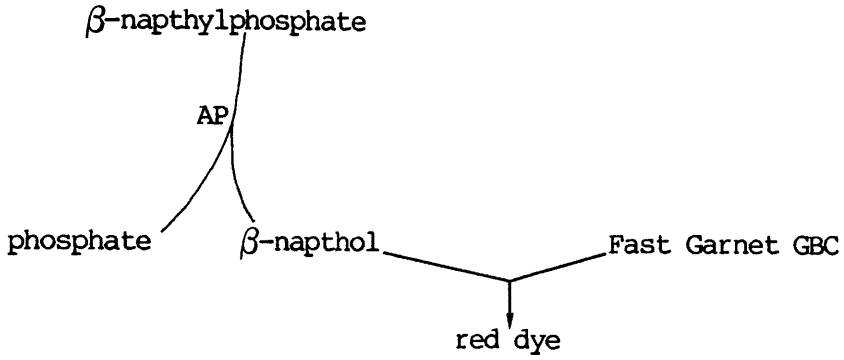
The enzyme activities are visualized by deposition of the insoluble dark purple formazan.

ASAT activity was stained by submerging the gel in a reaction mixture containing 100 mM Tris-HCl pH 8.2, 92 mM L-aspartate, 11.5 mM α-ketoglutarate, and 0.75 mg Fast Blue B ml^{-1}, and incubating for 2-4 hours. The reactions involved were:



Acid phosphatase activity was detected by submerging the gel in a reaction mixture containing 200 mM sodium acetate-acetic acid pH 5.0 for L.mexicana mexicana, 1 mg β-napthylacid phosphate ml^{-1} and, 1 mg

Fast Garnet GBC ml^{-1} and incubating for 0.5-2 hours. The reactions involved were:



GDH activity was studied by submerging the gel in a reaction mixture containing 100 mM Tris-HCl pH 8.5, 1 M L-glutamate, 0.25 mg NAD^+ ml^{-1}, 0.25 mg MTT ml^{-1}, and 0.025 mg PMS ml^{-1}, and incubating for 2-4 hours.

Glucose-6-phosphate dehydrogenase (G-6-PDH) (EC 1.1.49) activity was stained by overlaying the gel with a reaction mixture containing 100 mM Tris-HCl pH 8.0, 0.2 mg glucose-6-phosphate ml^{-1}, 20 mM $MgCl_2$, 0.2 mg $NADP^+$ ml^{-1}, 0.3 mg MTT ml^{-1}, 0.2 mg PMS ml^{-1} and incubating for 0.5-1 hour.

Glucose phosphate isomerase (GPI) (EC 5.3.1.9) activity was detected by overlaying the gel with a reaction mixture containing 100 mM Tris-HCl pH 8.0, 0.8 mg D-fructose-6-phosphate ml^{-1}, 0.5 U ml^{-1} Torula yeast G-6-PDH 1.5 mg $MgCl_2$ ml^{-1}, 0.2 mg $NADP^+$ ml^{-1}, 0.6 mg MTT ml^{-1}, and 0.2 mg PMS ml^{-1}, and incubating for 15-30 min.

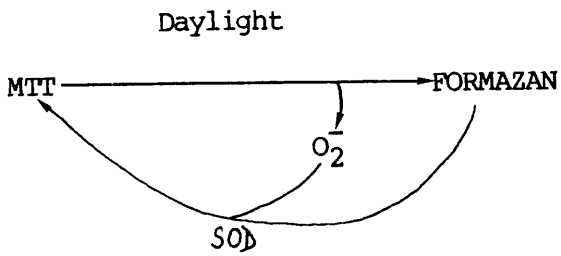
MDH activity was detected by submerging the gel in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 1 M malate, 0.25 mg NAD^+ ml^{-1}, 0.25 mg MTT ml^{-1}, and 0.25 mg PMS ml^{-1} and incubating for 2-2.5 hours.

6-Phosphogluconate dehydrogenase (6-PGDH) (EC 1.1.1.44) activity was detected by overlaying the gel with a reaction mixture containing

100 mM Tris-HCl pH 8.0, 0.33 mg 6-phosphogluconate ml^{-1}, 0.28 mg $NADP^+$ ml^{-1}, 2.5 mg $MgCl_2$ ml^{-1}, 0.30 mg MTT ml^{-1}, and 0.025 mg PMS ml^{-1} and incubating for 20-35 min.

Phosphoglucomutase (PGM) (EC 2.7.5.1) activity was detected by overlaying the gel with a reaction mixture containing 100 mM Tris pH 8.0, 0.8 glucose-1-phosphate ml^{-1}, 0.3 mg $NADP^+$ ml^{-1}, 0.75 U ml^{-1} Torula yeast G-6-PDH, 1.66 mg $MgCl_2$ ml^{-1}, 0.2 mg MTT ml^{-1}, and 0.01 mg PMS ml^{-1} and incubating for 20-35 min.

SOD (EC 1.15.1.1) activity was detected by overlaying the gel with a reaction mixture containing 100 mM Tris-HCl pH 8.0, 0.3 mg MTT ml^{-1}, 0.2 mg PMS ml^{-1}, exposing it to bright light for 15 min, and then incubating (in the dark) for 30-35 min. The reactions involved were:



2.15 DETERMINATION OF FREE AMINO ACID POOLS

2.15.1 Preparation of parasite homogenates for amino acid analysis

Parasite cells to be processed for in amino acid analysis were washed three times (see Section 2.11.1) in 250 mM sucrose and stored as pellets at -70°C until use. Parasite homogenates were prepared by resuspending the cells in 200 mM sodium citrate-citric acid buffer pH 2.2. To ensure total lysis of the cells the suspension was then sonicated as described in section 2.12.1. A sample was then taken for protein estimation and the remainder deproteinated by adding three volumes of 3% (w/v) sulphosalicylic acid. The precipitated protein was pelleted by centrifugation at 10,000 x g for 10 min, at 18°C and the supernatant removed and stored at 4°C until required.

2.15.2 Amino acid analysis

Accelerated amino acid analysis was kindly performed by J.Jardine (Department of Biochemistry, University of Glasgow) on a LKB 4400 Amino Acid Analyser employing an ion-exchange column packed with sodium form Ultrapac 8 resin. 25 µl of each sample (originally containing approximately equal amounts of protein, 100-200 µg) were loaded onto the column and analysis performed as outlined in the LKB Technical Publication, PCN 10. The resulting peaks were identified and quantified using a set of amino acid standards.

2.16 MEASUREMENT OF OXYGEN CONSUMPTION OF PROMASTIGOTES

2.16.1 Preparation of cells

Leishmania major promastigotes were washed three times in PBS pH 7.3 and resuspended in this buffer (for endogenous rate) or HOMEM medium supplemented with 10% (v/v) HIFCS (for stimulated rate) to a density of 2×10^7 cells ml^{-1}.

2.16.2 Measurement of oxygen consumption

Oxygen consumption was measured using a Clark-type oxygen electrode (Rank Bros., Cambridge, England) with a constant stirrer, and maintained at 25°C by a circulating water jacket. The electrode was calibrated each time before use according to the method of Robinson and Cooper (1970). 3 ml of the cell suspension was then added to the electrode chamber, the electrode allowed to equilibrate for about 2 min, and oxygen consumption followed for at least 20 min.

2.17 MEASUREMENT OF NITROBLUE TETRAZOLIUM REDUCTION BY PROMASTIGOTES

2.17.1 Preparation of cells

Leishmania major promastigotes were washed twice in HBSS pH 7.30, and resuspended in RPMI 1640 medium (see section 2.4.1) containing 0.5 mg NBT ml^{-1} to a density of 2.5×10^7 cells ml^{-1} in a total volume of

1.6 ml.

2.17.2 Measurement of nitroblue tetrazolium reduction

The cell suspension was incubated for 1 hour at 34°C with a gas phase of 5% CO_2, 95% air and the reaction terminated by centrifuging (2000 x g, 10 min, 4°C) the cells out of solution. They were then washed twice in PBS pH 7.3 and the NBT extracted by adding 1 ml pyridine to the pellet and boiling for 15 min (Haidaris & Bonventre, 1982). The precipitated protein was removed by centrifuging at 10,000 x g for 2 min at 18°C and the absorbance of the supernatant read at 515 nm on a MSE Spectroplus spectrophotometer. The amount of NBT reduced was calculated using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ (Haidaris & Bonventre, 1982).

2.18 TRANSFORMATION STUDIES

2.18.1 Determination of the effect of 3-methoxybenzamide on the transformation of amastigotes to promastigotes

Purified amastigotes of L.mexicana mexicana and L.donovani washed once in PSGEMKA pH 7.3 (see section 2.1.2) and crude preparations of L.major amastigotes (from mouse footpads) washed three times in this buffer were resuspended in HOMEM transformation medium (see section 2.1.1) supplemented with 10% (v/v) HIFCS to a density of 5 x 10^7 amastigotes ml^{-1}. 0.5 ml aliquots of this suspension were added to universals with various concentrations of 3-methoxybenzamide (3-MB) in a total volume of 5 ml (HOMEM transformation medium) and incubated for 48 hours at 25°C. The numbers of promastigotes (not transformation intermediates) present in control and treated cultures at the end of the incubation was estimated using a haemocytometer. The % inhibition of transformation was calculated from the formula: [1-(number of promastigotes in treated culture/number of promastigotes in control culture)] x 100.

2.18.2 Determination of the effect of 3-methoxybenzamide on the production of metacyclic forms

Leishmania major mid-log phase promastigotes were inoculated into universal tubes containing HOMEM standard medium (see section 2.1.1) with (treated) or without (control) the desired concentration of 3-MB in a fixed volume of 10 ml to give a starting density of $1x10^5$ cells ml^{-1}. The cultures were followed until they reached stationary-phase and then agglutinated with 100 µg PNA ml^{-1} using the agglutination assay described in section 2.3.2. The number of free, unagglutinated metacyclic promastigotes in treated cultured were counted and expressed as a % of the number in control cultures.

2.18.3 Assessment of *in vitro* growth of mid-log phase and metacyclic promastigotes

Leishmania major mid-log phase and metacyclic promastigotes were washed once in HBSS pH 7.3, resuspended in HOMEM standard medium, counted, and inoculated into universal tubes to give a starting density of 1×10^5 cells ml^{-1} in a final volume of 5 ml with air as gas phase. The tubes were incubated at 25°C and cell numbers monitored every 24 hours using a haemocytometer.

2.18.4 Determination of the effect of "spent" and fresh medium on the production of metacyclic forms

"Spent" medium from L.major stationary-phase cultures was obtained by centrifuging the cultures at 2000 x g for 10 min at 4°C to remvoe the cells, taking the supernatant and centrifuging again (as above) to ensure that no cells remained. The resulting supernatant was removed and designated "spent" medium, this was kept at 4°C until use.

Leishmania major mid-log phase promastigotes were washed once in HBSS pH 7.3 and resuspended in fresh HOMEM standard medium or "spent"

medium to a density of 1×10^7 cells ml^{-1}. "Smears" of cell cultures were made at the start of the experiment and then at 4 hour intervals up to 24 hours and finally at 48 hours. "Smears" were dried in air, fixed and stained, and the percentage of metacyclics present assessed by agglutination with 100 µg PNA ml^{-1} (as described in section 2.3.2) at the start of the experiment and then after 24 hours.

2.19 PROTEIN DETERMINATIONS

For the Gelatin Disc-PAGE studies, protein content was determined by the method of Sedmak and Grossberg (1977), and for all other studies by the method of Lowry et al (1951), with bovine serum albumin (BSA) being used as standard in both cases.

2.20 STATISTICAL ANALYSIS OF DATA

A two-way analysis of variance test or an unpaired Students t-test were used (as indicated) to establish the significance of data (with $P < 0.05$ being taken as significant).

2.21 MATERIALS

Growth culture medium for Leishmania promastigotes including foetal calf serum, MEM Eagle Suspension powder, MEM (50 x) amino acids solution and MEM (100 x) non-essential amino acids solution were obtained from Gibco-Biocult, Paisley, Scotland. Liver infusion and Tryptose for LIT medium were obtained from Difco, East Molesey, Surrey, England. RPMI 1640, foetal calf serum and Lab-Tek 8-chamber tissue culture slides for macrophage cultures were obtained from Flow Laboratories, Rickmansworth, Surrey, England. HBSS pH 7.3 without bicarbonate but with phenol red, was obtained from Gibco-Biocult, HBSS without bicarbonate, without phenol red, was obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England.

Agarose IEF (Cat. No. 17-0468-01), Gelbond, pI calibration kit

pH3-10 (Cat. No. 17-0471-01). CM-Sephadex C25, Ficol-Hypaque and Percoll were obtained from Pharmacia Fine Chemicals Ltd., Milton Keynes, England. Gelatin (Cat. No. G-2500), and high (Cat. No. SDS-6H) and low (Cat. No. SDS-7) molecular weight PAGE calibration kits were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England. Sorbitol was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, and leupeptin from the Protein Research Foundation , Osaka, Japan.

All other chemicals, enzymes and linkage enzymes were purchased from Sigma Chemical Co. Ltd., or BDH Chemicals Ltd., Poole, Dorset, England.

3.0 RESULTS

3.1 LEISHMANIAL METACYCLIC PROMASTIGOTES

3.1.1 Occurrence of metacyclic promastigotes of L.major and putative metacyclic promastigotes of L.mexicana mexicana in in vitro cultures

These studies were undertaken to confirm that the strain of L.major in this study produced metacyclic promastigotes as described previously for another strain, and that mid-log phase promastigotes were agglutinated by PNA (i.e. PNA^+) whereas metacyclic forms were not (i.e. PNA^-) (Sacks et al., 1985). Mid-log phase and stationary-phase promastigotes were treated with various concentrations of the lectin and the resultant agglutination measured, the results are given in Fig. 6A. The majority of the mid-log phase cells were agglutinated by PNA concentrations in the range 25-100 $\mu g\ ml^{-1}$. Repeat experiments using 100 μg PNA ml^{-1} showed that on average 95±1% of the mid-log phase cells were agglutinated whereas only 35±17% of the stationary-phase cells were (Fig. 6B). Subsequently, 100 ug PNA ml^{-1} was used routinely to purify metacyclic of L.major from stationary-phase cultures.

The metacyclic forms of L.major showed several characteristic features. They were extremely active and appeared to be more motile than mid-log phase cells although this was not quantified. The percentage of the cells motile was similar for mid-log phase and metacylic promastigotes. After 1 hour incubation at 34°C in PBSG pH 7.3, 80±6% (mean ± standard deviation, n=4) of the mid-log phase cells were assessed as "fast" compared to 83±3% for the metacyclics, whereas the figures for % motile were 96±4% and 94±2% for mid-log phase and metacyclic promastigotes, respectively. The metacyclic forms also have a characteristic morphology. Their cell bodies are smaller and narrower than mid-log phase cells, but the most dramatic structural

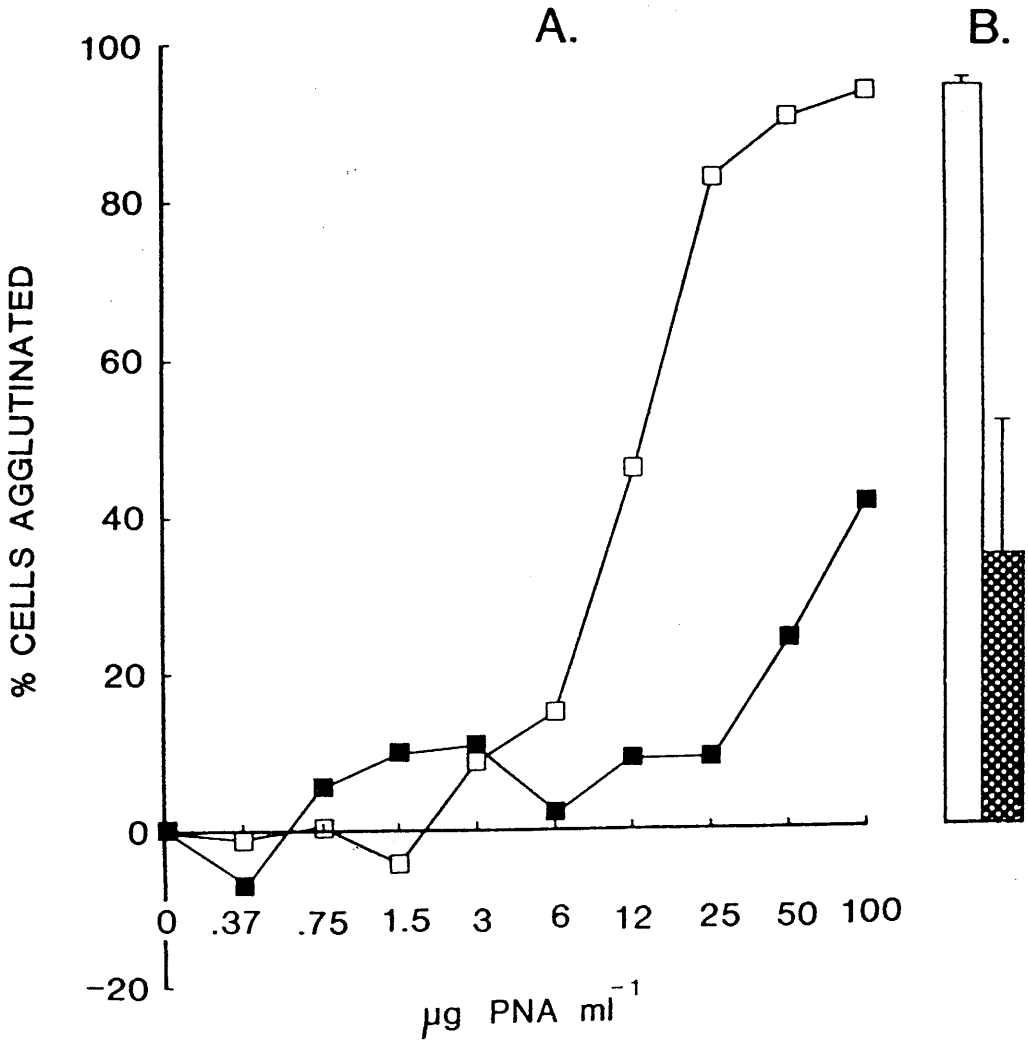


Fig. 6. Peanut agglutinin-mediated agglutination of L.major mid-log phase and stationary-phase promastigotes.

(A) Mid-log phase (■) and stationary-phase (□) promastigotes were agglutinated with a range of PNA concentrations. This experiment was carried out once only.

(B) Mid-log phase (□) and stationary-phase (▩) promastigotes were agglutinated with 100 μg PNA ml^{-1}. Each histogram represents the mean (± standard deviation) from five experiments.

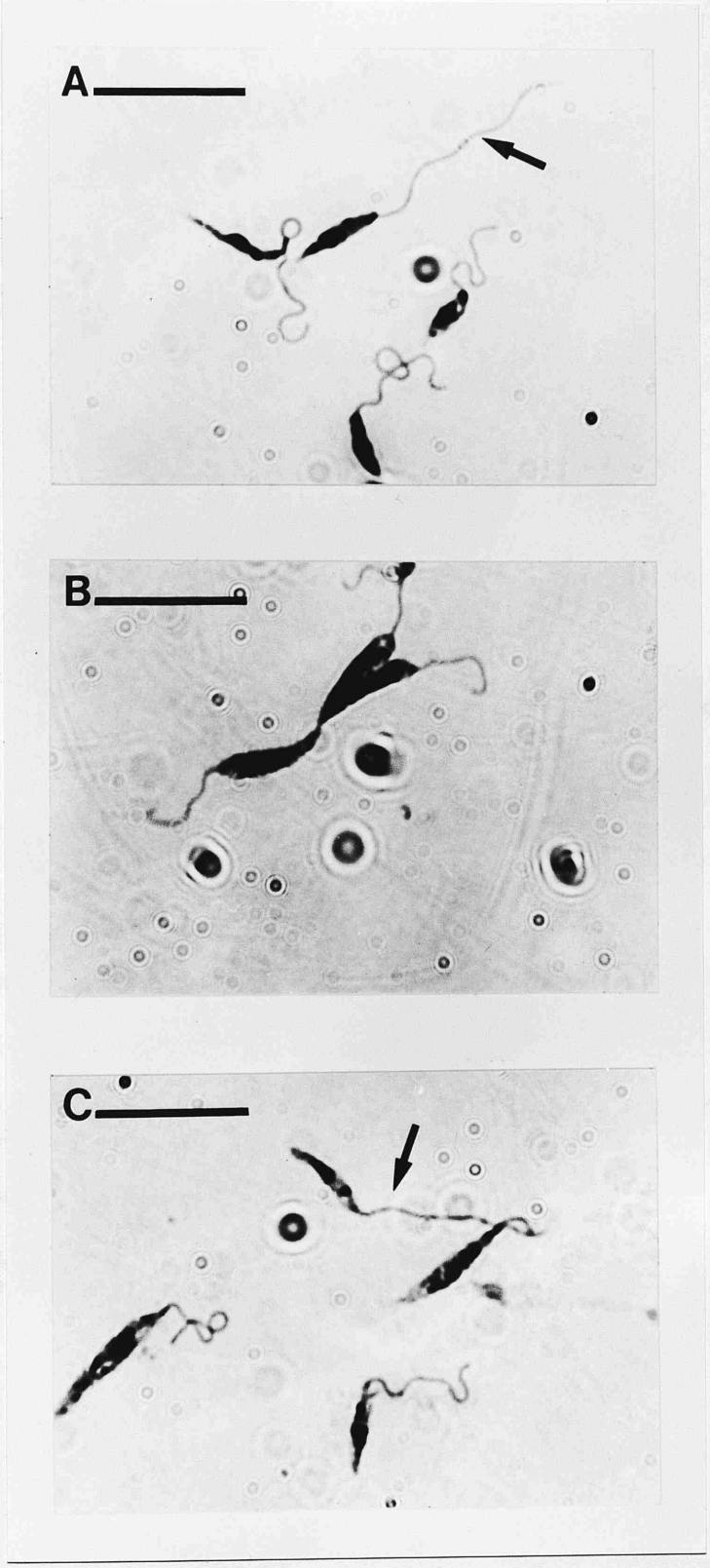


Fig. 7. Light micrographs of different promastigote forms of L.major and L.mexicana mexicana.

(A) L.major metacyclic promastigotes. Note length of flagellum (arrowed).

(B) L.major mid-log phase promastigotes.

(C) L.mexicana mexicana stationary-phase promastigotes. Note putative metacyclic promastigote (arrowed).

All scale bars = 10 μm.

Table 6A: Occurrence of metacyclics in mid-log phase and stationary phase populations of *L.major*.

Assessment method	% metacyclic forms[c]	
	Mid-log phase cultures	Stationary-phase cultures
Agglutination[a]	5± 1 (5)	65±17 (5)
Morphological criteria[b]	3± 1 (4)	52± 5 (4)

Table 6B: Occurrence of putative metacyclics in mid-log phase and stationary-phase populations of *L.mexicana mexicana*.

Assessment method	% metacyclic forms[c]	
	Mid-log phase cultures	Stationary phase cultures
Morphological criteria[b]	4± 2 (11)	43±11 (11)

[a] By agglutination using 100 μg PNA ml^{-1}, figures are percentage of promastigotes unagglutinated.

[b] In stained "smears" of cultures.

[c] The results are the means (± standard deviation) from the number of experiments given in parentheses.

change concerns the length of the flagellum compared to the cell body. Typical light micrographs of the two forms are shown in Fig. 7. Metacyclic promastigotes have an elongated flagellum which can be over twice the length of the cell body whereas the flagellum of mid-log phase promastigotes is slightly shorter or a similar size to the cell body. Using the morphological differences to identify the forms, their occurrence in mid-log phase and stationary-phase cultures was determined. The results for L.major are shown in Table 6A. It was found that the number of metacyclics in mid-log phase and stationary-phase cultures from morphological considerations was very similar to the results obtained using the agglutination method. Examination of stained "smears" of stationary-phase cultures of L.mexicana mexicana revealed that forms, similar in morphology to the metacyclics of L.major, were present in quite large numbers (Fig. 7). Examination of more cultures showed that stationary-phase cultures contained many more of these forms compared to mid-log phase cultures (Table 6B), the numbers of putative metacyclic forms in these two populations were quite consistent and moderately similar to the numbers in mid-log phase and stationary-phase populations of L.major.

3.1.2 Analysis of the susceptibility of L.mexicana mexicana promastigotes to agglutination by agglutinins

In an attempt to separate out the putative metacyclic forms of L.mexicana mexicana, as had been achieved for L.major, a battery of lectins and Cat F. The choice of lectins that were investigated was dictated by availability and, to a lesser extent, cost. The various agglutinins were tested once, although at a range of concentrations, for their ability to agglutinate mid-log phase and stationary-phase promastigotes of L.mexicana mexicana using the standard procedure described in the Materials and Methods Section 2.3.2. The results are

summarised in Table 7. Most of the lectins used did not agglutinate either population of promastigotes, ConA and LCA caused agglutination, but to similar extent with both mid-log phase and stationary-phase promastigotes. Both these lectins have affinities for glucose and mannose. So the results suggest that one or both of these sugars are exposed on the surface of L.mexicana mexicana promastigotes. Cat F also agglutinated both populations of L.mexicana mexicana promastigotes and to a similar extent, confirming the presence of negative changes on the surface of promastigotes. The agglutination due to ConA, LCA, and Cat F was studied in more detail (Fig. 8). In all cases, agglutination was dose-dependent and similar with both populations of promastigotes. Thus, it would appear that glucose/ mannose and negative charges are equally distributed on promastigotes from both populations.

3.1.3 Separation of L.mexicana mexicana promastigote forms by density gradient centrifugation

Density gradient centrifugation was investigated as a possible method of purifying the putative metacyclic forms of L.mexicana mexicana seen in stationary-phase cultures. Pre-formed gradients of 100, 75, 50, and 25% stock iso-osmotic Percoll (SIP) were loaded with stationary-phase promastigotes of L.mexicana mexicana and centrifuged for 5 min at 400 x g. This did not produce distinct bands (Fig. 9). Centrifuging for 10 min at 400 x g, however, resulted in a series of separate, discrete bands in the 50% and 25% SIP gradients that could be easily seen by eye. Examination of wet preparations from these bands, however, revealed populations of promastigotes of mixed morphologies (i.e. putative metacyclics and non-metacyclics).

Table 7: Susceptibility of mid-log phase and stationary-phase promastigotes of L.mexicana mexicana to agglutination by various agglutinins.

Agglutinin[a]	Affinity[b]	Parasite population	
		Mid-log phase cultures	Stationary-phase cultures
PNA (Peanut agglutinin)	β-D-galactose(1-3)-N-acetyl-D-galactosamine, -D-galactose	-	-
SBA (Soybean agglutinin)	N-acetyl-D-galactose	-	-
SJA (Sophora japonica agglutinin)	N-acetyl-D-galactosamine, D-galactose	-	-
VVA (Vicia villosa agglutinin)	N-acetyl-D-galactosamine,	-	-
DBA (Dolichos biflorus agglutinin)	N-acetyl-α-D-galactosamine,	-	-
WGA (Wheat germ agglutinin)	N-acetyl-β-D-glucosamine	-	-
UEA-I (Ulex europaeus agglutinin)	L-fucose	-	-
ConA (Concanavalin A)	α-D-glucose, α-D-mannose	+	+
LCA (Lens culinaris agglutinin)	"	+	+
CatF (Cationised ferritin)	negative charges	+	+

-, no apparent agglutination observed.
+, high levels of agglutination observed.

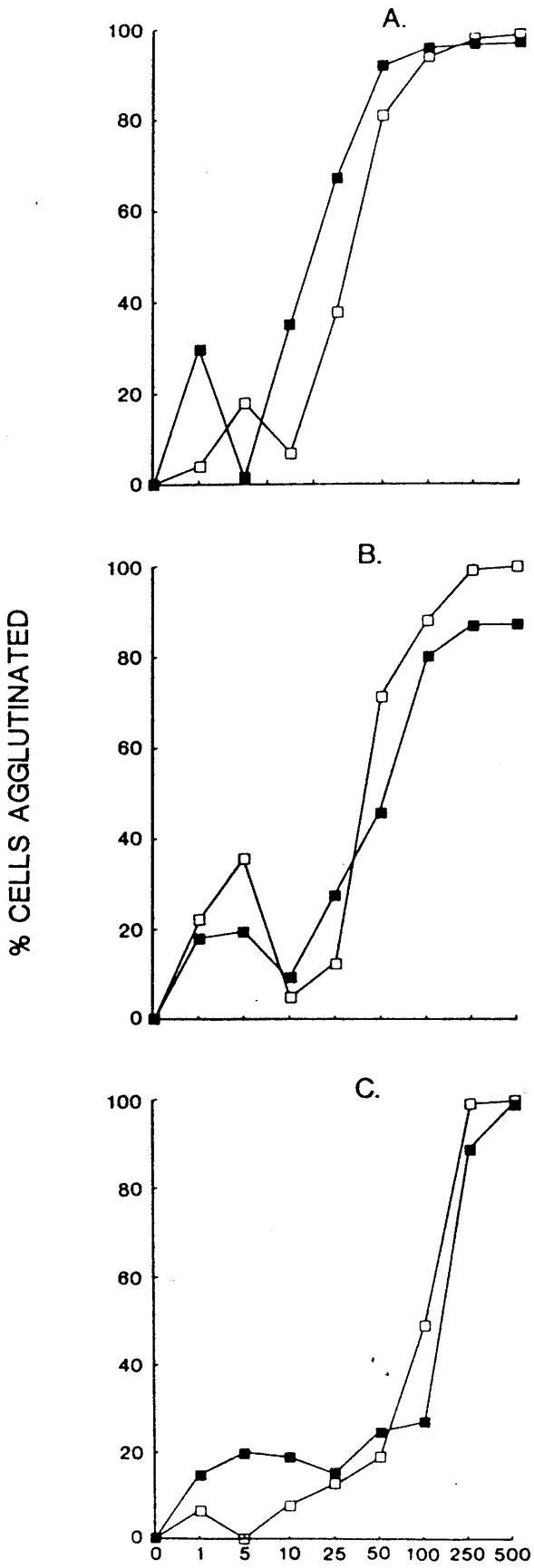
[a] Each agglutinin was tested once with each parasite population using a range of concentrations up to 500 μg ml^{-1}.

[b] from Sigma catalogue.

Fig. 8. Agglutinin-mediated agglutination of L.mexicana mexicana mid-log phase and stationary-phase promastigotes.

Mid-log phase (□) and stationary-phase (■) promastigotes were agglutinated with a range of concentrations of ConA (A), LCA(B) and CatF (C). Each agglutinin was tested once with each promastigote form.

Fig. 8.


A.
B.
C.
100
80
60
40
20
0
% CELLS AGGLUTINATED
0 1 5 10 25 50 100 250 500
AGGLUTININ CONCENTRATION (μg ml⁻¹)

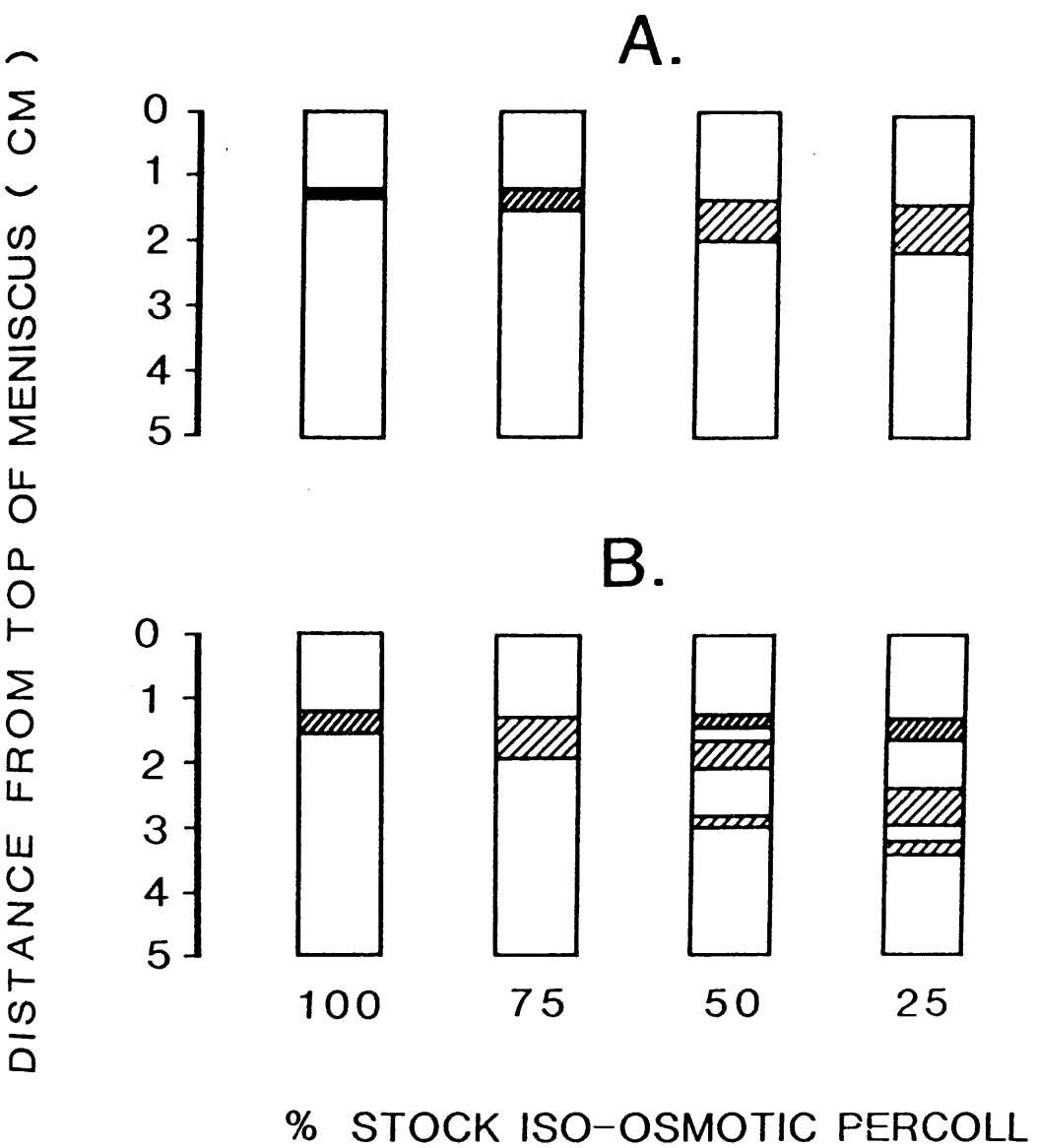


Fig. 9. Density gradient centrifugation of L.mexicana mexicana stationary-phase promastigotes.

(A) Centrifuged for 5 min at 400 x g.
(B) Centrifuged for 10 min at 400 x g.

Shading represents opacity of resulting bands.

3.2 THE INTERACTION OF L.MAJOR PARASITES WITH THE MAMMALIAN HOST AND WITH MACROPHAGES IN VITRO

3.2.1 The fate of different promastigote forms of L.major in mice, and in murine resident peritoneal macrophages in vitro

To measure their infectivity in vivo, metacyclic, stationary-phase and mid-log phase promastigotes were inoculated into the footpads of Balb/c mice. Two sizes of inoculi were used. Results representative of the three separate experiments performed are shown in Fig. 10. With an inoculum of 1×10^6 promastigotes per footpad, the metacyclics produced lesions more rapidly than mid-log phase cells. At this inoculum, however, stationary-phase promastigotes produced lesions as quickly as the purified metacyclics. When the inoculum was 1×10^4 cells per footpad, however, metacyclic promastigotes were not only seen to be more infective than mid-log phase promastigotes but also produced lesions more rapidly than stationary-phase cells.

The intracellular fate of mid-log phase and metacyclic promastigotes was also examined in vitro using Balb/c resident peritoneal macrophages, the results of these experiments are given in Fig. 11. The macrophages were exposed to promastigotes at parasite: macrophage ratios of 1:1, 10:1, and 20:1 for 2 hours, whereupon the parasite were removed by washing and the cultures incubated further. The 1:1 ratio resulted in only low levels of infection, both in terms of the % macrophages infected and numbers of intracellular parasites per 100 macrophages. Consequently, more parasites were used in order to obtain higher infection rates.

An important observation was that in all cases mid-log phase promastigotes resulted in higher initial infection rates than metacyclic promastigotes. This will be referred to in more detail in section 3.2.2.

Fig. 10. Infectivity of different promastigote forms of L.major to Balb/c mice.

1×10^6 (A) or 1×10^4 (B) mid-log phase (□), stationary-phase (■) and metacyclic (●) promastigotes were inoculated into the left-hind footpads of Balb/c mice. Groups of five mice were used for each promastigote form at each inoculum. Each point represents the mean (± standard deviation) footpad width from five mice apart from the 1×10^4 inoculum for metacylic promastigotes where the results are from four mice. The data presented are from one experiment which is representative of the three experiments carried out.

Fig. 11. The intracellular fate of mid-log phase and metacyclic promastigotes of L.major within Balb/c resident peritoneal macrophages in vitro.

Macrophage infections were initiated with mid-log phase (□) and metacyclic (●) promastigotes at parasite:macrophage ratios of 1:1(A), 10:1(B) and 20:1(C). Each point represents the mean (± standard deviation) from two to three experiments at the 1:1 ratio, and three to six experiments at the 10:1 and 20:1 ratios. Each experiment was carried out in duplicate.

Fig. 10.

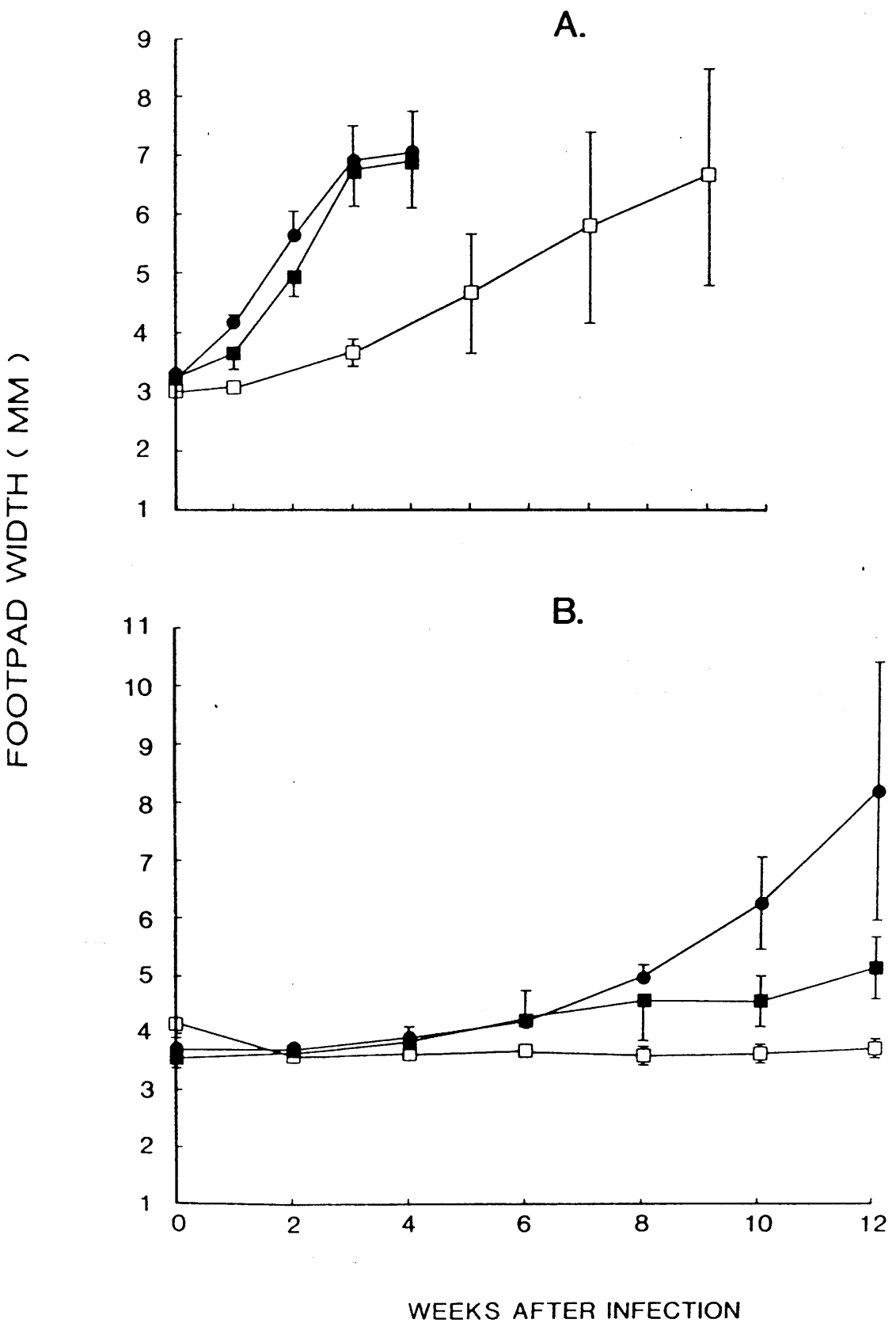
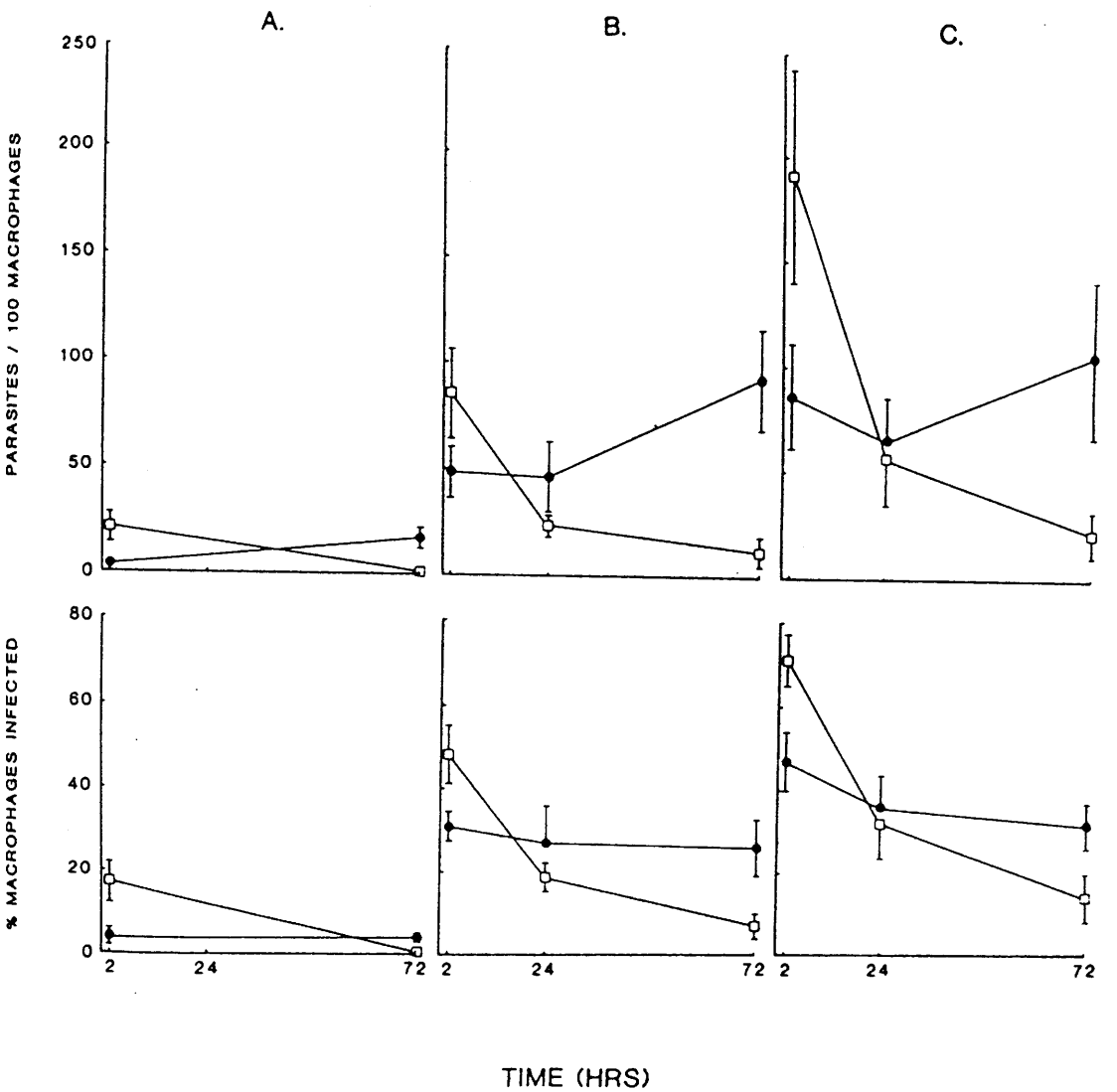

A.
B.
FOOTPAD WIDTH (MM)
WEEKS AFTER INFECTION
1
2
3
4
5
6
7
8
9
10
11
0
2
4
6
8
10
12

Fig. 11.


A.
B.
C.
PARASITES / 100 MACROPHAGES
250
200
150
100
50
0
% MACROPHAGES INFECTED
80
60
40
20
0
2
24
72
TIME (HRS)

Using the 1:1 ratio, infections resulting from mid-log phase promastigotes were almost completely cleared by 72 hours (% macrophages infected 0.5±0.4; number of parasites/100 macrophages, 1±1) In contrast, when metacyclic promastigotes were used the parasites survived and increased in number approximately 3-fold (to 17±5 parasites per 100 macrophages) over the 72 hour incubation. A similar picture was seen with the 10:1 ratio intracellular parasites originating from mid-log phase promastigotes were progressively killed over the 72 hour incubation period. Most of the destruction of the parasites occurred during the first 24 hours in culture, when there was a 4-fold reduction in the number of parasites/100 macrophages the % macrophages infected was reduced to less than half. It should be noted that there was still a residual infection remaining after 72 hours. On two occasions at this ratio, macrophage cultures were incubated for another 72 hours (a total infection time of 144 hours). There was a further reduction in parasite load during this time such that only a few parasites remained (% macrophages infected, 1±1; number of parasites 100 macrophages, 2±1. In contrast, metacyclic promastigotes lead to infections in which the parasite numbers remained stable for the first 24 hours in culture and then increased approximately 2-fold by 72 hours. At the 20:1 ratio, infections resulting from mid-log phase promastigotes declined in a similar manner as occurred with the 10:1 ratio, with an equivalent residual infection remaining at 72 hours. Further incubation up to 144 hours resulted in further reduction but not complete clearance of the infection (residual infection: % macrophages infected, 4±3; numbers of parasites per 100 macrophages, 6±4, n=2). When metacyclics were used at the 20:1 ratio, the situation was not as clear cut as with the lower ratios. There was a small apparent decline in the % macrophages

infected and the numbers of parasites/100 macrophages during the first 24 hours incubation. Subsequently up to 72 hours incubation, the % macrophages infected remained level and there was an apparent increase in the numbers of parasites/100 macrophages, although not to the same extent as seen when fewer parasites were used. Surprisingly, incubating macrophages infected by metacyclics (both 10:1 and 20:1 ratios) for longer periods resulted in small reductions in parasite load. The number of parasites/100 macrophages remained approximately constant but the % macrophages infected declined slightly (from 24±2 of 72 hours to 17±4 at 144 hours for the 10:1 experiment, n=2, and from 36±6 at 72 hours to 20±5 in the 20:1 experiments, n=2). Clearly, amastigotes were multiplying in some of the macrophages whereas others were killing the amastigotes.

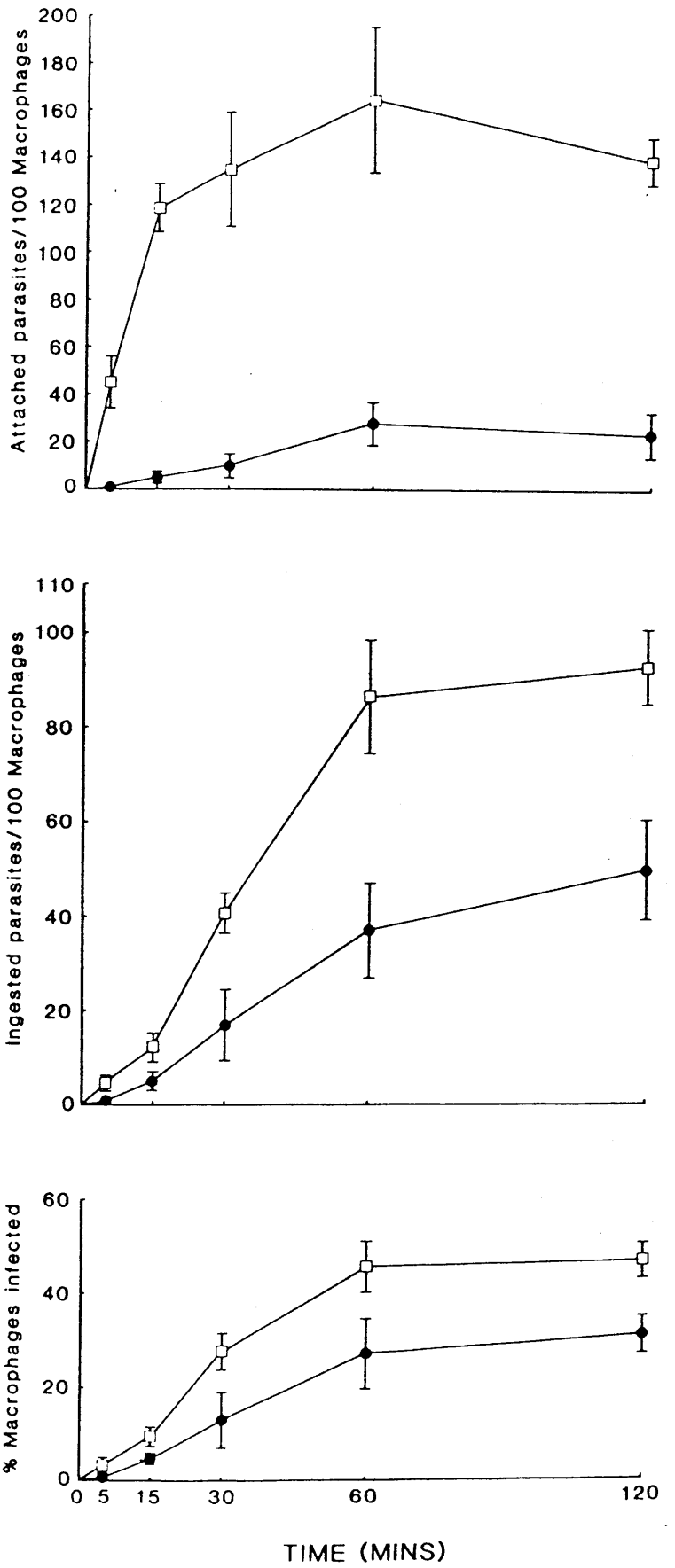
3.2.2 The kinetics of attachment and uptake of *L.major* mid-log phase and metacyclic promastigotes by murine resident peritoneal macrophages *in vitro*

To study the difference in uptake of mid-log phase and metacyclic promastigotes in more detail, Balb/c resident peritoneal macrophage cultures were incubated with promastigotes (at a parasite:macrophage ratio of 10:1) for time periods of up to 2 hours and the % macrophages infected, the numbers of promastigotes attached/100 macrophages and numbers of promastigotes/100 macrophages determined. This was carried out twice in duplicate and the results are shown in Fig. 12. For all the parameters studied mid-log phase promastigotes had greater interaction with macrophages than the metacyclics. The largest difference between the two promastigote forms was in the number of parasites attached. For all incubation periods, consistently greater numbers of mid-log promastigotes than metacyclics were attached. The difference varied from an apparent 170-fold at 5 min to 6-fold at 120

Fig. 12. The kinetics of attachment and uptake of mid-log phase and metacyclic promastigotes of L.major by Balb/c resident peritoneal macrophages in vitro.

Macrophage cultures were exposed to mid-log phase (□) and metacyclic (●) promastigotes at a ratio of 10 parasites:1 macrophage. Each point represents the mean (± standard deviation) from two experiments each carried out in duplicate. All experiments were carried out in the presence of 10% HIFCS.

Fig. 12.


Attached parasites/100 Macrophages
200
180
160
140
120
100
80
60
40
20
0
Ingested parasites/100 Macrophages
110
100
80
60
40
20
0
% Macrophages infected
60
40
20
0
0 5 15 30 60 120
TIME (MINS)

min. The kinetics of attachment also differed between the two forms. For mid-log phase promastigotes the numbers attached increase linearly and very rapidly up to 15 min. There was a further small increase up to 60 min, and thereafter the numbers attached remained essentially the same. The pattern with metacyclic promastigotes was somewhat different, the numbers attached increased apparently linearly, but at a much lower rate, for 60 min, and then similar to mid-log phase cells, the numbers attached after this time remained approximately constant.

The kinetics of ingestion differed from those of attachment. For both mid-log phase and metacyclic promastigotes there was a short lag period with subsequent rapid uptake over the first 60 min incubation, with the majority of the parasites being ingested during this period. Thereafter there was a slight trend to further apparent uptake over the remaing period of incubation. Overall, there were approximately half as many metacyclics ingested as mid-log phase cells. For both promastigote forms, the numbers of macrophages that were infected proceeded in a similar manner to uptake, the % macrophages infected at 60 min being essentially the same as that at 120 min indicating that most of the phagocytosis occurred within the first 60 min after exposure to parasites, metacyclics infected 1.5 to 2-fold less macrophages compared to mid-log phase cells.

The results for the interaction between mid-log phase promastigotes, metacyclic promastigotes and pure amastigotes of L.major and Balb/c resident peritoneal macrophages when incubated at various parasite:macrophage ratios are summarised in Table 8. The largest difference between uptake of mid-log phase and metacylic promastigotes was seen at the 1:1 ratio experiments; there were 4 to 5-fold more parasites ingested and macrophages infected by the mid-log phase cells compared to the metacyclics. This difference in uptake was

Table 8: The interaction of different forms of L.major with Balb/c resident peritoneal macrophages in vitro.

Parasite:Macrophage ratio	Parasite form[a]	Serum[b]	Parasites/100 Macrophages[c]			% Macrophages[c] infected
			Attached	Ingested	Total	
1:1	mid-log phase	+	9± 3	21± 7	30± 7	17± 5
	metacyclic	+	3± 1	4± 1	7± 2	4± 2
10:1	mid-log phase	+	113± 29	86± 17	201± 43	47± 6
	mid-log phase	-	53± 15	31± 5	84± 11	24± 5
	metacyclic	+	25± 9	49± 11	71± 19	31± 4
	metacyclic	-	18± 5	25± 7	43± 6	21± 5
	amastigote	+	19± 7	425± 62	445± 59	93± 1
20:1	mid-log phase	+	167± 50	191± 51	358± 94	71± 6
	mid-log phase	-	104± 37	60± 10	164± 46	36± 5
	metacyclic	+	44± 10	86± 25	130± 30	47± 7
	metacyclic	-	46± 12	52± 6	98± 16	37± 3
	amastigote	+	49± 10	729±117	778±123	97± 1
50:1	amastigote	+	136± 34	120± 66	1366± 95	100

[a] Mid-log phase and metacyclic promastigote results from 2 hour exposures, amastigote results from 1 hour exposures.

[b] Experiments performed in the presence (+) or absence (-) of 10% (v/v) HIFCS.

[c] The results are the means (± standard deviation) from two to three experiments each carried out in duplicate.

reduced to 1.5 to 2-fold at ratios of 10:1 and 20:1. At a ratio of 100 parasites:1 macrophage both promastigote forms had infected all macrophages after a 2 hour exposure time (data not shown) although for both promastigote forms there were too many parasites associated with the macrophages to enumerate accurately the numbers attached and ingested. In contrast to the metacyclics, amastigotes were phagocytosed avidly by the macrophages. At the 10:1 ratio the majority of macrophages were infected and contained large numbers of parasites (425±62 amastigotes/100 macrophages). Similar results were seen when the parasite:macrophage ratio was 20:1, although a 100% infection rate was achieved only if a 50:1 ratio was used.

There were also differences between the numbers of parasites attached. At the 1:1 ratio there were 3 times as many mid-log phase promastigotes attached compared to metacyclics, at the 10:1 ratio there was approximately 5 times as many and there was a slightly smaller difference at the 20:1 ratio. Interestingly, relatively few amastigotes were seen to be attached compared to the number intracellular.

The interaction of mid-log phase and metacylic promastigotes with macrophages in the absence of serum was also investigated using ratios of 10:1 and 20:1. Attachment of metacyclic promastigotes was unaffected by the absence of serum at both ratios, while the numbers of mid-log phase promastigotes attach ing were reduced approximately 2-fold. With mid-log phase promastigotes there was a 2 to 3-fold reduction in the % macrophages and the numbers of parasites ingested whereas the uptake of metacyclics was reduced less dramatically.

3.2.3 The respiratory burst of murine resident peritoneal macrophages upon exposure to different forms of *L.major* in vitro

Following the finding that metacyclic promastigotes were able to

survive and multiply in Balb/c resident peritoneal macrophages whereas mid-log phase promastigotes were progressively killed, I decided to determine if survival was related to the RB activity of the macrophages. The relative RB of Balb/c resident peritoneal macrophages elicited by the two promastigote forms and also pure amastigotes was assessed by measuring the production of O_2^-. In preliminary experiments, I was unable to detect O_2^- production using the ferricytochrome C assay (Haidaris & Bonventre, 1982) or lucigenin-enhanced CL. Attempts to measure H_2O_2 production using the method of Pick and Mizel (1981) were also unsuccessful. I found, however, that these macrophages stimulated by exposure to parasites readily reduced NBT to formazan (a reaction shown to be almost exclusively due to reduction by O_2^- [Baehner et al., 1976]), whereas only a few control macrophages (treated with medium only i.e. no stimulus) had formazan deposits (see below). This method was therefore chosen to assess O_2^- production qualitatively and quantitatively. The RB was measured qualitatively by assessing the % macrophages stimulated to reduce NBT, i.e. formazan positive and correcting the values with the medium only control as described in Materials and Methods section 2.5.5.1. The % macrophages stimulated in medium only controls after a 1 hour incubation was 6 ± 4 (n=18). In cases where 2 hour incubations were used control values were higher, 23 ± 6 (n=3) and for this reason the majority of incubations were carried out for 1 hour. Although the promastigotes themselves were also found to reduce NBT (see section 3.7.5 and below) this could not be visualised in individual parasites by the light microscopy method employed even after 2 hour incubations.

Figure 13 shows the results of experiments using various parasite:macrophage ratios. At the 1:1 ratio, the % macrophages stimulated to reduce NBT when exposed to either of the promastigote

forms, was similar to, or below levels for the medium only controls. When more parasites were used, macrophages were stimulated to reduce NBT and the percentage of macrophages increased with increasing numbers of both promastigote forms such that with the 100:1 ratio the majority of macrophages were stimulated. There was an apparent difference between promastigote forms, the metacyclics appeared to stimulate approximately half the number of macrophages stimulated by mid-log phase promastigotes at the 10:1, 20:1 and 50:1 ratios. It seems likely, however, that this difference is explained by the finding reported in section 3.2.2, that exposure of macrophages to mid-log phase promastigotes results in a 1.5 to 2-fold greater initial infection rate than exposure to metacyclics. Greater numbers of mid-log phase cells were also attached (see Table 8), although the role of attached parasites in stimulating the RB is unclear. The finding, however, that the % macrophages stimulated to reduce NBT by the metacyclics at a ratio of 20:1 is significantly lower ($P<0.05$, Students t-test) than the % stimulated by mid-log phase promastigotes at the 10:1 ratio (where the % macrophages infected and numbers of parasites ingested are roughly the same as when metacyclics were used at a 20:1 ratio), suggests that attached parasites can stimulate the RB. Attempts to investigate the importance of attached parasites in stimulation of the RB by determining the number of macrophages stimulated in the absence of serum (when less parasites attach) were unsuccessful as under these conditions the NBT agglutinated both promastigote forms.

The effect of time on the % macrophages stimulated was also investigated, results are shown in Fig. 14. At the 100 parasites:1 macrophage ratio, the kinetics of stimulation by mid-log phase and metacyclic promastigotes were similar, although the macrophages stimulated to reduce NBT at all times less than 60 min was apparently

Fig. 13. Nitroblue tetrazolium reduction by Balb/c resident peritoneal macrophages upon exposure to different forms of L.major in vitro.

Macrophage cultures were exposed to mid-log phase (□) and metacyclic (▩) promastigotes and amastigotes (▩) for 60 min. Each histogram represents the mean (± standard deviation) from two experiments for the amastigotes and five to seven experiments for the two promastigote forms. Each experiment was carried out in duplicate.

The reduction of NBT by Balb/c resident peritoneal macrophages upon exposure to zymosan (■) is shown for comparison. The histogram represents the mean (± standard deviation) from five single experiments.

Fig. 14. The kinetics of nitroblue tetrazolium reduction by Balb/c resident peritoneal macrophages upon exposure to mid-log phase and metacyclic promastigotes of L.major in vitro.

Macrophage cultures were exposed to mid-log phase (□) and metacyclic (●) promastigotes at parasite:macrophage ratios of 100:1 (A) and 20:1 (B). Each point represents the mean (± standard deviation) from three to six experiments. Each experiment was carried out in duplicate.

Fig. 13.

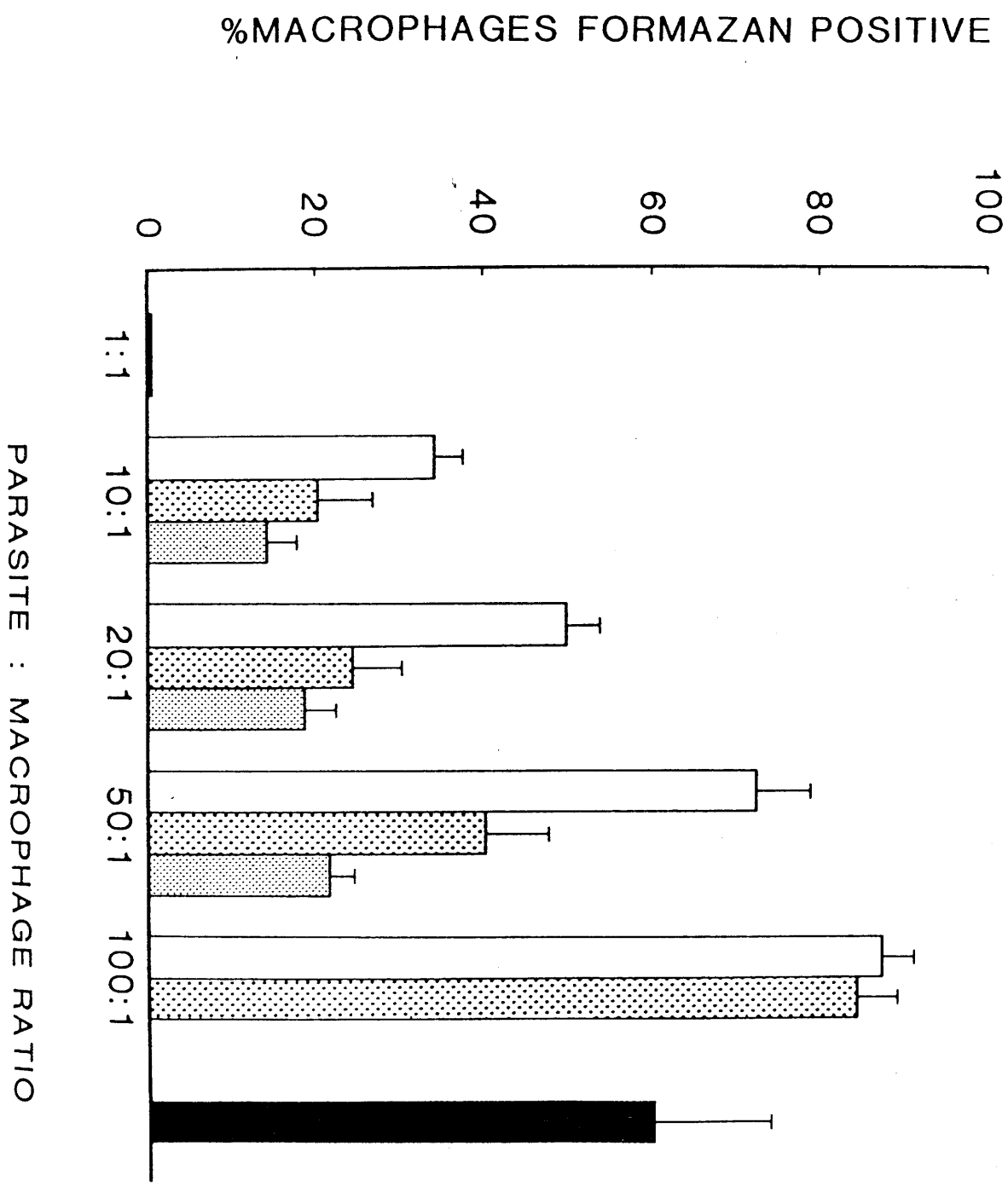
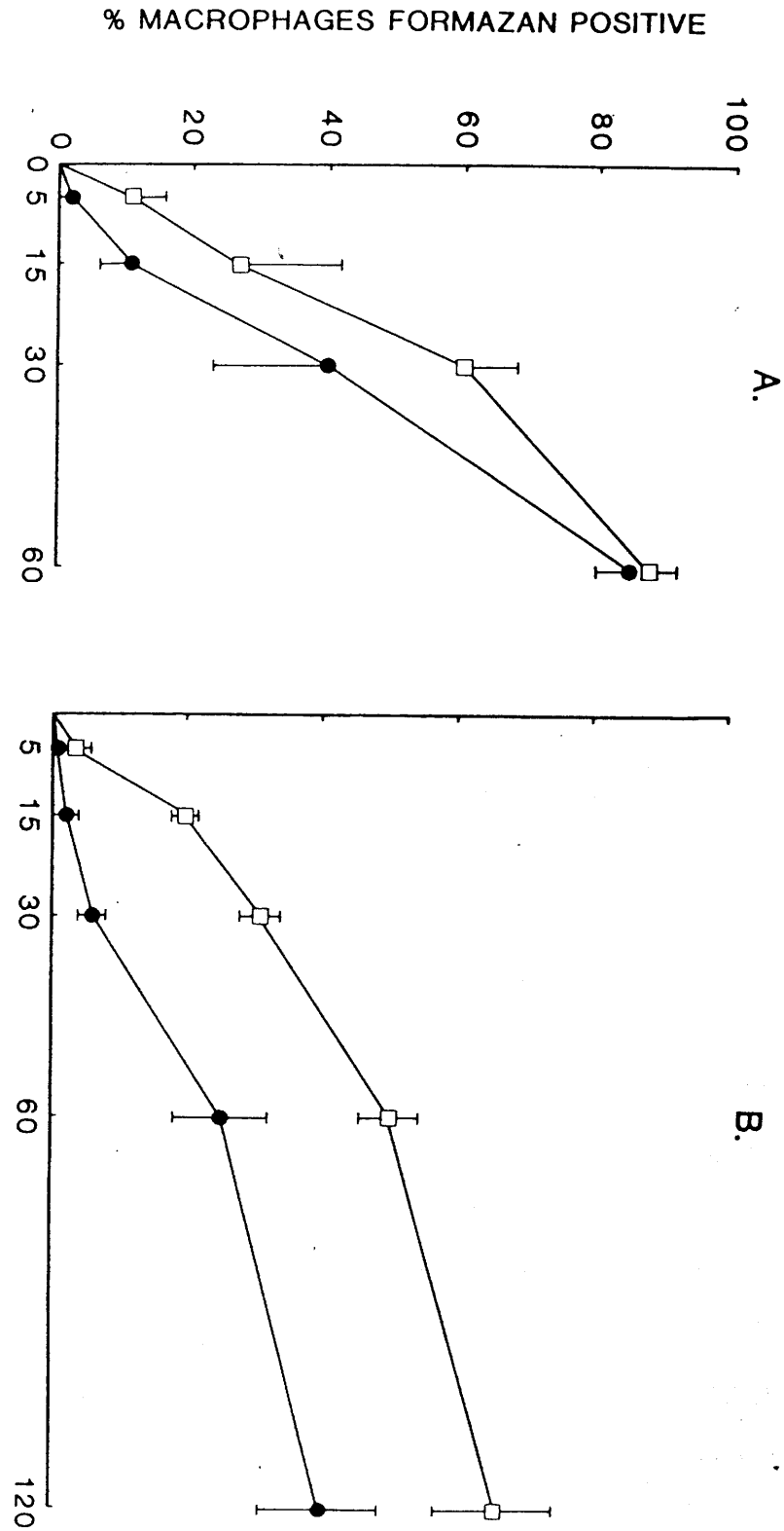

%MACROPHAGES FORMAZAN POSITIVE
0
20
40
60
80
100
1:1
10:1
20:1
50:1
100:1
PARASITE : MACROPHAGE RATIO

Fig. 14.


% MACROPHAGES FORMAZAN POSITIVE
0
20
40
60
80
100
0 5 15 30 60
A.
5 15 30 60 120
B.
TIME (MIN)

lower in the metacyclic infections. At the 20:1 ratio the situation was somewhat different. Fewer macrophages were stimulated by metacyclics at all times with the most noticeable differences occurring over the first 30 min during which time many macrophages were stimulated by mid-log phase promastigotes but very few by the metacyclics. The difference during this first 30 min was much greater than that found concerning the relative infection and uptake rates of the two promastigote forms at these times and may be explained by the large difference between the numbers of attached mid-log phase promastigotes and metacyclic promastigotes at these times (see Fig.12).

To determine whether the metacyclics stimulated a quantitatively lower RB compared to mid-log phase cells, the absolute amount of formazan produced was measured. The results are shown in Fig. 15A. The values are corrected for controls which were macrophage cultures treated with medium only (control values: 3 ± 1 nmoles 60 min^{-1} [$1x10^6$ macrophages]$^{-1}$; n=5). It was found that NBT was also reduced by promastigotes themselves and so their contribution was calculated and is shown in Fig. 15B. Taking this into account, the mid-log phase promastigotes gave rise to variable but apparently larger amounts of reduced NBT than did the metacyclics. When the differences in attachment and uptake rates are taken into account it appears that metacyclic promastigotes stimulate macrophages to a similar extent as mid-log phase promastigotes.

The number of macrophages stimulated to reduce NBT by purified amastigotes is also given in Fig. 13. In comparison to the promastigotes, purified amastigotes were markedly unstimulatory. In the 10:1 ratio experiment only 14% of the macrophages reduced NBT although 93% were infected with an average of 4 parasites/macrophage.

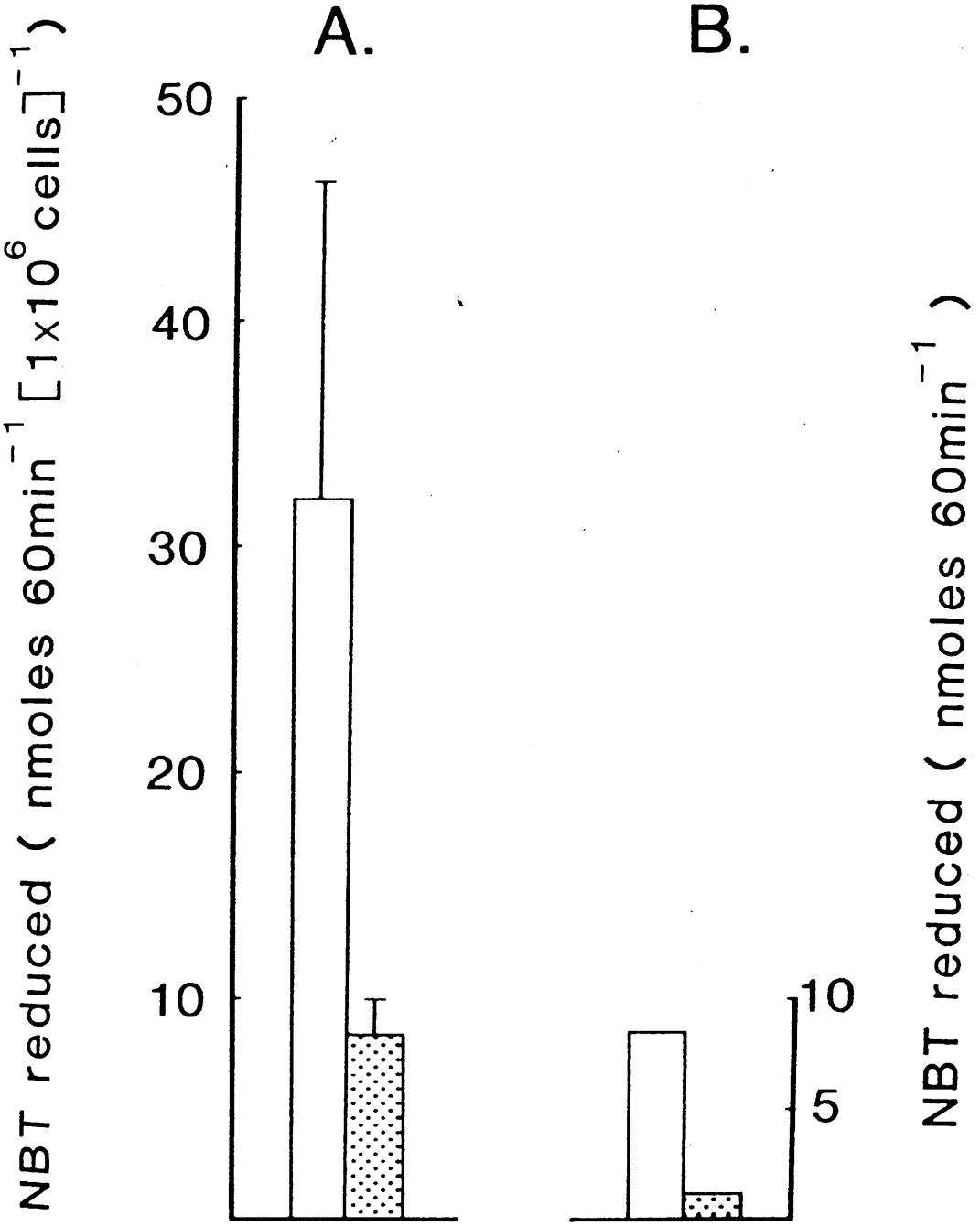


Fig. 15. Quantitative nitroblue tetrazolium reduction by Balb/c resident peritoneal macrophages upon exposure to L.major mid-log phase and metacyclic promastigotes.

(A) Amount of NBT reduced by macrophages upon exposure to mid-log phase (□) and metacyclic (⁘) promastigotes. Each histogram represents the mean (+ standard deviation) from five experiments.

(B) Amount of NBT reduced by mid-log phase (□) and metacyclic (⁘) promastigotes themselves. Calculated as described in Materials and Methods, Section 2.5.5.2.

Increasing the number of amastigotes used produced only slight increases in the % macrophages stimulated and at the 50:1 ratio the % macrophages stimulated was still considerably lower than for mid-log phase promastigotes used at a 10:1 ratio. To check the effect of contaminating material in the amastigote preparation, crude (washed twice) and pre-column (washed five times) amastigotes were also exposed to macrophages. Although there was no difference in uptake and % macrophages infected between these and pure amastigotes (data not shown) there were large differences in the % macrophages stimulated. The number of macrophages stimulated to reduce NBT decreased with increasing purity of the amastigote preparation. For example, at the 10:1 ratio the crude preparation stimulated 49% of the macrophages, the pre-column preparation 37% and the final preparation of pure amastigotes only 14%.

3.3 THE INTERACTION OF L.MEXICANA MEXICANA PARASITES WITH THE MAMMALIAN HOST AND WITH MACROPHAGES IN VITRO

The work described so far was carried out with L.major. One advantage of working with this species is that pure metacyclics can be obtained. A disadvantge is difficult to obtain pure amastigotes in large numbers. In contrast, large numbers of L.mexicana mexicana can be easily purified but as yet the putative metacyclics (in stationary-phase populations) have not been isolated. As I was interested in comparing metacyclics and amastigotes I decided to compare the interactions of L.mexicana mexicana with mice and macrophages with those found for L.major.

3.3.1 The fate of mid-log phase and stationary-phase promastigotes of L.mexicana mexicana in mice and in murine resident peritoneal macrophages in vitro

To test for differences in infectivity in vivo, mid-log phase and

stationary-phase promastigotes were inoculated into the footpads or rumps of mice using different inoculi and lesion production monitored. Results of parasite growth in footpads are shown in Fig. 16 for Balb/c mice and Fig. 17 for CBA mice. These are from one of the three experiments performed with each mouse strain and the one in which differences in infectivity were most apparent. They show stationary-phase promastigotes to be more infective than mid-log phase cells. In the other two experiments there were no significant differences between the lesions resulting from the two promastigote forms. For the Balb/c mice, at an inoculum of 1×10^6 promastigotes per footpad, both promastigote forms produced lesions. Stationary-phase promastigotes, however, produced lesions slightly more rapidly and of greater average size than mid-log phase promastigotes. At the lower inoculum, both promastigote forms again produced lesions but the difference between the size of the lesions that developed was greater. Stationary-phase promastigote-induced lesions grew more rapidly than those resulting from the use of mid-log phase promastogotes. Reducing the inoculum to 1×10^3 or 1×10^2 mid-log phase promastigotes per footpad did not prevent lesion production (data not shown). Lesions resulting from the innoculation of either promastigote form of L.mexicana mexicana took longer to develope lesions in Balb/c mice than did those produced by L.major promastigote forms of the same inoculum. (see Fig. 10 for comparison).

Similar experiments were performed with CBA mice and were similar to those reported for Balb/c mice. The lesions developed more slowly than Balb/c mice, however, and there was a less clear cut relationship between lesion size and inoculum size.

Similar experiments were also carried out inoculating L.mexicana mexicana subcutaneously in the shaven rumps of CBA mice. The results

Fig. 16. Infectivity of mid-log phase and stationary-phase promastigotes of L.mexicana mexicana to Balb/c mice.

1×10^6 (A) or 1×10^4 (B) mid-log phase (□) and stationary-phase (■) promastigotes were inoculated into the left-hind footpads of Balb/c mice. Groups of five mice were used for each promatigote form at each inoculum. Each point represents the mean (± standard deviation) foot pad width from five mice. The data presented are from the one experiment of the three carried out where the differences in infectivity between the two promastigote forms were most apparent.

Fig. 17. Infectivity of mid-log phase and stationary-phase promastigotes of L.mexicana mexicana to CBA mice - footpad infections.

Legends for promastigote forms, inocula and experimental procedures as for Fig. 16. The results are the means (± standard deviation) from five mice in each case, apart from the 1×10^6 and 1×10^4 inocula for stationary-phase promastigotes where the results are from four mice. The data presented are from one experiment of the three carried out where the differences between the two promastigote forms were most apparent.

Fig. 16.

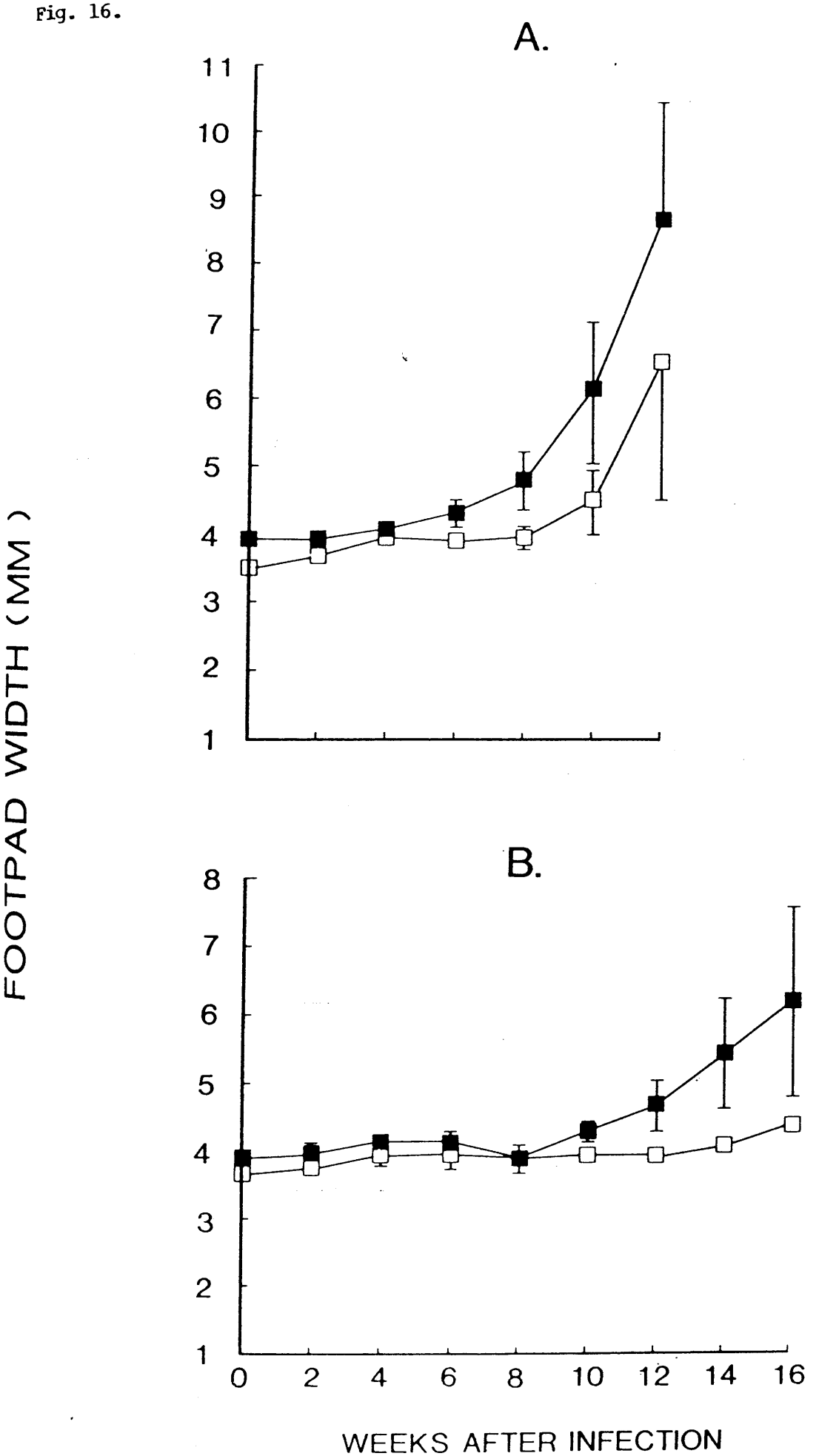
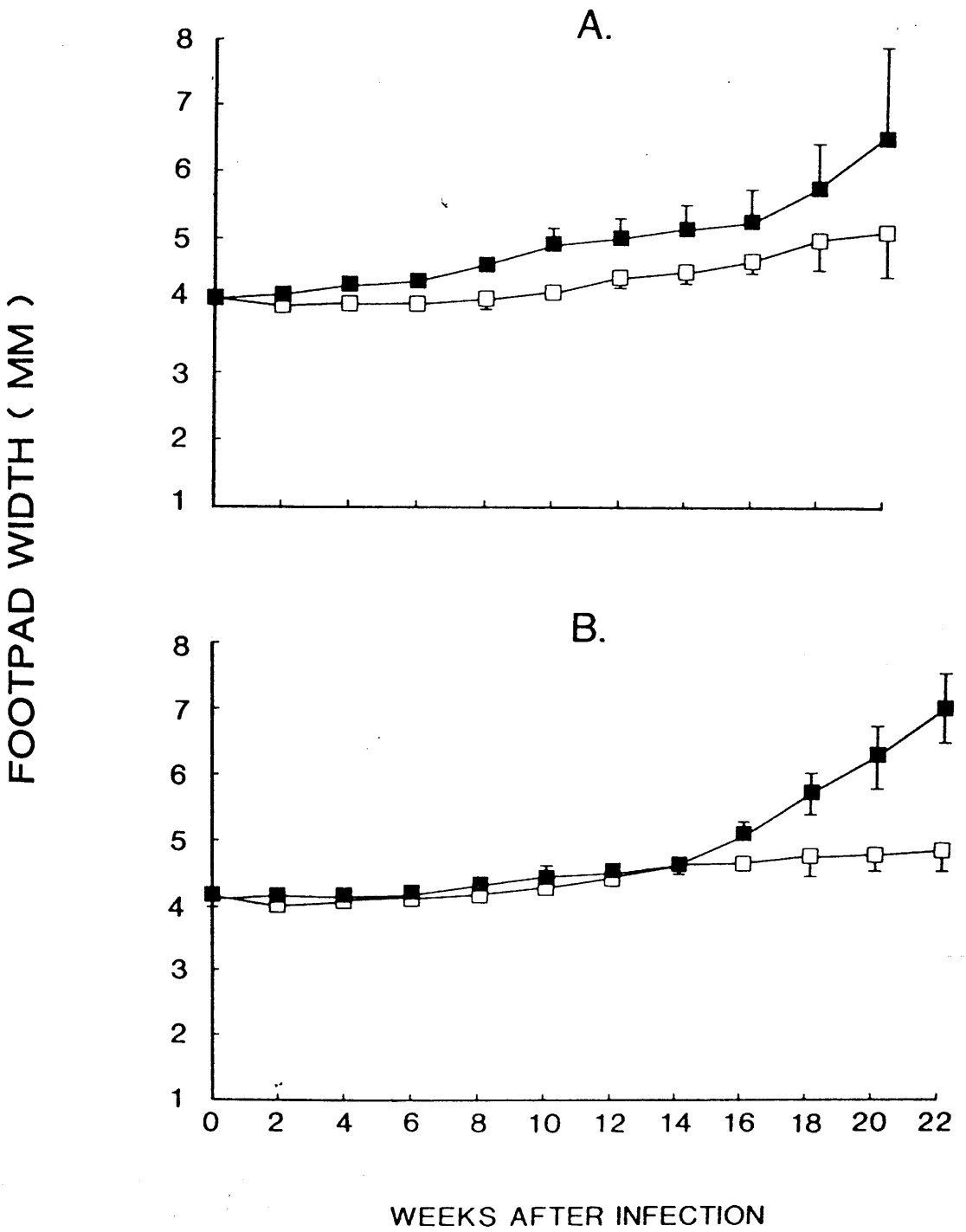

A.
B.
FOOTPAD WIDTH (MM)
WEEKS AFTER INFECTION
1
2
3
4
5
6
7
8
9
10
11
0
2
4
6
8
10
12
14
16

Fig. 17.


A.
B.
8
7
6
5
4
3
2
1
0
2
4
6
8
10
12
14
16
18
20
22
FOOTPAD WIDTH (MM)
WEEKS AFTER INFECTION

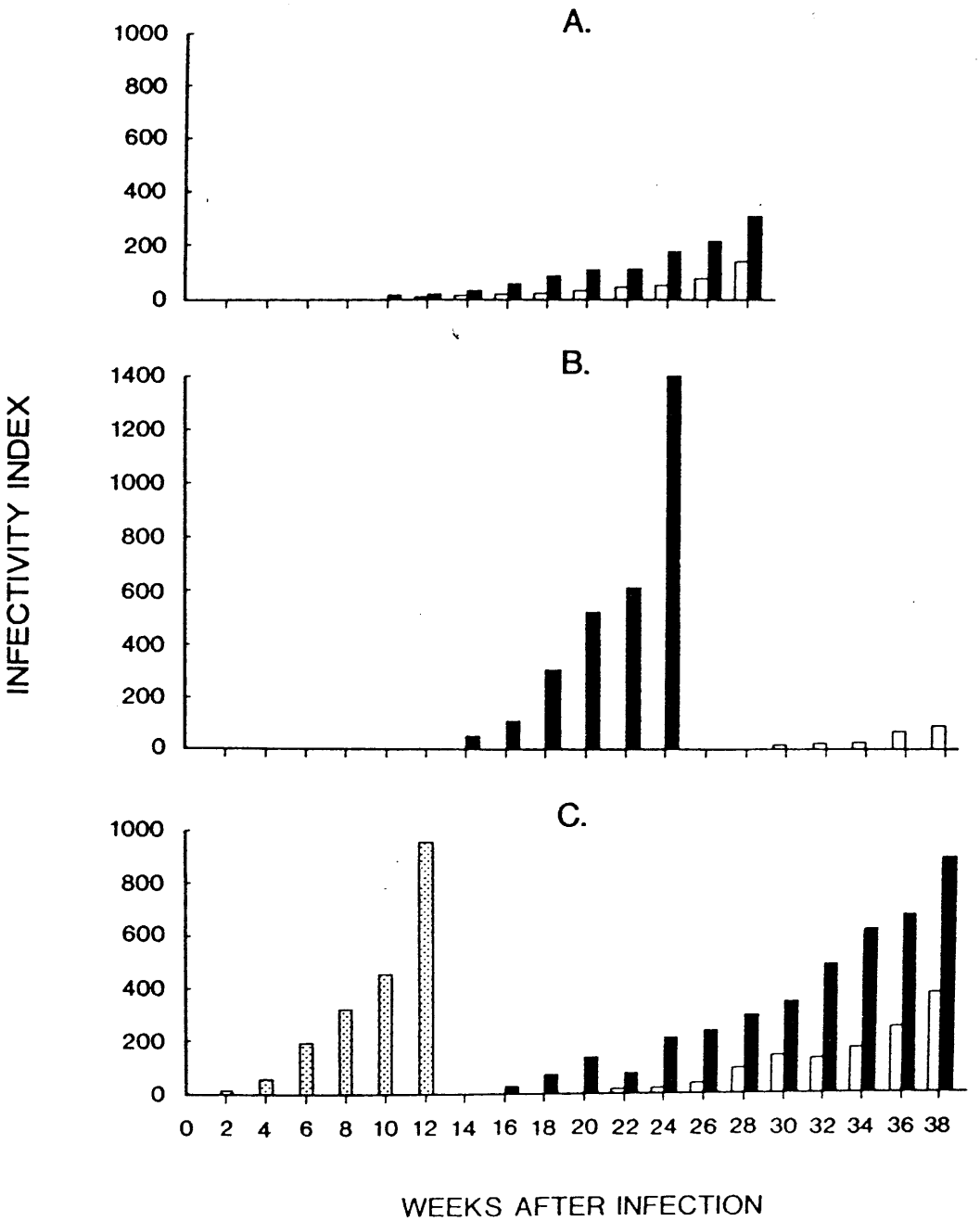


Fig. 18. Infectivity of different forms of L.mexicana mexicana to CBA mice - rump infections.

3×10^5 (A), 3×10^6 (B) and 3×10^7 (C) mid-log phase (□) and stationary-phase (▩) promastigotes, and 3×10^7 (C) amastigotes (▨) were inoculated into the rumps of CBA mice. Groups of five mice were used for the two promastigote forms at each inoculum, and groups of ten mice for the amastigote infections. The data presented for the promastigotes are from the one experiment of the three carried out where the differences in infectivity between the two forms were most apparent. The data presented for the amastigotes are from one experiment which is representative of the three carried out.

are shown in Fig. 18. The infectivity index for stationary-phase promasigotes was higher than for mid-log phase promastigotes at all the inoculi used. The greatest difference was apparent when the inoculum was 3×10^6 promastigotes per rump. In this case the lesions due to stationary-phase cells had grown to their maximum size and began ulcerating by week 24 and so the experiment had to terminated before lesions due to the mid-log phase cells had appeared. An interesting point to note is that the lowest inoculum used (3×10^5 cells) gave rise to lesions earlier with both promastigote forms than when more parsites were innoculated although in general smaller lesions were produced. The largest lesions resulted from an inoculum of 3×10^6 stationary-phase promastigotes. The corresponding infectivity of amastigotes at an inoculum of 3×10^7 is also shown in Fig. 18. Lesions were produced much more rapidly than with promastigotes of either form.

The intracellular fate of mid-log phase and stationary-phase promastigotes was also examined after engulfment in vitro by Balb/c and CBA resident peritoneal macrophages. The macrophages were exposed to the promastigoes for 2 hours at parasite:macrophage ratios of 1:1 and 5:1. The parasites were washed off and the cultures re-incubated for up to 72 hours. Two separate experiments were performed (each in duplicate) for macrophages from each mouse strain, and the results are shown in Figs. 19 and 20 for Balb/c and CBA macrophages, respectively. Both promastigote forms of L.mexicana mexicana were more infective to Balb/c macrophages in vitro than were L.major promastigotes, in that fewer (4 to 10-fold) parasites resulted in similar initial infection rates (compare Fig. 11 and Fig. 19). This contrasts with the in vivo results which showed that equivalent lesions were produced by fewer L.major than L.mexicana mexicana (see Figs. 10 and 16). The results for Balb/c macrophages exposed to promastigotes at 1:1 and 5:1 ratios

Fig. 19. The intracellular fate of mid-log phase and stationary-phase promastigotes of L.mexicana mexicana within Balb/c resident peritoneal macrophages in vitro.

Macrophage infections were initiated with mid-log phase (□) and stationary-phase (■) promastigotes at parasite:macrophage ratios of 1:1 (A), and 5:1 (B). Each point represents the mean (± standard deviation) from two experiments each carried out in duplicate.

Fig. 20. The intracellular fate of mid-log phase and stationary-phase promastigotes of L.mexicana mexicana within CBA resident peritoneal macrophages in vitro.

Legends for promastigote forms and parasite:macrophage ratios as for Fig. 19. Each point represents the mean (± standard deviation) from two experiments each carried out in duplicate.

Fig. 19.

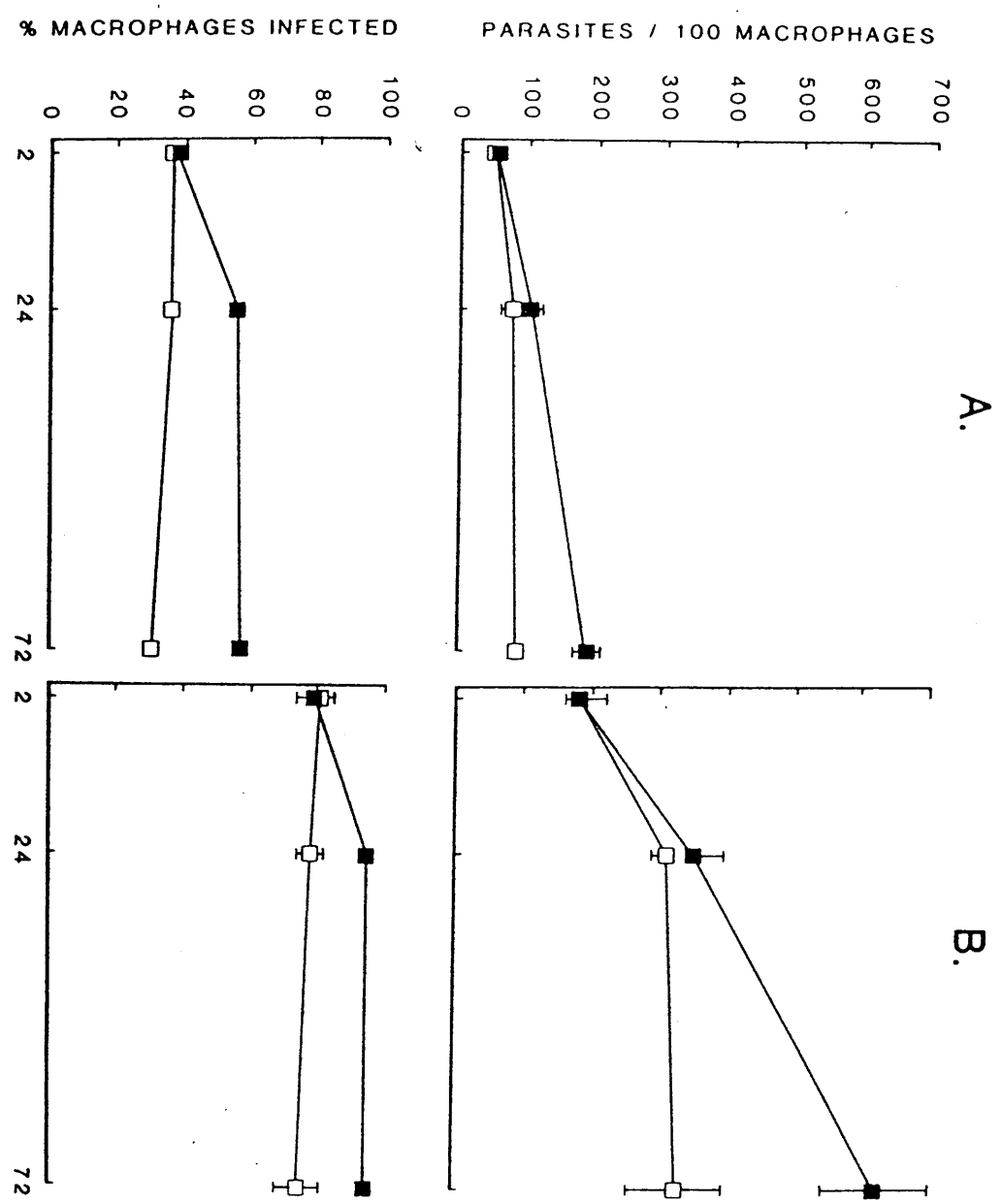
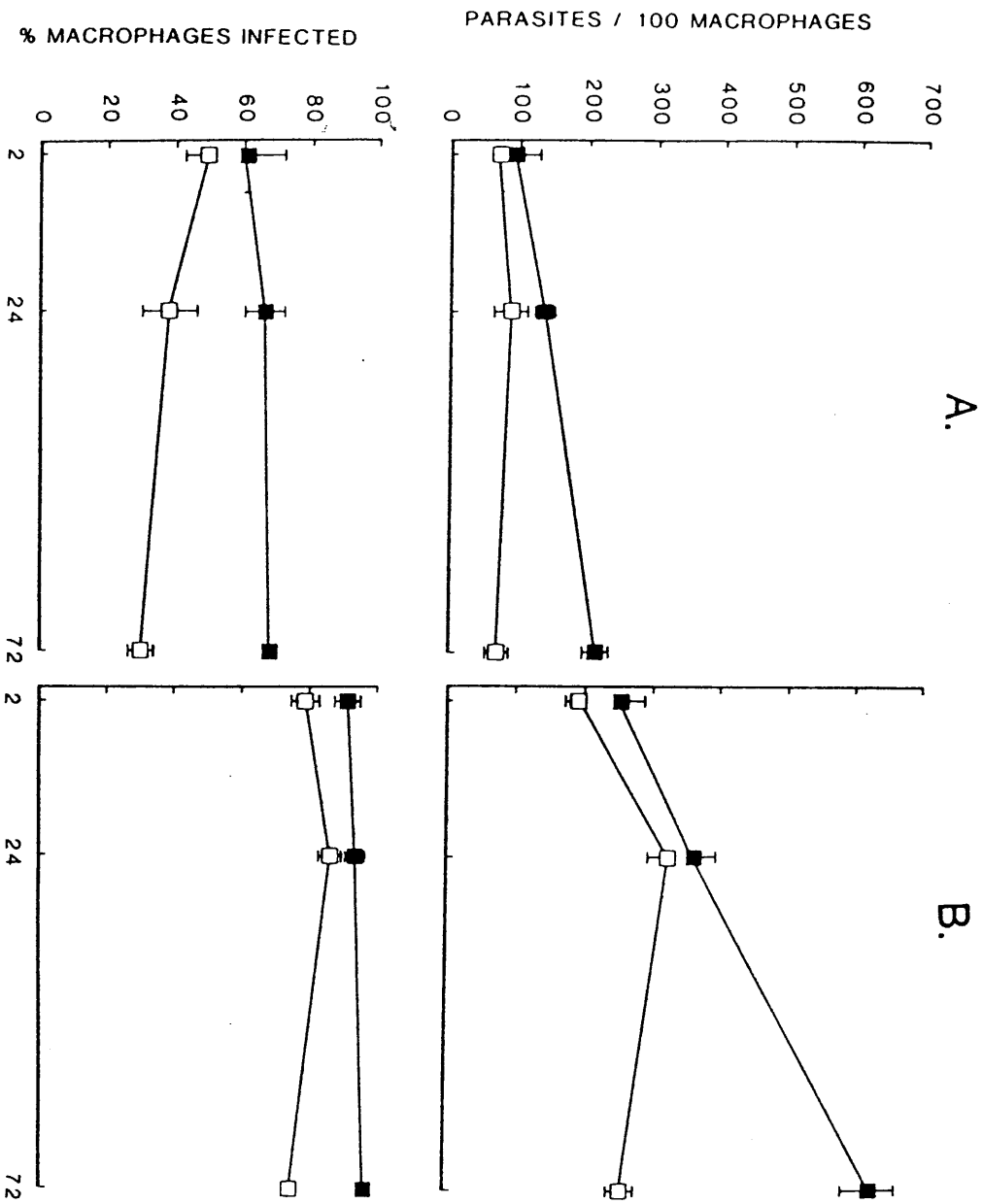

% MACROPHAGES INFECTED
PARASITES / 100 MACROPHAGES
0
20
40
60
80
100
0
100
200
300
400
500
600
700
2
24
72
A.
B.
TIME (HRS)

Fig. 20.


% MACROPHAGES INFECTED
PARASITES / 100 MACROPHAGES
0
20
40
60
80
100
0
100
200
300
400
500
600
700
2
24
72
A.
B.
TIME (HRS)

showed essentially the same trend. For mid-log phase promastigotes, the number of parasites/100 macrophages increased over the first 24 hours in culture, whereas the % macrophages infected changed little. Over the next 48 hours (72 hours in total) in culture, the number of parasites/100 macrophages and % macrophages infected remained relatively stable. For stationary-phase promastigote infections, the numbers of parasites/100 macrophages also increased over the first 24 hours in culture, more so than for mid-log phase cells, and the % macrophages infected also increased. Moreover, during a further 48 hours incubation, the % macrophages remained static, but the numbers of parasites/100 macrophages increased 2-fold so that it was over 3-fold higher than the initial level, indicating that amastigote multiplication had occurred. At the 72 hour incubation time point the numbers of parasites/100 macrophages resulting from stationary phase infections was 2-fold higher than that for mid-log phase infections.

The results for CBA macrophages also show a similar pattern for both ratios. For stationary-phase promastigotes, the results are quite similar to those with Balb/c macrophages. Over the 72 hour incubation period, the numbers of parasites/100 macrophages increased steadily resulting in over a 2-fold increase compared to the starting infection. For a mid-log phase promastigotes the results were a little different to those with Balb/c macrophages. There was only a very slight or no net increase in the numbers of parasites/100 macrophages during the course of the 72 hour incubation, although there was an apparent transient increase at 24 hours. The % macrophages infected also decreased in the 1:1 ratio experiment over 72 hours incubation. At the 72 hour time point the numbers of parasites/100 macrophages resulting from stationary-phase infections was over 2-fold higher than that for mid-log phase infections.

It should be noted, however, that results given in Table 9 for Balb/c resident peritoneal macrophages suggest (similar to the *in vivo* results) that after phagocytosis by macrophages *in vitro* stationary-phase promastigotes do not always produce higher infection rates after 72 hours incubation than mid-log phase cells. In this case, after phagocytosis they appear to behave in a similar manner to mid-log phase promastigotes (see section 3.3.2 for further details).

A summary of attachment and uptake data for the interaction of mid-log phase and stationary-phase promastigotes, and amastigotes of *L.mexicana mexicana* with murine resident peritoneal macrophages is given in Table 10. When the ratio of parasites:macrophages was 1:1 with Balb/c macrophages, the numbers of promastigotes attached and ingested/100 macrophages and the % macrophages infected were the same with 1 hour and 2 hour exposure times for both mid-log phase and stationary-phase cells. Also at both time points the numbers ingested/100 macrophages and % of macrophages infected with mid-log phase and stationary-phase promastigotes were the same, although many more (3 to 6-fold) of the former were attached. The results comparing mid-log phase and stationary-phase promastigote interactions with CBA macrophages were very similar. When a 5:1 ratio was used with Balb/c macrophages, the 2 hour exposure to either promastigote form produced slight apparent increases in the numbers attached and ingested/100 macrophages and the number of macrophages infected compared to the 1 hour exposures. At both 1 hour and 2 hour exposures more mid-log phase cells were attached than stationary-phase cells, but the difference was not as great as at the 1:1 ratio. Similar differences were seen in the numbers attached/100 macrophages with CBA macrophages. Also, with CBA macrophages, stationary-phase promastigotes gave rise to slightly larger numbers of parasites ingested/100 macrophages and macrophages infected compared to mid-log phase cells. Interestingly, at the 1:1

Table 9: Infection of Balb/c resident peritoneal macrophages by mid-log phase and stationary-phase promastigotes of L.mexicana mexicana[a].

Parasite:Macrophage ratio	Parasite form	Number of parasites/ 100 macrophages[b]	% macrophages infected[b]
1:1	Mid-log phase	89± 5	23± 2
	Stationary phase	89± 3	34± 4
5:1	Mid-log phase	417±51	67± 7
	Stationary phase	312±33	71±10
25:1	Mid-log phase	1432±149	98± 3
	Stationary phase	864±119	94± 5

[a] Macrophage cultures were exposed to parasites for 2 hours, free parasites washed off and the cultures reincubated for a further 72 hours.

[b] The results are the means (± standard deviation) from three experiments each carried out in duplicate.

Table 10: Interaction of different forms of L.mexicana mexicana with murine resisdent peritoneal macrophages in vitro[a].

Parasite: Macrophage ratio	Parasite form	Mouse strain	Exposure time	Parasites/100 Macrophages[b]			% Macrophages infected[b]
				Attached	Ingested	Total	
1:1	Mid-log phase	Balb/c	1 hr	16± 6	45± 7	61±13	35± 5
	Mid-log phase	Balb/c	2 hr	20± 7	51± 3	71± 7	37± 3
	Stationary-phase	Balb/c	1 hr	5± 2	54± 9	59±10	38± 3
	Stationary-phase	Balb/c	2 hr	3± 1	55± 5	58± 5	38± 3
	Mid-log phase	CBA	2 hr	36±10	71± 9	107±19	49± 7
	Stationary-phase	CBA	2 hr	12± 6	93±32	105±38	60±12
5:1	Mid-log phase	Balb/c	1 hr	65± 5	143±16	208±18	71± 4
	Mid-log phase	Balb/c	2 hr	81±15	180±18	261±24	81± 4
	Stationary-phase	Balb/c	1 hr	39± 3	169±30	208±28	77± 5
	Stationary-phase	Balb/c	2 hr	50± 9	182±43	232±35	79± 5
	Mid-log phase	CBA	2 hr	94±19	189±19	283±38	77± 4
	Stationary-phase	CBA	2 hr	62±13	253±40	315±51	91± 4
	Amastigote	Balb/c	2 hr	38±11	681±73	718±82	97± 5
25:1	Amastigote	Balb/c	2 hr	164±38	1825±116	1989±121	100
50:1	Amastigote	Balb/c	2 hr	286±60	2198±225	2484±258	100

[a] All experiments were carried out in the presence of 10% (v/v) HIFCS.

[b] The results are the means (± standard deviation) from two to three experiments each carried out in duplicate.

ratio, and to a lesser extent at the 5:1 ratio, CBA macrophages appeared to phagocytose both promastigote forms better than Balb/c macrophages, and also greater numbers were attached.

Balb/c macrophages readily phagocytosed amastigotes of L.mexicana mexicana. At a parasite:macrophage ratio of 5:1, this resulted in nearly all the macrophages becoming infected and over three times as many ingested parasites/100 macrophages compared to that found with the two promastigote forms. At higher ratios, 100% of macrophages were infected and there were over 1000 ingested parasites/100 macrophages, numbers attached were low in comparison. For example, in the 5:1 ratio experiment there were similar numbers of amastigotes and stationary-phase promastigotes attached to Balb/c macrophages. For all three parasite forms increasing the parasite:macrophage ratio resulted in increased numbers of parasites attached and ingested/100 macrophages and a higher percentage of the macrophages being infected, but it was not a linear correlation.

3.3.2 The respiratory burst of murine resident peritoneal macrophages upon exposure to different forms of L.mexicana mexicana

The relative macrophage RB stimulated by different parasite forms was assessed qualitatively as before (see section 3.2.3). The results for CBA macrophages are shown in Fig. 21 (% macrophages stimulated to reduce NBT in medium only controls was 6±4; n=4). At all parasite:macrophage ratios a RB was detected and was similar with both mid-log phase and stationary-phase promastigotes, and the % macrophages stimulated to reduce NBT was greater when more parasites were used. It is interesting to note, however, that considerably fewer macrophages were stimulated than were infected; 3 to 6-fold fewer at the 1:1 ratio, and approximately 2-fold less at the 5:1 ratio. The results for Balb /c macrophages are given in Fig. 22 (% macrophages

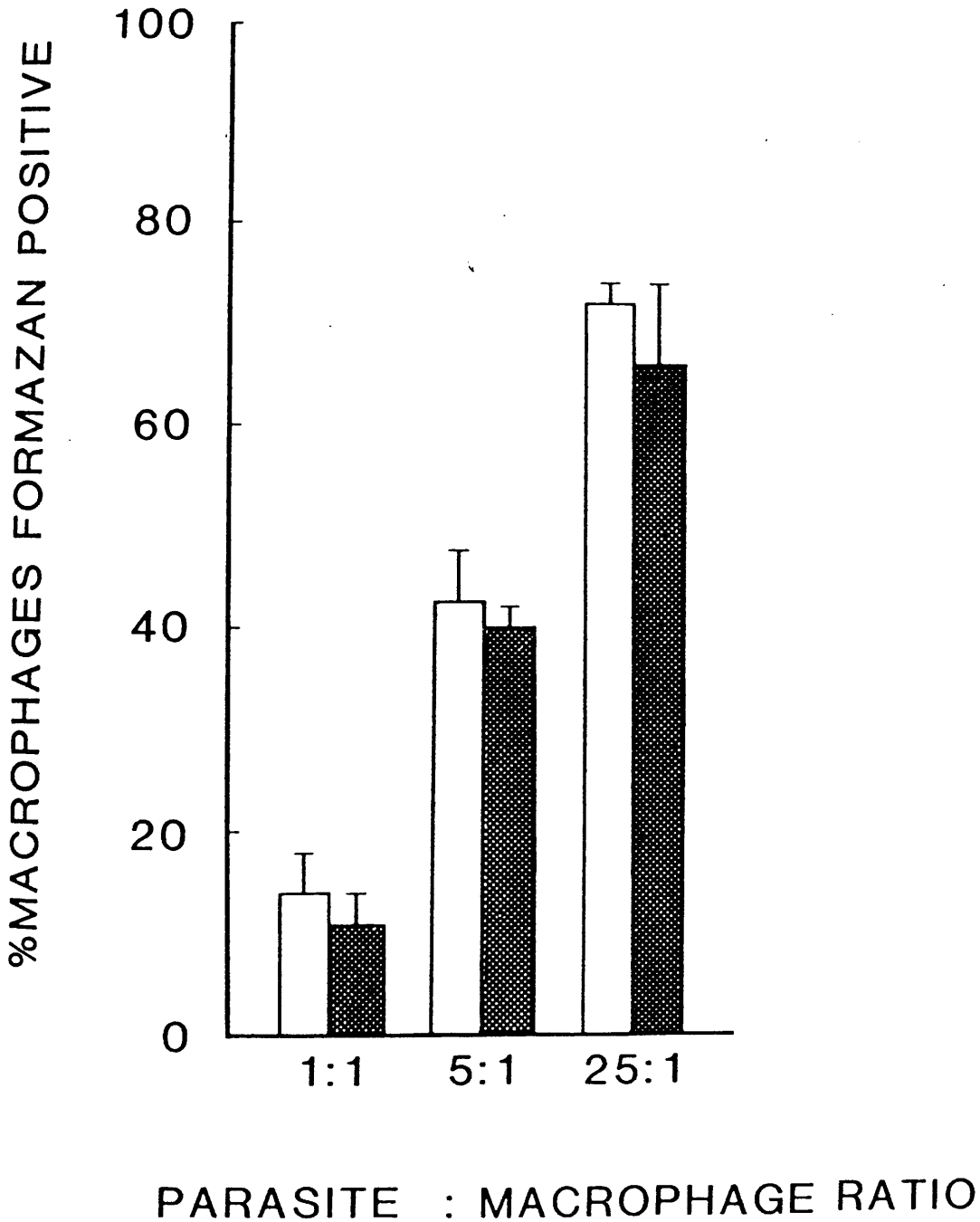


Fig. 21. Nitroblue tetrazolium reduction by CBA resident peritoneal macrophages upon exposure to mid-log phase and stationary- phase promastigotes of L.mexicana mexicana in vitro.

Macrophage cultures were exposed to mid-log phase (□) and stationary-phase (▩) promastigotes for 60 min. Each histogram represents the mean (± standard deviation) from two experiments each carried out in duplicate.

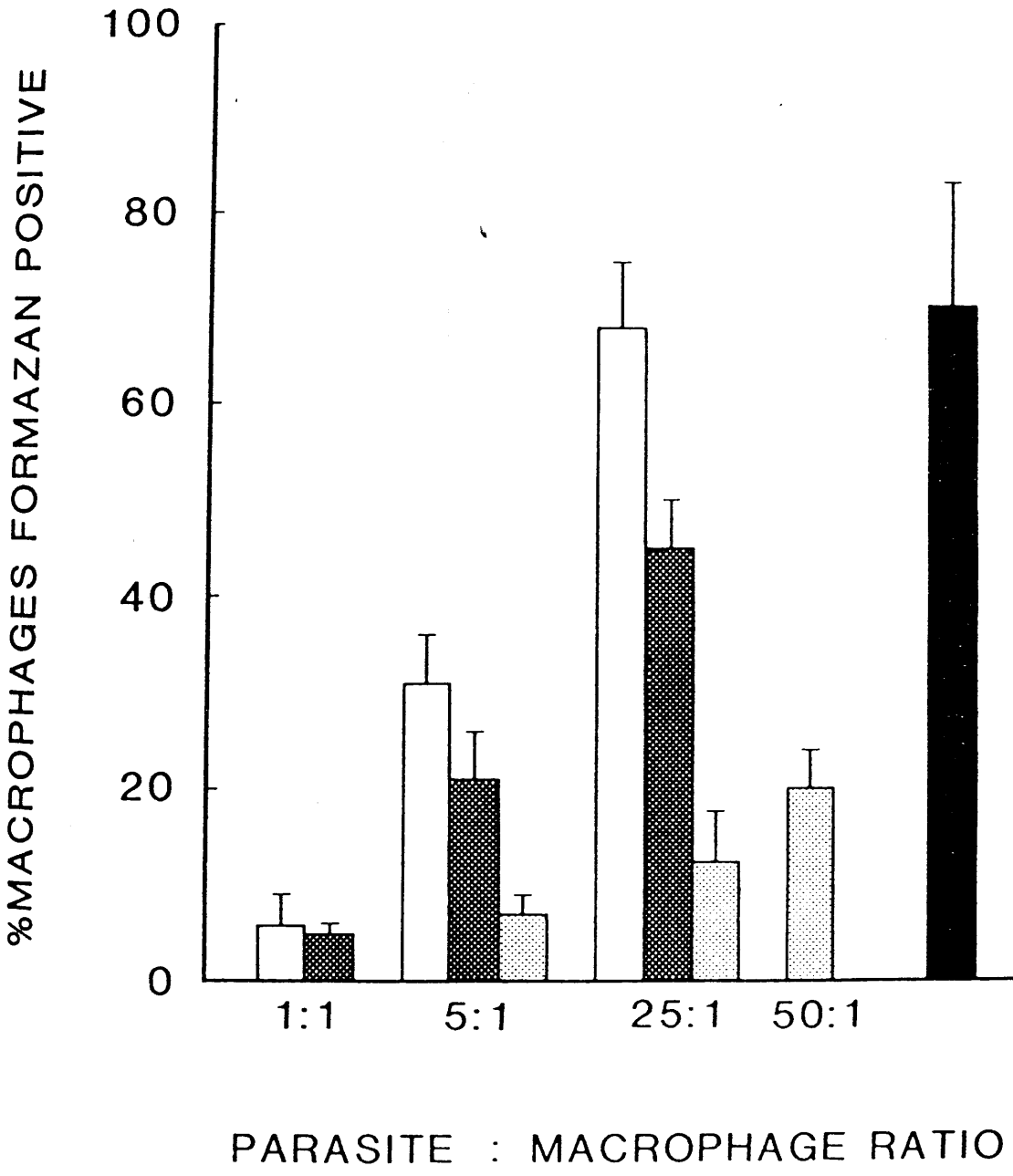


Fig. 22. Nitroblue tetrazolium reduction by Balb/c resident peritoneal macrophages upon exposure to different forms of L.mexicana mexicana in vitro.

Macrophage cultures were exposed to mid-log phase (□) and stationary-phase (▩) promastigotes and amastigotes (▨) for 60 min. Each histogram represents the mean (± standard deviation) from three experiments each carried out in duplicate.

The reduction of NBT by Balb/c resident peritoneal macrophages upon exposure to zymosan (■) is shown for comparison. The histogram represents the mean (± standard deviation) from five single experiments.

stimulated in medium only controls was 2±1; n=9). Again, mid-log phase and stationary-phase promastigotes stimulated a detectable RB at all ratios, although a lower percentage of the macrophages were stimulated than with the CBA macrophages. Amastigotes were much less stimulatory in comparison and the numbers of macrophages stimulated increased only slightly with increased parasite challenge. For example, at the 50:1 ratio 20% of the macrophages were stimulated by the amastigotes whereas 31% were stimulated by mid-log phase promastigotes at the much lower ratio of 5:1.

The results for Balb/c macrophages and stationary-phase promastigotes suggest that they may be less stimulatory at 5:1 and 25:1 ratios than mid-log phase cells. No uptake or attachment studies were performed at this time, although the resulting infections after incubation of the macrophages for 72 hours were quantified and the results are shown in Table 9. Taking these results together with those reported in section 3.3.1 on the fate of L.mexicana mexicana promastigotes in macrophages, it seems likely that fewer stationary-phase promastigotes than mid-log phase promastigotes at ratios of 5:1 and 25:1 and that this could explain why fewer macrophages were stimulated to produce a RB in this case.

3.4 THE INTERACTION OF PARASITIC CELLS WITH RABBIT PERITONEAL NEUTROPHILS IN VITRO

Lucigenin-enhanced CL is a flexible technique for measuring the RB response, not only because it allows the RB to be quantified, but also because it allows the response to be easily monitored with respect to time. Unfortunately, attempts to measure the RB of murine resident peritoneal macrophages to parasites with this technique were unsuccessful probably due to the relatively low amounts of O_2^- produced by these cells (see Introduction, section 1.4.2.3.3.1). Preliminary

studies, however, had shown that rabbit peritoneal neutrophils produced a large oxidative response which was detectable using the above technique. Further, it appears that neutrophils are well represented in the initial cellular infiltrate that occurs after inoculation of promastigotes into a mammal (Marsden & Jones, 1985; Wilson et al., 1987). Neutrophils may therefore play a role in determining the outcome of an infection. Thus, it was decided to investigate (using lucigenin-enhanced CL) the RB elicited by different forms of Leishmania species and other trypanosomatids on contact with rabbit peritoneal neutrophils, which can readily be obtained in large numbers.

3.4.1 The kinetics attachment and uptake of different forms of Leishmania species by rabbit peritoneal neutrophils in vitro

The attachment and uptake experiments were performed with two different neutrophil preparations and were carried out within 24-72 hours after preparation of the neutrophils. Due to variations in staining, even on the same slide, and although apparently ingested organisms were seen in every preparation examined, it was not possible in many cases to distinguish between ingested parasites and attached parasites. Therefore the figures for interaction were expressed as parasites associated (= attached and ingested)/100 neutrophils. The results for L.mexicana mexicana are shown in Fig. 23. Leishmania mexicana mexicana mid-log phase promastigotes were associated rapidly to a maximum value over the first 15 min contact with the neutrophils (assumed zero). This was accompanied by a reduction in the numbers of free extracellular promastigotes. Thereafter the numbers of parasites associated with the neutrophils remained level as did the numbers removed from the medium. Amastigotes of L.mexicana mexicana were also associated rapidly during the first 15 min. Subsequently there was an

Fig. 23. The kinetics of attachment and uptake of L.mexicana mexicana mid-log phase promastigotes and amastigotes by rabbit peritoneal neutrophils in vitro.

(A) Rabbit peritoneal neutrophils were exposed to mid-log phase promastigotes (□) and amastigotes (▲) at a ratio of 1 parasite:1 neutrophil. Each point represents the mean (± standard deviation) from two experiments each carried out in duplicate.

(B) Rabbit peritoneal neutrophils were exposed to mid-log phase promastigotes and amastigotes (symbols as in A) at a ratio of 5 parasites:1 neutrophil. Each point represents the mean (± range) from two single experiments.

The neutrophil preparations used in these experiments had been stored in exudate solution at 4°C for approximately 48-72 hours prior to use.

Fig. 24. The kinetics of attachment and uptake of L.major mid-log phase and metacyclic promastigotes by rabbit peritoneal neutrophils in vitro.

(A) Rabbit peritoneal neutrophils were exposed to mid-log phase (□) and metacyclic (●) promastigotes at a ratio of 1 parasite:1 neutrophil. Each point represents the mean (± standard deviation) from two experiments each carried out in duplicate.

(B) Rabbit peritoneal neutrophils were exposed to mid-log phase and metacyclic promastigotes (symbols as in A) at a ratio of 5 parasites:1 neutrophil. Each point represents the mean (± range) from two single experiments.

The neutrophil preparations used in these experiments had been stored in exudate solution at 4°C for approximately 24-48 hours prior to use.

Fig. 23.

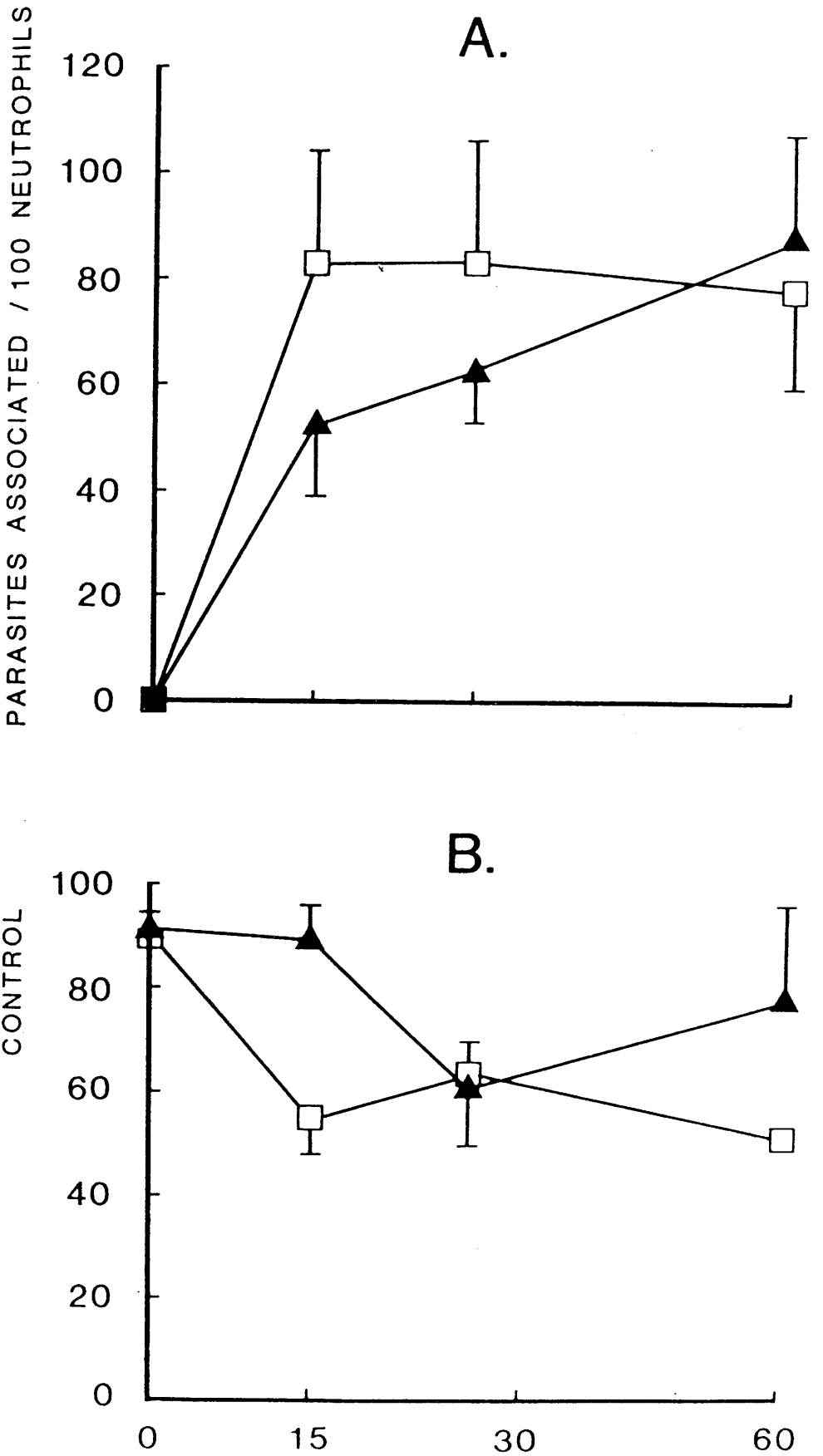
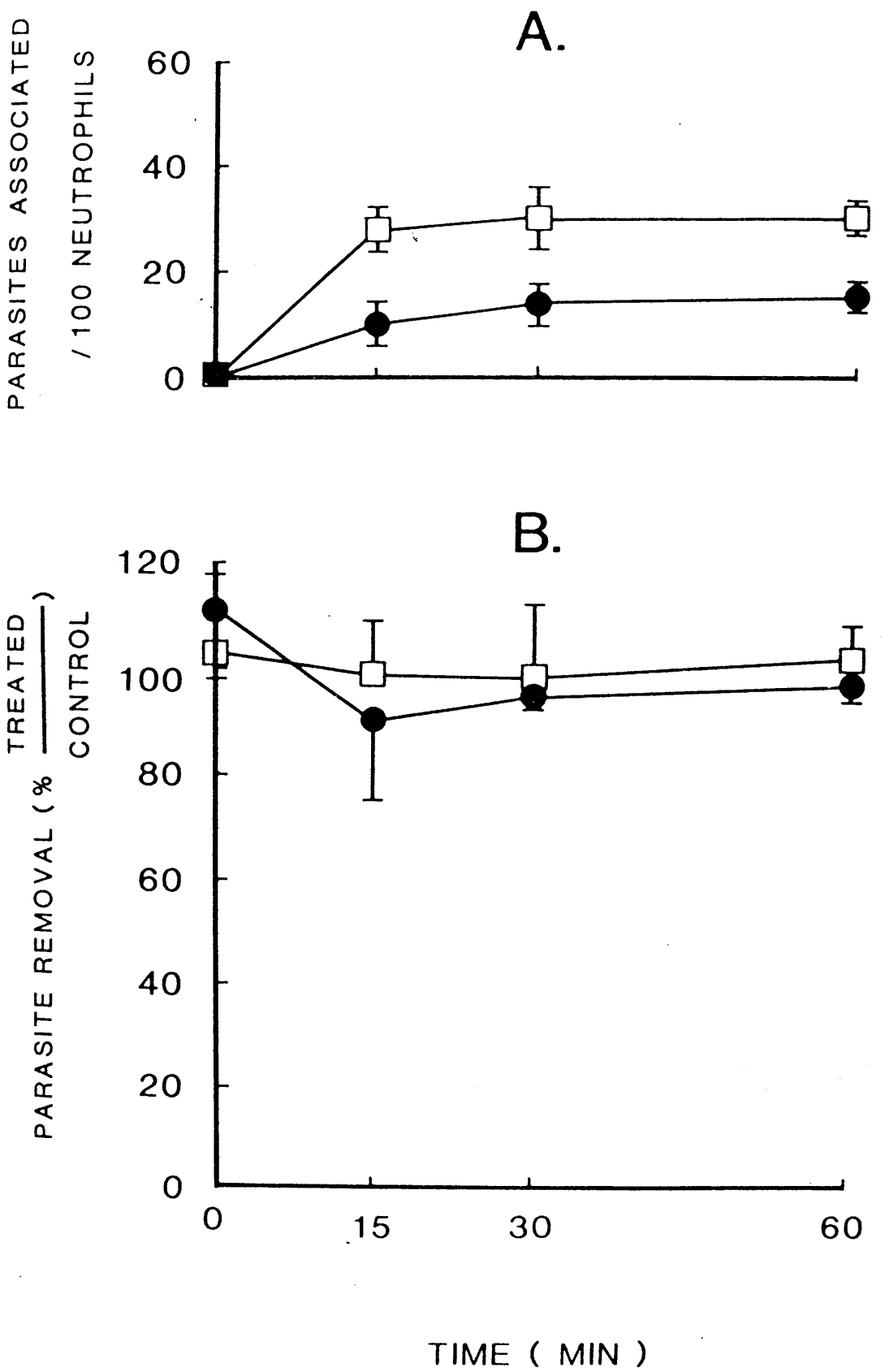

A.
PARASITES ASSOCIATED / 100 NEUTROPHILS
120
100
80
60
40
20
0
B.
PARASITE REMOVAL (% TREATED / CONTROL)
100
80
60
40
20
0
0
15
30
60

Fig. 24.


A.
PARASITES ASSOCIATED
/100 NEUTROPHILS
60
40
20
0
B.
PARASITE REMOVAL (% TREATED / CONTROL)
120
100
80
60
40
20
0
0
15
30
60
TIME (MIN)

apparent increase in numbers of amastigotes associated with the neutrophils to levels similar to that for mid-log phase promastigotes. This was not accompanied, however, by an overall reduction in the number of free extracellular parasites, probably because even after 15 min amastigotes had clumped together in the control and experimental samples to such an extent that it made them difficult to count. The inaccuracies in the counts may have obscured any removal of the extracellular amastigotes by the neutrophils. The results for L.major promastigotes are shown in Fig. 24. The majority of association of mid-log phase promastigotes with neutrophils occurred during the first 15 min, and thereafter numbers increased steadily. The numbers of L.major mid-log phase promastigotes associated with the neutrophils at 15 min was nearly 3-fold less than that for L.mexicana mexicana mid-log phase cells. L.major metacyclic promastigotes were associated even less well than mid-log phase cells, and at a lower rate, although similar to these latter cells, the majority had become associated with neutrophils after 15 min. Overall, there were approximately half as many metacyclics associated with neutrophils with neither promastigote form, however, was any detectable concomitant reduction in the numbers of free extracellular parasites over the 60 min incubation period.

3.4.2 The respiratory burst of rabbit peritoneal neutrophils upon exposure to different forms of *Leishmania* species and also other trypanosomatids *in vitro*

The ability of the neutrophils to produce CL as a response to external stimuli varied considerably between different neutrophil preparations (compare Fig. 26A and Fig. 29C) making it difficult to compare absolute values between preparations. This effect has also been reported by other workers (Lackie & Lawrence, 1987). Thus, the

results presented below in Figs. 25, 26 and 28 are all from experiments performed with the same neutrophil preparation carried out within 4-55 hours after preparation of the neutrophils. The CL due to stimulation by PMA is also included as an indication of the general responsiveness of the neutrophils at the time of the experiment.

Several controls were included in each experiment. Parasites without neutrophils produced very low levels of CL which never went above 1 mV at any time point in any experiment. Neutrophils alone, without an external stimulus, produced some CL. These values, however, reached a maximum of 4 mV and in the majority of experiments was much lower. The CL response of neutrophils with DMSO was similar to the latter. Experimental CL values were corrected using the neutrophil alone control.

A preliminary experiment using L.major mid-log phase promastigotes at a ratio of 10 parasites:1 neutrophil was performed in the presence of SOD (600 U) and catalase (600 U). Addition of SOD inhibited the CL response of the neutrophils to the promastigotes by 79%. In contrast, catalase inhibited the response by only 9%. These results confirmed that the majority of lucigenin-enhanced CL response was due to neutrophil production of O_2^- (Williams & Cole, 1981a,b; Baxter et al., 1983).

The PMA stimulated CL responses in all experiments were quite similar (compare Fig. 25C [4-6 hours after preparation of neutrophils], Fig. 26C [28-31 hours] and Fig. 28D [50-55 hours]) which confirmed previous reports (Lackie, 1977 and pers. comm., Lackie & Lawrence, 1987) that storage of the neutrophils for up to 72 hours in exudate solution at 4°C had little detrimental effect on their responsiveness to PMA and indicated that cell viability and function remained constant throughout the time course of the experiments with

each neutrophil preparation.

The results obtained for the interaction of L.major with neutrophils are given in Fig. 25. They show the largest differences observed between mid-log phase and metacyclic promastigotes although the trend was similar in the three experiments carried out with different neutrophil preparations. At all the parasite:neutrophil ratios used, mid-log phase promastigotes elicited CL responses and in all cases the CL response increased to a peak and then declined. The size and duration of the CL response was dependent on the number of parasites added. For example, at the 50:1 ratio the peak CL response occurred after approximately 13 min, whereas with the 10:1 and 5:1 ratios there was a similar peak response but it was reached only after approximately 30 min. The maximum CL response at the 1:1 ratio also occurred at this time but the peak was much lower. Metacyclic promastigotes also elicited a CL response at all the ratios, the size and/or time to reach the peak was again dependent on parasite load. In comparison to mid-log phase promastigotes, however, the metacyclic stimulated lower CL responses. For example, at the 50:1 ratio the peak was lower and was reached only after about 30 min. With the 10:1 and 5:1 ratios there was a similar delay in the peak response being reached and again they were lower (95±5 mV at the 5:1 ratio; and 103±9 mV at the 10:1 ratio after approximately 60 min). At the 1:1 ratio the peak response (63±18 mV) was similar to that for mid-log phase promastigotes but was only reached at after approximately 70 min. In the other two experiments performed with different neutrophil preparations which were less responsive, a similar situation as outlined above was seen although differences between the two promastigote forms in the peak response and/or time to reach the peak were not as great. It seems likely that, as with murine resident peritoneal macrophages (see Section 3.2.2), the differences in CL

response elicited by the two promastigote forms may be explained by the differences in the number of parasites that became associated with neutrophils as reported in Section 3.4.1. The response of the neutrophils to PMA was much lower than that to the promastigote forms.

The CL response of neutrophils to L.mexicana mexicana is shown in Fig. 26. The most striking feature which was consistently seen (in all four experiments with different neutrophil preparations) with mid-log phase promastigotes was the very small CL response that occurred when large numbers of promastigotes were added (the 50 parasites:1 neutrophil ratio). In all cases there was a rapid rise to a small peak (between 20 and 60 mV), thereafter the CL response declined quickly and remained at just above the control level. In an attempt to confirm that I was not missing a large, fast rising CL peak during the first few minutes of interaction of neutrophils with large numbers of promastigotes. On one occasion, the number of samples was reduced from the normal 24 to 3 (neutrophil alone control, promastigotes and neutrophils at a ratio of 50:1, neutrophils and PMA). This allowed the CL response to be monitored at more frequent time points, the results are shown in Fig. 27. Although the peak CL response was still apparently missed even under these conditions, the results do show that there was not a large, very rapid CL response during the first few minutes of interaction between neutrophils and promastigotes. With other parasite:neutrophil ratios (Fig. 26), the CL response was much greater and interestingly that caused by the parasites at the 5:1 ratio was consistently larger than that caused by parasites at 10:1, although the time to reach the peak was similar. In addition, mid-log phase promastigotes of L.mexicana mexicana at the 10:1, 5:1 and 1:1 ratios usually produced larger peak CL more rapidly than L.major mid-log phase promastigotes. This is apparent with these set of results

only with the 1:1 ratio, probably because this particular neutrophil preparation was very responsive. Although the peak CL was similar for the two species at the 10:1 and 5:1 ratios, that as a response to L.mexicana mexicana mid-log phase promastigotes occurred much more quickly. In contrast to the mid-log phase promastigotes (with the exception of the 50:1 ratio), L.mexicana mexicana amastigotes (in all three experiments with different neutrophil preparations) consistently elicited a very small CL response, which at ratios below 50:1 was almost negligible. This confirmed the lack of stimulatory activity of these cells towards phagocyte oxidative metabolism. The response to PMA of the neutrophils used in the amastigote experiment was apparently a little higher, at least over the first 20 min. This confirmed that the neutrophils were fully responsive.

The CL response of neutrophils to the addition of Herpetomonas muscarum ingenoplastis, H.muscarum muscarum or L.donovani mid-log phase promastigotes are shown in Fig. 28. Herpetomonas muscarum ingenoplastis stimulated a considerable CL response at all the parasite:neutrophil ratios (for the three experiments performed with different neutrophil preparations) apart from the 50:1 ratio. At this ratio the results were more variable and could be similar to that at the 10:1 and 5:1 ratios (see Fig. 28) or much lower (see Fig. 29), reduced to a small peak. This appeared to depend on the responsiveness of the neutrophil preparation. The response to H.muscarum ingenoplastis was, however, greater than that which occurred with H.muscarum muscarum at the same ratio. Herpetomonas muscarum muscarum also stimulated a considerable CL response at all ratios in the three experiments performed, apart from the 50:1 ratio. In this case, however, the results were more consistent, H.muscarum muscarum always elicited a small (see Fig. 28) to negligible (see Fig. 29) CL response which peaked at very low levels and then declined to a value just

Fig. 25. The kinetics of the chemiluminescence response of rabbit of peritoneal neutrophils upon exposure to L.major mid-log phase and metacyclic promastigotes in vitro.

Rabbit peritoneal neutrophils were exposed to mid-log phase (A) and metacyclic (B) promastigotes at parasite:neutrophil ratios of 1:1 (□), 5:1 (○), 10:1 (■) and 50:1 (●). The responses of the neutrophils to PMA (C) during the mid-log phase ( ) and metacyclic ( ) promastigote experiments are shown for comparison. Each point represents the mean (± standard deviation) of triplicate determinations. The data presented are from one experiment, with each parasite form, which is representative of the three performed.

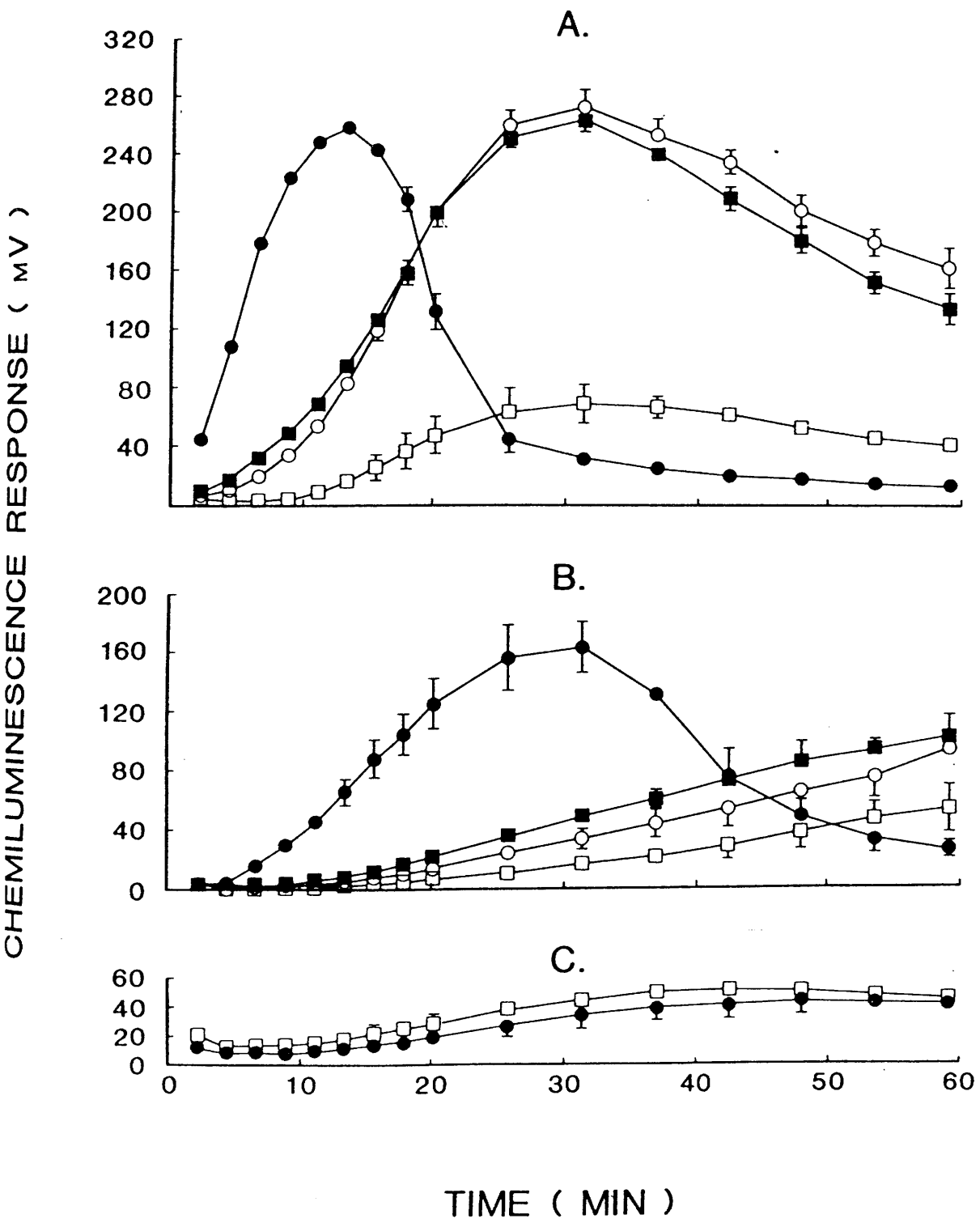
The neutrophil preparation used in these experiments had been stored in exudate solution at 4°C for approximately 4-6 hours prior to use.

Fig. 26. The kinetics of the chemiluminescence response of rabbit peritoneal neutrophils upon exposure to L.mexicana mexicana mid-log phase promastigotes and amastigotes in vitro.

Rabbit peritoneal neutrophils were exposed to mid-log phase promastigotes (A) and amastigotes (B) at parasite:neutrophil ratios of 1:1, 5:1, 10:1 and 50:1 (symbols as in Fig. 25). The responses of the neutrophils to PMA (C) during the mid-log phase promastigote (□) and amastigote (▲) experiments are shown for comparison. Each point represents the mean ± (standard deviation) of triplicate determinations. The data presented are from one experiment, with each parasite form, which is representative of the three performed.

The neutrophil preparation used in these experiments had been stored in exudate solution at 4°C for approximately 28-30 hours prior to use.

Fig. 25.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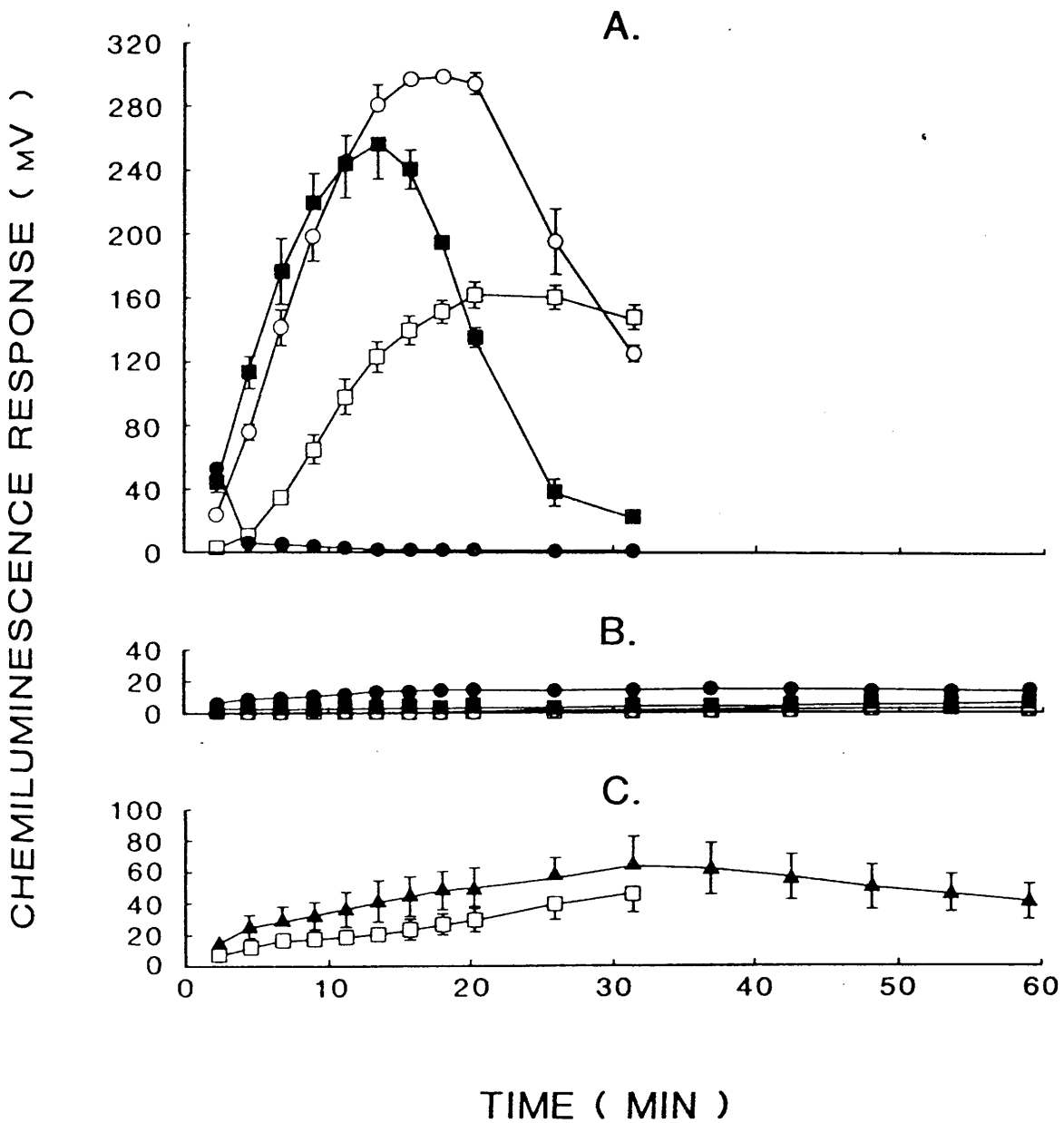
CHEMILUMINESCENCE RESPONSE (mV)
TIME (MIN)

Fig. 26.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40
50
60
CHEMILUMINESCENCE RESPONSE (mV)
TIME (MIN)

Fig. 27. The initial kinetics of the chemiluminescence response of rabbit peritoneal neutrophils to L.mexicana mexicana mid-log phase promastigotes in vitro.

Rabbit peritoneal neutrophils were exposed to mid-log phase promastigotes (□) at a ratio of 50 parasites:1 neutrophil. The response of the neutrophils to PMA (▲) during the experiment is shown for comparison. Each point represents the value from one sample. This experiment was carried out once only.

The neutrophil preparation used in this experiment had been stored in exudate solution at 4°C for approximately 6 hours prior to use.

Fig. 28. The kinetics of the chemiluminescence response of rabbit peritoneal neutrophils to insect trypanosomatids and L.donovani mid-log phase promastigotes in vitro.

Rabbit peritoneal neutrophils were exposed to Herpetomonas muscarum ingenoplastis (A), H.m.muscarum (B) and mid-log phase promastigotes of L.donovani (C) at parasite:neutrophil ratios of 1:1 (□), 5:1 (○), 10:1 (■) and 50:1 (●). The responses of the neutrophils to PMA (D) during the H.m.ingenoplastis (●), H.m.muscarum (▲) and L.donovani (□) experiments is shown for comparison. Each point represents the mean (± standard deviation) of triplicate determinations. The data presented for the insect trypanosomatids are from one experiment, with each parasite, of the two performed. The data for L.donovani are from the one experiment performed.

The neutrophil preparation used in these experiments had been stored in exudate solution at 4°C for approximately 50-55 hours prior to use.

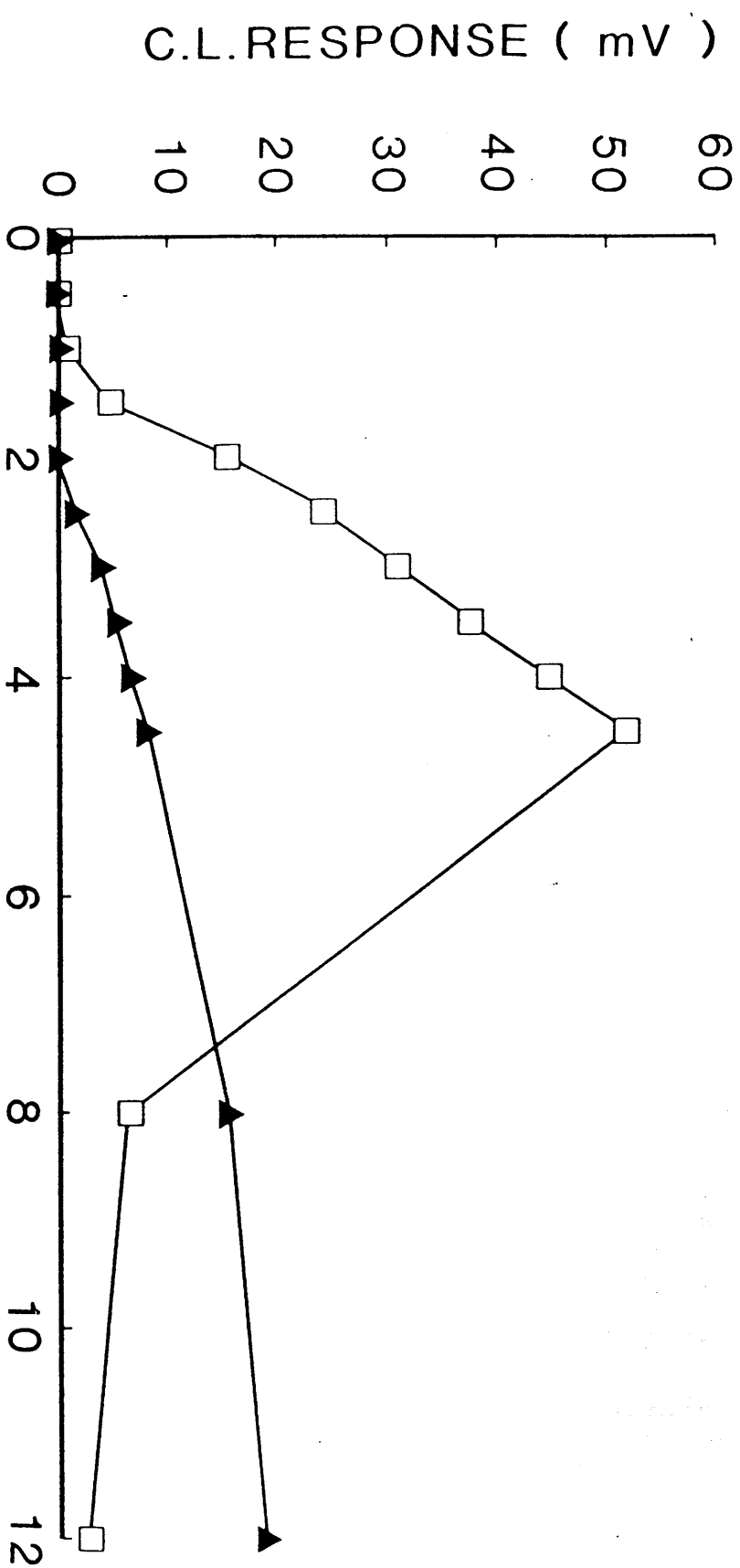
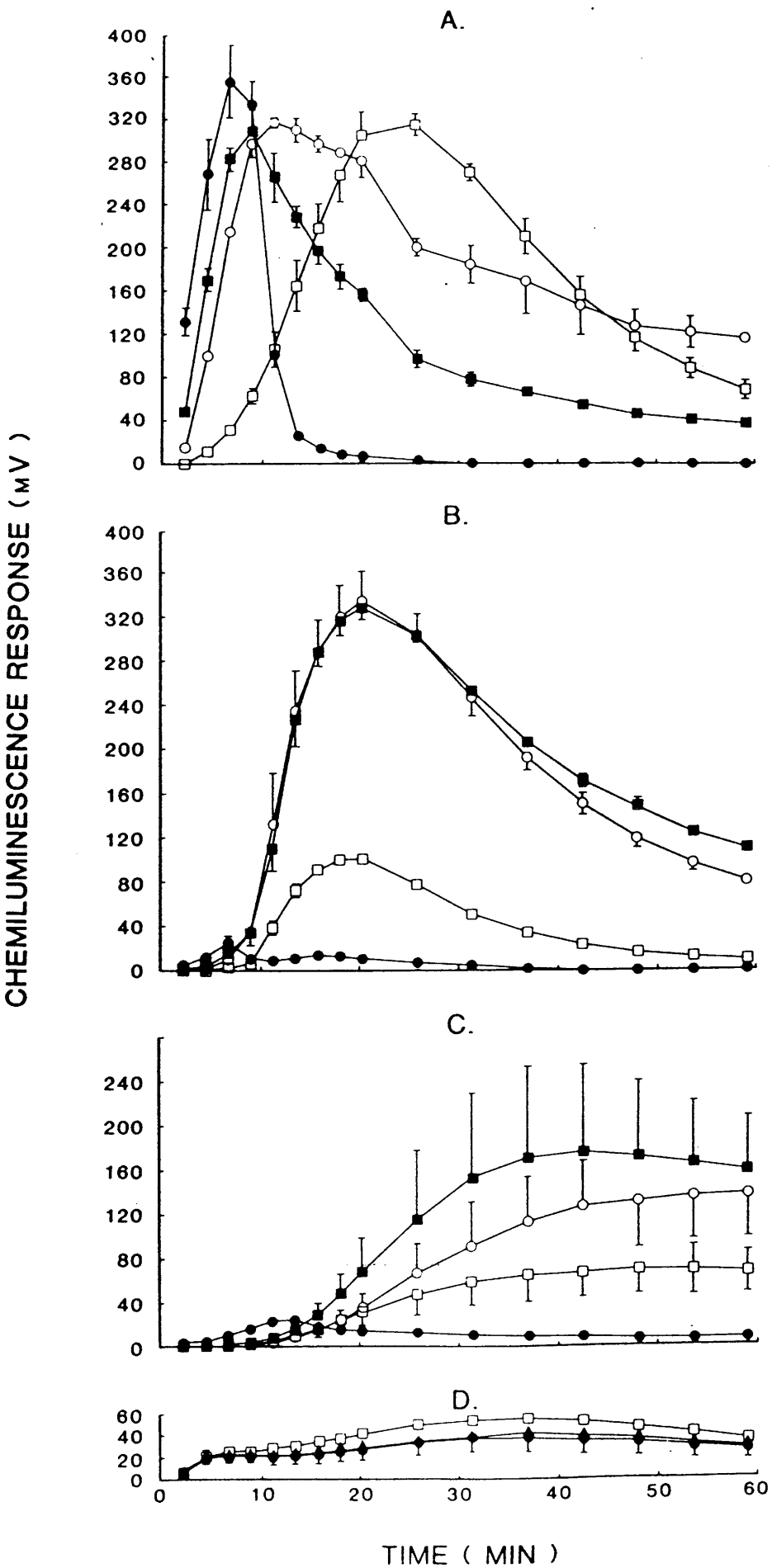


Fig. 27.

Fig. 28.


A.
B.
C.
D.
CHEMILUMINESCENCE RESPONSE (MV)
TIME (MIN)
0
40
80
120
160
200
240
280
320
360
400
0
10
20
30
40
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above the control. This was very similar to the situation that occurred with L.mexicana mexicana mid-log phase promastigotes (see Fig. 26). The CL response to Leishmania donovani mid-log phase promastigotes was also dose dependant although the shape of the CL response curves at ratios of 10:1 and 5:1 (and in some cases the peak response) was different from that for the insect trypanosomatids and mid-log phase promastigotes of L.mexicana mexicana and L.major. Interestingly, like H.muscarum mascarum and mid-log phase promastigotes of L.mexicana mexicana, L.donovani mid-log phase promastigotes at the 50:1 ratio stimulated at CL response that was restricted to only a small peak and which then slowly declined to low levels. The CL response of the neutrophils to PMA was similar in all these three experiments.

3.4.3 The respiratory burst of rabbit peritoneal neutrophils upon exposure to glutaraldehyde-fixed, sodium fluoride-treated, or untreated parasites in vitro

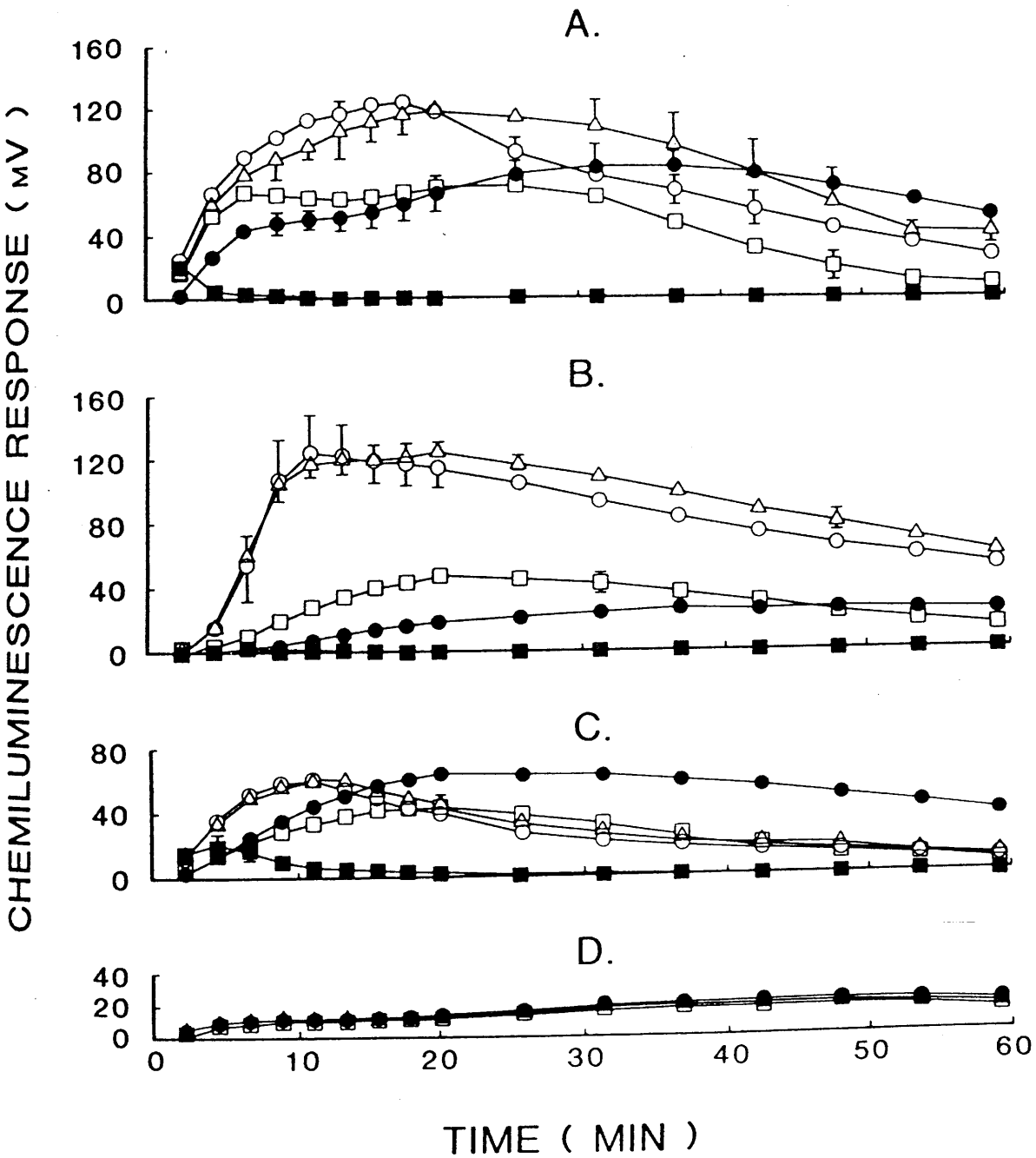
There seemed to be an approximate correlation between the presence of surface acid phosphatase on the trypanosomatids (i.e. present on L.mexicana mexicana [see Section 3.6.3] and L.donovani [Gottlieb & Dwyer, 1981a,b,c] mid-log phase promastigotes, and H.muscarum muscarum [Coombs et al., 1987], but apparently not present on L.major mid-log phase and metacyclic promastigotes [see Section 3.6.3] and H.muscarum ingenoplastis [Coombs et al., 1987]) and their ability at high density to dramatically reduce the CL response (due to O_2^- production) of neutrophils. This was investigated in more detail by studying the response of neutrophils to parasites treated with glutaraldehyde which killed them and therefore prevented any secretion but retained surface acid phosphatase activity (Gottlieb & Dwyer, 1981; Hassan & Coombs, 1987). Other parasite cells were treated with

Fig. 29. The kinetics of the chemiluminescence response of rabbit peritoneal neutrophils to glutaraldehyde-fixed, sodium fluoride-treated, or untreated parasites *in vitro*.

Rabbit peritoneal neutrophils were exposed to *H.muscarum ingenoplastis* (A), *H.muscarum muscarum* (B), *L.mexicana mexicana* mid-log phase promastigotes (C). Key to treatments and parasite:neutrophil ratios: 5:1, untreated, (○); 5:1, glutaraldehyde-fixed, (●); 5:1, NaF-treated, (△); 50:1, untreated, (■); 50:1, glutaraldehyde-fixed, (□). The responses of the neutrophils to PMA during the *H.muscarum ingenoplastis* (●), *H.muscarum muscarum* (▲) and *L.mexicana mexicana* (□) experiments are shown for comparison. Each point represents the mean (± standard deviation) of triplicate determinations. The data presented are from the one experiment carried out with each parasite.

The neutrophil preparation used in these experiments had been stored in exudate solution for 24-28 hours prior to use.

Fig. 29.


A.
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40
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60
CHEMILUMINESCENCE RESPONSE (mV)
TIME (MIN)

5 mM NaF which would inhibit all acid phosphatase activities (Hassan & Coombs, 1987). The results are shown in Fig. 29.

The neutrophil preparation used in this set of experiments were quite unresponsive in comparison to other preparations, as was confirmed by their response to PMA. The results for all three species/ subspecies at the parasite:neutrophil ratio of 50:1 were quite similar. The glutaraldehyde-fixed cells stimulated a greater (larger peak, longer duration) CL response than live untreated cells but less than that for live untreated cells at the 5:1 ratio. At the 5:1 ratio the CL response stimulated by NaF-treated cells was the same as that for live untreated cells in all cases. The results with glutaraldehyde-fixed cells at this ratio were a little variable, but similar for the three species in that there was a reduced CL response of the neutrophils compared with their response to living untreated cells.

3.5 THE RESPIRATORY BURST OF HUMAN BLOOD MONOCYTES UPON EXPOSURE TO L.MAJOR MID-LOG PHASE AND METACYLIC PROMASTIGOTES IN VITRO

In an attempt to quantitate the RB of a cell that is infected by Leishmania, the oxidative response of human blood monocytes to L.major was investigated using lucigenin-enhanced CL. This was carried out twice, representative results from one experiment are shown in Fig.30. The response to both forms was much lower than with rabbit peritoneal neutrophils. Metacyclic promastigotes appeared to be more stimulatory than mid-log phase promastigotes, the former producing a CL response that increased more rapidly and to a larger peak. Unfortunately, no data were obtained on the relative uptake and attachment of the two forms to monocytes. The response of the monocytes to PMA was similar to that with rabbit peritoneal neutrophils and was larger than the response to either promastigote form.

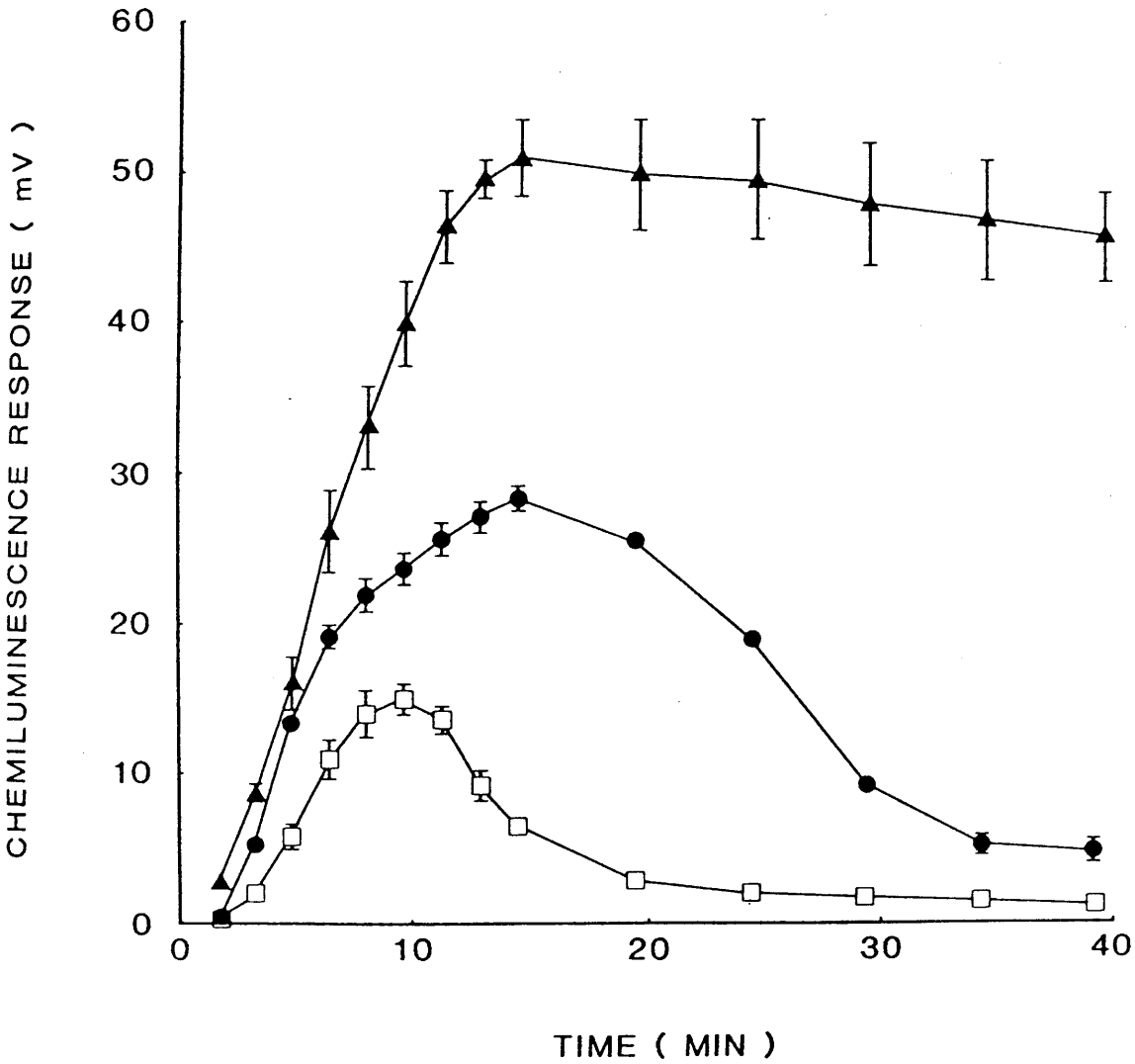


Fig. 30. The kinetics of the chemiluminescence response of human blood monocytes upon exposure to L.major mid-log phase and metacyclic promastigotes in vitro.

Human blood monocytes were exposed to mid-log phase (□) and metacyclic (●) promastigotes at a parasite:monocyte ratio of 50:1. The response of the monocytes to PMA (▲) is shown for comparison. Each point represents the mean (± standard deviation) of triplicate determinations. The data presented are from one experiment which is representative of the two performed.

3.6 INVESTIGATIONS OF SOME OF THE MECHANISMS OF SURVIVAL OF METACYCLICS

Investigations were made into the mechanisms enabling metacyclics to survive in macrophages.

3.6.1 Comparison of the susceptibility of L.major mid-log phase and metacyclic promastigotes to hydrogen peroxide and to hydrogen peroxide/lactoperoxidase/potassium iodide in a phagocyte-free system

Following the finding that the metacyclics of L.major stimulated an equivalent RB to mid-log phase promastigotes in a variety of phagocytic cell types. Both promastigote forms were tested for their susceptibility to H_2O_2 and to H_2O_2/LPO/KI combinations (the latter are thought to mimic the MPO system present in polymorphonuclear leukocytes and monocytes) to determine of the better survival of the metacyclics could be limited to a lowered suseceptibility to some of the products of the RB. The results are shown in Fig. 31. It was found that metacyclic promastigotes were more resistant than mid-log phase promastigotes to the effects of H_2O_2 over a narrow range of concentrations. This was true whether the % "fast" or % motile were measured. For example, at a H_2O_2 concentration of 0.18 mM, 85% of the metacyclics were motile and 60% were classed as "fast" compared to figures of 11% and 5%, respectively, for mid-log phase promastigotes. When the % motile were measured, the concentration of the H_2O_2 that would reduce the number motile to 50% (LD_{50}; as calculated from the graph) were calculated to be 0.28 mM and 0.09 mM for metacyclics and mid-log phase promastigotes, respectively. A similar difference when the % "fast" were measured, the LD_{50}'s were 0.20 mM and 0.06 mM H_2O_2 for mid-log phase and metacyclic promastigotes, respectively.

With the H_2O_2/LPO/KI system the situation was reversed, mid-log

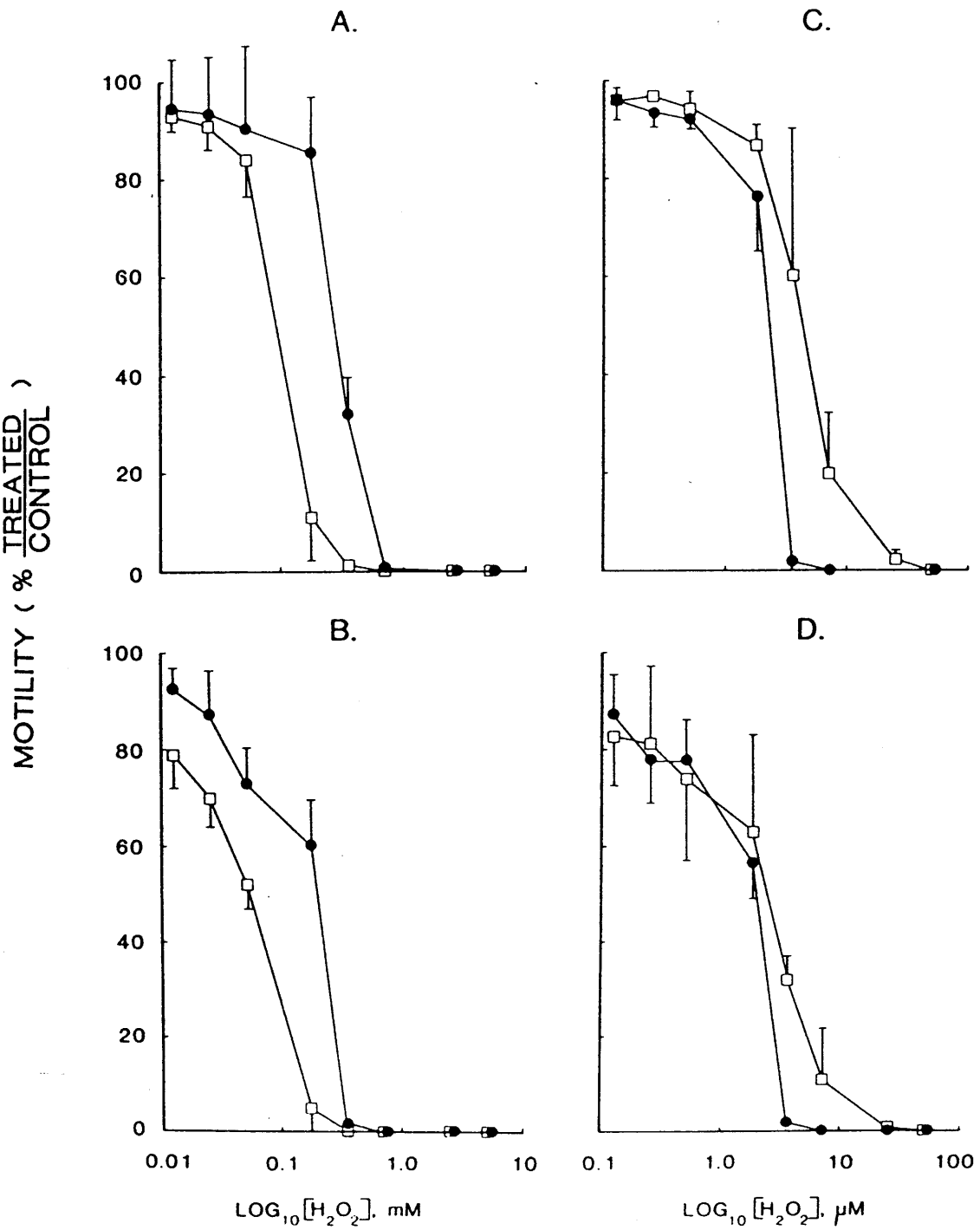


Fig. 31. The susceptibility of L.major mid-log phase and metacyclic promastigotes to hydrogen peroxide, and to hydrogen peroxide/lactoperoxidase/potassium iodide combinations, in vitro.

(A) % promastigotes motile. }
(B) % promastigotes "fast". } H_2O_2

(C) % promastigotes motile. }
(D) % promastigotes "fast". } H_2O_2/LPO/KI

Mid-log phase promastigotes, (□); metacyclic promastigotes, (●). Each point represents the mean (± standard deviation) from four experiments.

phase promastigotes appeared to be more resistant than metacyclic promastigotes. With respect to % motile, the LD_{50} for mid-log phase cells was 4.4 μM, approximately double that for metacyclics which was 2.3 μM. Less differences was seen with the % "fast" results, mid-log phase and metacyclic promastigotes having LD_{50}'s of 2.3 μM and 1.9 μM, respectively.

Comparison of LD_{50}'s revealed that much lower levels (approximately 100-fold) H_2O_2 were toxic to metacyclic promastigotes when LPO and KI was also present. In contrast the difference with mid-log phase promastigotes was only approximately 20-fold.

Addition of LPO and KI without H_2O_2 had no effect on the viability of promastigotes. Presence of catalase (3000 U) in the reaction mixture with the highest H_2O_2 used in either assay completely prevented leishmanicidal activity. Control values for mid-log phase and metacyclic promastigotes in PBSG, pH 7.3 only for % "fast", and % motile are given in section 3.1.1.

3.6.2 The sensitivity of different promastigote forms of *L.major* and *L.mexicana mexicana* to lysis by normal human serum *in vitro*

My results had shown that stationary-phase promastigotes of *L.mexicana mexicana* and, stationary-phase and metacyclic promastigotes of *L.major* were more infective *in vivo* than mid-log phase cells. It has also been reported that the more infective stationary-phase promastigotes of *L.braziliensis panamensis* were also more resistant to lysis by normal human serum than log-phase cells (Franke *et al.*, 1985). Thus, the effect of normal human serum on the different promastigote forms used in this study was investigated. The results for *L.major* are shown in Fig. 32A. All the promastigote populations were lysed by normal human serum, none survived in 100% serum. There were however, differences in the susceptibility of different

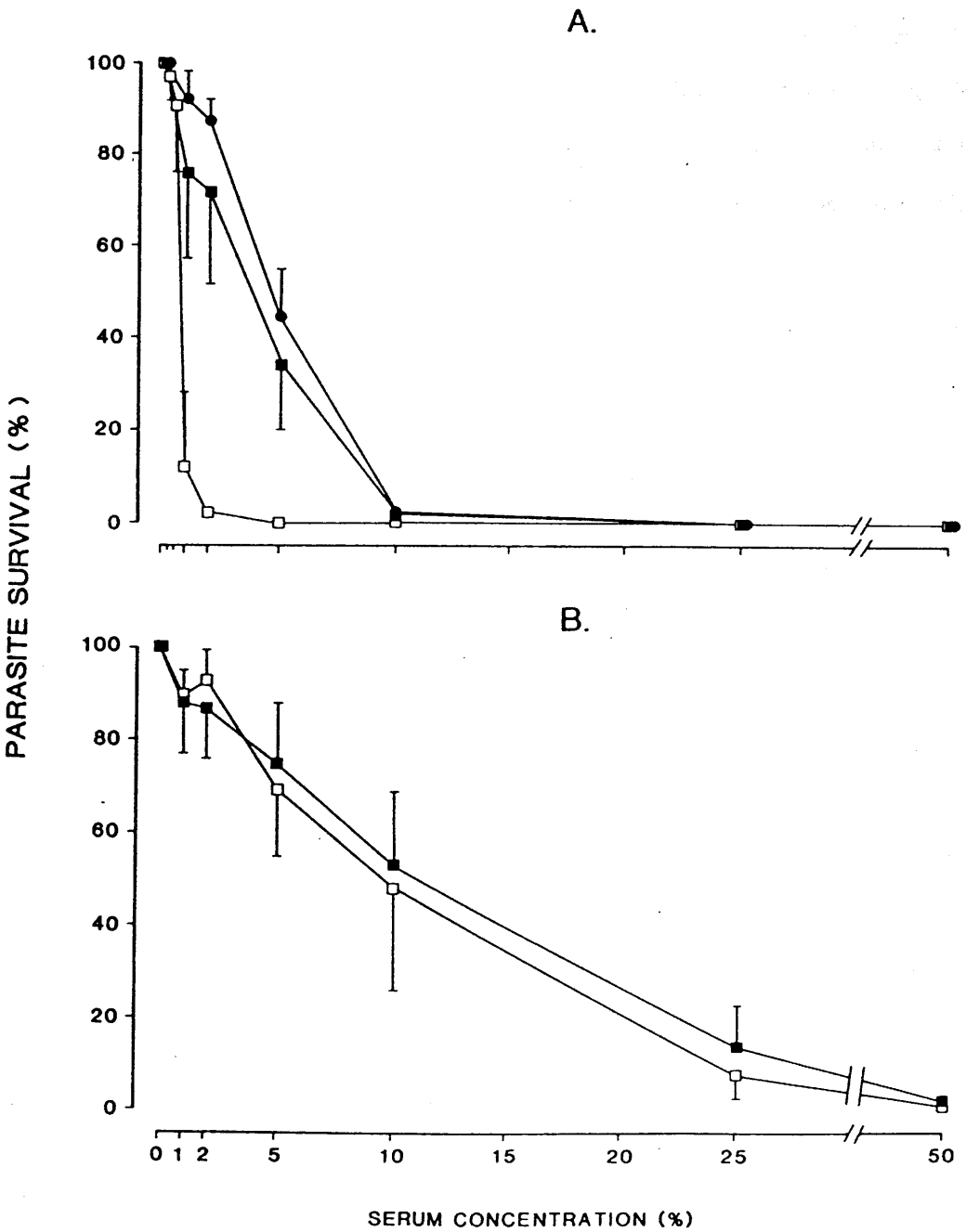


Fig. 32. The sensitivity of different promastigote forms of L.major and L.mexicana mexicana to lysis by normal human serum in vitro.

(A) L.major: mid-log phase promastigotes, (□); stationary-phase promastigotes, (■); metacyclic promastigotes (●).

(B) L.mexicana mexicana: mid-log phase promastigotes, (□); stationary-phase promastigotes, (■).

Each point represents the mean (± standard deviation) from three experiments.

promastigote forms. Some of the metacyclic and stationary-phase promastigotes survived at a concentration of 10% serum, and there was good survival at 5%. The survival rates of metacyclic and stationary-phase promastigotes at concentrations of 10% serum and below were similar. The LD_{50}'s (as calculated from the graph) of metacyclic and stationary-phase promastigotes were 4.6% and 3.8% serum, respectively. In contrast, mid-log phase promastigotes were highly susceptible to lysis, all being lysed at 5% serum, and the LD_{50} being approximately 0.8%. Heat-inactivating serum almost completely abolished its lytic activity, 93±6% (n=3) metacyclics surviving in 10% HI serum. Unfortunately HI serum at this concentration caused mid-log phase promastigotes to agglutinate to such an extent that it was impossible to accurately count numbers unlysed. The majority of them, however, appeared unlysed and intact under these conditions. Similarly, some agglutination occured with mid-log phase cells, but it was still to count the cells and in this case 87±13% were unlysed. Interestingly agglutination was not observed with normal human serum.

The results with L.mexicana mexicana promastigotes are shown in Fig. 32B. Both mid-log phase and stationary-phase promastigotes were susceptible to lysis, no promastigotes survived in 100% serum. At least some of both promastigote forms survived at concentrations of 50% serum and below, the percentage survival increasing steadily as serum concentration was reduced below 25%. Unlike L.major promastigotes, however, the survival rates of mid-log phase and stationary-phase promastigotes were similar. Both populations of promastigotes of L.mexicana mexicana were more resistant to lysis than any of the L.major promastigote forms; mid-log phase and stationary-phase promastigotes having LD_{50}'s of 9.5% and 11.2% serum, respectively. Similar to L.major, testing either of the promastigote

populations with 25% HI serum caused them to agglutinate to such an extent that they could not be counted accurately, although the majority of the parasites appeared to be intact. Again, agglutination was not observed with normal human serum.

3.6.3 Analysis of acid phosphatase activities of different forms of *Leishmania* species

Several recent reports have suggested that acid phosphate activities present in leishmanias may play a crucial role in their survival in macrophages (Remaley *et al.*, 1984; Remaley, Glew *et al.*, 1985; Katakura, 1986). In addition, preliminary experiments involving short term incubations (up to 45 min) of *L.major* mid-log phase promastigotes with PNPP showed that they were able to hydrolyse this substrate to PNP at a rate equivalent to 6 nmoles min^{-1} (mg $protein^{-1}$). This suggested the presence of surface located or secreted activities of acid-phosphatase, although the possibility that PNPP was being taken up, hydrolysed within the cells, and then PNP released could not be ignored. I therfore examined the acid phosphatase activities of *L.major* mid-log phase and metacyclic promastigotes and for comparison the activities in different forms of *L.mexicana mexicana* to see if there was a correlation between changes in acid phosphatase activities and infectivity.

The acid phosphatase activities detected in homogenates of *Leishmania* species and their partition between the sedimentable and soluble parts of the cell are given in Table 11. The specific activities in mid-log phase and metacyclic promastigotes of *L.major* were similar to each other and also to those present in mid-log phase and stationary-phase promastigotes of *L.mexicana mexicana*. Amastigotes of this latter species had lower activity in comparison. More interesting are the differences found concerning the distribution of

Table 11: Acid phosphatase activities of different forms of L.major and L.mexicana mexicana.

Enzyme	L.major		L.mexicana mexicana		
	Mid-log phase promastigotes	Metacyclic promastigotes	Mid-log phase promastigotes	Stationary-phase promastigotes	Amastigotes
Specific activity[a]	66± 9	50± 7	52	49	28
% sedimentability[b]	19± 3(107±8)	28±13 (88±26)	66 (102)	47 (131)	51 (61)
Protein, % sedimentability[b]	57± 4(99±6)	64± 6 (94± 1)	61 (102)	58 (97)	58 (97)

[a] The activities are given in nmoles min^{-1} (mg protein)$^{-1}$ for parasite homogenates incubated in succinic acid - NaOH, pH 6.0.

[b] The activity or protein sedimented in the 105,000 x g pellet as a % of the total activity or protein recovered. The % activity or protein recovered is given in parentheses. The results for L.major are means (± standard deviation) from three experiments, those for L.mexicana mexicana are from one experiment.

acid phosphatase between particulate and soluble fractions of the two species. For both promastigote forms of L.major the majority of the activity was recovered in the soluble fraction. In contrast, aproximately 50% of all forms of L.mexicana mexicana was found to be sedimentable.

Lyophillised medium from L.major promastigote cultures of various ages, from which the cells had been removed by centrifugation, were examined for the presence of acid phosphatase activity. I was, however, unable to detect any activity even in medium from 168 hour cultures. This suggested that the enzyme was either not secreted or secreted only in very limited amounts from L.major during in vitro culture.

The pH profiles of the acid phosphatase activities of the different fractions of L.major promastigotes were determined in order to characterise them further and to see if there were differences between the activities of the two forms. The results are shown in Fig. 33. The pH profiles were similar for the crude homogenates (data not shown) and supernatant fractions of both promastigote forms with a well defined maximal activity at pH 6.0. The pH profiles of the particulate fraction of the mid-log phase and metacyclic promastigotes were also similar to each other, but different from the soluble activities in having rather ill-defined maxima at pH 5.5 and pH 5.0, respectively. These results suggested the presence of a distinct particulate activity.

To test whether the particulate activity was surface located, promastigotes of L.major, and L.mexicana mexicana, as a positive control (Hassan and Coombs 1987), were stained from acid phosphatase activity (using disodium-2-glycerol phosphate as substrate and lead as the capture agent) and processed for the electron microscope. Typical electron micrographs are shown in Fig. 34. Considerable surface-

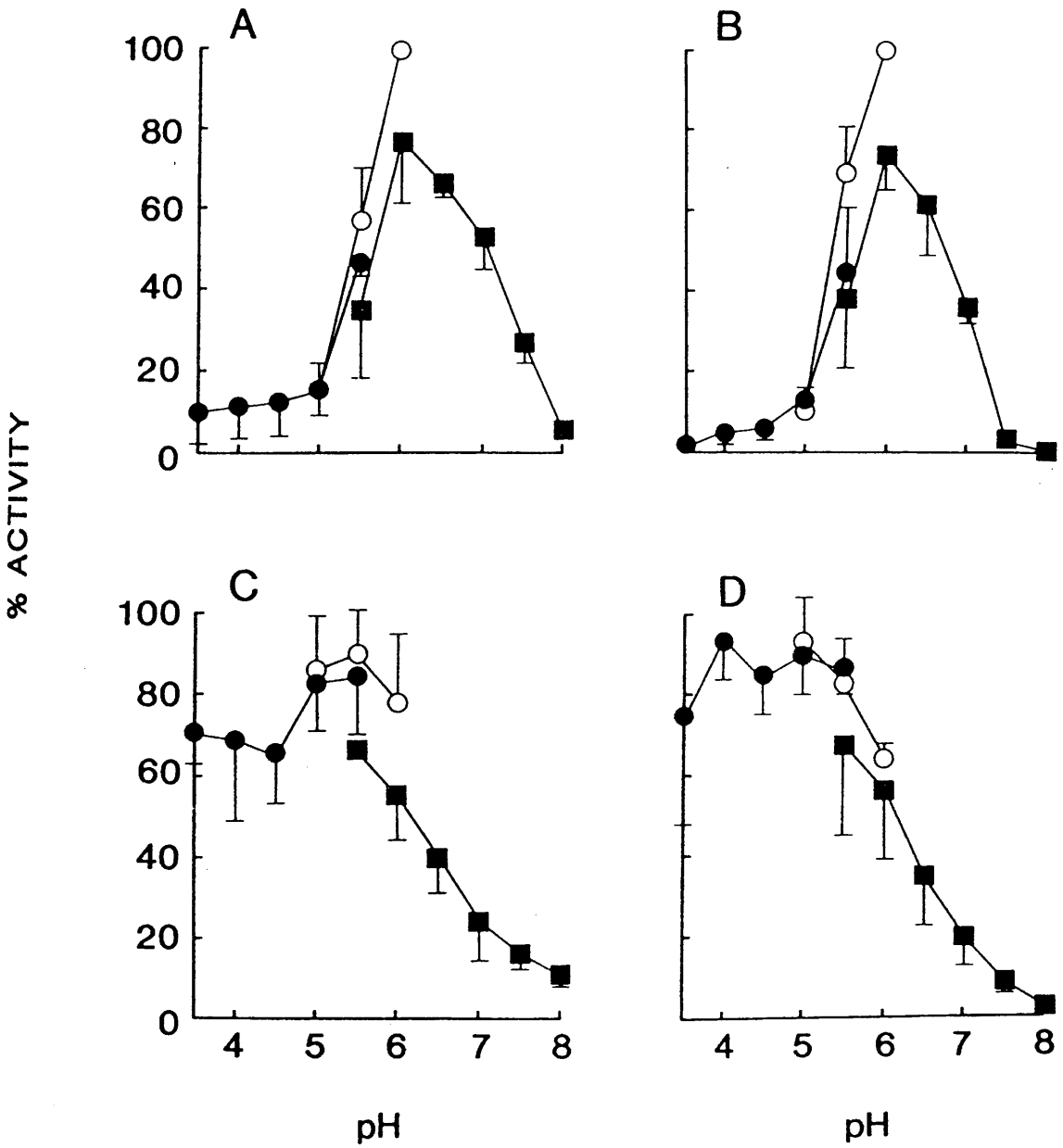
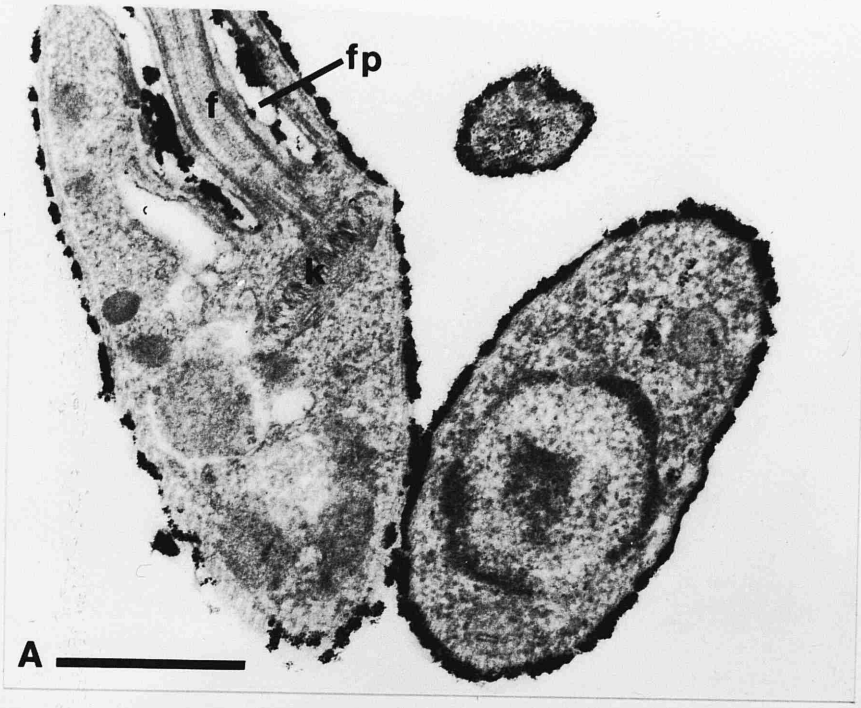


Fig. 33. The pH profiles of acid phosphatase activities of L.major mid-log phase and metacyclic promastigotes.

The buffers used (all 50mM) were: sodium acetate-acetic acid, ●; succinic acid-NaOH, (○); Tris-maleate, (■). Key: mid-log phase promastigote supernatant fraction (A); metacyclic supernatant fraction (B); mid-log phae promastigote particulate fraction (C); metacyclic particulate fraction (D). The activities at different pH's are expressed as a % of the maximum activity found, and each point represents the mean (± standard deviation) from three experiments.



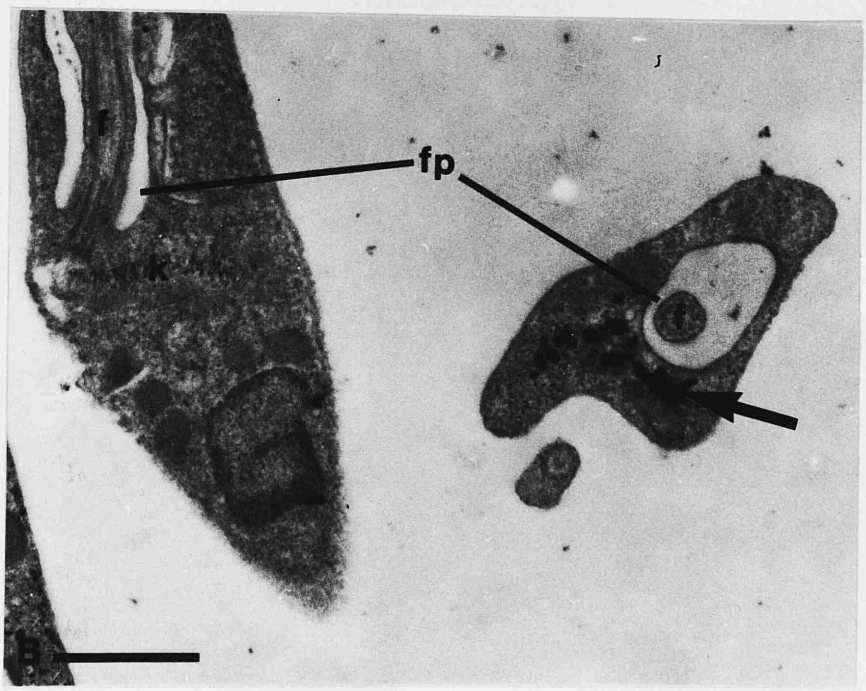


Fig. 34. Ultrastructural localization of acid phosphatase in mid-log phase L.mexicana mexicana promastigotes and stationary-phase L.major promastigotes.

(A) L.mexicana mexicana mid-log phase promastigotes. Electron micrograph courtesy of H.F. Hassan. Scale bar = 1.0 μm.

(B) L.major stationary-phase promastigotes. Note the absence of reaction product from the surface yet the presence of such within the parasite (arrowed). Scale bar = 0.75 μm.

Table 12: The effects of inhibitors on the acid phosphatase activities of mid-log phase and metacyclic promastigotes of *L.major*[a].

Inhibitor	Concentration	Mid-log phase promastigotes	Metacyclic promastigotes
Ammonium molybdate	10	82	72
	5	89	83
	1	95	88
	0.05	90	89
	0.01	85	84
Sodium fluoride	10	94	91
	5	95	96
	1	92	93
	0.05	NI	10
	0.01	NI	9
Sodium tartrate	80	78	75
	40	68	74
	20	60	68
	10	45	65
	5	45	64
	1	22	48
	0.05	9	18
	0.01	15	13

[a] The results given are the % inhibition related to the activity in the absence of inhibitors and are the values from one experiment or the means from two experiments. Homogenates were used as the source of the enzyme.

NI, no inhibition detected.

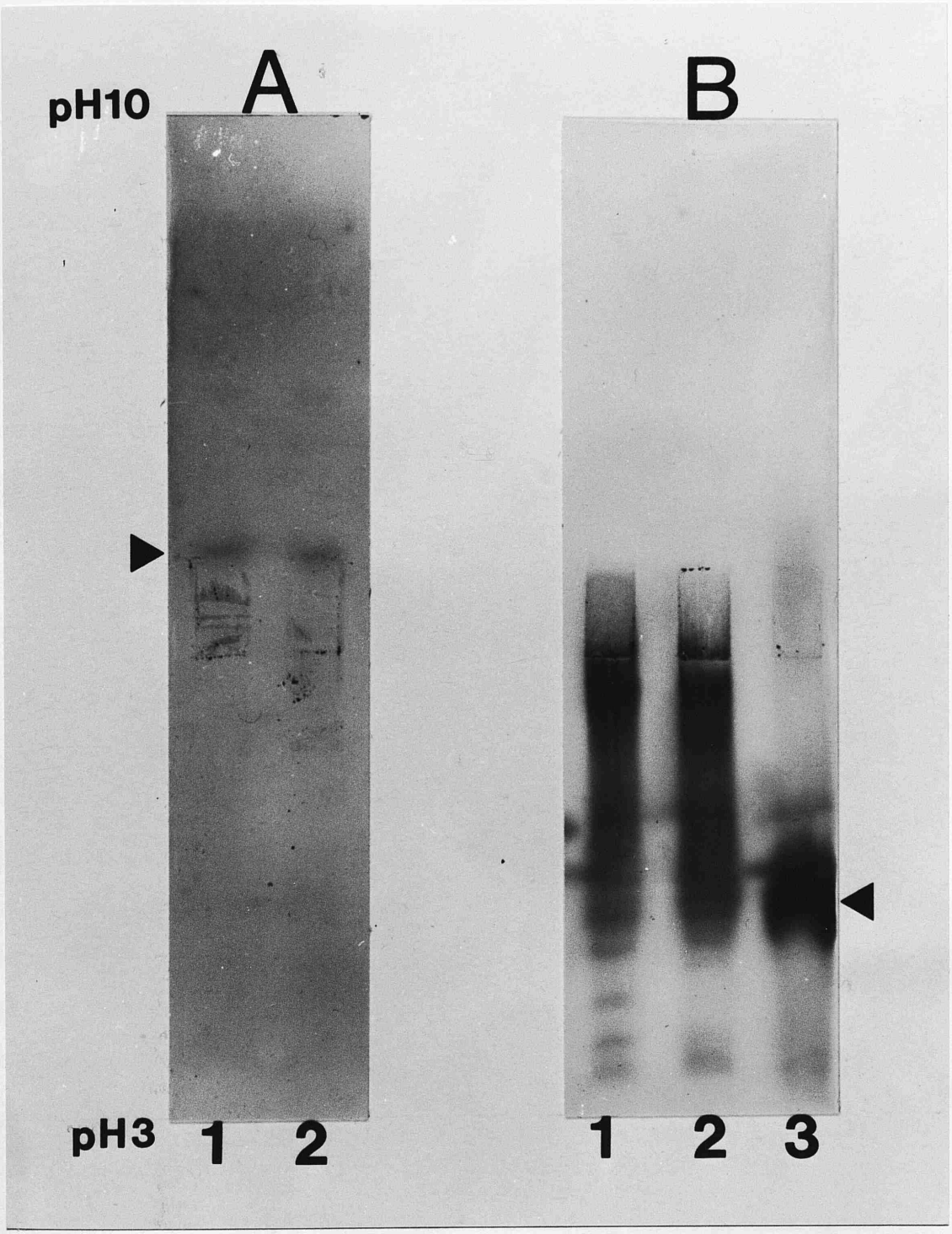


Fig. 35. Acid phosphatase isoenzyme patterns in different forms of L.major and L.mexicana mexicana.

(A) L.major. Key to tracks: mid-log phase promastigotes, (1); metacyclic promastigotes, (2). Arrow indicates the single isoenzyme present, focussing in the same position in both forms.

(B) L.mexicana mexicana. Key to tracks: mid-log phase promastigotes, (1); stationary-phase promastigotes, (2); amastigotes, (3). Arrow indicates the major isoenzyme present in amastigotes.

located activity was detected in L.mexicana mexicana mid-log phase promastigotes, whereas stationary-phase promastigotes of L.major appeared to lack any surface located activity, although staining can be seen within the cell. Similar results were found with mid-log phase and metacyclic promastigotes of L.major (data not shown). No staining was seen when the substrate was excluded from the incubation medium.

The inhibitor sensitivity of acid phosphatase of L.major was determined using crude homogenates of mid-log phase and metacyclic promastigotes. The activities in the two promastigote forms were similarly affected by sodium-L(+)-tartrate, NaF and ammonium molybdate over a range of concentrations as is shown in Table 12.

Homogenates of the different forms of the two species were subject to IEF and the gels strained for acid phosphatase activity. The results are shown in Fig. 35. IEF of L.major homogenates revealed just one band of acid phosphatase activity focussing in the same position with both forms at an apparent pI of approximate 6.0. In contrast, complex banding patterns of activities were found with L.mexicana mexicana homogenates. The enzymes of amastigotes were very different from those of mid-log phase promastigotes having one major broad band with an apparent pI of approximately 5.2. The banding pattern of stationary-phase promastigotes of L.mexicana mexicana although having minor similarities to that of amastigotes was much more like the banding pattern found for mid-log phase promastigotes .

3.6.4 Analysis of the proteolytic activities of different forms of *Leishmania* species by Gelatin-Disc-PAGE

Leishmanias possess relatively high proteinase activity and a role in allowing them to survive in macrophages has been proposed (Coombs, 1982). Recently, one of these activities has been shown to occur on the surface of promastigotes and roles in attachment to,

uptake by, and survival within macrophages has been suggested (Chaudhuri & Chang, 1986; Etges et al., 1986; Russell & Wihelm, 1986). Thus, the proteolytic activities of different forms of Leishmania species were examined to determine if there was a correlation between changes in proteolytic activities and infectivity. At least three independent samples of each form of each species were analysed, apart from L.major amastigotes where two independent samples were analysed. Figures are representative results, no variation between samples was observed except where stated.

Proteinases were detected in all forms of the Leishmania species examined (Figs. 36 and 39). Highest proteinase activity was always obtained at pH 5.5 although every proteinase activity could be detected at pH 4.0 and pH 8.0. Very interestingly, a proteinase activity running between apparent molecular weights 63-68KDa was found to be consistently present in all the homogenates tested irrespective of form or species used (Figs. 36 and 39). Apart from the L.mexicana mexicana samples (see below) all proteinases detected in homogenates were of 60KDa or greater.

The highest activities were found in L.mexicana mexicana amastigotes as a series of low molecular weight (around 30KDa) enzymes. These could be resolved into several separate bands if the gel was incubated for 1 hour, rather than the 4 hours required for the other proteinases (Fig. 37). These enzymes were uniformly absent from mid-log phase promastigotes, but with stationary-phase promastigotes the situation was not so clear. The low molecular weight enzymes were either apparently absent (see Fig. 40) or present up to the amounts shown in Fig. 36. Even when present they were not identical in molecular weight to those in amastigotes and were at lower activity. The other proteinases in L.mexicana mexicana were present in all

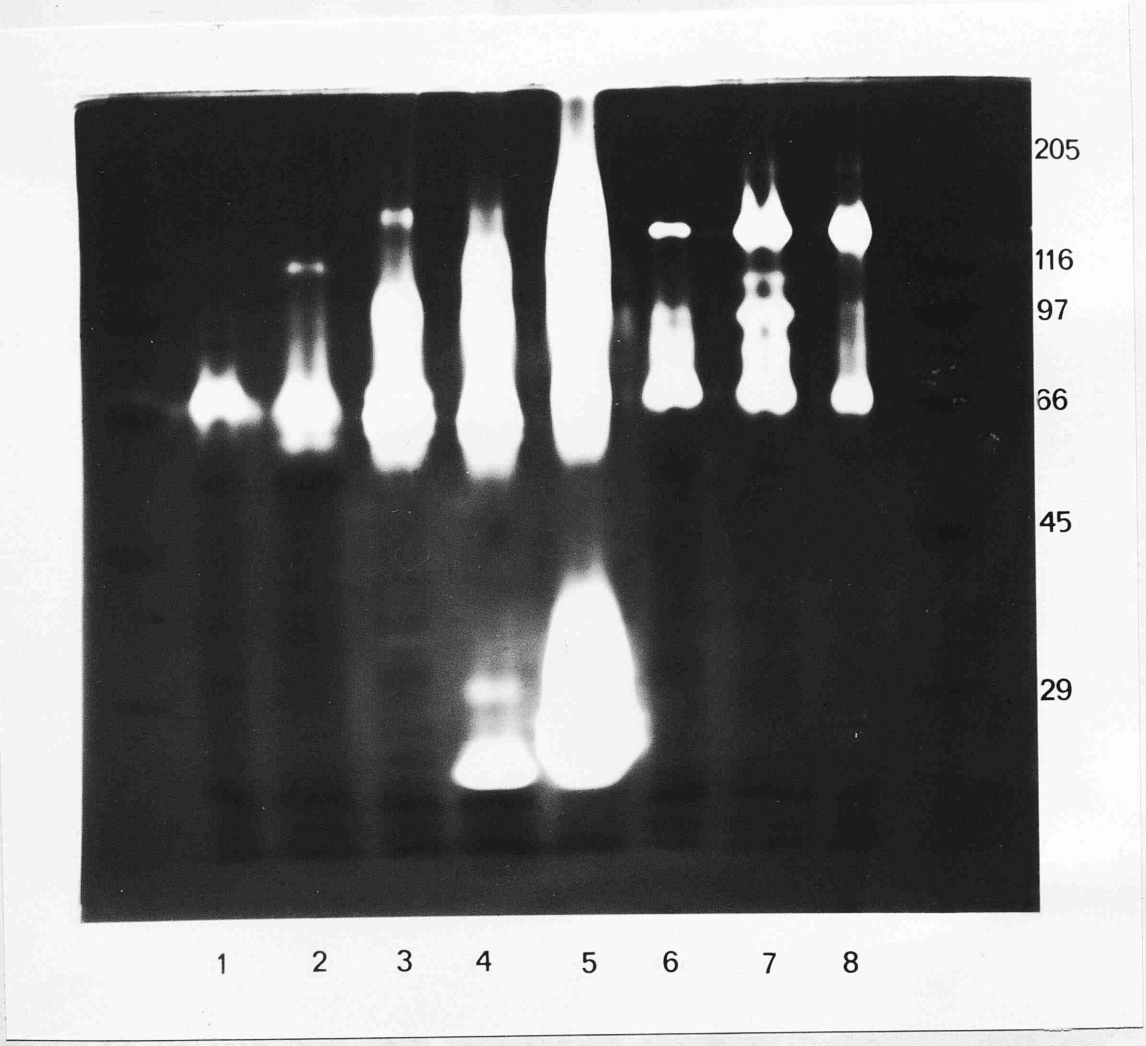


Fig. 36. Gelatin Disc-PAGE analysis of the proteinases of different forms of Leishmania species.

Key to tracks: Tracks 1-2, L.major: mid-log phase promastigotes, (1); metacyclic promastigotes, (2). Tracks 3-5, L.mexicana mexicana: mid-log phase promastigotes, (3); stationary-phase promastigotes, (4); amastigotes, (5). Tracks 6-8, L.donovani: mid-log phase promastigotes, (6); stationary-phase promastigotes, (7); amastigotes, (8). The positions of the molecular weight markers (in KDa) are given on the right of the figure.

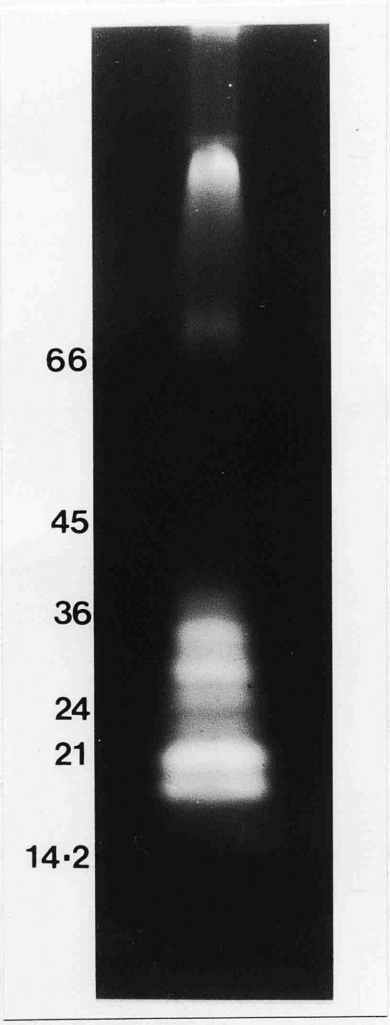


Fig. 37. Gelatin Disc-PAGE analysis of the proteinases of L.mexicana mexicana amastigotes.

Gel was incubated for 1 hour. The positions of the molecular weight markers (in KDa) are given on the left of the figure.

forms as a series of enzymes of molecular weight 60KDa or above which produced a broad band of activity. The activities were somewhat higher in stationary-phase promastigotes than in mid-log phase promastigotes, and amastigotes had the highest activity of all. Specific activities of proteinases active on azocasein detected in promastigotes and amastigotes of L.mexicana mexicana also differed as described in Section 3.7.2.

Differences between forms were also found for L.donovani (Fig. 38). There was a high molecular weight enzyme (at an apparent molecular weight of approximately 130KDa) which had much higher activity than either promastigote form; although within the promastigote samples it appeared to have higher activity in stationary-phase promastigotes in comparison to mid-log phase promastigotes. Two proteinases with apparent molecular weights of approximately 60KDa and 90KDa were more active in stationary-phase promastigotes than in mid-log phase cells and absent from amastigotes. There was also a proteinase with an apparent molecular weight of approximately 100KDa in stationary phase promastigotes which was apparently absent from mid-log phase promastigotes.

The pattern for L.major proteolytic enzymes was generally much simpler (Fig. 39). The amastigotes had a distinct proteinase activity with an apparent molecular weight of approximately 120KDa which was also present in metacyclic promastigotes although at lower activity. This enzyme was also present in mid-log phase promastigotes in variable amounts from apparent absence (Fig. 36) to present up to the amounts shown in Fig. 39, which was still lower than that in metacyclic promastigotes. The other proteinases present in amastigotes appeared to form a broad band of activity between apparent molecular weights 63 and 120KDa. To further characterise the proteolytic activities of L.major mid-log phase and metacyclic promastigotes, an

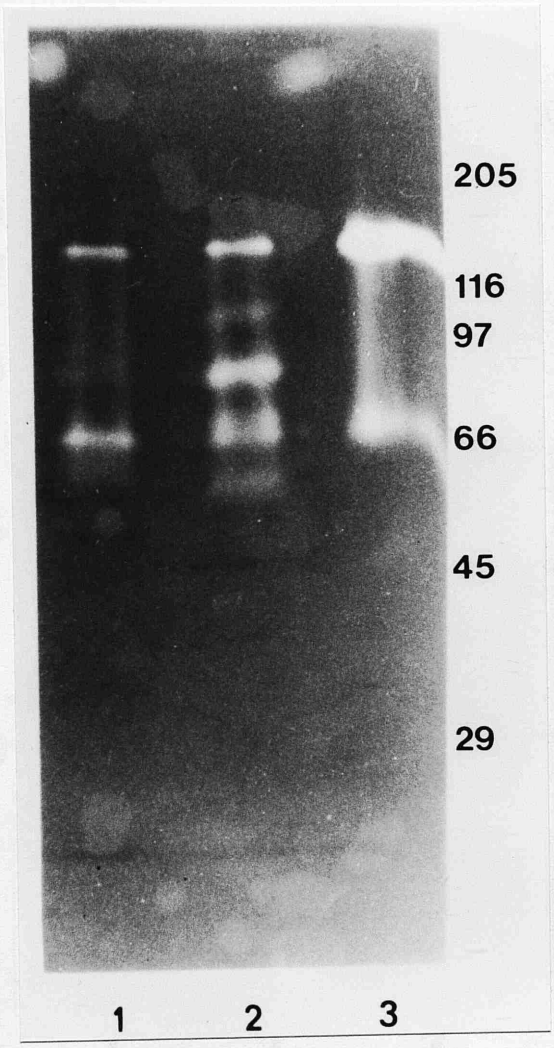


Fig. 38. Gelatin Disc-PAGE analysis of the proteinases of different forms of L.donovani.

Key to tracks: mid-log phase promastigotes, (1); stationary-phase promastigotes, (2); amastigotes, (3). The positions of the molecular weight markers (in KDa) are given on the right of the figure.

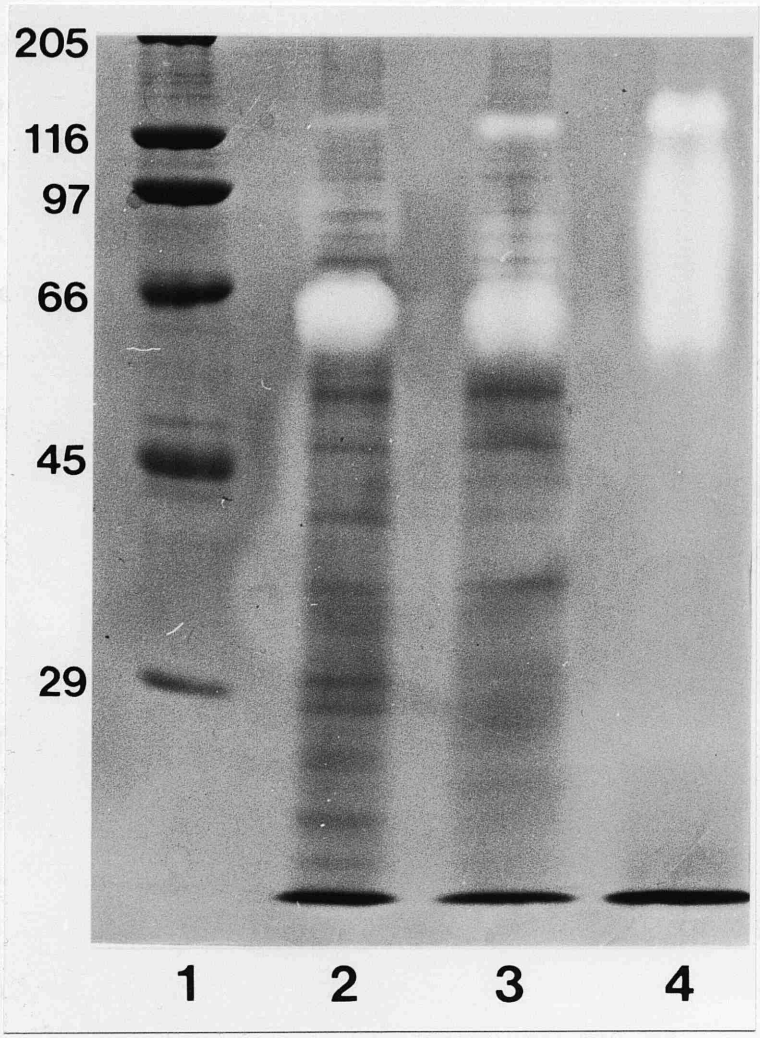


Fig. 39. Gelatin Disc-PAGE analysis of the proteinases of different forms of L.major.

Key to tracks: molecular weight markers (in KDA), (1); mid-log phase promastigotes, (2); metacyclic promastigotes, (3); amastigotes, (4).

experiment was carried out where homogenates of these two promastigote forms were centrifuged at 100,000 x g at 4°C for 1 hour and the resulting particulate and soluble fractions subject to electrophoresis. None of the proteinase activities present (see above), however, partitioned exclusively into either fraction (data not shown).

Interestingly, it was found that a number of proteinases, including the 63-68KDa proteinase common to all species were able to bind to conA conjugated to agarose. This is shown in Fig. 40 for the proteinases of L.mexicana mexicana stationary-phase promastigotes. The presence of unbound enzyme was probably due to saturation of the conA-agarose.

The proteinases of all samples apart from L.major amastigotes were characterised further by incubating the gels in buffer containing a series of proteinase inhibitors. The aspartic proteinase inhibitor pepstatin, the metalloproteinase inhibitor EDTA and the serine proteinase inhibitor phenylmethylsulphonylfluoride (PMSF) had no effect on any of the band patterns. Differential effects were observed with cysteine proteinase inhibitors. With L.mexicana mexicana, antipain and leupeptin inactivated the low molecular weight proteinases but not any of the other enzymes; this is shown in Fig. 41 for amastigotes. Neither of these compounds inhibited any of the proteinases of L.major or L.donovani (data not shown). Iodoacetic acid had no effect on the higher molecular weight enzymes in any of the species. Phenanthrolene, a metalloproteinase inhibitor which also inactivates many cysteine proteinases of protozoa (North, 1982), inhibited the higher molecular weight but not the lower molecular weight enzymes of L.mexicana mexicana (this is shown in Fig. 41 for amastigotes) and all of the proteinases of the other two species.

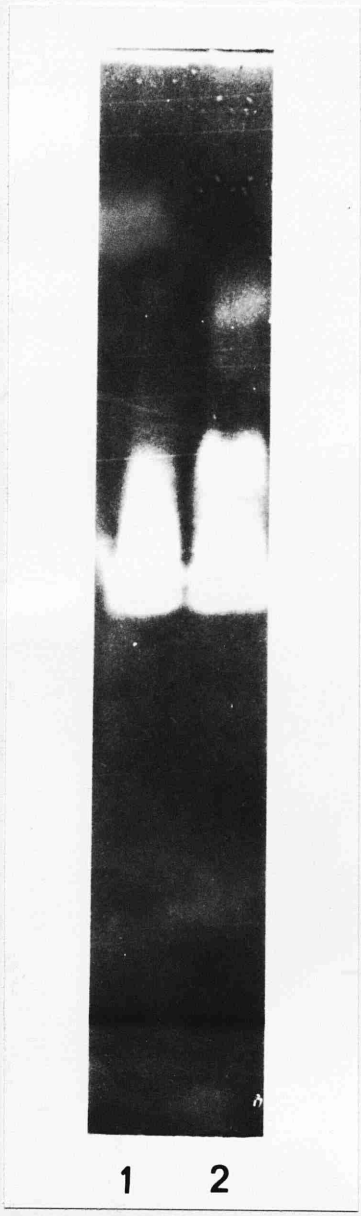


Fig. 40. Binding of leishmania proteinases to Concanavalin A-agarose.

A homogenate prepared from L.mexicana mexicana stationary-phase promastigotes was incubated with ConA-agarose. The bound and unbound fractions were analysed by Gelatin disc-PAGE. Key to tracks: proteinases eluted after binding to ConA-agarose, (1); non-binding proteinases, (2).

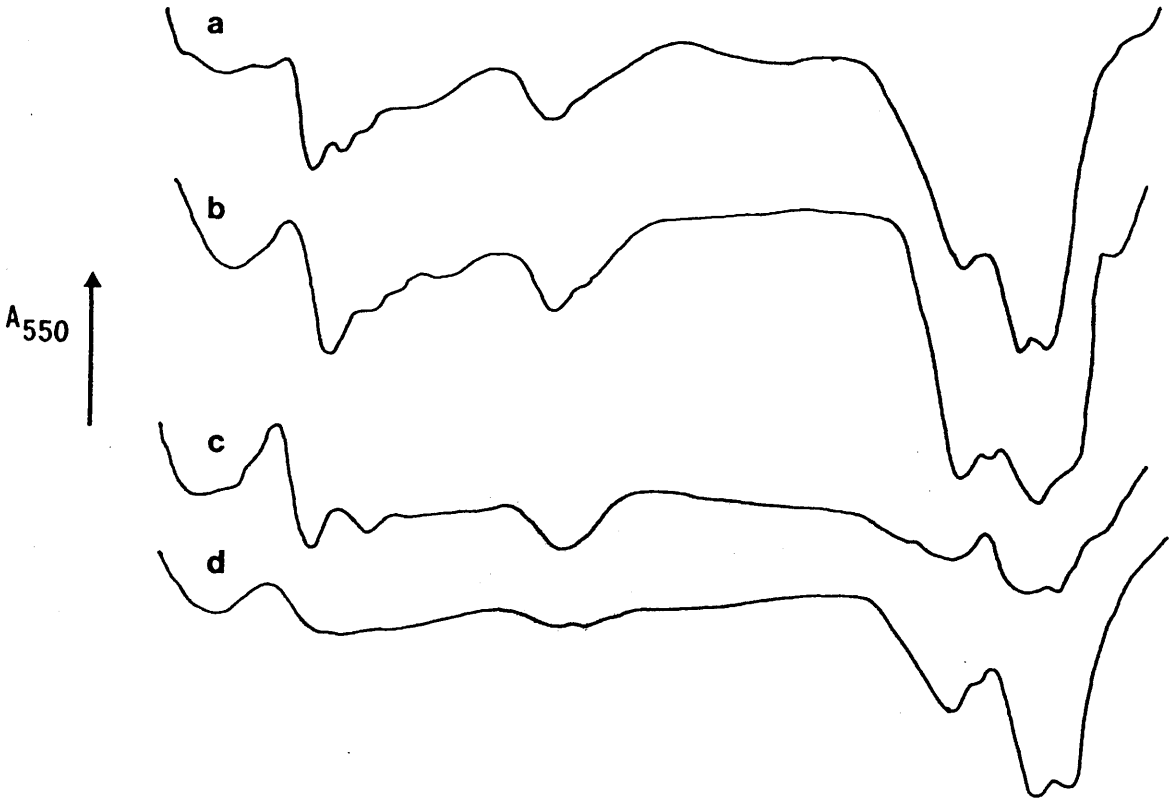


Fig. 41. Inhibition of L.mexicana mexicana amastigote proteinase activity.

Strips of gel were incubated in: buffer alone, (a) or buffer containing 1mM EDTA (b); 20 µg leupeptin ml^{-1} (c); 1 µm phenanthroline, (d). Densiometric scans of gels run from the cathode (left) to the anode (right) are shown.

3.7 CELL BIOLOGY OF THE DIFFERENT FORMS OF LEISHMANIAS

To further characterise the different forms of L.major and L.mexicana mexicana they were investigated in a number of other ways.

3.7.1 Electron microscope studies

Mid-log phase and stationary-phase populations and metacyclics of L.major, and mid-log phase and stationary-phase populations of L.mexicana mexicana were examined briefly using electron microscopy to see if differences in infectivity could be correlated with changes in ultrastructural features. Some apparent differences were seen when mid-log phase and stationary-phase promastigotes of L.mexicana mexicana were examined (Fig. 42). Stationary-phase promastigotes appeared to have more lipid inclusions than mid-log phase promastigotes although this apparent difference was not quantified. No other differences were apparent. Neither stationary phase nor mid-log phase promastigotes, however, possessed "megasomes".

Similarly, metacyclic promastigotes of L.major had more lipid inclusions than mid-log phase cells (Fig. 43). More interestingly, more detailed examination of these two promastigote forms using the electromicroscope (Fig. 44), revealed that metacyclic promastigotes and many but not all stationary-phase promastigotes possessed an irregular, finely grained "surface coat" which was present over their entire cell surfaces but which was absent from mid-log phase cells. The coat was absent at junctions between flagellum and flagellum pocket suggesting it was exogenous in origin, although repeated washing did not remove the coat.

5.7.2 Enzyme content

In an attempt to characterise the infective stages of the molecular level and to see what other pre-adaptations occur upon

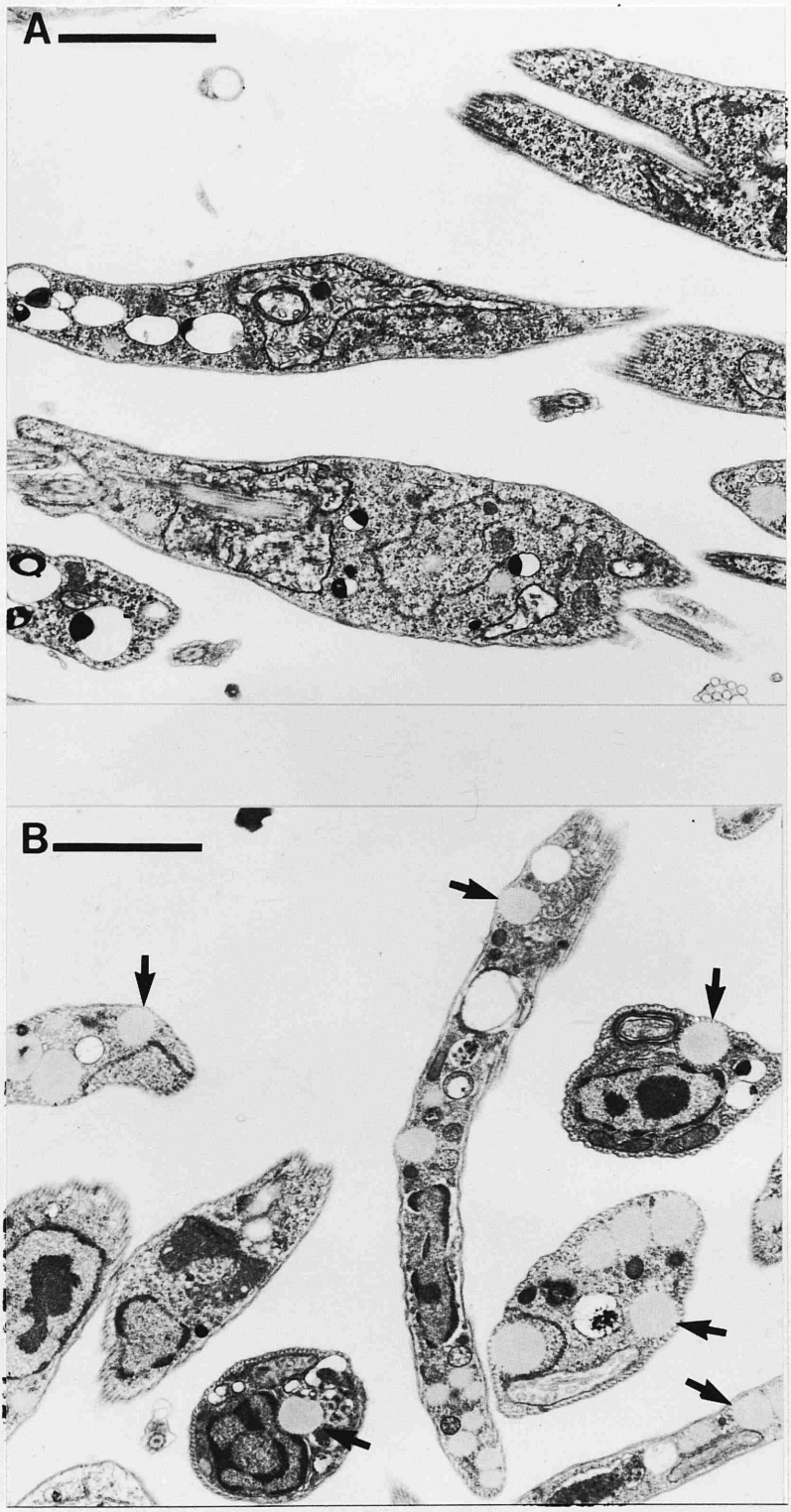


Fig. 42. Low power transmission electron micrographs of L.mexicana mexicana mid-log phase and stationary-phase promastigotes.

(A) Mid-log phase promastigotes. Scale bar = 2.0 μm.
(B) Stationary-phase promastigotes. Note the large number of lipid inclusions within the cells; an example is arrowed in each of the cells where they are present. Scale bar = 2.0 μm.

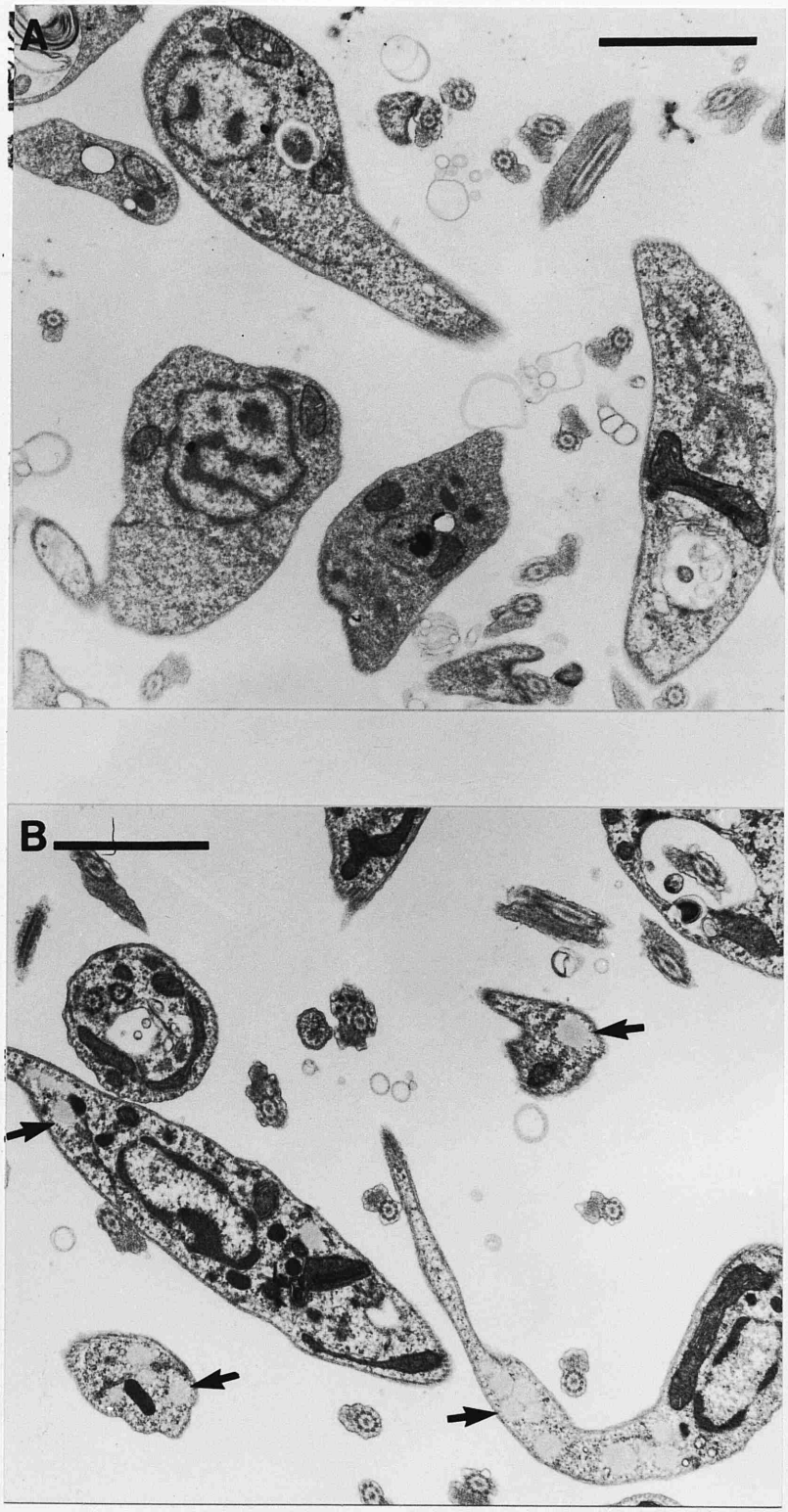


Fig. 43. Low power transmission electron micrographs of L.major mid-log phase and metacyclic promastigotes.

(A) Mid-log phase promastigotes. Scale bar = 2.0 μm.
(B) Metacyclic promastigotes. Note the large number of lipid inclusions within the cells; an example is arrowed in each of the cells where they are present. Scale bar = 2.0 μm.

Fig. 44. High power transmission electron micrographs of different promastigote forms of L.major showing the presence of the "surface coat".

(A and B) Metacyclic promastigotes. Note presence of the "surface coat" (arrows).

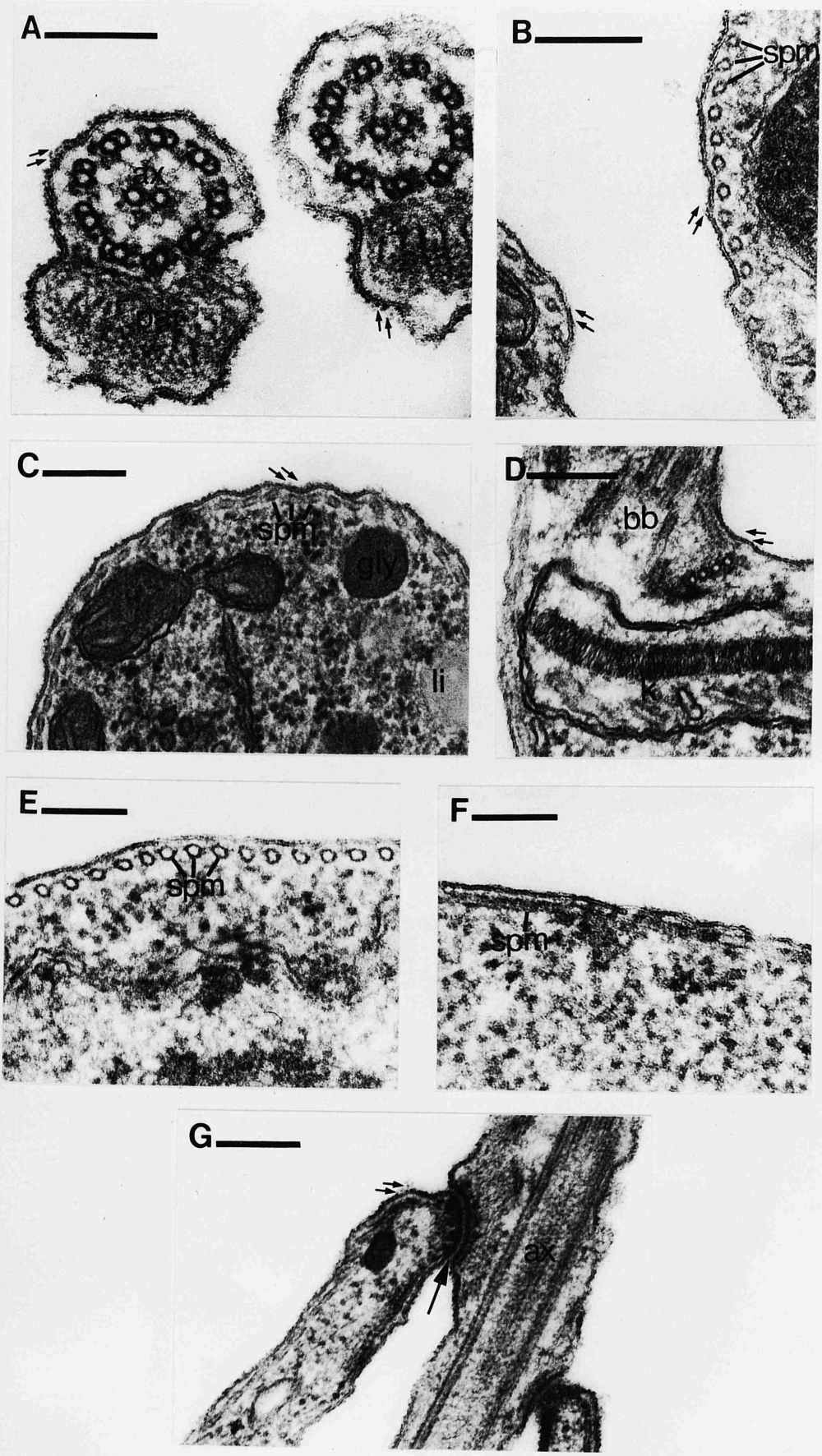
(C and D) Stationary-phase promastigotes. Note the presence (arrows, C) and absence (arrows, D) of the "surface coat".

(E and F) Mid-log phase promastigotes. Note the absence of the "surface coat".

(G) Stationary-phase promastigote. Note presence of the "surface coat" (small arrows), but absence of "surface coat" between junction of flagellum and flagellum pocket (large arrow).

All scale bars = 0.1 μm. Abbreviations: axoneme, (ax); basal body, (bb); glycosome, (gly); lipid inclusion, (li); nucleus, (n); paraxial rod, (par); subpellicular microtubules, (spm).

Fig. 44.



change from mid-log phase promastigotes to the more infective stationary-phase or metacyclic promastigotes, the levels of certain enzymes in the different forms of L.major and L.mexicana mexicana were determined. The enzymes selected for use in this study were all known to be at much higher or lower activity in mid-log phase promastigotes compared to amastigotes of L.mexicana mexicana (Mottram & Coombs, 1985b). Because of the difficulty in obtaining large numbers of pure L.major amastigotes there is little or no data concerning enzyme activities in these amastigotes and I have, for the purpose of this part of the study, presumed similar differences occur with this species. The results are given in Table 13.

In the studies with L.major, significant differences ($P<0.05$, Students t-test) in activity between mid-log and metacyclic promastigotes were only found with GDH and PK. Both were at less than half the activity in the metacyclic promastigotes compared to mid-log phase, which is consistent with the lower activities of these enzymes found in L.mexicana mexicana amastigotes. The other enzymes tested were equally active in both promastigote forms, suggesting that in L.major they are not involved in the pre-adaptations of metacyclic promastigotes and illustrating species differences in the requirements of this form. More of the enzymes of L.mexicana mexicana showed significant differences in levels between mid-log phase and stationary-phase cells. The results suggested that the stationary-phase promastigote populations were intermediate between mid-log phase promastigotes and amastigotes. Both ASAT and GDH were at lower activity in stationary phase promastigotes. More interestingly, however, some enzymes (MDH, PEP carboxykinase and proteinase) were at higher activities in stationary-phase promastigotes compared to mid-log phase promastigotes. These differences correlate well with the enzyme activities detected in the amastigotes.

Table 13: Enzyme activities[a] in different forms of L.mexicana mexicana and L.major.

Enzyme	L.mexicana mexicana			L.major	
	Mid-log phase promastigotes	Stationary-phase promastigotes	Amastigotes	Mid-log phase promastigotes	Metacyclic promastigotes
Alanine aminotransferase (ALAT)	285± 47 (3)	270± 39 (3)	52± 10 (5)	271± 40 (3)	244± 18 (3)
Aspartate aminotransferase (ASAT)	158± 18 (3)	61± 13 (c)[c]	23± 4 (4)	98± 13 (3)	90± 7 (3)
Glutamate dehydrogenase (GDH)	927±178 (3)	504±148 (3)[c]	122± 37 (3)	555±137 (5)	223± 48 (4)[c]
Malate dehydrogenase (MDH)	138± 57 (3)	434± 79 (3)[c]	986±280[b](3)	335± 97 (4)	489±108 (5)
Phosphoenolpyruvate carboxykinase (PEP carboxykinase)	2± 1 (3)	8± 2 (3)[c]	55± 19 (3)	3± 1 (3)	6± 3 (3)
Pyruvate kinase (PK)	54± 5 (3)	37± 1 (3)	17± 10 (3)	93± 13 (4)	41± 14 (4)[c]
Proteinase	4± 1 (3)	11± 2 (4)[c]	108± 15 (3)	ND	ND

[a] Specific activities are given in nmoles min^{-1} (mg protein)^{1}, except for proteinase activity which is in mU (mg protein)$^{-1}$. The results are the means (± standard deviation) from the number of experiments given in parentheses.

[b] Enzyme activity determined in the presence of 100 ug leupeptin ml^{-1}.

[c] Activity in stationary-phase or metacyclic promastigotes significantly differed from mid-log phase cells, $p < 0.05$.

ND, not determined.

3.7.3 Isoenzyme and protein profiles

To characterise the different forms with respect to isoenzyme and protein content, homogenates were subject to IEF and stained for protein or enzyme activities. The results presented are representative of the two or more (L.mexicana mexicana) or three (L.major) samples of each form that were studied. Differences are highlighted by arrows. No variation between samples was apparent. The results for L.major and L.mexicana mexicana are shown in Figs. 45 and 46, respectively.

Staining for total protein indicated both qualitative and quantitative differences between the protein profiles of mid-log phase and metacyclic promastigotes of L.major. There were protein bands that were present in one promastigote form but not the other, and also differences in the levels of certain protein bands present in the two promastigote forms. Not surprisingly, when enzymes were stained for there were distinct differences between the isoenzyme profiles of the two promastigote forms. Significant qualitative differences were seen with ASAT, GPI and GDH. Metacyclic promastigotes possessed activity bands with apparent pI's of approximately 5.2 for ASAT, 5.6 for GPI, and 6.4 for GDH) which were apparently not present in mid-log phase promastigotes. There were also qualitative differences found. Metacyclic promastigotes had two closely opposed G-6-PDH isoenzymes (between apparent pI's of 6.50-6.60), which were at higher activity than in mid-log phase cells. In contrast, an isoenzyme of GDH (at an apparent pI of approximately 5.8), isoenzymes of 6-PGDH at apparent pI's of approximately 4.7 and 5.2), PGM at apparent pI's of approximately 5.3 and 5.6) and to a lesser extent an isoenzyme of SOD (at an apparent pI of approximately 6.1) were at higher activity in mid-log phase promastigotes than in metacyclics. There were no apparent differences between either promastigote form for MDH.

Fig. 45. Isoenzyme and protein profiles of L.major mid-log phase and metacyclic promastigotes.

Key to tracks: mid-log phase promastigotes, (1); metacyclic promastigotes, (2). Enzymes: aspartate aminotransferase, (ASAT); glucose phosphate isomerase, (GPI); glutamate dehydrogenase, (GDH); glucose-6-phosphatase dehydrogenase, (G-6-PDH); 6-phosphogluconate dehydrogenase, (6-PGDH); phosphoglucomutase, (PGM); superoxide dismutase, (SOD); malate dehydrogenase, (MDH).

Closed arows indicate quantitative differences between the two promastigote forms, open arrows indicate qualitative differences.

Fig. 46. Isoenzyme profiles of different forms of L.mexicana mexicana. Key to tracks: mid-log phase promastigotes, (1); stationary-phase promastigotes, (2); amastigotes, (3). Enzymes: Abbreviations as in Fig. 45.

Closed arrows indicate quantitative differences between the different parasite forms, open arrows indicate qualitative differences.

Fig. 45.

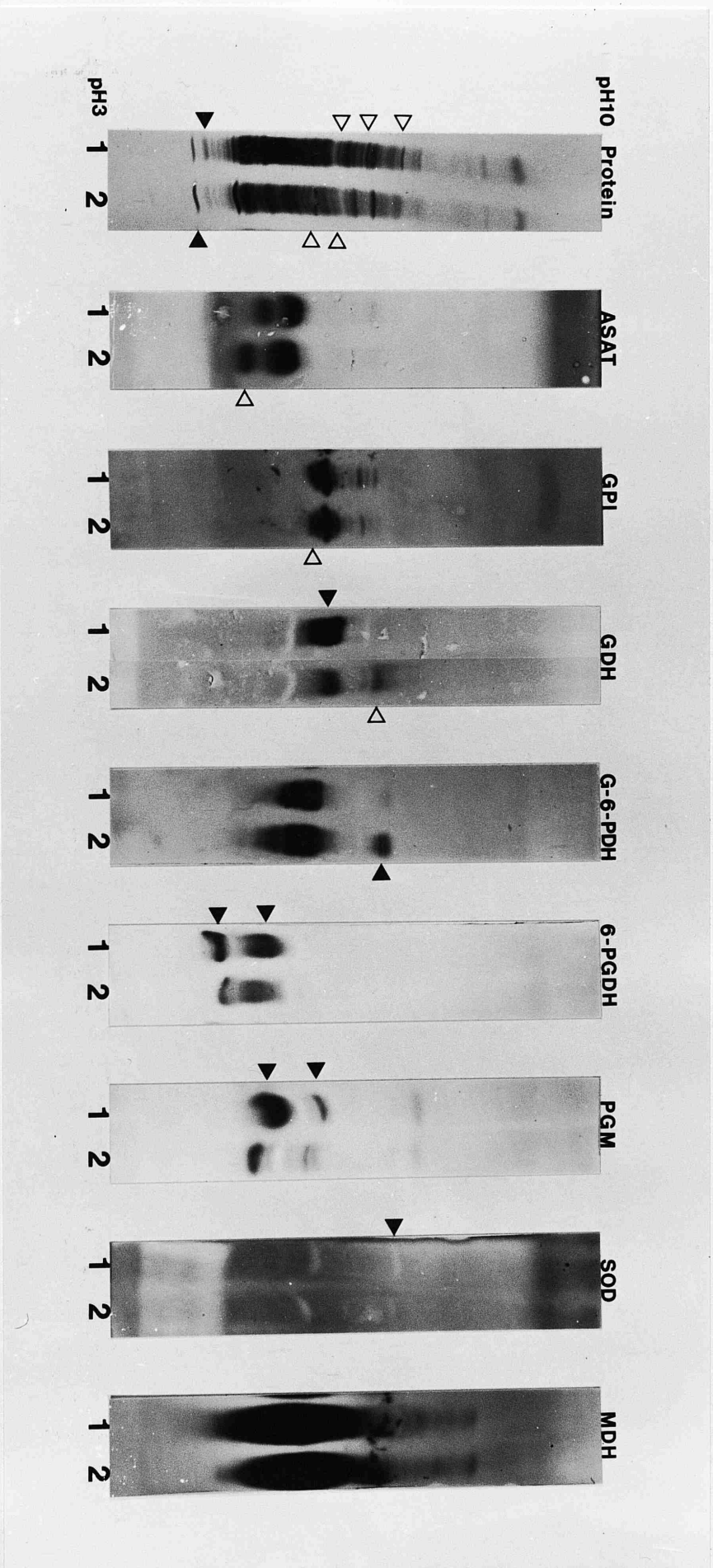
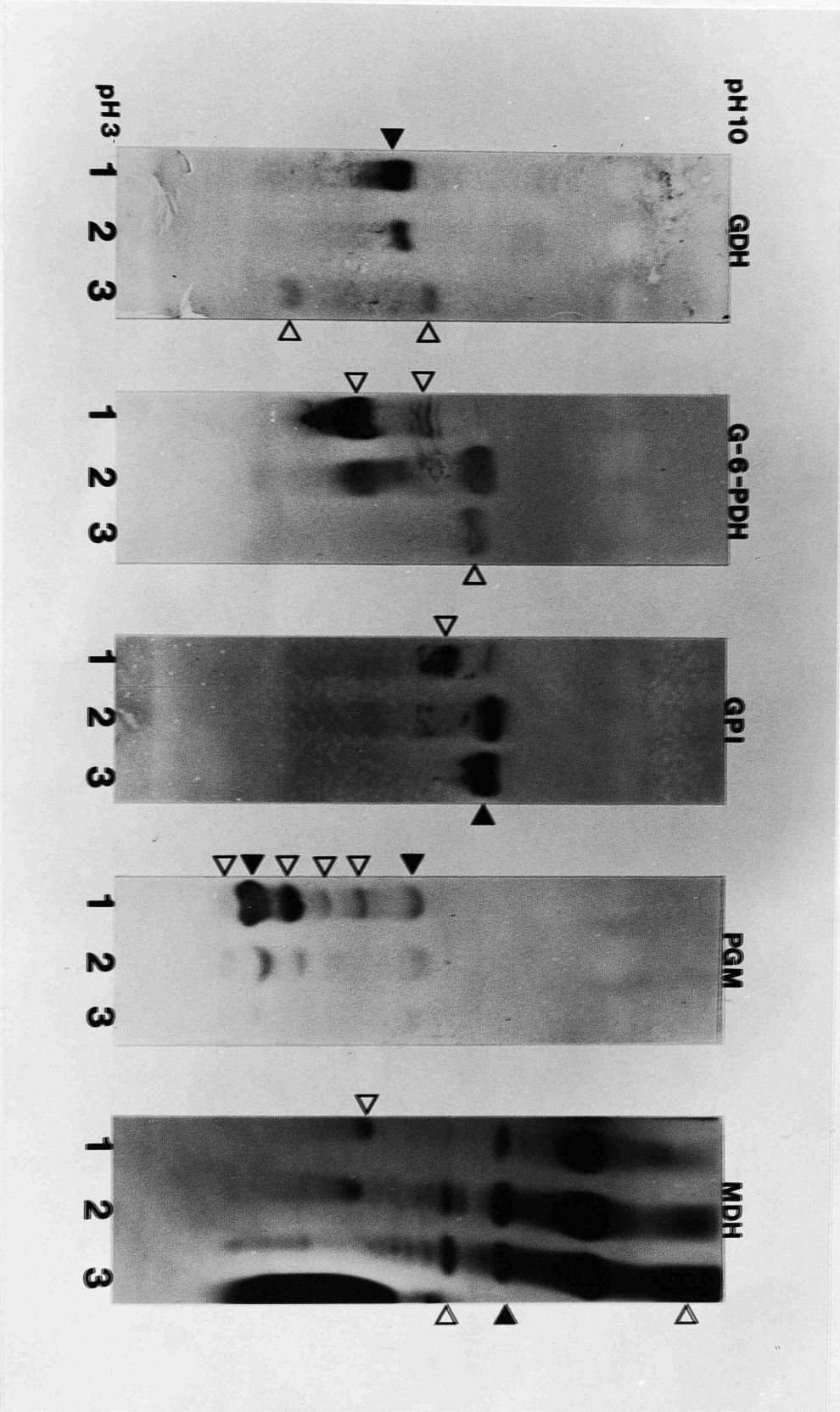


Fig. 46.



The occurrence of isoenzymes of some of these enzymes in the different forms of L.mexicana mexicana was also examined. All those studied showed significant differences in the profiles of the stationary-phase promastigotes compared to mid-log phase promastigotes and amastigotes. Mid-log phase and stationary-phase populations of promastigotes possessed a single isoenzyme of GDH which focussed at the same position (at an apparent pI of approximately 5.8), although this latter population had lower GDH activity confirming the results reported in Section 3.7.2 (see Table 13). In contrast, amastigotes had two isoenzymes of lower activity focussing in different positions (at apparent pI's of approximately 5.0 and 6.0) to those of the promastigotes. With all the other enzymes studied the stationary-phase promastigotes had isoenzyme profiles with charcteristics of both mid-log phase promastigotes, and also amastigotes. This is well demonstrated by the results with G-6-PDH. A band with an apparent pI of approximately 6.6 is present in amastigotes and also stationary-phase promastigotes, whereas there is little or no activity in the mid-log phase cells. In contrast, a broad band with an apparent pI of approximately 5.4 is present in mid-log phase and stationary-phase promastigotes, but apparently absent in amastigotes.

3.7.4 Amino acid content

The composition of the intracellular amino acid pools of different forms of L.major and L.mexicana mexicana were examined and the results are shown in Table 14. There were differences between levels in the various forms of both species. Proline and glutamic acid were at approximately at 2-fold higher concentration in L.major metacyclics compared to mid-log phase cells. In contrast, the concentrations of threonine, alanine, valine, methionine, isoleucine, lysine and arginine were 2 to 5-fold lower in L.major metacyclic

Table 14: The free amino acid[a] pool of different forms of L.mexicana mexicana and L.major.

Amino Acid[b]	L.mexicana mexicana			L.major	
	Mid-log phase promastigotes	Stationary-phase promastigotes	Amastigotes	Mid-log phase promastigotes	Metacyclic promastigotes
Threonine	16 ± 4	9 ± 3	10 ± 3	9 ± 1	5 ± 1
Serine	4 ± 1	10 ± 1	11 ± 5	7 ± 1	10 ± 2
Glutamic acid	24 ± 9	18 ± 5	34 ± 3	9 ± 1	25 ± 1
Proline	33 ± 5	17 ± 2	< 5	12 ± 1	23 ± 6
Glycine	29 ± 5	16 ± 1	13 ± 1	54 ± 3	21 ± 3
Alanine	129 ± 26	69 ± 3	58 ± 7	233 ± 7	102 ± 8
Valine	31 ± 3	10 ± 1	13 ± 1	22 ± 2	4 ± 1
Methionine	10 ± 1	7 ± 2	15 ± 11	15 ± 1	5 ± 1
Isoleucine	21 ± 2	4 ± 1	< 8	12 ± 1	< 3
Leucine	18 ± 2	7 ± 1	13 ± 8	9 ± 1	4 ± 1
Tyrosine	2 ± 1	2 ± 1	3 ± 2	< 2	< 2
Phenylalanine	3 ± 1	5 ± 1	3 ± 3	< 1	< 1
Histidine	11 ± 3	2 ± 1	9 ± 1	1 ± 1	2 ± 1
Lysine	75 ± 28	17 ± 1	34 ± 1	28 ± 2	12 ± 3
Arginine	95 ± 30	10 ± 1	12 ± 7	39 ± 1	9 ± 2

[a] The results are given in nmoles (mg protein)$^{-1}$ and are the means (± range) from two independent determinations.

[b] For amino acids which were detected in one sample but not another the levels are given as being less than the amount detected in the one. Amino acids not present (aspartic acid, cysteic acid, cysteine) were below the limit of detection (0.1 nmole).

promastigotes compared to mid-log phase promastigotes. Similarly, in L.mexicana mexicana proline, glycine, alanine, valine, isoleucine, lysine, and arginine were 2 to 8-fold lower in amastigotes compared to mid-log phase promastigotes; levels in stationary-phase promastigotes were either intermediate between mid-log phase promastigotes and amastigotes (e.g. proline), or more similar to that in amastigotes (e.g. valine). There were also differences between the concentrations of amino acids in mid-log phase promastigotes of the two species. Levels of isoleucine, arginine, lysine, and glutamic acid were approximately 2-fold higher in L.mexicana mexicana mid-log promastigotes compared to L.major mid-log phase cells, whereas alanine and glycine were 2-fold lower. It is noteworthy that alanine was the most abundant amino acid in all forms of both species.

3.7.5 Respiration studies

A characteristic of L.major metacyclic promastigotes is that they appear to move more rapidly than mid-log phase cells. Experiments were performed to determine if this is reflected in differences between respiration rates. The results are shown in Table 15.

The respiration rates for mid-log phase promastigotes were much more variable than for metacyclic promastigotes making interpretation of the data difficult. Therefore, the data for respiration (when expressed in a per cell basis or when related to cell protein) were analysed using a 2-way analysis variance test. The outcome was the same whether respriration was expressed on a per cell basis or related to cell protein. The endogenous and stimulated respiration rates for mid-log phase cells were significantly higher ($P<0.05$) than those for metacyclic promastigotes, and interestingly the stimulated rates for both promastigote forms were significantly different ($P<0.05$) from their respective endogenous rates, although the extent of the

Table 15: Respiration rates of mid-log phase and metacyclic promastigotes of L.major.

	Rates	
	Mid-log phase promastigotes	Metacyclic promastigotes
Endogenous rate		
nmole O_2 $min^{-1}(10^8$ cells$)^{-1}$	25±10 (4)	10± 1 (4)
nmole O_2 min^{-1}(mg protein)$^{-1}$	21± 8 (4)	17± 2 (4)
Stimulated rate		
nmole O_2 $min^{-1}(10^8$ cells$)^{-1}$	51±21 (4)	14± 2 (4)
nmole O_2 min^{-1}(mg protein)$^{-1}$	44±18 (4)	23± 4 (4)

[a] The results are the means (± standard deviation) from the number of experiments given in parentheses.

Promastigotes resuspended in PBS, pH 7.3.

Promastigotes resuspended in HOMEM medium with 10% (V/V) HIFCS.

Table 16: The protein content of mid-log phase and metacyclic promastigotes of L.major.

	Mid-log phase promastigotes	Metacyclic promastigotes
Protein content[a] $\mu g(4x10^7$ cells)	470±81 (14)	239±80 (13)

[a] The results are the means (± standard deviation) from the number of experiments given in parentheses.

Table 17: Nitroblue tetrazolium reduction by mid-log phase and metacyclic promastigoes of *L.major*[a].

	Mid-log phase promastigotes	Metacyclic promastigotes
nmoles $60min^{-1}$ $(10^8$ cells$)^{-1}$	294±66 (5)	68±40 (5)
nmoles $60min^{-1}$ (mg protein)$^{-1}$	255±59 (5)	114±67 (5)

[a] The results are the means (± standard deviation) from the number of experiments given in parentheses.

stimulation for mid-log phase promastigotes was not significantly different ($P>0.05$) from metacyclic promastigotes.

The protein content and rates of NBT reduction were also measured for the two forms and the results are shown in Tables 16 and 17, respectively. Metacyclics had only approximately half the protein content of mid-log phase cells. Similarly, there were differences with respect to NBT reduction. On a cell to cell basis, the rate of reduction by metacyclic promastigotes was some 4-fold less than that for mid-log phase cells, and when related to cell protein there was still more than a 2-fold difference.

3.7.6 Transformation of leishmanial forms

The transformation of amastigotes to promastigotes, and the change of mid-log phase promastigotes to metacyclic promastigotes and vice versa were studied in a variety of ways. In particular it was hoped to ascertain whether the change of mid-log phase promastigotes to metacyclics was a true transformation similar to that occurring when promastigotes change to amastigotes.

Amastigote to promastigote transformation was investigated using: 3-MB, an inhibitor of adenosine diphosphate ribosyl transferase (ADPRT) and a known inhibitor of differentiation in related parasite systems (Williams, 1983, 1984): and hydroxyurea, an inhibitor of mitosis and an inhibitor of transformation of L.mexicana mexicana amastigotes to promastigotes (Hart et al., 1981c). The results are shown in Table 18. The effect of 3-MB on the production of L.major metacyclics was also examined, and the results are shown in Table 19. 10 mM hydroxyurea inhibited transformation of L.major and L.donovani amastigotes to promastigotes by over 90%. The transformation of L.mexicana mexicana and L.donovani was also inhibited by 3-MB. The effect was concentration dependent with 2.5 mM 3-MB inhibiting

Table 18: The effects of inhibitors on the transformation of amastigotes to promastigotes in different species of *Leishmania*[a].

Inhibitor	Concentration (mM)	*L.mexicana mexicana*	*L.donovani*	*L.major*
3-Methoxybenzamide	2.5	62 (1)	81 (1)	ND
	1.25	62 (1)	ND	-52±35 (3)
	0.625	19 (1)	33 (1)	-11± 5 (3)
	0.3125	2 (1)	11 (1)	ND
Hydroxyurea	10	ND	99 (1)	96± 3 (3)

[a] The results are given as % inhibition of transformation which was calculated from the reduction in the number of promastigotes compared to control and are the values or means (± range or standard deviation) from the number of experiments given in parentheses. Negative figures indicate there was a stimulation. In the controls 26% of *L.mexicana mexicana*, 23% of *L.donovani* and 51±3% (mean ± standard deviation) of *L.major* amastigotes transformed to promastigotes over the period of incubation.

ND, not done.

Table 19: Effect of 3-methoxybenzamide on the production of metacyclics during *in vitro* culture.

3-Methoxybenzamide concentration (mM)	Metacyclic production as a % of the control[a]
1.25	110±34 (4)
0.3	102±28 (2)

[a] Treated and untreated (control) stationary phase cultures were agglutinated with 100 ug PNA ml^{-1} and the number of free promastigotes in treated cultures expressed as a % of the number in the control. The results are the means (± range or standard deviation) from the number of experiments given in parentheses.

greatly. With L.mexicana mexicana a similar inhibition was soon with 3-MB at the lower concentration of 1.25 mM. In contrast, this concentration did not inhibit the transformation of L.major amastigotes to promastigotes and indeed appeared to have a stimulatory effect (Table a). Similarly, 3-MB had no effect on the production of metacyclics during in vitro culture.

To determine if the metacyclics of L.major could divide and/or change back to "mid-log phase" promastigotes, populations of the two promastigote forms were incubated in standard HOMEM medium and their numbers and morphology monitored over time. The results are shown in Fig. 47. Mid-log phase promastigotes multiplied rapidly and grew almost logrithmically for 72 hours, whereupon the division rate decreased and the numbers began to stabilise as the culture went into stationary-phase. In contrast, there was little or no multiplication of the metacyclic promastigotes over the first 24 hours in culture; there was a definite and reproducible lag-phase where apparently no division occurred. After 24 hours the promastigotes still had the characteristic morphology of metacyclics (data not shown). Cell numbers increased during the next 24 hours, however, and by 48 hours the cells had typical mid-log phase morphology (data not shown). Subsequently, the cell numbers changed in very similar way to the mid-log phase initiated cultures. A similar, but less pronounced, lag-phase was also observed when stationary-phase promastigotes of L.mexicana mexicana were used to initiate cultures although this was not quantified.

In an attempt to discover the trigger for the production of metacyclics, I studied the effect of suspending L.major mid-log phase promastigotes in "spent" medium taken from stationary-phase cultures of L.major or in fresh HOMEM standard medium. The results from a representative experiment are shown in Fig. 48. The starting

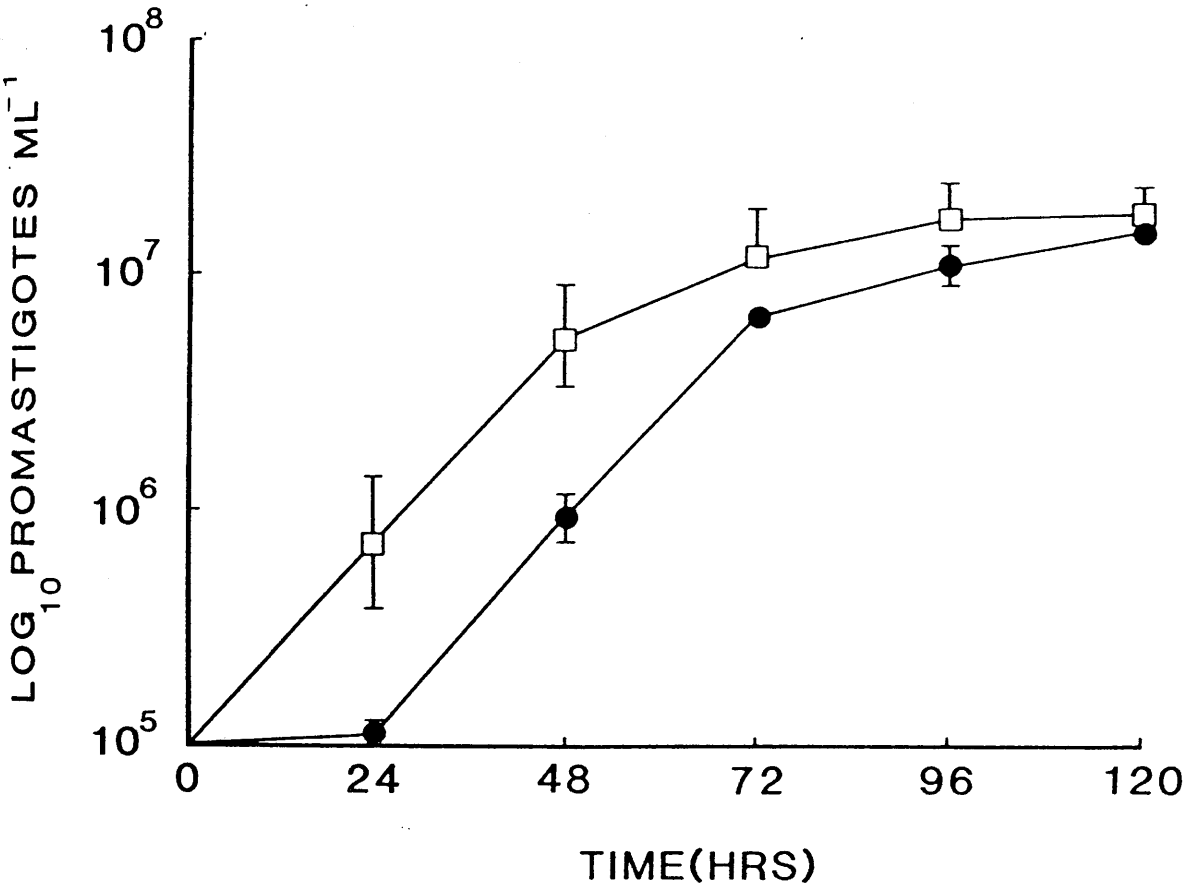


Fig. 47. Growth *in vitro* of *L.major* mid-log phase and metacyclic promastigotes.

Mid-log phase promastigotes,(□); metacyclic promastigotes,(●). Each point represents the mean (± standard deviation) from three experiments.

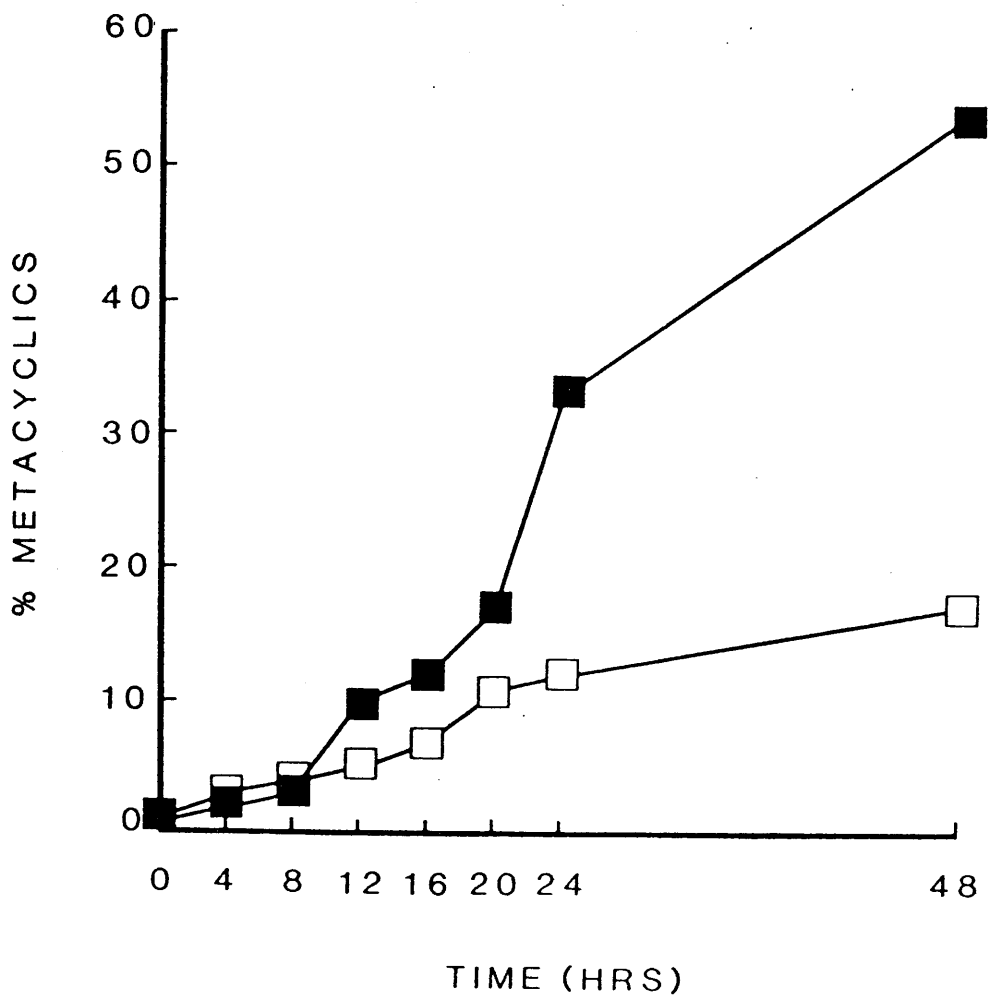


Fig. 48. The effect of "spent" and fresh medium on the production of L.major metacyclic from mid-log phase promastigotes.

"Spent" medium, (■); fresh medium, (□). The data presented are from one experiment which is representative of the two performed.

population of mid-log phase promastigotes contained 1% metacyclics as judged by morphology, and 6% determined by agglutination with PNA. The numbers of metacyclics (as judged by morphology) in both cultures steadily increased and similarly up to 8 hours. Subsequently, the rate of transformation to metacyclics began to increase more rapidly in the "spent" medium cultures. After 24 hours, 33% (by morphology) and 27% (by agglutination) of the cells had transformed to metacyclics in the "spent" medium culture, whereas in the fresh medium culture only 12% (by morphology) and 14% (by agglutination) of the cells had similarly transformed. The changes continued over the next 24 hours, such that by 48 hours the fresh medium culture contained 17% metacyclics and the "spent" medium culture contained 54% metacyclics, a similar number to the maximum produced in stationary-phase culture (see Section 3.1.1).

4.0 DISCUSSION

4.1 METACYCLIC PROMASTIGOTES

4.1.1 Metacyclics in cultures of leishmanias

My results regarding the agglutination of different promastigote populations of L.major with PNA (Fig. 6) are very similar to, and essentially confirm, those of Sacks et al (1985) using cloned L.major promastigotes. This indicates that the production of PNA (metacyclic) population in stationary-phase cultures is a general feature of L.major promastigotes.

The metacyclic forms of L.major that were produced in my cultures had characteristic morphology and were very motile. Sacks et al (1985) also observed both these characteristics and the morphology of these in vitro forms is very similar to that of the forms found in the probosis and anterior part of the alimentary canal of sandflies (Killick-Kendrick, 1986). Although the metacyclic promastigotes appeared more "active" than mid-log phase cells, there was no difference between the two forms with respect to the % "fast" (assessed as the flagellum appearing as a blur) or a % motile this suggested that the metacyclics were moving overall more quickly or also perhaps changing direction more than the mid-log phase cells, thus giving the impression of being more active. To be sure, however, quantitative studies of movement of the two forms are required.

An interesting difference between my results and these of Sacks et al (1985) is that these workers reported that 100% of log-phase cells were agglutinated by 100 μg PNA ml^{-1}. I found consistently that approximately 5% of the population were unagglutinated. This difference may reflect the methodology used in the two studies. In particular, the agglutination assay I used was less sensitive than that of Sacks et al (1985). Nevertheless, the consistency of my results, and the fact that assessing the % metacyclics by a different

method (by morphology) also suggested that there was a low percentage of metacyclics consistently present, indicates that the differences were real and is probably explained by innate differences between the two strains; metacyclics being produced earlier with mine. This finding may also be important in explaining discrepancies between my results and those of Sacks et al (1985) regarding infectivity of the different forms (see section 4.2).

Forms similar morphologically to the metacyclics of L.major were consistently seen during my studies at low numbers in mid-log phase and high numbers in stationary-phase populations of L.mexicana mexicana. It is not certain that these forms represent the infective metacyclic form (which apparently occurred as indicated by infectivity, see section 4.2) of this species. The similarity of these forms, however, in terms of morphology, and numbers present in different populations, in comparison to L.major would suggest that this is likely. There is circumstantial evidence supporting this conclusion, most notably their morphology. These forms are very similar morphologically to those reported to occur in the probosis and anterior foregut of Leishmania-infected sand flies (Adler & Theodor, 1931; Hertig & McConnel, 1963; Laison et al., 1977; Killick-Kendrick, 1979; 1986; Warburg et al., 1986), in the fluid seeping from a fresh bite (Adler & Ber, 1941) and in the fluid of force-fed and membrane-fed sand flies (Adler & Theodor, 1929; Adler & Theodor, 1931; Warburg & Schlein, 1986). These forms have been seen with L.tropica, L.major, L.donovani, L.infantum,L.chagasi, and members of the L.braziliensis and L.mexicana complexes, although the only detailed study of L.mexicana mexicana in sandflies reported no such forms (Walters et al., 1987). The occurence of this specific morphological form in vitro cultures of the various species has not been well documented. Berbian (1939) reported the presence of different morphological forms of L.

tropica in NNN medium after 8 days, the majority being " short infective metacyclics", and as already discussed Sacks et al (1985) reported the occurrence of the characteristic metacyclics in stationary-phase cultures of L.major. With L.donovani (Keithly & Bienen, 1981) and L.chagasi (Rizvi, pers comm.), however, analysis of the morphological forms present in log-phase and stationary-phase populations did not reveal the occurence of a specific morphological form in the latter population. Interestingly, Greenblatt et al (1985) measuring cell body length and size distribution in log-phase and stationary-phase cultures of a virulent clone of L.major did not detect a particular size of promastigote that was more prominent in the stationary-phase population,, although there was a shift to smaller size with time in culture. Further, some avirulent clones had the same size distribution as the virulent clones, while others were different. When the sizes of the cell of L.donovani a virulent (high sub-passage) and virulent (low sub-passage) promastigotes were measured variable results were produced. For example, Katakura (1986) found no difference in size, whereas Nolan and Herman (1985) reported that virulent promastigotes were slightly shorter than the avirulent cells. Thus there seems little concensus on the occurrence of forms of typical "metacyclic" morphology in cultures of different species; in my experience, however, such forms are present in cultures of L.mexicana mexicana as well as the better characterised L.major. Confirmatory evidence that these forms are truly metacyclics awaits the development of a method of purifying them. Nevertheless, information obtained concerning their infectivity, isoenzyme and enzyme content and free amino acid pool composition suggest that they are (see below).

The % metacyclics occurring in stationary-phase cultures has only

been examined in a few studies. My results, based on morphology (L.mexicana mexicana, L.major) and agglutination with PNA (L.major) suggested that mid-log phase populations of both L.major and L.mexicana mexicana contained low numbers of metacyclics (approximately 5%), and that the stationary-phase populations contained approximately 50% metacyclics. Sacks et al (1985) reported the presence of about 50% metacyclics in stationary-phase populations in a cloned strain of L.major but none in log-phase populations (by agglutination with PNA). The numbers in other species in stationary-phase populations appear to be lower: Doran and Herman (1981) calculated from infectivity studies determining parasite burdens, that approximately 2% of promastigotes from 3-day-old cultures, and 15% from 10-day-old promastigotes from stationary-phase cultures became established as amastigotes. Interestingly, Franke et al (1985) found from serum sensitivity studies that about 10% of L.donovani stationary-phase promastigotes were metacyclics and that L.braziliensis panamensis stationary-phase populations contained approximately 30% metacyclics. With neither of these species were metacyclics detected in log-phase cultures. The apparent variability in numbers of metacyclics between species may reflect differences in the methods of detecting them and growth conditions, as well as real species differences (see Section 4.1.2, for fuller discussion on the production of metacyclics).

The use of PNA to purify metacyclics of L.major was very successful and formed the basic method in my study of this form. My attempts at purifying metacyclics/morphological subpopulations of L.mexicana mexicana using similar methods were unsuccessful. My studies using lectins and CatF suggested that only sugar residues accessible on the surface of L.mexicana mexicana promastigotes, both from mid-log and stationary-phase cultures, were similar to

glucose/mannose and that the cells have a negative charge. Other workers have reported that L.mexicana promastigotes have other sugar residues on their surface, including β-D-galactose (binds to RCA) and possibly N-acetyl-D-galactosamine (binds to SBA) (Jacobson et al., 1982). Similarly, Russell and Wilhelm (1986) using stationary-phase promastigotes of the same strain of L.mexicana mexicana as this study, could detect in two-dimensional gels of radiolabelled surface proteins sugars that bind to ConA (glucose/mannose), Helix pomatia lectin (N-acetylgalactose) and WGA (N-acetylglucosamine). All these were present on the major surface glycoproteins of apparent molecular weight 63KDa. It is likely that the additional binding obtained by these workers was due to the use of partially purified membrane preparations which had residues exposed which are not normally accessible on intact promastigotes. However, labelling intact promastigotes with galactose via galactosyl transferase indicated that at least N-acetylglucosamine residues were present on the surface and normally exposed. This method has much greater sensitivity than simply measuring agglutination, indeed it was possible that there are insufficient N-acetyglucosamine residues to cause agglutination. Stokes (1984) did report, however, from lectin-mediated agglutination studies that L.mexicana mexicana promastigotes had galactose and mannose residues on their surface. Also, Gueugnot et al (1985) using a wide range of lectins found that L.mexicana mexicana promastigotes had glucose/mannose, B-galactose-lactose, N-acetyl-D-galactosamine and lactose-like residues on their surface. Similarly, lactose-like residues have been detected on L.mexicana mexicana promastigotes using Ulex europeaus II-lectin (Greenblatt et al., 1984). The reasons for the differences from my studies are unclear, although they probably result from differences in methodology including the lectins used and the different strains used;

quantitative and qualitative variations in the binding of lectins (and therefore presence of sugars) to different strains of the same species have been noted (Jacobson et al., 1982; Greenblatt et al., 1984; Gueugnot et al., 1985). Different media were also used and this apparently can have an effect on lectin-binding (Stephenson, pers. comm.). Nevertheless, from the positive point of view the sugar residues found to be present on mid-log and stationary-phase L.mexicana mexicana in this study are the same as those which have consistently been found to be present on promastigotes regardless of species or strain (see Introduction section 1.2.2.3 for details and references).

My results suggest that L.mexicana mexicana promastigotes differ from L.major in that no difference was detected between the sugar residues on mid-log phase and the more infective stationary-phase promastigotes. In contrast, infective stationary-phase cultures of L.braziliensis panamensis were found to possess more ConA and LCA-binding molecules on their surfaces that promastigotes from essentially non-infective log-phase cultures (Grogl et al., 1987). Similarly, Doran and Herman (1981) demonstrated that 3-day-old promastigotes showed greater agglutination with RCA_{60} (binds to β-D-galactose, and N-acety-D-galactosamine) than the more infective 10-day-old promastigotes. Differences in carbohydrate residues between avirulent and virulent strains of L.braziliensis (Ayesta et al., 1985), L.mexicana amazonensis (Saraiva et al., 1986) and L.tropica (Ebrahimzadeh & Jones, 1983) have also been reported, although Handman et al (1983) could find no difference in lectin-binding of parental promastigotes and avirulent and virulent clones isolated from it. Interestingly, differences in the number of surface mannose and galactose residues between amastigotes and promastigotes of L.mexicana mexicana have been reported (Stokes, 1984) and qualitative and

quantitative differences in lectin-binding between promastigotes and amastigotes of L.mexicana amazonensis (Saraiva et al., 1986) and L.donovani (Wilson & Pearson, 1984) have also been described. Thus it appears fairly common that different parasite forms possess different sugar residues and that there are also species differences.

The differences reported, for instance for L.braziliensis panamensis, may well provide the basis of a method for purifying metacyclics, similar to the one developed for L.major. Unfortunately, however, a lectin that could be used in this way for L.mexicana mexicana has not yet been found. Further studies using sensitive methods for assessing binding may help to identify dfferential binding between forms than can be exploited.

Attempts at separating the different morphological forms apparently present in stationary-phase populations of L.mexicana mexicana using density-gradient centrifugation were also unsuccessful, the bands separated containing mixed populations with respect to morphology. It is possible, however, that by further manipulation of experimental conditions, that density-gradient centrifugation could be used to separate out the different morphological forms as has been achieved for broad and slender trypomastigotes of Trypanosoma cruzi (Schmatz et al., 1983).

4.1.2 Transformation studies

My results from investigating the effect of inhibitors on the transformation of amastigotes to promastigotes showed that 3-MB, an inhibitor of ADPRT, prevented transformation of L.mexicana mexicana as previously reported (Capaldo, 1984), and also L.donovani confirming that it has a widespread effect as an inhibitor of differentiation (Williams, 1983, 1984). An interesting finding, however, was that it had no such effect on L.major transformation, suggesting that

transformation of this species involves different processes from the other two species. However, hydroxyurea did prevent the transformation of L.donovani, and L.major, similar to that reported for L.mexicana mexicana (Hart et al., 1981c), showing that, as has been described in the sandfly (Shortt et al., 1926; Strangways-Dixon & Laison, 1966; Warburg et al., 1986), division is an integral part of the transformation of amastigotes to promastigotes.

3-MB had apparently no effect on the production of L.major metacyclic promastigotes. This became less surprising when I subsequently found that it had no effect on amastigote to promastigote transformation in this species. However, it then became clear that the use of this compound would not provide an answer as to whether the change from mid-log phase promastigotes to metacyclic promastigotes was a true transformation. The metacyclics of L.major apparently can change back into "mid-log phase" promastigotes, although this appears to take between 24 and 48 hours to occur (see Fig. 47). Although there is a possibility that the dividing promastigotes in metacyclic initiated cultures could have resulted from contamination of the metacyclics with PNA^+ ("mid-log phase") promastigotes, it would seem unlikely, as this effect was reproducible (whereas the number of PNA^+ promastigotes probably would not have been), and the lag period before dividing promastigotes predominated should have been much greater if they had arisen from a few contaminating PNA^+ promastigotes that had the "normal" doubling time. This does not, however, exclude the possibility that only some of the metacyclics were changing back into "mid-log phase" promastigotes although the apparent absence of metacyclics after 48 hours suggests that this was not the case. A distinct lag period was also noted when stationary-phase cultures of L.mexicana mexicana were used to initiate a fresh culture whereas this

was not apparent when mid-log phase cells were used. Similar results have also been reported with 6 other sub-species or species, including L.major and L.mexicana mexicana (Mosser et al., 1986). There is no firm information regarding whether metacyclic promastigotes can change back into non-metacyclics. Similar to my results with purified metacyclics of L.major, Adler and Theodor (1929) found that metacyclic promastigotes of L.tropica resulting from the deposition into cultures medium during the feeding of P.sergenti through an artificial membrane, could apparently produce positive culture although the numbers were not monitored with time. The findings of Giannini (1974) with L.donovani differ a little. She showed that promastigotes subcultured repeatedly from stationary-phase cells declined in infectivity whereas those subcultured from log-phase did not, suggesting that the metacyclic promastigotes were progressively lost rather than changing back into "log-phase" cells. Whether this reflects true species differences or just differences in methodology and conditions, such as culture medium, is not known at present.

Rather like is known about the factors involved in triggering the change into metacyclic promastigotes. My results using fresh and "spent" medium suggest, that the presumably nutrient-depleted environment of "spent" medium triggers the production over 48 hours of more metacyclics (up to levels that occur in stationary-phase cultures) than fresh, nutrient rich, medium (see Fig. 48). The slight increase in metacyclics occurring in fresh medium cultures was probably due to the use of high initial cell densities (1×10^7 cells ml^{-1}) which was required to give the necessary numbers for the agglutination method. This number of parasites would be likely to deplete nutrients from the medium rapidly and this could trigger the production of some metacyclics. The majority of the change to metacyclics appeared to occur between 20-48 hours. Whether it is

possible, by increasing the incubation time beyond 48 hours to produce populations that were all metacyclics is not known, as the experiments were terminated after this time. However, results from examining the numbers of metacyclics in stationary-phase cultures (see Table 6A) indicate that this is unlikely using this system. Sacks and Perkins (1985) reported that suspension of log-phase promastigotes in "spent" medium for 4-6 hours was sufficient to generate metacyclics although no data on numbers were given. In my system little or no metacyclic production was triggered over this time period. These differences could be due to different numbers of promastigotes being suspended although this is not possible to judge in the absence of more detailed information. The key ingredients of "spent" medium involved in the triggering of metacyclic production have not been identified although it is likely that factors other than nutrients may be involved. Changes in pH and the build-up of excretion products are clear candidates. The findings with L.major in P.papatasi, however, that the most infective promastigote population was produced after the flies had passed their bloodmeals, in fact day 5 promastigotes from sandflies that had passed their bloodmeals were more infective than those at day 5, 6 or 7 that had retained their bloodmeals, suggests that nutrient-depletion is important.

That metacyclics are produced in stationary-phase cultures is consistent with other parasitic protozoa. Trypanosoma cruzi epimastigotes transform into infective trypomastigotes during stationary-phase (Fernandes & Castellani, 1966). The production of metacyclics of leishmanias appears to be similar to T.cruzi in that although both have attachment phases in the insect vector (Killick-Kendrick, 1979; Vickerman, 1985), in neither does in vitro attachment appear to be necessary for the production of metacyclics (Vickerman,

1985). This contrasts with the situation with salivarian trypanosomes where attachment *in vitro* appears to be necessary for the production of metacyclics (Vickerman, 1985). This may explain why with *Leishmania* there is incomplete transformation to metacyclics. Indeed *Leishmania* promastigotes can be induced to attach to plastic *in vitro* by altering the surface properties by scratching or by bombarding with electrons (Maraghi *et al.*, 1987; Wallbanks, 1987). In these conditions leishmania attach and produce hemidesmosome-like plaques (Maraghi *et al.*, 1987; Wallbanks, 1987) similar to those seen in the sandfly vector. It would be interesting to see whether under these conditions greater numbers of metacyclics were produced. Indeed, my results (see Table 6A-6B) and those of others (Doran & Herman, 1981; Franke *et al.*, 1985; Sacks *et al.*, 1985) have shown with a variety of species in a variety of media that the maximum percentage of metacyclics produced is between 50-65% of the total population, suggesting that factors other than nutrient conditions, such as attachment, may be important.

4.2 INFECTIVITY STUDIES *IN VIVO* AND *IN VITRO*

My results regarding the infectivity (*in vivo* and *in vitro*) of different promastigote forms of *L.major* (see Figs. 10 and 11) essentially confirm those of Sacks and Perkins (1984) and Sacks *et al* (1985) with this parasite. However, Mitchell *et al* (1985) found that the LRC-L137 line of *L.major* was not more infective in stationary-phase than in log-phase of growth, although a clone which was more infective than this parental isolate did show this phenomenon, whereas an avirulent clone did not produce lesions in mice whether log-phase or stationary-phase promastigotes were used. This suggests that some strains of *L.major* may not be able to produce metacyclics, and indeed it does appear that not all leishmanial strains are equally capable of transforming to the metacyclic stage *in vitro* (Sacks, pers. comm. in

Scott & Sher, 1986). A similar situation has also been reported for T.cruzi (Vickerman, 1985). My results with L.mexicana mexicana mid-log phase and stationary-phase promastigotes (see Figs. 16-20), however, and those of other workers with other Leishmania species (Giannini, 1974; Doran & Herman, 1981; Keithly & Bienen, 1981; Sacks & Perkins, 1984; Franke et al., 1985; Rizvi et al., 1985; Scott & Sher, 1986; Kweider et al., 1987; Wozencraft & Blackwell, 1987) do confirm that an increase in infectivity with age in culture is a frequently occurring phenomenon.

Metacyclic promastigotes and stationary-phase promastigote populations of L.major were both more infective to mice than mid-log phase cells at the inoculi used (see Fig. 10). When stationary-phase cells were inoculated in large numbers ($1x10^6$/footpad), the rate of lesion growth was similar to that resulting from the use of metacyclics, whereas when a lower inoculum was used ($1x10^4$/footpad), differences were observed. These results can probably be accounted for by the composition of the stationary-phase population. Over half of this population (see Table 6A) were metacyclics, so that when a high inoculum was used, there were sufficient numbers of metacyclics to produce a lesion very rapidly. At the lower inoculum, the difference between the numbers of metacyclics present in the stationary-phase population and pure metacyclic preparation meant that lesions were produced at different rates. I also observed differences in the time for lesions to become apparent after inoculation of differing numbers of promastigotes, confirming that it was dose-dependent within limits. This has also been reported for rump infections with L.major promastigotes (Behin et al., 1979). The $1x10^6$ inoculum was probably on or above the upper limit, as suggested by the results for stationary-phase and metacyclic promastigotes.

Comparison of these results with other work is not possible, as

there has been no published comparison of this sort (i.e. relative infectivity of stationary-phase promastigotes and metacyclics). I found, however, that a similar limit to the dose-dependent response with L.mexicana mexicana promastigote infections in the rumps of CBA mice (see Fig. 18) and this has also been reported when using amastigotes of this latter species (Laurie, pers. comm.). In this latter case, however, it was found that lesions grew more slowly after inoculation of $3x10^7$ amastigotes compared to $3x10^6$ or $3x10^5$. This may have been due to the increased antigenic stimulus with the higher challenge, and to a subsequent, partially effective immune response. The infectivity experiments using different promastigote populations of L.mexicana mexicana in Balb/c or CBA mice footpads were not as clear cut as with L.major. The situation at the of $1x10^6$ inoculum was similar to L.major stationary-phase VS metacyclics. The greatest differences were apparent when the lower inoculum ($1x10^4$) was used, but even then mid-log phase cells produced lesions although a little less rapidly then stationary-phase cells. These results suggest that metacyclics must be produced much earlier during in vitro growth of L.mexicana mexicana than with L.major (but see forward).

An interesting result was that infections in the footpads of CBA mice did not appear to be as dose-dependent as similar infections in Balb/c mice with L.major or L.mexicana mexicana. In general footpad lesions developed more slowly in CBA mice than in Balb/c with both of the L.mexicana mexicana promastigote populations. It is well known that the course of cutaneous leishmaniasis in mice varies with inbred mouse strain, it being subject to genetic regulation (Handman et al., 1979; Behin et al., 1979; Nacy et al., 1983; Howard, 1985). It has been shown specifically for L.mexicana (no subspecies given) (Perez et al., 1978; Perez et al., 1979) that Balb/c mice are highly susceptible

to infection in comparison with most other strains. With reference to my results, it may be related in part, to the fact that CBA macrophages *in vitro* upon stimulation with PMA produce significantly larger amounts of O_2^- and H_2O_2 than do Balb/c macrophages (Buchmuller-Rouiller & Mauel, 1986). Comparison of my results with CBA (see Fig. 21) and Balb/c (see Fig. 22) macrophages stimulated with mid-log phase cells indicates that this may also be the case in my study. It should be noted, however, that infections in the mouse will be affected by many other factors, including other components of the immune system.

Rump infections in CBA mice with *L.mexicana mexicana* generally took larger than footpad infections in CBA mice to become apparent and to reach maximum size (see Fig. 17 and Fig. 18, compare $1x10^6$ footpad with $3x10^6$/rump). It has recently been reported that the site of inoculation of *L.major* and *L.mexicana amazonensis* promastigotes in different strains of mice as well as golden hamsters can affect the rate of skin-lesion development (Kirkpatrick *et al.*, 1987). Indeed, an earlier report demonstrated that fewer lesions due to *L.mexicana mexicana* developed after inoculation of promastigotes in the flank than when other inoculation sites, including footpads, were used. In contrast to my results, however, it was also reported that there were no differences between the time of appearance of lesions at various sites after inoculation of *L.mexicana mexicana* promastigotes (Wilson *et al.*, 1979).

I found that amastigotes of *L.mexicana mexicana* were much more infective than either promastigote population, and it is generally accepted that amastigotes are more infective *in vivo* than promastigotes of *L.donovani*, *L.mexicana mexicana*, *L.mexicana amazonensis*, and *L.braziliensis* (Keithly, 1976; Wilson *et al.*, 1979; Doran & Hernon, 1981; Wozencraft & Blackwell, 1987).

Comparison of the infections in Balb/c mice resulting from the inoculation of L.major promastigote forms and the respective promastigote forms of L.mexicana mexicana showed that in general (although this was not so apparent with the $1x10^6$ inocula) the lesions due to L.mexicana mexicana took longer to develop than those due to L.major. A similar situation in vivo has been reported with infections due to L.major and L.mexicana amazonensis, where the former parasite gave rise to lesions much more rapidly that the latter in the strains of mice tested (Stokes, 1981). These in vivo results of mine are also in contrast to the situation seen with in vitro infections of macrophages (see below, and section 4.3). While the reasons for these differences between species in animals are not immediately obvious, clearly these are unlikely to be due to differences in uptake and survival in macrophages (although macrophage heterogeneity must not be forgotten), but they may be related to the general and specific immunosuppression which has been shown to be produced by several Leishmania species during infection in experimental animals (Perez, 1983; Howard, 1985; Nickol & Bonventre, 1985; Mofleh, 1987); this may possibly be greater with L.major infections than with those of L.mexicana mexicana. There are other possible explanations, however, but as yet there appears to be no experimental evidence available.

An interesting feature of the mid-log phase populations of promastigotes of both L.major and L.mexicana mexicana is that despite having apparently very low numbers of metacyclics present (as already discussed in Section 4.1.1, see also Table 6), approximately 10-fold less than stationary-phase cutures, their inoculation into mice results in lesion development, albeit at a slower rate (although with the $1x10^6$ inoculation of L.mexicana mexicana lesion development due to mid-log phase and stationary-phase populations was quite similar). Other workers examining log-phase and stationary-phase infectivity in

vivo have also found that with L.tropica (L.major) (Sacks & Perkins, 1984), L.braziliensis panamensis (Franke et al., 1985) and L.donovani (Giannini, 1974; Doran & Herman, 1981; Keithly & Bienen, 1981; Wozencraft & Blackwell, 1987) that although promastigotes from stationary-phase populations were more infective than those from log-phase populations, this latter population was able to produce infections. Where the same strains were examined in vitro in macrophages, however, it was reported that the log-phase cells were cleared from the macrophages by 72 hours (Sacks & Perkins, 1984). It is unclear at present whether the very low numbers of metacyclics present in log-phase populations (for a fuller discussion see Section 4.1.1) can account for the infections in experimental animals. Nevertheless, the fact that my in vitro infectivity studies and those of most other workers (Sacks & Perkins, 1984; Rizvi et al., 1985; Sacks et al., 1985; Scott & Sher, 1985) show that stationary-phase or metacyclic promastigotes survive and multiply whereas mid-log phase or log-phase cells are killed or produce non-multiplying infections, whereas in vivo production of lesions by log-phase promastigotes is common to all species (see above), suggests that in vivo, other factors of the immune system are involved and that these infections caused by log-phase promastigotes could be the result of immunosuppression as described above. Further, it has recently been reported that L.major infections in vivo inhibit macrophage phagocytic function in vivo (Mofleh, 1987) and in vitro inhibit subsequent RB to further stimuli, thus encouraging superinfection (Buchmuller-Rouiller & Mauel, 1987). This latter effect would be of significance to mid-log phase promastigotes of L.major as my results indicate they are more susceptible to products of the RB (see Fig. 31, and Section 4.4.3 for a fuller discussion). Also, Kutish and Janovy (1981) showed that

concurrent infection of macrophages with L.donovani and non-pathogenic protozoa can result in the persistence of the latter. Thus metacyclics may "protect" non-metacyclics to some extent. Taking these points together, it would seem that the low numbers of metacyclics present in mid-log phase cultures of L.major and L.mexicana mexicana could initiate an infection in vivo which due to subsequent immunosuppression and/or inhibition of macrophage function allows the infection due to non-metacyclics to flourish in those macrophages infected, whereas in the simplified in vitro system this does not occur.

My results examining in vitro infectivity of different promastigote forms of L.major and L.mexicana mexicana (see Figs. 11, 19 and 20) highlighted what may be fundamental differences between the two species with respect to mid-log phase promastigotes. In the case of L.major many parasites were killed by 72 hours (see Fig. 11) and incubation to 144 hours resulted in parasite clearance unless very high numbers had been used originally. This is similar to published results for L.tropica (L.major) (Sacks & Perkins, 1984) and L.major (Sacks et al., 1985; Scott & Sher, 1986). The situation with L.donovani and L.chagasi appears to be slightly different, it was found that even when low numbers of promastigotes were used they were not completely cleared, although they were substantially reduced (Rizvi et al., 1985). My results with L.mexicana mexicana are rather different as I found that over 72 hours infections that had been initiated with mid-log phase promastigotes were not reduced but remained essentially the same (with CBA macrophages, see Fig. 20 or slightly increased (with Balb/c macrophages, see Fig. 19). Leishmania mexicana amazonensis log-phase infections were also reported to remain steady over a 72 hour incubation (Scott & Sher, 1986), suggesting that this better survival may be a feature of the L.mexicana complex and not just an artifact of my system. Other evidence from my studies on

L.mexicana mexicana, for instance regarding agglutinin-binding (as already discussed in Section 4.1.1) attachment and uptake by macrophagess (see Section 4.3.1) stimulation of the RB (see Section 4.3.2) and serum sensitivity (see Section 4.6.2) suggest that mid-log phase promastigotes are similar to stationary-phase cells in some ways, although there are distinct differences, for example morphology (as already discussed in Section 4.1.1), enzyme and isoenzyme content (see Section 4.4.6). It is tempting to speculate that mid-log phase populations of L.mexicana mexicana (and possibly L.mexicana amazonensis) contain a significant proportion of cells that are intermediate between non-infective promastigotes and the infective metacyclic - cells that have started to change to metacyclics and can survive in macrophages. A situation perhaps similar to the premetacyclics of salivarian trypanosomes (Vickerman, 1985) which are similar to but not the same as metacyclics of these parasites. If such forms occur with leishmanias, their appearance earlier during the growth phase in culture of L.mexicana mexicana than L.major would explain some of the results observed. At present, however, this is highly speculative; especially as the metacyclic forms of L.mexicana mexicana has not yet been definitely identified.

I have also found differences in the kinetics of infections in macrophages initiated with metacyclic promastigotes of L.major and the stationary-phase promastigotes of L.mexicana mexicana. There was a lag of approximately 24 hours in the former case before multiplication occurred, but none was apparent with L.mexicana mexicana. A lag-phase over the first 24 hours has also been noted with in vitro infections resulting from stationary-phase promastigotes from L.major and also L.mexicana amazonensis (Scott & Sher, 1986). In contrast, and similar to may results for L.mexicana mexicana, no lag was observed with

stationary-phase promastigotes of L.donovani, L.chagasi (Rizvi et al., 1985), and L.braziliensis (Kweider et al., 1987). These differences may reflect differences in the time taken for the parasites to tranform to amastigotes; the kinetics of this transformation have been little studied with most species.

A possible explanation for the variability in infectivity found with L.mexicana mexicana stationary-phase populations in vivo, and also possibly in vitro, and the difference in infectivity between mid-log phase and stationary-phase populations is that these populations differed with respect to proteolytic activities in terms of absolute activity (see Section 4.4.6) and also isoenzyme pattern (see Section 4.4.4). L.mexicana mexicana stationary-phase promastigotes contained variable amounts of low molecular weight proteinases, that were similar but not identical to those in amastigotes. They were not present in mid-log phase cells. These proteinases in amastigotes have been shown to make up the bulk of the proteolytic activity in this form (Coombs, 1982; Pupkis & Coombs, 1984; Pupkis et al., 1986) and it has been suggested that they play a key part in the survival of the amastigotes in macrophages. Inhibitors of these proteinases are potently antileishmanial in vitro (Coombs & Baxter, 1986). It is possible that these enzymes may be the crucial factor enabling the parasites to multiply extensively in macrophages. The variability in their occurrence in stationary-phase promastigotes may have given rise to the variable results obtained with this population. Unfortunately parallel studies on proteolytic content and infectivity of promastigote populations were not carried out.

4.3 PARASITE-PHAGOCYTE INTERACTIONS IN VITRO

4.3.1 Attachment and uptake

Examining the association with murine resident peritoneal

macrophages of different promastigote forms of L.major and L.mexicana mexicana revealed some interesting differences between the two species. In order to obtain, after a 2 hour exposure, similar infection rates (in terms of % macrophages infected and number of parasites/100 macrophages) with Balb/c macrophages, 4 to 10-fold fewer L.mexicana mexicana mid-log phase or stationary-phase promastigotes were required than for L.major mid-log phase or metacyclic promastigotes (compare Fig. 11 and Fig. 19, and Table 8 and Table 10). Even amastigotes of L.major, which are phagocytosed avidly compared to the two promastigote forms (see Table 8) were taken up less well than amastigotes of L.mexicana mexicana, giving rise to similar % macrophages infected but with approximately half the number of parasites/100 macrophages. Thus it seems that differences in uptake are common to all forms of these two species. Similar differences with respect to promastigotes were reported by Scott and Sher (1986). These workers found that approximately five times more of L.major than L.mexicana amazonensis (mid-log phase or stationary-phase promastigotes with both species) were required to give similar initial infection rates in C3H/HeN resident peritoneal macrophages. Interestingly, however, in contrast to my results, this difference did not persist through to the amastigote form when the same strains of these two species were compared. Although in the same study using amastigotes of another L.mexicana amazonensis strain gave rise to much lower initial infection rates than L.major and to the former L.mexicana amazonensis strain. In addition, uptake of L.major promastigotes does also seem to vary with strain, as Sacks et al (1985) reported a strain of L.major that gave rise to similar infection rates to those I obtained with L.mexicana mexicana and to those obtained by Scott and Sher (1986) with L.mexicana amazonensis. Further, Ardehali and Koubyar (1978) reported that L.donovani and

L.enrietti promastigotes gave rise to greater % macrophages infected (outbred murine resident peritoneal macrophages) than did L.tropica promastigotes. However, Stokes (1981) found that the initial infection rates of NMRI resident peritoneal macrophages by promastigotes of L.major, L.mexicana mexicana and L.mexicana amazonensis were similar, although those of L.donovani were lower. Thus, there appears to be a lot of variation between species and also strains with respect to uptake into macrophages; in most cases this cannot be explained at present.

My results regarding the association of leishmania parasites with rabbit peritoneal neutrophils showed that despite the quite different conditions employed when examining uptake by these phagocytic cells the differences found between the uptake of L.major and L.mexicana mexicana parasites by macrophages also persisted with neutrophils (see Figs. 23 and 24). There appears to have been no other studies directly comparing the uptake of different Leishmania species by different phagocytic cell types.

The reason for this substantial difference in uptake of L.mexicana mexicana and L.major by macrophages and neutrophils that I found is unclear. It is tempting to speculate that the presence of acid phosphatase activity on the surface of L.mexicana mexicana promastigotes and its apparent absence from L.major promastigotes (see Fig. 34) may be involved. For it has been suggested that acid phosphatase on the surface of the parasite is involved in the process of parasite-macrophage recognition and uptake whereby parasite cells may be attached and phagocytosed by existing scavenger receptors on macrophages normally involved in the retrieval of secreted lysosomal enzymes (Chang, 1983; Chang & Fong, 1983; Dwyer & Gottlieb, 1983; Saraiva et al., 1987). The finding that amastigotes of L.mexicana

mexicana also have surface located acid phosphatase activity, but they possess less than promastigotes (Hassan & Coombs, 1987) and yet they are phagocytosed more readily by macrophages, would appear to argue against this enzyme being central to the parasite-macrophage interaction. However, it has been shown that amastigotes of L.donovani (Channon et al., 1984; Blackwell et al., 1985) and L.mexicana amazonensis (Saraiva et al., 1987) appear to employ different receptor-ligand interactions from promastigotes for attachment and uptake by macrophages and it could be that surface acid phosphatase is not required with this parasite form. Indeed, Saraiva et al (1987) found that L.mexicana amazonensis amastigotes were avidly phagocytose (better than promastigotes) despite not having detectable surface acid phosphatase activity (Pimenta & deSouza, 1986). This is not confirmatory, however, for as Hassan and Coombs (1987) pointed out, lack of detection of surface enzyme on amastigotes must be interpreted with caution, as this form of the parasites seems particularly able to cap its surface.

Examining the interaction of leishmania parasites with phagocytic cells also highlighted other interesting aspects. After a 2-hour exposure using Balb/c resident peritoneal macrophages, L.major mid-log phase promastigotes gave rise to higher (approximately 2 to 5-fold) initial infection rates compared to metacyclic promastigotes (see Fig. 11 and Table 8), although this difference could apparently be abolished by adding large numbers of metacyclics. Such differences were not apparent with mid-log phase and stationary-phase promastigotes of L.mexicana mexicana under similar conditions (see Figs. 19 and 20, and Table 10), although some data with Balb/c macrophages (see Fig. 15 and Table 9, and Results) indicated that this may also occur with this species in certain circumstances. A most interesting finding was that the numbers of L.major mid-log phase

promastigotes attached wsas much greater (3 to 5-fold) than with the metacyclics, and in this case similar differences were found with L.mexicana mexicana using stationary-phase promastigotes with Balb/c and CBA macrophages (see Table 10). Interestingly, differences of the same order were found when the association of L.major mid-log phase and metacyclic promastigotes with rabbit peritoneal neutrophils was examined (see Fig. 24). Thus differences in attachment and uptake seem to be a general feature of the two promastigote forms. Related results have recently been reported for L.major by Sacks et al (1985). Although no data were given, it was stated that L.major mid-log phase and metacyclic promastigotes were taken up by murine resident peritoneal macrophages by the same extent, whereas Scott and Sher (1986) found, similarly to me, that the uptake of L.major stationary-phase promastigotes by C3H/HeN resident peritoneal macrophages was lower than that of log-phase promastigotes. These workers also reported, similar to my results with L.mexicana mexicana, that L.mexicana amazonensis log-phase and stationary-phase promatigotes were taken up to a similar extent. In contrast, Wozencraft and Blackwell (1987) found that significantly more stationary-phase promastigotes were attached and ingested by Balb/c resident peritoneal macrophages the log-phase promastigotes (in the absence of serum), whereas Rizvi et al (1985) found that attachment to Balb/c resident peritoneal macrophages was similar; nevertheless, the initial infection rate due to stationary-phase promastigotes was approximately double that due to log-phase promastigotes. These latter workers also found similar results with L.chagasi as did Kweider et al (1987) with L.braziliensis. My data and the results of other workers thus suggest that there are clear species differences with respect to the interaction of different promastigote forms with macrophages. The

reasons for these differences is as yet unclear. However, with respect to the differences I found in attachment and uptake of L.major mid-log phase and metacyclic promastigotes by macrophages. The fact that attachment and uptake of metacyclics was less affected by the absence of HI serum compared to mid-log phase cells indicates that these two promastigote forms may interact with phagocytes via different receptors which may explain the differences in attachment and uptake observed. Interestingly, Blackwell et al (1985) reported a similar effect when examining the attachment and uptake of L.donovani amastigotes and promastigotes by macrophages in the absence and presence of of HI serum; the attachment and uptake of amstigotes was less affected by the absence of serum than it was for promastigotes.

Comparison of uptake of different forms also demonstrated that amastigotes of both L.mexicana mexicana and L.major were phagocytosed by Balb/c resident peritoneal macrophages for more efficiently than either mid-log phase, stationary phase, or metacyclic promastigotes (see Tables 9 and 10). The increased uptake of amastigotes compared to promastigotes has also been reported for L.mexicana amazonensis amastigotes with NMRI resident peritoneal macrophages (Stokes, 1981) and Swiss mice resident peritoneal macrophages without serum (Saraiva et al., 1987) and for L.mexicana mexicana with murine resident peritoneal macrophages (Grimaldi et al., 1983). The literature regarding L.donovani is equivocal, Channon et al (1984) found amastigotes gave rise to higher initial infection rates than did promastigotes with (57BL/10ScSm resident peritoneal macrophages (in the absence of serum), whereas Scott et al (1983) with C3H/HeN resident peritoneal macrophages and Pearson et al (1982) with human monocyte-derived macrophages, reported no difference in initial infection rates between amastigotes and promastigotes of L.donovani. This was also seen with respect to monocytes (Murray & Cartelli, 1983;

Pearson, Harcus et al., 1983). A somewhat surprising finding of my studies was that murine resident peritoneal macrophages appeared to phagocytose L.mexicana mexicana mid-log phase promastigotes and L.major mid-log phase and metacyclic promastigotes as efficiently as rabbit peritoneal neutrophils (compare Tables 8 and 10 with Figs. 23 and 24). Neutrophils are generally thought to be more phagocytic than macrophages within the same species (Parker, 1984), indeed it has been reported that human blood neutrophils took up approximately twice as many L.donovani amastigotes as did human blood monocytes (Chang, 1981b,c) and a similar situation has been reported for Trypanosoma dionisii epimastigotes (Thorne et al., 1979). Another surprising finding was that with the neutrophils there was no greater phagocytosis of L.mexicana mexicana amastigotes compared to promastigotes. Similarly, it was reported that there was no difference in the uptake of L.mexicana amazonensis amastigotes and promastigotes by rat peritoneal neutrophils (Pimenta et al., 1987). The observed differences in relative uptake of L.mexicana mexicana amastigotes between murine resident peritoneal macrophages and rabbit peritoneal neutrophils suggests that this form may be taken up by different mechanisms by these two phagocytic cell types.

The kinetics of the interaction of leishmania with macrophages were studied in more detail using L.major promastigotes and Balb/c resident peritoneal macrophages (see Fig. 12). My results showed that the differences in attachment and uptake persisted with incubation periods in the range 5 min to 120 min. The kinetics of attachment of mid-log phase cells were similar to those reported for L.donovani, with hamster resident peritoneal macrophages at 37^{o}C in the absence of serum; the majority of attachment occurring over the first 15-20 min (Chang, 1981a). In this system used by Chang (1981a) attachment was

saturated by 30 min (when the parasite:phagocyte ratio was 10:1), whereas in my system further apparent attachment occurred between 15 min and 60 min. This difference may be due to the higher temperature employed in Chang's system as this worker showed that attachment was temperature dependent and at 25°C attachment had reached saturation between 30 min and 60 min. However, Hernandez et al (1986) have recently reported that at a ratio of 5:1 J774 cells are saturated with L.mexicana amazonensis promastigotes by 30 min at 35°C in the absence of serum. In contrast, under similar conditions at a ratio of 10:1, Chang and Chang (1986) found attachment of this species to be saturated by 10 min. Because of the different phagocytic cells and incubation conditions used, for example there being differences in temperature, which has been shown to be important in attachment (Chang, 1981a; Hernandez et al., 1986), the use of HI serum or no serum (my results indicated in the system I used that this may be important), and the numbers of parasites added (my results indicated that this also has an effect), it is difficult to relate other workers results with mine. Overall, however, they agree in several aspects well, attachment appearing to be time-dependent and saturable. This is indicative of a receptor-mediated interaction, as has been shown to occur with other Leishmania species and macrophage and macrophage-like cell systems (Chang, 1981a; Bray, 1983a; Klempner et al., 1983; Wyler & Suzuki, 1983; Zehavi et al., 1983; Channon et al., 1984; Blackwell et al., 1985; Chang & Chang, 1986; Hernandez et al., 1986; Wilson & Pearson, 1986). The same appears to hold for the attachment of metacyclic promastigotes, although this proceeds at a lower rate and the kinetics are different from those for mid-log phase promastigotes.

Concerning phagocytosis, the kinetics for mid-log phase and metacyclic promastigotes of L.major were similar, and different from those for attachment; phagocytosis of the two promastigote forms was,

however, also time-dependent and saturatable, with most phagocytosis having taken place by 60 min. Similar results were seen with L.mexicana mexicana mid-log phase and stationary-phase promastigotes (see Table 10) for both Balb/c and CBA macrophages. Again there are problems in comparing my results with those of other workers, as discussed above in relation to attachment. However, where phagocytosis has been studied with respect to time, it has been shown to be time-dependent and saturatable for human blood monocytes and L.donovani amastigotes (Chang, 1981b,c), and L.mexicana amazonensis amastigotes and promastigotes with Swiss mice resident peritoneal macrophages (Saraiva et al., 1987); most of the uptake apparently occurring over the first 60 min of exposure.

In studying the interaction of L.mexicana mexicana amastigotes and mid-log phase promastigotes, and L.major mid-log phase and metacyclic promastigotes with rabbit peritoneal neutrophils, it was only possible to determine numbers of cells associated (= attached + ingested) with the neutrophils. In an attempt to confirm that phagocytosis was occuring, the reduction in numbers of extracellular parasites during parasite-neutrophil interaction was also monitored (see Figs. 23 and 24); a technique used successfully by Pearson and Steigbigel (1981) to confirm phagocytosis of L.donovani promastigotes by human blood neutrophils. Only in the case of L.mexicana mexicana mid-log phase promastigotes, however, did I find tha there was a significant reduction in the number of extracellular parasites that was consistent with the numbers of parasites associated with the neutrophils. Although similar numbers of amastigotes were apparently associated with the neutrophils, I was unable to confirm an equivalent reduction in numbers of amastigotes in suspension. This experiment was complicated by the clumping of the amastigotes in both control

(without neutrophils) and experimental (with neutrophils) samples - this made counting them difficult and inaccuracies in the counts probably masked the removal of amastigotes that occurred. It has previously been reported that amastigotes have a tendency to clump and which can be minimised by keeping them at low densities ($1-5x10^7$ ml^{-1}), in a special suspension buffer (PSGEMKA, see Materials and Methods Section 2.1.2; Hart et al., 1981b). This buffer was not asked in this study because I wanted to keep conditions as similar to those employed in the chemiluminescence studies, also because this buffer contains substances such as EDTA which could potentially interfere with parasite-neutrophil interaction (Chang, 1981a). Similarly to my results with L.mexicana mexicana amastigotes, I found that L.major mid-log phase and metacyclic promastigotes, although apparently associated with neutrophils, were not apparently removed from the medium during this interaction (see Fig. 24). This could have been due to the low numbers being associated with the neutrophils, but also that the removal experiment were done using a parasite:neutrophil ratio of 5:1 which made it more difficult to detect a small reduction in the number of free parasites. This relatively large number of parasites were used as the expected rapid and extensive removal of parasites from the medium would have soon reduced their numbers to below the sensitivity of the haemocytometer. If it had been practical to use a lower number of parasites, it may have been possible to monitor their attachment, as Pearson and Steiebigel (1981) observed an approximately 200-fold reduction in the number of extracellular parasites (they were reduced to 0.5% of the control figures by 15 min) when the interaction of L.donovani promastigotes and human blood neutrophils, at a 1:1 ratio, was examined. This gave rise to similar numbers of promastigotes being associated with the neutrophils as occurred in my experiments with L.mexicana mexicana mid-log phase

promastigotes.

With all the parasite forms I used, the majority of association occurred in the first 15 min of interaction (see Figs. 23 and 24), Pearson and Steigbigel (1981) found a similar situation with L.donovani promastigotes and human blood neutrophils, but only in the presence of fresh serum; no phagocytosis occurred in HI serum. Recently, Pimenta et al (1987) also observed that L.mexicana amazonensis promastigotes and amastigotes were readily attached to and ingested by rat neutrophils in vivo in the peritoneal cavity, but these interactions in vitro required the presence of fresh serum, no interaction occurring in the presence of HI serum. On the other hand, Chang (1981b,c) observed that uptake of L.donovani amastigotes by human blood neutrophils could occur in the presence of HI serum, although the infection rates were increased in the presence of fresh serum. Under both conditions, however, ingestion proceeded more slowly than I found in my studies or reported by other workers, taking 60 min to reach maximum levels. Studies with other members of the Trypanosomatidae have also shown that human blood neutrophils can ingest the parasites in the presence of HI serum (Thorne et al., 1979). The exudate solution from rabbit peritoneum that I used was not lytic over the time of the attachment/uptake assay at the dilution used and although it was not tested for complement components the results of Pimenta et al (1987) suggest that complement components do occur in such fluid in the peritoneum of rats anyway. It is also thought that complement is a component of normal rabbit peritoneal fluid, but that the peritoneal exudate solution resulting from the eliciting of neutrophils with glycogen contains complement components probably in inactivated form and not as a full cascade (Lackie, pers comm.). Further work is needed to ascertain the precise requirements

with respect to complement for the interaction to occur, although from other aspects my results provided the information required for my study.

It is interesting that over the period of the incubation of parasites with rabbit peritoneal neutrophils no killing of the parasite cells could be detected by light microscopy. Neutrophils have been reported to kill amastigotes and promastigotes of leishmania rapidly (Chang, 1981c; Pearson & Steigbigel, 1981; Pimenta et al., 1987). Pearson and Steigbigel (1981) and Chang (1981c) reported that L.donovani promastigotes and amastigotes were killed by human blood neutrophils and that this could be detected by light microscopy, this was not apparent, however, until after 120 min incubation of L.donovani promastigotes with neutrophils (Pearson & Steigbigel, 1981). Electron microscope studies, however, have shown parasite damage to occur to L.donovani promastigotes and amastigotes by 10 to 30 min after the start of the interaction. Similarly, it has been reported for L.mexicana amazonensis promastigotes and amastigotes that digested parasites were seen as early as 5 min after interaction, although undigested and digested parasites were seen in the same cell (Pimenta et al., 1987). It seems likely, therefore, that parasite killing also occurred in my system but that light microscopy was not sufficiently sensitive to detect it, as it is difficult to discriminate between live and dead organisms by this method.

4.3.2 Stimulation of the respiratory burst

The results of my infectivity studies (as already discussed in Section 4.2) showed that stationary-phase promastigotes of L.mexicana mexicana, and stationary-phase and metacyclic promastigotes were more infective to mice than mid-log phase cells, and that they were able to survive and multiply in resident peritoneal macrophages in vitro,

whereas mid-log phase cells were either killed or simply survived without multiplying. A possible explanation for this could have been that they stimulated a lower RB in a similar way that the more infective amastigotes do (Haridaris & Bonventre, 1981; Stokes, 1981; Pearson et al., 1982; Murray, 1982a; Murray & Cartelli, 1983; Channon et al., 1984; Alexander & Russell, 1985; Scott et al., 1985). Indeed it has been reported that L.major metacyclic promastigotes from the proboscis of an infected sandfly do not stimulate a RB (Alexander & Russell, 1985; Killick-Kendrick, 1987). Several interesting findings have come out of this series of experiments. Firstly, that upon exposure to murine resident peritoneal macrophages amastigotes of both L.major and L.mexicana mexicana stimulate very low RB responses in comparison to mid-log phase and stationary-phase or metacyclic promastigotes (see Fig. 13, and Fig. 22). The differences are even more pronounced when relative infectivity rates of amastigotes and promastigote forms are taken into account. For instance, with L.mexicana mexicana, even at a ratio of 50 amastigotes:1 macrophage, the % macrophages stimulated to reduce NBT was still lower when mid-log phase promastigotes were used at the 5:1 ratio. These differences are further highlighted by the RB produced upon exposure of these two parasite forms to the very oxidatively reactive rabbit peritoneal neutrophils. With L.mexicana mexicana, for which amastigote and promastigote uptake were similar, the amastigotes stimulated a negligible RB in comparison to that stimulated by promastigotes (see Fig. 26). Clearly, amastigotes of L.mexicana mexicana are very poor stimulators of the RB with both types of phagocytes. My results with murine resident peritoneal macrophages confirms the findings of most other workers using this phagocytic cell type; they found that the amastigotes of L.mexicana mexicana (Stokes, 1981, 1984; Alexander & Russell, 1985) and L.donovani (Murray, 1982a; Channon et al., 1984;

Scott et al., 1985) stimulated a much lower RB, both qualitatively and quantitatively, than promastigotes of these species. The published reports concerning other phagocytic cell types are equivocal and were all carried out with L.donovani. Pearson, Harcus et al (1983) found with human blood monocytes that amastigotes (at a ratio of 20 parasites:1 monocyte and above) were as stimulatory as promastigotes when the RB was measured by luminol-enhanced CL They only apparently stimulated about half the monocytes, however, as assessed by qualitative NBT reduction. In addition, the amastigotes were significantly less stimulatory than promastigotes when examined at a ratio of 10:1 using luminol-enhanced CL. If monocyte-derived macrophages were used, however, the RB stimulated by amastigotes, as measured by this latter method, was significantly lower than that stimulated by promastigotes at all the ratios used (Pearson et al., 1982). This suggested that the more oxidatively reactive monocytes masked the less stimulatory nature of the amastigotes, although this does not correspond to my results. Indeed, other workers using L.donovani amastigotes and promastigotes have reported that with both human blood monocytes and macrophages derived from them, amastigotes stimulated the secretion of significantly lower amounts of H_2O_2 than did promastigotes (Murray & Cartelli, 1983). The reasons for such apparently contradictory results are not clear, but they may reflect different methodologies. The only studies apart from mine examining the RB of neutrophils resulting from interaction with leishmania involve the use of L.donovani promastigotes and human blood neutrophils (Pearson & Steigbigel, 1981; Remaley, Glew et al., 1985); in these no comparison with amastigotes were performed. In these studies the promastigotes stimulated a RB when added, as was also found in my studies (see Figs. 25, 26 and 28).

One of the main findings of my investigations is that metacyclic promastigotes of L.major, in contrast to amastigotes, do stimulate a RB and when differences in attachment and uptake between mid-log phase and metacyclic promastigotes are taken into account, it appears that the metacyclics stimulate similar RB response to that of mid-log phase cells when mixed with murine resident peritoneal macrophages or rabbit peritoneal neutrophils (see Figs. 13 and 25, and Results Sections 3.2.3 and 3.4.2). In my studies with human blood monocytes, the RB detected was of longer duration and larger than that due to mid-log phase cells (see Fig. 30). It is not known, however, whether this reflects increased uptake of the metacyclics as uptake studies were not carried out. When L.mexicana mexicana stationary-phase promastigotes were mixed with murine resident peritoneal macrophages, they also appeared to stimulate (with CBA macrophages, and also probably Balb/c macrophages) a similar RB to that stimulated by mid-log phase cells. These results with L.major and L.mexicana mexicana contrasts with the only report regarding stimulation of the RB by log-phase and stationary-phase promastigotes. This involved L.donovani and it was found that more O_2^-, as detected by the ferricytochrome C assay, was produced in response to log-phase promastigotes than to stationary-phase cells (Blackwell et al., 1986). This may simply reflect species differences in the strategies employed by the infective metacyclic promastigotes to survive the antimicrobial defences of the macrophage. Indeed, although L.major metacyclics apparently stimulate a similar RB to mid-log phase cells, this former promastigote form is also more resistant to H_2O_2 in vitro than the latter (see Section 4.4.3 and Fig. 31) and this species may have employed this strategy to allow them to survive in macrophages. However, it has also been reported that preliminary studies using qualitative NBT reduction showed that L.major promastigotes (the same

strain as this study) from the midgut of P.papatasii stimulated murine macrophages to reduce NBT, whereas those from the proboscis (metacyclic promastigotes did not (Alexander & Russell, 1986; Killick-Kendrick, 1987). This could be interpreted in terms of the metacyclic promastigotes produced during in vitro culture, although apparently similar to those produced in the sandfly, not being identical to them. Perhaps they do not fully change to metacyclics in culture, certain constituents in the sandly being necessary for this to occur. Presumably, conditions have to be correct to allow complete change to metacyclics; variation in these conditions could lead to partial change to metacyclics and so preadaptation in some areas but not others. I have already discussed how this may have occurred with L.mexicana mexicana (see Section 4.2). Nevertheless, it is imporant that work with sandly-derived promastigotes is repeated using fully controlled conditions and a more thorough study before these "preliminary results" which may not be accurate, become accepted dogma.

A most interesting finding with the rabbit peritoneal neutrophils was the apparent "inhibition" of C.L. produced by some parasites when added at high numbers (see Figs. 26, 28 and 29). The feature of this was that there was an approximate correlation with the presence of surface acid phosphatase activity and this "inhibition". The effect was not found with either promastigote form of L.major with the parasite numbers used (see Fig. 25) and these apparently lack surface acid phosphatase (see Fig. 34; discussed more fully in Section 4.4.5). It would be interesting to know if "inhibition" could be obtained if larger numbers of these parasites were used. Herpetomonas muscarum ingenoplastis although apparently lacking surface activity (Coombs et al., 1987) did produce some apparent "inhibition", although this was

more dependent on the reactivity of the neutrophil preparation than in the other cases (see Figs. 28 and 29). Attempts to show that surface acid phosphatase was involved by NaF-treating (which would inhibit all acid phosphatase activities [Hassan & Coombs, 1987]), or glutaraldehyde-fixing the cells (which would prevent secretion but retain surface activity [Gottlieb & Dwyer, 1981a,b,c; Hassan & Coombs, 1987]) produced equivocal results (see Fig. 29). NaF-treatment had no effect on CL; this may be because the cells were not treated long enough or that when they were taken out of the NaF its effect was reversed. Unfortunately their acid phosphatase activities after the experiment were not tested. The effect of glutaraldehyde-fixation appeared to depend on the parasite:neutrophil ratio used; overall it is difficult to come to any conclusion about these results. However, the fact that H.muscarum ingenoplastis does produce inhibition does suggest that some mechanism other than acid phosphatase may be involved. There are several other possible explanations: **(1)** By adding such large numbers of parasite cells, they could simply absorb or "quench" any light produced before it could be detected by the luminometer. This, however, seems unlikely as when similar numbers of L.major promastigotes were added no such "inhibition" was seen. It could be that the absorption occurred only when there was close opposition of the parasite with the neutrophil which would explain why inhibition was not seen with L.major. Unfortunately, no uptake experiments were performed with L.donovani mid-log phase promastigotes, H.muscarum muscarum or H.muscarum ingenoplastis to test this theory although it seems rather unlikely. **(2)** The endogenous SOD present in leishmanial cells (Ghafoor & Coombs, 1981; Meshnick & Steiger, 1981; Murray 1981a; Channon & Blackwell, 1985b) and the insect trypanosomatids (Ghafoor & Coombs, 1981; Meshnick & Eaton, 1981) could compete with the lucigenin for any extracellular O_2^-

released. The SOD activity in the number of L.mexicana mexicana promastigotes added ($5x10^7$) using Ghafoor and Coombs (1981) figures for SOD activity in L.mexicana mexicana promastigotes (23 U [mg protein]$^{-1}$) works out as 13.5 U. This is much lower than the 600 U of exogenous SOD that was found to be required to reduce the RB by 79%. The lack of inhibition by L.major is also hard to explain using the SOD theory. It therefore seems unlikely that SOD is involved. **(3)** Another possibility is that the parasites are releasing soluble factors which either damages or inhibits the RB. Pearson et al (1982) found that if the filtered supernatant fluid from an incubated suspension of L.donovani promastigotes was added to human blood mnocyte-derived macrophages, the luminol-enhanced CL response was not stimulated. They concluded that CL was due to the presence of the parasites rather than parasite factors. These studies, however, did not preclude the presence of inhibitory factors in the filtrates. This theory could be tested relatively easily by adding such filtrates (from "inhibiting" parasites) to L.major or PMA-stimulated neutrophils and following the RB response. **(4)** Neutrophils upon contact with microorganisms can degranulate, produce regative oxygen intermediates (via the RB) and during active phagocytosis lysosomal enzymes may also be released into the extracellular medium (Parker, 1984). All these events could produce potentially damaging substances in the extracellular medium around the neutrophils which could in turn damage the neutrophils themselves so that their RB response was curtailed. In such a case one would expect the same situation to occur with L.major as well, although cell contact might be required to initiate the reaction and I have shown that L.major binds less. It is also possible that parasites damaged by the neutrophils (via degranulation release of lysosomal enzymes and, initially, products of the RB) could locally

inhibit the RB directly, such as by soluble acid phosphotase activity, and indirectly by the presence of substances that would damage the neutrophils. Indeed, cell free extracts of Trypanosoma cruzi, and L.donovani and L.mexicana promastigotes contain a lytic factor that disrupts membranes of some mammalian erthrocytes and Vero cells. Similar extracts from peritoneal cells from mice and vero cells lacked this factor (O'Daly & Aso, 1979). Nevertheless, the differential effect could only be explained if it was shown that L.major lacked such factors or that they were not released in such large quantities due to reduced parasite-neutrophil interaction.

Clearly there are several other possible explanations but none preclude acid phosphatase from being involved. Indeed it has been shown that a purified tartrate-resistant enzyme present on the surface of L.donovani promastigotes (one of three activities) inhibits O_2^- and H_2O_2 production by f-Met-Leu-Phe stimulated human blood neutrophils (Remaley et al., 1984; Remaley, Glew et al., 1985), whereas using purified secreted acid phosphatase no inhibition was seen (Das, Saha, Remaley et al., 1986). Further, Remaley, Glew et al (1985) examined the RB produced by human blood neutrophils on contact with L.donovani promastigotes and zymosan in the presence and absence of a specific inhibitor of the tartrate-resistant leishmanial surface acid phosphatase (Complex E). They found that in the presence of Complex E the O_2^- production stimulated by zymosan was similar to that in the absence of this compound, whereas O_2^- production stimulated by L.donovani promastigotes was increased 2 to 3-fold by the presence of Complex E indicating specifically that this surface acid phosphatase was involved. Thus, in this system, at least, acid phosphatase appears to be clearly implicated. However, the significance of this in enabling the parasite to survive is unclear as no details were given of the phase of growth of the promastigotes used in this study. In my

study mid-log phase cells were capable of this "inhibition". Unfortunately stationary-phase cells were not tested, and it seems strange that cells that are less infective (see Figs. 16-20; and also Rizvi et al., 1985; Wozencraft & Blackwell, 1987) are still capable of inhibiting the RB. The fact that L.mexicana mexicana mid-log phase cells could produce "inhibition" of the neutrophil RB may explain why considerably fewer macrophages were stimulated than were initially infected by either promastigote form of this species (see Table 10 and Fig. 21 for CBA macrophages). These results again suggest, as already discussed in Section 4.2, that the mid-log phase populations of L.mexicana mexicana used in this study are similar to stationary-phase cells in some ways, but not others and may represent a population of intermediate or partial metacyclics.

4.4 ADAPTATIONS TO LIFE IN DIFFERENT ENVIRONMENTS

4.4.1 Electron microscope studies

There is no published work on the ultrastructure of infective forms of Leishmania. My electron microscope studies of stationary-phase promastigotes of L.mexicana mexicana and metacyclic promastigotes of L.major revealed that they apparently contained more lipid inclusions than their respective mid-log phase promastigotes although this was not quantified (see Figs. 42 and 43). The increase in lipid inclusions in stationary-phase populations is also a feature of members of the Trypanosoma brucei complex (Tetley, pers. comm.), and may be a characteristic of no physiological significance. However, Brun and Krassner (1976) reported that the ratio of lipid inclusions to cytoplasmic volume was identical in promastigotes and amastigotes of L.donovani, which means that the promastigotes would have five times as many lipid inclusions as amastigotes but would suggest that mid-log phase and stationary-phase metacyclic promastigotes would be

likely to possess similar amounts. Further, it has recently been postulated that parasite lipids might detoxify H_2O_2 by serving as substrates for harmless peroxidation reactions (Channon & Blackwell, 1985b), and so more lipid inclusions in metacyclics (of L.major) would correlate with their ability to withstand greater concentrations of H_2O_2 than mid-log phase cells (see Fig. 31) and hence this characteristic may have a physiological role (for a fuller discussion, see Section 4.4.3).

No promastigote form of L.mexicana mexicana appeared to have "megasomes", even though stationary-phase promastigotes were found to possess low molecular weight cysteine proteinases similar to those in amastigotes, one of which has been localised to these organelles (Pupkis et al., 1986) (see Fig. 36 and Section 4.4.4). Serial sections were not taken, however, and hence low numbers of "megasomes" in the stationary-phase cells may have been missed. Alternatively, the proteinases may be present in small organelles that become "megasomes" upon transformation of the cells to amastigotes. Such organelles could be difficult to detect.

A most interesting finding from the electron microscope studies was the irregular, finely grained "surface coat" which was present on some stationary-phase cells (presumably metacyclics) and all metacyclics of L.major (see Fig. 44). The observation that this "coat" was absent from junctions between flagellum and flagellum pocket suggests it was of exogenous origin, but its presence on stationary-phase cells confirms that it was not an artifact of the metacyclic purification procedure (see Materials and Methods Section 2.1.2). A similar "surface coat" has recently been demonstrated on L.mexicana amazonensis promastigotes but only after exposure to sublytic dilutions of fresh serum; it was thought that this was due to

complement components (Pimenta et al., 1987). This could not explain the situation with L.major, as HI serum was used in the medium the "surface coat" may, however, be due to absorption of (as yet unknown) components from the serum. Whatever the origin of the coat, its presence confirms that there are distinct surface differences between the two forms. It has been shown that the more substantial glycoprotein surface coat on bloodstream trypomastigotes of Trypanosoma congolense and T.brucei brucei protects these forms from lysis by human serum (Ferrante & Allison, 1983). The presence in L.major metacyclics of the "surface coat" correlates with increased resistance of this form to lysis by human serum compared to mid-log phase cells (see Fig. 32) and hence the "surface coat" may also have a physiological role (for a fuller discussion, see below).

4.4.2 Serum Sensitivity

My results showed that the different promastigote forms of L.major and L.mexicana mexicana were all susceptible to lysis by normal human serum (see Fig. 32). Lysis of promastigotes by serum from various sources including humans has been shown to be mainly due to the action of complement (Hindle et al., 1926; Rezai et al., 1975; Mosser and Edelson, 1984; Mosser et al., 1986) which correlates well with my results as heat-inactivating the sera for 30 min at 56°C abrogated the majority of its lytic activity. It has been shown that for promastigotes of all species of Leishmania, apart from L.donovani, complement in human serum is activated via the alternative pathway (Pearson & Steigbigel, 1980; Mosser et al., 1986) and it appears likely that this was the mechanism occurring in my studies, although this was not investigated. In my hands L.mexicana mexicana promastigotes, both mid-log phase and stationary-phase forms, were much more resistant to the effects of serum than any of the L.major

promastigote forms. Similar findings were reported by Mosser et al (1986) for L.major 252 strain and L.mexicana mexicana promastigotes, although susceptibility to serum does appear to depend on the strain of parasite used as these workers also found that promastigotes of another L.major strain (1-S) were as resistant as the L.mexicana mexicana promastigotes.

I found that all promastigote forms of L.major and L.mexicana mexicana tested were killed by concentrations of 50% serum and above, none suvived on 100% serum. Comparison with other studies is difficult because even using sera from the same source the absolute amount of lysis achieved is apparently dependent on a number of variables including the buffer used to suspend the parasites, the concentrations of divalent cations, and the temperature at which the lysis assays are performed (Mosser et al., 1986). Nevertheless my results are similar to the findings of Mosser et al (1986) and Pearson and Steigbigel (1980) with L.major, L.mexicana mexicana, L.mexicana amazonensis, L.donovani and L.braziliensis guyanensis stationary-phase promastigotes, where concentrations of 10-20% serum and above were found to cause lysis of all cells. This, however contrasts with the reports of Russell (1987), who found 40-60% of stationary-phase L.mexicana mexicana promastigotes (the same strain as this study) were resistant to lysis with 80% normal human serum, although no comparison with mid-log phase promastigotes were carried out, and Franke et al (1985), who found that up to 30% of L.braziliensis panamanensis and 10% of L.donovani stationary-phase promastigotes survived in 100% normal human serum. An interesting feature of this last study was that log-phase cells were all killed under these conditions. The serum-resistant cell in stationary-phase populations of L.braziliensis guyanensis, thought to be metacyclics, were highly infective to animals whereas those from log-phase cultures were essentially non-

infective. Thus there was a sequential development in terms of serum lysis from susceptible to resistant as the cultures became stationary and more infective. Likewise, my results comparing L.major mid-log phase, and stationary-phase and metacyclic promastigotes showed a similar development of resistance to complement with growth phase. By comparing the LD 50's, I showed that stationary or metacyclic promastigotes require 5- to 6-fold higher concentrations of serum for lysis than mid-log phase cells. Hence this suggests that metacyclics are also preadapted survive the effects of the serum, although the significance of this in vivo is difficult to estimate as the concentration of serum at the site of the sandfly bite is unknown, but it is likely to be less than 100% due to dilution with sandfly saliva. It is interesting that both metacyclic and stationary-phase promastigotes of L.major were similarly susceptible to serum lysis. This is probably because the stationary-phase population is mixed being approximately 50-65% metacyclics (see Table 6A). Thus, a rather suprising finding was that, unlike L.major, stationary-phase and mid-log phase promastigotes of L.mexicana mexicana were similar with respect to serum sensitivity. This also contrasts with the finding of Franke et al (1985) (see above) but agrees with the findings of Mosser et al (1986), who also could not detect differences in susceptibility to lysis by human serum between mid-log phase and stationary-phase promastigotes of L.major (in contrast to my results), L.mexicana amazonensis and L.donovani. Pearson and Steigbigel (1980) also found that L.donovani from days 3 through to 7 in culture were equally susceptible to lysis by human serum. It is difficult to explain the discrepancies between studies although the methodology variables outlined above may be important. However, in my study the differences between the response of L.major and Lmexicana mexicana under the same

conditions clearly suggest species variation. In view of my other results concerning L.mexicana mexicana mid-log phase and stationary-phase promastigotes, which indicate that the mid-log phase promastigotes used in this study were already preadapted in some ways such that I suggested they may be partial or intermediate metacyclics, it would appear that serum sensitivity was one of these preadaptations.

How the metacyclics of L.major are more resistant to lytic effects of serum is not known. There have been no reports fron other workers regarding this and it was not investigated in this study. Work with other species has produced conflicting results. Franke et al (1985) found that both log-phase and stationary-phase promastigotes of L.braziliensis panamensis fixed complement on their surfaces. Thus the survival of resistant forms was not due to these forms not activating the complement cascade, as C3 was visualised on their suface although this was not quantified in comparison to log-phase cells. These results suggest that the cells may be able to prevent the cascade from proceeding to cell lysis. Moreover, Wozencraft and Blackwell (1987), using conditions where only activation of the alternative pathway was measured, reported that stationary-phase promastigotes of L.donovani were more efficient activators of this pathway and bound more C3 than log-phase cells. They did not examine, however, susceptibility to lysis of the two promastigote forms. In contrast, the serum-resistant promastigote forms in L.mexicana mexicana stationary-phase populations were found to bond 3 to 6-fold less C3, and bond it more slowly, than the lysed cells (Russell, 1987). The metacylics of L.major may therefore be more resistant because: **(1)** they can prevent the complement cascade proceeding to cell lysis possibly by cleaving the C3 to its inactive form, C3bi which would inhibit further activation of the cascade. Indeed this has been proposed as the function of the

suface protease which is apparently present on promastigotes of all species so far studied (Bouvier et al., 1987) and also is the major surface glycoprotein on these cells with an apparent molecular weight of 63-68KDa (see Introduction, Section 1.2.2.3 for references). It is of interest, therefore, that I detected a proteinase activity present in L.major metacyclics (and amastigotes and mid-log phase cells of this species also all forms of L.mexicana mexicana and L.donovani) which had similar characteristics to and strongly resembled this protein (see Figs. 36 and 39 and Section 4.4.4 for details). **(2)** The metacyclics, like amastigotes (Wozencraft & Blackwell, 1987), may be less efficient activators of the alternative complement pathway, and hence bind less C3 than mid-log phase cells. This could possibly be mediated by the "surface coat" which was present on all L.major metacyclics but apparently absent from mid-log phase cells (see Fig. 44 and Section 4.4.1 for details). Indeed, it has been shown that bloodstream trypomastigotes of Trypanosoma brucei brucei and T.congolense are re sistant to lysis by normal human serum whereas procyclics are highly susceptible because the former cell type possess the well characterised surface coat which masks components present on the plasma membrane which activate the alternative pathway of complement (Ferrante & Allison, 1983). **(3)** It is possible that both these mechanisms are involved, that they bind less C3 and the surface protease then cleaves this to its inactive form. Mid-log phase cells also apparently possess the surface protease (suggested by results see above and Section 4.4.4 and also Etges et al., 1986), but as they bind more complement (due to lacking a "surface coat") same could proceed to the latter parts of the cascade and hence lysis before it could be cleaved to the inactive form via the protease.

Several workers have reported that at high concentrations non-

immune normal human serum and serum from other sources agglutinates leishmania promastigotes (Schmunis & Herman, 1970; Bray, 1983a; Mosser et al., 1986). This has been attributed to the presence of "natural" antibodies (Pearson & Steigbigel, 1980; Pearson, Wheeler et al., 1983). I did not detect such an effect. However, agglutination was seen with L.major mid-log phase and to a lesser extent in stationary-phase promastigotes and also with L.mexicana mexicana mid-log phase and stationary-phase promastigotes when HI serum was used. It is unclear why agglutination occurred only after heat-inactivating the serum, although it may be that lysis masked this effect as the cells were killed quickly before they could interact with each other. Agglutination by HI serum but not fresh serum has been reported to occur with human serum and L.donovani promastigotes (Pearson & Steigbigel, 1980) and this was suggested to be due to the presence of "natural" antibodies. None of the sera used in this study were tested for antibodies to Leishmania, but it would seem unlikely that antibodies were present due to prior exposure to the parasites. That L.major mid-log phase but not metacyclic promastigotes were agglutinated suggests that there are surface differences between these two forms; interestingly, at least one surface antigen unique to L.major metacyclics has also been shown by Sacks et al (1985). Surface antigen differences have also been reported for L.chagasi between log-phase and stationary-phase promastigotes and also late sandfly promastigotes (Rizvi et al., 1985), and increasing expression, from log to stationary-phase promastigotes of L.braziliensis, of a surface antigen has been reported by Kweider et al (1987). The fact that both mid-log phase and stationary-phase promastigotes of L.mexicana mexicana were agglutinated supports my hypothesis that these two promastigote forms are similar with respect to surface components. That mid-log phase L.major were also agglutinated suggests that the

"natural" antibodies were probably recognising a common conserved epitope.

4.4.3 Sensitivity to products of the respiratory burst

Although I was unable to detect any H_2O_2 production by murine resident peritoneal macrophages upon exposure to Leishmania promastigotes, this has shown to be released into the extracellular medium in response to such contact by human-blood monocytes and macrophages (Murray & Cartelli, 1983) and murine resident peritoneal macrophages (Murray, 1981a, 1982a) and has been reported to be the microbicidal product of murine peritoneal macrophages which is most toxic to leishmania (Haidaris & Bonventre, 1981; Murray, 1981a, 1982a; Szuro-Sudol et al., 1983). Leishmanial promastigotes are known to be susceptible to reagent and generated H_2O_2 in vitro, whilst amastigotes are more resistant (Murray, 1981a, 1982a; Locksley & Klebanoff, 1983; Pearson, Harcus et al., 1983; Thorne & Blackwell, 1983; Channon & Blackwell, 1985a). My results showed that the more infective metacyclic promastigotes of L.major were more resistant to H_2O_2 than mid-log phase cells over a narrow range of concentrations (see Fig. 31). Direct comparison with other workers results is of limited value because of the different methodologies used and the fact that assay conditions, such as numbers of cells used, sub-passage number, incubation temperature, degree of agitation of the reaction mixture, and treatment time, can apparently influence the outcome of the experiment (Thorne & Blackwell, 1983). Nevertheless, Table 4 (see Introduction) gives the results for reagent H_2O_2 for promastigotes and amastigotes where the LD_{50}'s have been calculated; the values reported for promastigotes are of the same order (0.1 mM - 0.5 mM) as those I found for the two promastigote forms of L.major. Further, the difference I found between the LD_{50}'s for mid-log phase and metacyclic

promastigotes of L.major are within the range (2 to 10-fold) reported by other workers to be the difference in susceptibility between promastigotes and amastigotes (Pearson et al., 1983; Thorne & Blackwell, 1983; Channon & Blackwell, 1985a). My results also agree well with those of other workers comparing in vitro susceptibility of L.donovani low and high sub-passage promastigotes (Channon & Blackwell, 1985a), and virulent (low subpassage) and avirulent (high sub-passage) promastigotes (Katakura, 1986); in these studies it was shown that the high sub-passage and avirulent promastigotes were more susceptible to the effects of reagent H_2O_2 than the low sub-passage and virulent promastigotes, respectively. It should be noted, however, that other workers using avirulent and virulent L.donovani promastigotes claim to have indirectly shown by catalase pre-treating macrophage cultures that the avirulent promastigotes were not deficient in their defense against the oxygen-dependent mechanisms of the macrophage (Nolan & Herman, 1985). These different results cannot be explained at present.

My finding that metacyclics of L.major are less susceptible to H_2O_2 than mid-log phase cells could explain in part at least, why despite stimulating a similar RB, (see Section 4.3.2. and Results Sections 3.2.3. and 3.4.2) metacyclics survive in murine resident peritoneal macrophages in vitro whereas mid-log phase cells do not (see Fig. 11). However, whether the differences in susceptibility, in vitro, between the two promastigote forms of L.major are relevant to the situation within the macrophage cannot be answered definitely, as the precise concentration of H_2O_2 to which the parasites are exposed to in macrophages has yet to be determined. That H_2O_2 is produced in the parasitophorous vacuole has been demonstrated by Chang (1981c), who showed that H_2O_2 was restricted to the leishmania-containing

vacuoles of human monocytes infected with L.donovani amastigotes, so it would appear likely that intracellular leishmanias are exposed to this product.

Addition of exogenous peroxidase and a halide to the in vitro system resulted in both promastigote forms of L.major being killed by much lower levels of reagent (see Fig. 31) H_2O_2. A similar effect for reagent H_2O_2 has been reported for several Leishmania species, with both amastigotes and promastigotes (Haidaris & Bonventre, 1981; Locksley & Klebanoff, 1983; Pearson, Harcus et al., 1983; Thorne & Blackwell, 1983; Katakura, 1986), for bacteria (Locksley & Klebanoff, 1983), for other trypanosomatids such as Trypanosoma dionisii (Thorne et al., 1981) and for other intracellular protozoa such as Toxoplasma gondii (Murray & Cohn, 1979). The enhancement in leishmanicidal activity I found is of the same order (5 to 100-fold) as that reported for amastigotes and promastigotes of other Leishmania species (see Table 4, Haidaris & Bonventre, 1981; Pearson, Harcus et al., 1983; Thorne & Blackwell, 1983), and where promastigotes and amastigotes have been examined within the same assay system the latter were still more resistant than the former (Thorne & Blackwell, 1983). A somewhat surprising result from my studies, therefore, was that in the presence of peroxidase and a halide mid-log phase promastigotes are more resistant than (when assessed as % motile) or similarly susceptible (when assessed as % "fast") as the metacyclics to the effects of H_2O_2. A similar situation was reported for L.donovani avirulent and virulent promastigotes, when comparing their susceptibility to H_2O_2 and H_2O_2/LPO/KI (Katakura, 1986). The significance of my results and this author's findings is unclear although it may be, for the metacyclics at least, that they are only preadapted to live in mononuclear phagocytes which can produce H_2O_2, but do not possess the MPO system. As these are the cells that leishmanial parasites normally live in,

this would seem to make sense. This result would also suggest that the mechanism by which H_2O_2 mediates damage to both promastigote forms of L.major is different from that which occurs in the presence of peroxidase and a halide. Unfortunately, the mechanisms of damage by either system are not clear. Excess H_2O_2 in other systems has been shown to mediate damaging peroxidation reactions resulting in the production of lipid hydroperoxides (Tappell, 1973) whereas the MPO system is thought to mediate damage (via the production of hypohalous acid) by halogenation and oxygenation of cell surface components (Badwey & Karnovsky, 1980; Klebanoff et al., 1983; Locksley & Klebanoff, 1983). The mechanism by which metacyclics protect themselves from the effects of H_2O_2 alone were not investigated, although there are several possibilities, some of which could be tested experimentally. It has been suggested that L.donovani amastigotes are more resistant to H_2O_2 than promastigotes due to the higher catalase activity present in the former cells (Murray, 1982a; Channon & Blackwell, 1985b), it is possible that L.major metacyclic promastigotes also contain higher levels than mid-log phase promastigotes. Alternatively, it has also been postulated that an unsaturated lipid sink, present in the parasite as a normal component of membranes or incorporated into lipid inclusions, could remove or scavenge H_2O_2 by acting as substrates for harmless peroxidation reactions (Channon & Blackwell, 1985b). Interestingly, I found that L.mexicana mexicana stationary-phase (see Fig. 42) and L.major metacyclic promastigotes (see Fig. 43) had apparently more lipid inclusions than their respective mid-log phase promastigotes (see Section 4.4.1). This, therefore, correlates with the increased resistance of L.major metacyclic promastigotes to H_2O_2 in comparison to mid-log phase promastigotes and hence this characteristic may be

mediated in this way. Finally, it has recently been suggested that L.donovani amastigotes are more resistant to H_2O_2 than promastigotes because they have higher intracellular concentrations of trypanothione on a cell to cell basis (Kennedy, pers. comm.). The same may be true for L.major metacyclic promastigotes although the concentration of trypanothione within these cells has not yet been examined.

4.4.4 Proteolytic activities

Gelatin Disc-PAGE was found to be a highly sensitive and useful technique for analysing the proteolytic activities of other pathogenic protozoa such as Trichomonas species (Lockwood et al., 1987) and some trypanosomatids (Lockwood, 1987). My studies have also shown it to be an appropriate technique for the analysis of different forms of Leishmania. Developmental changes were apparent in the three species tested and the results provide confirmation of the great differences in the proteinase activity and pattern of activity between amastigotes and promastigotes of the three species examined. The isoenzyme patterns had been shown previously only for L.mexicana mexicana (North & Coombs, 1981). Furthermore, the study has highlighted the difference between mid-log phase and metacyclic promastigotes (L.major) and also between mid-log phase and stationary-phase promastigotes (L.mexicana mexicana, L.donovani) which my results (as already discussed in Sections 4.4.1 and 4.2) and those of other workers (Giannini, 1974; Doran & Herman, 1981; Keithly & Bienin, 1981; Sacks & Perkins, 1984; Rizvi et al., 1985; Wozencraft & Blackwell, 1987) have shown to be mixed populations containing metacyclics.

The most active proteinases in amastigotes of L.mexicana mexicana had high electrophoretic mobility, but there were also slower moving lower activity enzymes. the lower molecular weight amastigote enzymes were in the 16-36KDa range (see Figs. 36 and 37) and so included the

31KDa enzyme characterised previously (Pupkis & Coombs, 1984). The latter enzymes were absent from all forms of L.major and L.donovani and from mid-log phase L.mexicana mexicana promastigotes (see Fig. 36) confirming the previous report of Pupkis and Coombs (1984). Significantly, similar, although not identical enzymes were variably present in stationary-phase cells (see Fig. 36) and this may explain (as already discussed in Section 4.2) the variability in infectivity of these forms found in this study. The high mobility L.mexicana mexicana promastigote enzymes described previously (North & Coombs, 1981) were probably due to a significant proportion of metacyclics in the preparations used.

There has been no detailed analysis of the proteinases of different forms of L.major and L.donovani, although the total proteinase activities determined previously for promastigotes and amastigotes (Pupkis et al., 1986) are in accord with the levels of activity detected in the gels. Peptidase activity in 3-day and 10-day-old L.donovani were studied by Doran and Herman (1981) using a low molecular weight substrate, it is unclear, however, if and how the enzymes they detected are related to those I have studied. My studies have revealed differences between different promastigote forms and also amastigotes for L.major and L.donovani. Stationary-phase promastigotes of L.donovani had proteinases specific to this form (see Fig. 38). In both species there were enzymes which increased in activity from mid-log phase promastigotes (which possessed the lowest activity), there being greater activity in the stationary-phase or metacyclic promastigotes, with the highest activity being in the amastigotes. This was true for L.major for a proteinase activity running at an apparent molecular weight of approximately 120KDa (Fig. 39) and also for L.donovani for a proteinase running at 130KDa (see Fig. 38). At present one can only speculate on the function of these

enzymes but the increasing activity correlating with increasing infectivity suggests some role in the infection process or survival once within the macrophage.

It is not yet known whether the higher molecular weight enzymes detected in gels represent an entirely different group from the high activity, lower molecular weight enzymes present in L.mexicana mexicana amastigotes (and also stationary-phase promastigotes). The lack of sensitivity of the former to various inhibitors suggests that they are not cysteine proteinases. The inhibitory effect of phenanthrolene, which was also reported by Pupkis and Coombs (1984) for the 67KDa proteinase purified from L.mexicana mexicana, suggests that they may be metalloproteinases, although EDTA, on inhibitor of many enzymes of this class, failed to have any effect. Whether distinctions between types of proteinase reflect other differences such as intracellular location must await further investigation. The finding, however, that at least one of the high activity enzymes of L.mexicana mexicana amastigotes is located within lysosomal-like organelles ("megasomes"), which do not apparently occur in any form of other species (Pupkis et al., 1986) or in mid-log phase and stationary-phase promastigotes of L.mexicana mexicana (see Section 4.4.1 and Pupkis et al., 1986) shows that there must be some differences.

An important finding from this study was that there was an enzyme activity running at between 63-68KDa which was present in all forms of all the species examined (see Figs. 36 and 39), which suggests that this enzyme may be highly conserved. This coincides with recent reports for L.major (Etges et al., 1986) and L.mexicana mexicana (Chaudhuri & Chang, 1987) concerning a protein with an apparent molecular weight of 63KDa. This is one of the major cell surface

proteins and has proteolytic activity. A similar surface protease activity has recently been identified in 7 species and subspecies of Leishmania and in most species the position of proteolytic activity corresponded to a surface protein of approximate molecular weight 63KDa (Bouvier et al., 1987). The reported inhibitor sensitivity for the L.major promastigote proteinase and its ability to bind to ConA (Bouvier et al., 1985; Etges et al., 1986) are shared by the common proteinase activity I detected, suggesting that they are one and the same. Further, the molecular weight of the major surface antigen of promastigotes has been variously reported to be in the range 63-68KDa (Fong & Chang, 1982; Lepay et al., 1983; Gardiner et al., 1984; Etges et al., 1985; Colomer-Gould et al., 1985; Misle et al., 1985; Chang et al., 1986; Heath et al., 1987). The common proteinase activity I detected although apparently varying in molecular weight falls within this range. The mobility of the major surface protein has also been reported to vary within a single study, and this appears to depend on the conditions employed for electrophoresis (Bouvier et al., 1985; Colomer-Gould et al., 1985; Heath et al., 1987) especially the reducing conditions i.e. complete, incomplete, none. This may explain (although conditions were apparently the same in each experiment) the variability seen with my results and also those of others. It is unclear, however, why this only apparently affects this protein. The fact that this is reported to be a surface proteinase corresponds with a previous report showing the proteinse activity of L.mexicana mexicana promastigotes to be mainly particulate in nature (Coombs, 1982). This corresponds to my preliminary results with L.major promastigotes which indicated that the common activity found to run at 63-68KDa did not partition exclusively into either supernatant or particulate fractions resulting from a 100,000 x g centrifugation. It is possible that the Triton X-100 used for lysis may have solubilised

some of the surface activity resulting in apparently cytosolic activity. It has been shown by Gardiner et al (1984) and Heath et al (1987) that this can occur.

Another important finding was that the proteolytic activity of 63-68KDa was also present in amastigotes of all species tested. There are differing reports as to the presence of this major surface protein in amastigotes. Colomer-Gould et al (1985) detected it on amastigotes of 6 species or subspecies of Leishmania, similarly Russell (1987) reported its presence on L.mexicana mexicana amastigotes. In contrast, Fong and Chang (1982) and Chang et al (1986) could not detect it on L.mexicana amazonensis amastigotes. Likewise, Heath et al (1987) could not detect it on L.donovani amastigotes, although in this case they thought it may be present but in a different form.

The major surface protein with proteolytic activity has had many functions attributed (see Introduction Section 1.2.2.3) and some of these have already been discussed in respect to my results (see Section 4.4.2). The finding that it is apparently present in all forms of the species examined in this study at approximately the same activity contrasts to the report of Chaudhuri and Chang (1987) who found that virulent promastigotes L.mexicana amazonensis had 2.5 times as many molecules of this protein on their surface compared to avirulent promastigotes. Clearly there are several aspects of this interesting protein that are still to be clarified. Nevertheless, my results suggest that the activity is important for each of the forms and species used in this study. However, its role may not be related to the specific envrionments in which they live but rather it may perform some more basic function essential to leishmanias in each environment. It seems likely that much more will be reduced concerning this protein over the next few years and already many groups are

studying it with the aim of exploiting it as a vaccine or in diagnosis.

4.4.5 Acid phosphatase activities

My preliminary studies with live cells in the presence of PNPP indicated that L.major mid-log phase promastigotes were able to hydrolyse this substrate. Subsequent work, however, showed that such forms apparently do not secrete or possess any detectable surface acid phosphatase activity. This suggests that the promastigotes was able to take up the substrate, hydrolyse it within the cell, and release the product back into the medium. PNPP is thought to be a non-permeable substrate (Gottlieb & Dwyer, 1981b) and is likely to have taken up via pinocytosis in the flagellar pocket as has been demonstrated for Trypanosoma brucei procyclics (Langreth & Balber, 1975).

A significant finding was that both promastigote forms of L.major apparently lack surface acid phosphatase activity (see Fig. 34) and also promastigotes do not appear to secrete this enzyme during growth in vitro. It is possible that L.major possesses a surface acid phosphatase so different from that of other leishmanias that it was not detected by the method used. Nevertheless, in this respect the L.major strain used in this study differs from all other leishmanias studied, as L.mexicana mexicana (Hassan & Coombs, 1987; see also Fig. 34) and L.donovani (Gottlieb & Dwyer, 1981a,b,c; Dwyer & Gottlieb, 1983) promastigotes and amastigotes, and promastigotes of L.mexicana amazonensis (but apparently not amastigotes) (Pimenta & De Souza, 1986) all possess surface activity. Interestingly, at least one strain of L.major promastigotes has been reported to possess surface activity, albeit at low levels (Lovelace, pers. comm.). The reason why some strains possess activity whilst others do not is unclear, but the results obtained may reflect different detection techniques or growth

conditions, or it may simply be that there is a range of L.major strains, some having the surface enzyme whilst others do not. This appears to be the case with respect to secretion of this activity (see below).

Secretion of acid phosphatase into medium during growth of promastigotes in vitro is a characteristic of all leishmanias studied apart from most L.major strains (Lovelace & Gottlieb, 1986), which is consistent with my findings. Lovelace and Gottlieb (1986) found that only one of the seven strains of L.major tested secreted the enzyme, and this secretion produced only low amounts of the enzyme. Whether the strain that secretes activity is the same as the one which possesses surface activity is not known at present; nor is the functional significance of the variation.

The acid phosphatase activities of homogenates of L.major mid-log phase and metacyclic promastigotes were similar, as were those in L.mexicana mexicana mid-log phase and stationary phase promastigotes; amastigotes possessed lower activities (see Table 11). This is consistent with the report of Hassan and Coombs (1987) that acid phosphatase activity in L.mexicana mexicana promastigotes was slightly higher than that in amastigotes. These results contrast with the situation in Trypanosoma cruzi, epimastigotes and trypomastigotes of this species have 4 to 5-fold less activity than amastigotes (Nagakura et al., 1985), and also the findings of Katakura (1986) who reported that virulent L.donovani promastigotes possess twice as much activity as avirulent promastigotes.

Another way in which L.major appears to differ from other species with respect to acid phosphatase activity is that the majority of the activity was recovered in the soluble fraction of the cell whereas with L.mexicana mexicana (see Table 11), L.mexicana amazonensis and L.donovani over 50% of the acid phosphatase activity was sedimentable

(Hassan & Coombs, 1987). The pH profiles of the activities in the different fractions of L.major mid-log phase and metacyclic promastigotes were similar although the pellet activity profiles were different from those of the supernatant fractions (see Fig. 33); this indicates that the parasites possess multiple activities. The pellet activity, apparently not due to surface membrane activity, probably resulted from membranous systems within the cell as, acid phosphatase activity has been detected in the microsomal and also mitochondria plus nuclei fractions of T.cruzi epimastigotes (Letelier et al., 1985). The pH optima, 6.0, found for the crude homogenate and supernatant fraction is slightly higher than that reported for crude homogenates of L.mexicana mexicana promastigotes (5.5) and amastigotes (4.5) (Hassan & Coombs, 1987). The pellet fraction optimum being in the range 5.0-5.5 was similar to that reported by Hassan and Coombs (1987) for L.mexicana mexicana and also to those of L.donovani plasma membrane and secreted activities (4.5) (Gottlieb & Dwyer, 1981c). The acid phosphatase activities of both promastigote forms were also similar with respect to inhibitor sensitivity (see Table 12). In most cases comparison with other workers results are different because of their use of purified enzyme preparations or particular fractions. The majority of the activity in either promastigote form of L.major was inhibited by concentrations of 1mM NaF and above, similar to the results with L.mexicana mexicana. The three partially purified surface activities from L.donovani promastigotes were also reported to be almost totally inhibited by 5 mM NaF (Glew et al., 1982; Remaley, Das et al., 1985). Ammonium molybdate was a potent inhibitor of L.major acid phosphatase activity. Similar to the reports for L.mexicana mexicana promastigotes and amastigotes (Hassan & Coombs, 1987), and for L.donovani promastigote partially purified surface (tartrate-

resistant) acid phosphatase activity (Glew et al., 1982). The sensitivity to 2 mM sodium -L-(+)-tartrate of L.major secreted acid phosphatase has been reported to be low (7.5%) (Lovelace & Gottlieb, 1986) compared to all other Leishmania species (70-80%) and this is consistent with my findings (for activity from cells) that the activities of L.major are relatively resistant to this compound. When crude homogenates have been used as a source of enzyme, (eg. with L.donovani [Katakura, 1986] and L.mexicana mexicana [Hassan & Coombs, 1987]), the activities have been found to be similarly sensitive to sodium-L-(+)-tartrate as I have found for L.major. So in this respect L.major seems typical of other leishmanias.

Study of acid phosphatases of L.major and L.mexicana mexicana promastigotes by IEF, however, revealed very different patterns for the two species (see Fig. 35). The single isoenzyme found with both promastigote forms of L.major, pI 6.0, not surprisingly appears quite different in this respect to the enzyme secreted by L.donovani promastigotes, which has a pI of 4.5 (Lovelace et al., 1986) and also to the major surface enzyme of this species which was reported to have a pI of 4.1 (Glew et al., 1982). It is not possible to relate the complex pattern found with the L.mexicana mexicana to known enzymes from the other sources.

The purified tartrate-resistant surface acid phosphatase activity of L.donovani promastigotes has been shown to inhibit neutrophil oxidative metabolism Remaley et al., 1984) and this ability has been shown to extend to promastigotes themselves (Remaley, Glew et al., 1985). My results with L.major showing that the activities of the mid-log phase and metacyclic promastigotes are apparently similar and that both forms lack surface activity and the ability to secrete activity strongly suggests that surface acid phosphatase plays no part in the survival of this Leishmania species in macrophages, or in mammals. My

findings counter any suggestion that surface acid phosphatase is vital for the survival of all leishmanias. The findings of Hassan and Coombs (1987) that L.mexicana mexicana amastigotes have lower acid phosphatase activity present on their surface than promastigotes also points to a role for enzymes in the insect host. This suggestion is supported by the findings that an insect trypanosomatid, Herpetomonas muscarum muscarum, has high activity of acid phosphatase on its surface (Coombs et al., 1987). However, my results concerning the RB of rabbit peritoneal neutrophils show an approximate correlation between presence of surface acid phosphatase activity on a parasite cell and ability of the cells to "inhibit" the RB when they are added in large numbers (as already discussed in Section 4.3.2). That this also occurs with insect trypanosomatids makes its role difficult to elucidate at present. However, inhibition of neutrophil oxidative metabolism has been reported to also occur with purified prostatic acid phosphatase, this being as effective as leishmanial acid phosphatase (Das, Saha, Remaley et al., 1986), indicating that this is a property of several, but not all, acid phosphatase from a wide variety of sources. It will probably turn out that the various acid phosphatases have several functions for the parasite in its different environments; that one of them is the mediation of survival against the microbicidal activity of macrophages cannot be ruled out at this stage.

4.4.6 Enzyme content, and isoenzyme and protein profiles

My results from the examination of the enzymes content and isoenzyme profiles of different forms of Leishmania (see Table 13 and Figs. 45 and 46) indicate the stationary-phase promastigotes of L.mexicana mexicana and metacyclic promastigotes of L.major are preadapted with respect to certain enzymes for life in the macrophage,

and as changes in some of the enzyme contents are specific to either L.major or L.mexicana mexicana it appears that there may be species differences in the requirements involved in changing to the infective forms. In most cases the physiological significance of the changes in enzyme content and isoenzyme pattern are unclear at present.

There is little information regarding isoenzyme patterns in different forms of Leishmania ie. promastigotes and amastigotes. Those pertaining to proteolytic activities have already been discussed in section 4.4.4. Gardner and Chance (1972) using starch-gel electrophoresis failed to detect lactate dehydrogenase (LDH) and MDH activity in partially purified amastigotes of L.mexicana amazonensis and L.donovani. In constrast, Mottram (1984) using IEF detected MDH in both amstigotes and promastigotes of L.mexicana mexicana and found substantial qualitative and quantitative differences between the two forms. Similarly, Grimaldi et al (1982) also using IEF, detected variation in the number of staining intensity of the bands for GPI between L.mexicana mexicana promastigotes and amastigotes. I also found differences in the isoenzyme profiles of MDH and GPI when comparing mid-log phase promastigotes and amastigotes of L.mexicana mexicana. My results and those of the latter two workers suggest that changes in isoenzyme profiles seen between different parasite forms may be adaptations to the different environments they encounter. This also appears to be true in other related trypanosomatids as qualitative and quantitative differences in isoenzyme profiles have been reported to occur between epimastigotes, trypomastigotes and amastigotes of Trypanosoma cruzi (Bogliolo & Godfrey, 1987) and also between procyclic forms and bloodstream trypomastigotes of T.brucei and T.b.rhode siense (Kilgour, 1980; Betschart et al., 1983), further indicating that changes in isoenzyme patterns frequently occur as

adaptations to life in different environments.

There have been no previous reports on the enzyme content or isoenzyme of purified promastigotes and only a few studies on the changes in enzyme activities and isoenzyme content during the in vitro growth of Leishmania promastigotes (Doran & Herman, 1981; Cazzulo et al., 1985). Doran and Herman (1981) detected in both 3-day-old and 10-day-old promastigotes of L.donovani (by PAGE), two bands of different activity for G-6-PDH and a single band for peptidase. The single peptidase band in the more infective 10-day-old promastigotes was at 4-fold higher activity compared to that in the less infective 3-day-old cells, rather similar to the change I detected in proteinase activity between mid-log phase and stationary phase promastigotes of L.mexicana mexicana (see Table 13). Clearly, however, my results from Gelatin Disc-PAGE show that different enzymes must be involved (see Fig. 36). These workers suggested that the increase in activity reflected on increased catabolism of external and internal proteins as the medium became depleted of nutrients. A marked increase in proteolysis and intracellular protein degradation during nutrient deprivation of L.tropica promastigotes has also been reported (Simon & Mukkada, 1983). Although the increased activity could be simply due to the requirement for energy substrates by autodegradation. This may also be an essential trigger for the change to the metacyclic form. Indeed, as discussed in Section 4.1.2, depletion of nutrients in the sandfly and in stationary-phase promastigotes is thought to be involved in the production of metacyclics (Sacks & Perkins, 1984, 1985) and my results regarding the production of metacyclics agree with this. Doran and Herman (1981) also found that there were changes in the activity of the two bands for G-6-PDH between 3-day-old and 10-day-old promastigotes; one band increasing whilst the other decreasing. It is interesting that I also detected changes in the G-

6-PDH isoenzyme pattern between L.major mid-log phase and metacyclic promastigotes (see Fig. 45) and also between L.mexicana mexicana mid-log phase and stationary-phase promastigotes (see Fig. 46). These results suggest that changes in the isoenzyme content of G-6-PDH may be crucial in preparing metacyclic or stationary-phase promastigotes for survival inside macrophages of the host. I also detected changes in the isoenzyme pattern for GPI between L.major mid-log phase and metacyclic promastigotes (see Fig. 46) and between L.mexicana mexicana mid-log phase and stationary-phase promastigotes. Interestingly, Grimaldi et al (1982) using IEF, examined the isoenzyme pattern for GPI and found differences between virulent (low sub-passage) and avirulent (high sub-passage) L.mexicana mexicana promastigotes suggesting that this enzyme may also be important. Overall, my results and those of Doran and Herman (1981) and Grimaldi et al (1982) support the view that changes in isoenzyme patterns or content may be important in determining virulence or infectivity. With respect to metacyclic and stationary-phase promastigotes these changes appear to reflect preadaptations to life within the macrophage.

Not surprisingly, in view of my results with isoenzymes. I also found quantitative and qualitative differences in the soluble protein profile of L.major mid-log phase and metacyclic promastigotes (see Fig. 45). Grogl et al (1987) using SDS-PAGE similarly reported diffeences in the soluble polypeptide profiles between log-phase and stationary-phase promastigotes of L.braziliensis panamensis.

With regard to enzyme activities during growth in vitro, Cazzulo et al (1985) reported that the activities of a number of enzymes (MDH, ASAT, GDH, PK AND PEP carboxykinase) did not change significantly during growth in vitro of L.mexicana mexicana promastigotes, although as they did not examine truly stationary-phase cultures these findings

may not be relevant.

4.4.7 Respiration, nitroblue tetrazolium reduction and protein content

Respiration (endogenous and stimulated) in terms of both numbers of cells and also protein was significantly higher L.major mid-log phase cells than metacyclics (see Table 15). The endogenous rate for mid-log phase cells expressed per mg protein is similar to the figure obtained by Hart et al (1981a) for L.mexicana mexicana promastigotes under the same conditions. However, if the rate is expressed on a per cell basis, then the rate for L.major mid-log phase promastigotes is higher than that reported for L.mexicana mexicana promastigotes. This may be because there are real differences in protein content between the two species, or alternatively it may be because the cells used by Hart et al (1981a) were close to stationary-phase when studied and therefore would probably have a lower protein content per cell. An interesting finding, contrary to those of Hart et al (1981a) with L.mexicana mexicana promastigotes and amastigotes, was that under the same conditions the respiration of both promastigote forms was stimulated when they were suspended in HOMEM medium with 10% (v/v) FCS. This possibly suggests that L.mexicana mexicana parasites possess more energy reserves than L.major parasites. The lower respiration rate (endogenous and stimulated) in L.major metacyclic promastigotes compared to mid-log phase cells, even though the former appear more "active" than these latter cells, may be an adaptation to the apparently barren environment of the sandfly anterior foregut and proboscis (Killick-Kendrick, 1979). It may also simply relate to the lack of division of the metacyclics. Stationary-phase populations of Trypanosoma cruzi have also been shown to have a lower respiration rate than log-phase cells (Fernandes & Castellini, 1966).

The lower stimulated respiration rate of L.major metacyclic

promastigotes compared to that for mid-log phase cells is reflected in the lower rate of NBT reduction (whether expressed on a per cell basis or in terms of protein; see Table 17). In rat liver, NBT interacts almost completely with one site on the respiratory chain, possibly ubiquinone (Slater et al., 1963) and this may be the site of reduction with Leishmania. Leishmania donovani log and stationary-phase promastigotes have also been reported to be capable of significant cytochrome C reduction (Blackwell et al., 1986).

The protein content of L.major mid-log phase promastigotes was twice that of the metacyclics (see Table 16) which corresponds to their apparently smaller cell bodies and also correlates with Hart et al (1981a) finding that L.mexicana mexicana amastigotes have approximately 5-fold less protein per cell than promastigotes. Thus the metacyclics of L.major appear to be intermediate in protein content between mid-log phase promastigotes and amastigotes.

4.4.8 Amino acid content

My results examining the free amino acid pools of different forms of Leishmania species (see Table 14) showed that there were differences between species (compare mid-log phase promastigote values) and also within species between the different forms. With L.mexicana mexicana comparison of the values for mid-log phase and stationary-phase promastigotes and amastigotes would appear to suggest that (as with certain enzymes, see Section 4.4.6) the more infective stationary-phase promastigotes are pre-adapated for life in the macrophage with respect to free amino acid pool content; the same can be said for L.major metacyclic promastigotes. The more infective forms of L.mexicana mexicana (stationary-phase promastigotes and amastigotes) and L.major (metacyclic promastigotes) generally have lower levels of amino acids than their repective mid-log phase

promastigotes. For stationary-phase and metacyclic promastigotes this could be due to increased use of amino acids from the medium, as has been reported by Hart and Coombs (1982) and also to the consumption of intracellular amino acids in this situation. This could lead to depletion of pool sizes. Other workers have shown that the composition of the free amino acid pool in L.tropica promastigotes if the cells are starved for 3 hours; same amino acids increased whilst others decreased (Simon et al., 1983). The cells I analysed had not been starved, although they were washed three times (by centrifugation at 2000 x g for 15 min at 4°C), a process that took about 1 hour. However, the fact that changes in L.mexicana mexicana stationary-phase promastigotes correlate with those in amastigotes suggests that these changes and those in L.major metacyclic promastigotes may be of some physiological significance. There is no published work on the comparative amino acid content of different forms of Leishmania, although the free amino acid pool composition I have found appears to be quite similar to those reported for L.tropica promastigotes (Simon et al., 1983) and Trypanosoma cruzi epimastigotes (O'Daly et al., 1983). Qualitative and quantitative differences were found between the content of T.cruzi epimastigotes and mammalian cells (O'Daly et al., 1983). An interesting feature of the above trypanosomatids and those of Williamson and Desowitz (1960) examining several trypanosomes is that in all cases, alanine was the most abundant amino acid present. My results confirm that this is also true for the various forms of leishmania I have analysed, as yet though the significance of this is unclear. Intriguingly, however, it could relate to the use of proteinaceous substrates in energy metabolism (Blum et al., 1987). This is an area worthy of further study.

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