STUDIES ON NEW APPROACHES TO THE CHEMOTHERAPY OF LEISHMANIASIS

by

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ADH; N-acryloyl-1,6,-diaminohexane

BzPPANan; N-benzoyl-proline-phenylalanine-arginine nitroanilide

CHOL; cholesterol

DCP; dicetyl phosphate

DMSO; dimethyl sulfoxide

DPPC; dipalimotyl

DTT; dithiothreitol

E 64; L-leucyl amido-(4-guanidino)-butane

HIFCS; heat inactivated calf serum

IAA; iodoacetic acid

i.p.; intraperitoneal

i.v.; intravenous

leuOMe; L-leucine methyl ester

MTT; (3-(4,5,)-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide

PAGE; polyacrylamide gel electrophoresis

PEC; peritoneal exudate cell

PNA; peanut agglutinin

RES; reticuloendothelial system

Sb; antimony

SDS; sodium dodecyl sulphate

TLCK; N-p-tosyl-l-lysine chloromethyl ketone

TPCK; N-tosyl-L-phenylalanine chloromethyl

WHO; World Health Organisation

Summary

The ability of three different 'physical' drug delivery systems: liposomes, niosomes and starch microparticles, to increase the efficacy of sodium stibogluconate in clearing *L.donovani* from the liver of mice have been characterised. The efficacy of free sodium stibogluconate in this model has been used as the standard against which to measure the effectiveness of the drug delivery systems.

The two vesicular systems; liposomes and niosomes, gave an approximate ten-fold increase in efficacy compared with the free drug. Modifying the properties of the vesicles by varying their cholesterol content and hence their permeability to the encapsulated drug or varying their surface charge by the inclusion of dicetyl phosphate appeared to have little effect on their efficacy as carriers of sodium stibogluconate. Surprisingly, treatment of infected mice with empty vesicles also led to a small decrease in the parasite load.

Testing of the vesicular systems against *Leishmania* infected peritoneal exudate cells (PECs) *in vitro* gave results similar to those obtained using the *in vivo* model in that they increased the efficacy of the sodium stibogluconate. Interestingly, *L.m.mexicana* and *L.donovani* were equally susceptible to sodium stibogluconate in vesicles. Whereas *L.m.mexicana* is relatively unaffected by the free drug as with the *in vivo* model, empty vesicles had an effect on the parasites in exposed PECs, although this effect was difficult to quantify as there was also considerable toxicity to the PECs.

The third drug delivery system tested *in vivo*, starch microparticles, resulted in a 100-fold increase in efficacy compared with the free drug, thus proving much more potent than either of the vesicular systems. This difference could be due to the mechanism of drug release within the host cell. Nevertheless, all three drug delivery systems tested show potential for the treatment of

leishmaniasis. Their use ensures that less drug is required, so overcoming the problems of drug toxicity associated with sodium stibogluconate.

The efficacy of tubercidin in combination with inhibitors of nucleoside transport in mammalian cells, nitrobenzoylthioinosine (NBMPR) and dipyridamole, in the treatment of experimental leishmaniasis was investigated both *in vitro* and *in vivo*. The two nucleoside transport inhibitors, at concentrations which would protect mammalian cells, did not protect promastigotes of *L.donovani* or *L.m.mexicana* from the toxic effects of tubercidin. Tubercidin, both alone or in combination with these inhibitors, was toxic to the promastigotes of the two species with approximate LD_{50}s of 0.1 μM and 0.2 μM , respectively, and an approximate MLC of 1 μM for both species. When used alone, tubercidin proved highly toxic to PECs at all concentrations tested (1-10 μM). These cells were protected from the toxicity of tubercidin by nitrobenzoylthioinosine and dipyridamole. Treatment of infected peritoneal exudate cells with the drug combination did not result in a reduction in total parasite numbers or the number of cells infected. *In vivo* studies using tubercidin alone or in combination with either dipyridamole or NBMPR confirmed that these inhibitors lessened the toxicity of tubercidin to mice although there was no reduction in the parasite load of infected animals treated with the combination. These results indicate that, unlike the situation reported for malaria parasites, *Leishmania* does not appear to change the mechanism of uptake of nucleosides into the host cell.

Investigations of conditions prevailing in the parasitophorous vacuole of *Leishmania* infected cells and their importance to parasite survival were carried out by the use of methlyamine, ammonium chloride

and dextran sulphate to modify them. The use of methylamine and ammonium chloride to raise the pH of the parasitophorous vacuoles did not affect the growth of *L.m.mexicana* in PECs but both the growth and survival of *L.donovani* amastigotes was greatly impaired. In similar experiments dextran sulphate affected the growth of both species of *Leishmania* equally, but the cause of this effect remains unclear.

In a preliminary study, the effect of pH on the uptake and incorporation of adenosine and leucine by different species and stages of *Leishmania* was also determined. Interestingly, the optimum pH for uptake by metacyclics of *L.major* (pH 4-5) differed from that of the other promastigotes investigated (pH 6-7) and could represent a preadaption of these forms to life in the lysosomal system of macrophages. Taken together the results of the uptake and incorporation studies and the results of manipulating the lysosomal pH, suggest that *L.m.mexicana* amastigotes may be adapted for life at a higher pH than *L.donovani*. This may reflect differences in the survival stratagies of these two species of *Leishmania*.

The antileishmanial activities of several amino acid esters, particularly L-leucine methyl ester (leuOMe), and amino acid amides have been characterised in several respects. LeuOMe and tryptophanamide both have an effect on the growth *in vitro* of promastigotes of *L.major*, *L.m.mexicana* and *L.donovani*, although the effect of leuOMe is more transient. However, using both an *in vitro* viability assay based on the reduction of the tetrazolium salt MTT and the *Leishmania*-infected PECs model, it was shown that leuOMe is considerably more potent against amastigotes of *L.m.mexicana* than against promastigotes or *L.donovani* amastigotes. Tryptophanamide was found to have a broader spectrum of activity, although longer exposure times and higher concentrations were required to produce similar effects as leuOme against intracellular parasites.

The onset of activity of leuOMe against promastigote-initiated infections of PECs was found to be time-dependent. It was shown by the use of electron microscopy that susceptibility appears to correlate well with the appearance of megasomes as promastigotes transform to amastigotes. The electron microscopy studies also confirmed the lysosomal nature of these organelles as they were shown to arise from the trans golgi network, in line with current theories on lysosomal packaging.

Interest in the basis of the antileishmanial activity of leuOMe led to the investigation of the esterases of *Leishmania*. Several isoenzymes were identified but there appeared to be no correlation between their presence and susceptibility to leuOMe. A potential assay for following the hydrolysis of leucine benzyl ester involving determining the release of phenol was developed. Difficulties with this system led to a different approach being followed. This involved separation of released amino acid from the parent ester by paper electrophoresis. It proved a useful if unwieldly method of assay for amino acid ester hydrolysis.

The role of low molecular weight proteinases of *L.m.mexicana* in the action of leuOMe was then investigated. These enzymes were purified by an improved two step process involving gel filtration and ion exchange chromatography using FPLC methodology to partially purify the proteinases. The enzymes were confirmed to be of the cysteine type. These were shown, using paper electrophoresis, to be responsible for the majority of the hydrolysis of leuOme by the amastigotes of *L.m.mexicana*. Other species and stages of *Leishmania* could also hydrolyse leuOMe, although at a much lower rate. The partially purified enzymes from *L.m.mexicana* amastigotes and homogenates of amastigotes of *L.donovani* and *L.m.mexicana* were also tested for their

ability to hydrolyse other amino acid esters and amino acid amides, some of which are reported as having antileishmanial activity. Whilst most of the compounds were hydrolysed to some degree by the amastigote homogenates the purified enzymes appeared only to hydrolyse leuOMe and leucinamide, an amino acid amide which is also reported as having antileishmanial activity. Tryptophanamide was not hydrolysed by these enzymes, but was hydrolysed by the amastigote homogenates. This suggests that its antileishmanial activity is mediated in ways different from leuOMe. The results show that the cysteine proteinases specific to amastigotes of *L.m.mexicana* are responsible for the potent antileishmanial action of leuOMe and confirms the suggestion that these enzymes could be exploited as activators of appropriate pro-drugs.

1.0 INTRODUCTION

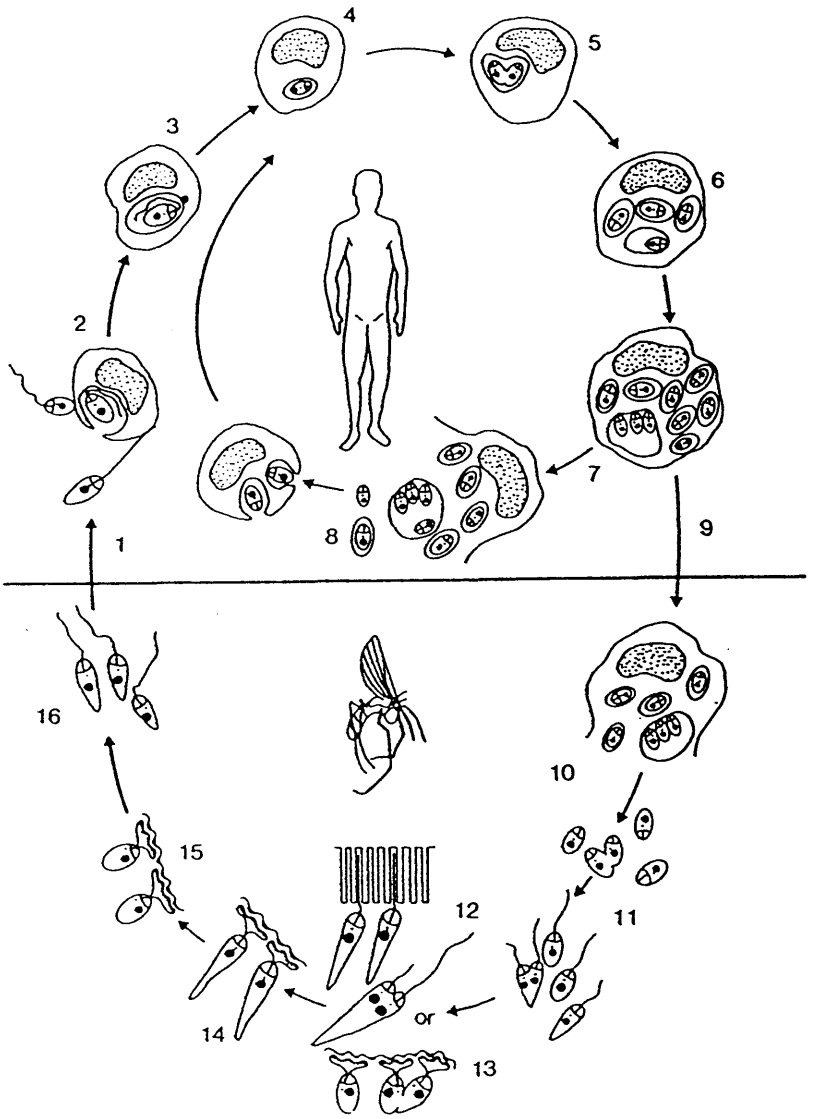
1.1. *LEISHMANIA* AND THE LEISHMANIASES

The disease leishmaniasis is a result of infection by one of a group of parasitic protozoa which are members of the genus *Leishmania*. The disease is widespread throughout large areas of the tropics and sub-tropics, affecting the health of many millions of people (Chance, 1981). The importance of this parasitic disease has been acknowledged by its inclusion in the list of six major diseases for study in the UNDP/WHO Special Program for Research and Training in Tropical Diseases.

This group of parasitic protozoa cause a wide spectrum of diseases. These can be classed broadly into three groups; cutaneous, mucosal and visceral leishmaniasis. The manifestation of the disease depends upon the species of *Leishmania* infecting and to some extent the immune status of the host (Turk and Bryceson, 1971). Although each species is similar morphologically, there are large differences between them in many aspects including metabolism, development, infection and transmission routes and geographical distribution. All of these characteristics have been used as aids to classification.

The typical life cycle of leishmania is outlined in figure 1. The disease is initiated in the vertebrate host by the bite of an infected phlebotomine sandfly in search of a bloodmeal. Extracellular motile flagellated forms, called promastigotes, are inoculated into a mammal whereupon they are rapidly phagocytosed by mononuclear phagocytes. Current evidence suggests that there are at least two types of promastigotes one of which, the metacyclic, is particularly adapted for surviving in mammals. Having been taken into mononuclear phagocytes the promastigotes transform into the non-motile stage, known as the amastigote. The amastigote divides by binary fission eventually causing the infected cell to rupture and release amastigotes which can be phagocytosed and so infect further

Figure 1. Lifecycle of *Leishmania*



1. Delivery of promastigotes into human skin by the bite of 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 a 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 gut of the sandfly; 11. replication of amastigotes and their differentiation into promastigotes; 12. replication of promastigotes in the abdominal midgut or depending on species; 13. in the pylorus and ileum of the sandfly hindgut; 14. forward movement of promastigotes to thoracic midgut; 15 sessile paramastigotes attached to the stomadeal valve, pharynx and cibarium; 16. motile promastigotes found in mouthparts of the sandfly.

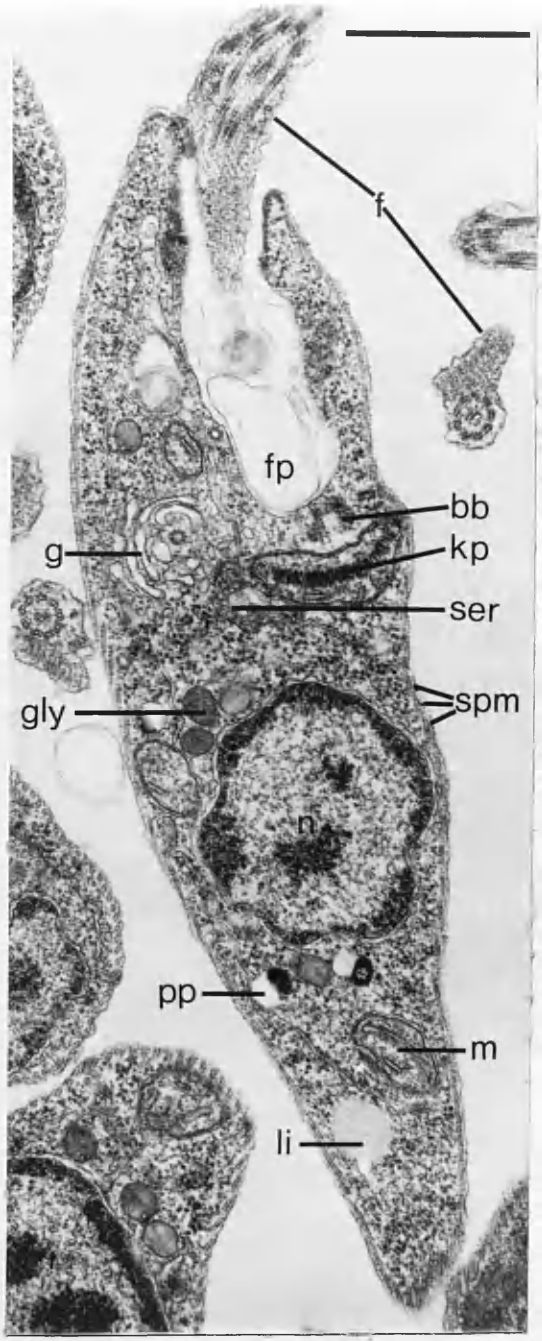
macrophages. Amastigotes taken up by a feeding sandfly transform back to promastigotes within the midgut of the sandfly. They then multiply, undergo developmental changes and migrate to the anterior foregut and proboscis of the sandfly where they are found as metacyclics. Some deviations from the typical pattern are found with a few species of *Leishmania*.

1.1.1. Ultrastructure of *Leishmania*

The promastigotes of all *Leishmania* species are fairly similar in size and morphology, possessing a long anterior flagellum and having the approximate dimensions of 2 x 20 μm. However, morphologically distinct forms do occur in both the sandfly (Killick-Kendrick *et al.*, 1974) and *in vitro* (Sacks *et al.*, 1985; Howard *et al.*, 1988) and these are thought to correspond to different parts of the developmental cycle. Amastigotes are typically much smaller (2 x 5 μm) than the promastigotes although there are differences in size between species. The typical ultrastructure of promastigotes and amastigotes are shown in figures 2 and 3, respectively.

Both of these stages are typical of all trypanosomatids, in having many sub-cellular structures in common with most eukaryotic cells. They do however, have some unique features (Vickerman and Preston, 1976). A large single mitochondrion, approximating to 10% of the total cell volume in the amastigote (Coombs *et al.*, 1986) is present and contains the kinetoplast, which lies just posterior to the basal body of the flagellum. The kinetoplast comprises DNA in the form of maxi- and mini-circles arranged in a regular manner (Chang *et al.*, 1985). Molyneux and Killick-Kendrick (1987) suggested that a connection between the basal body and the kinetoplast exists as they appear to remain in a constant spatial relationship throughout the lifecycle. Ultrastructural evidence for this relationship has not been

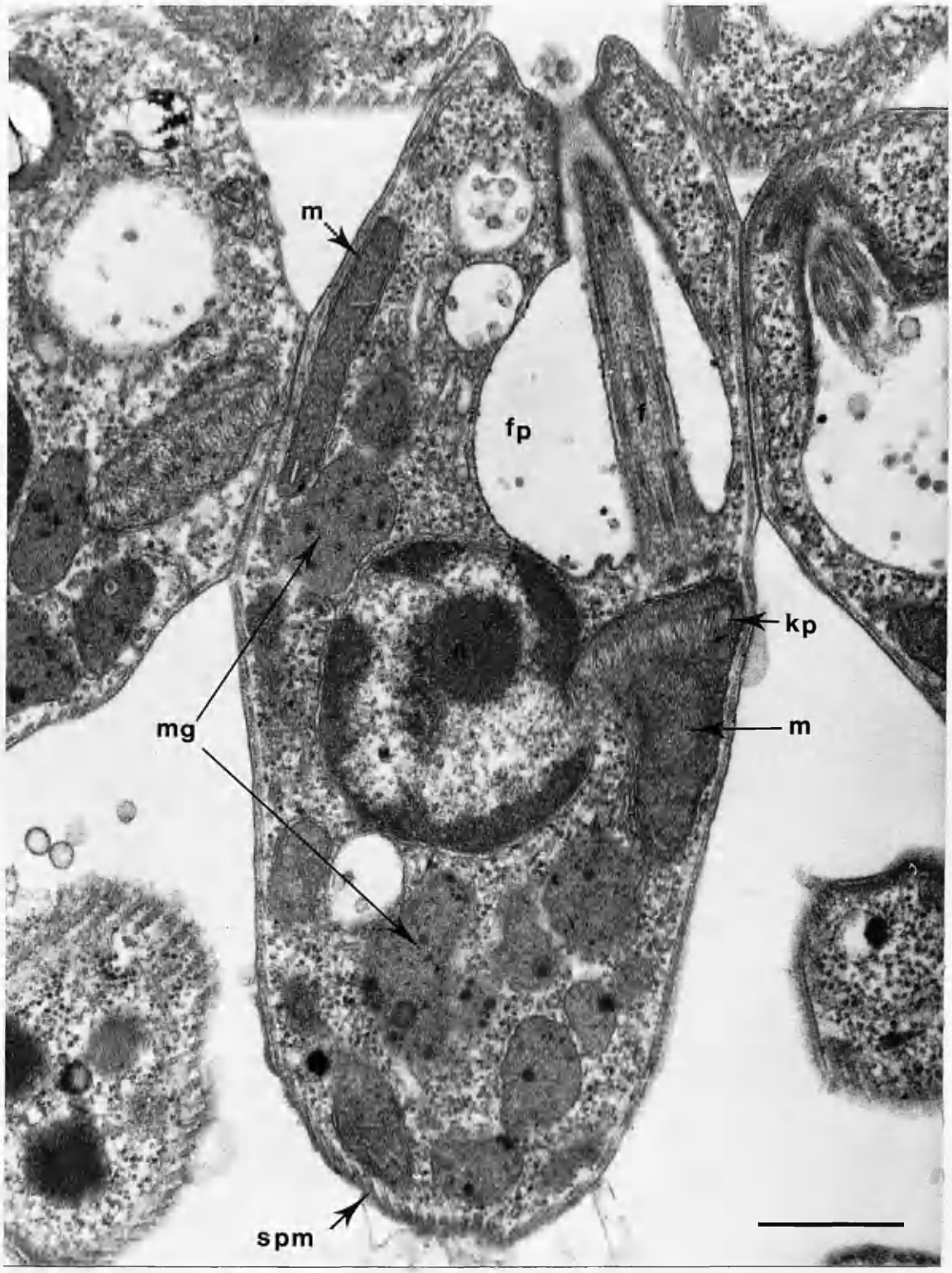
Figure 2. A transmission electron micrograph of a *Leishmania major* promastigote



Scale bar = 0.5 um. (Micrograph courtesy of Dr. L. Tetley)

Key to subcellular stuctures: Basal body, (bb); flagellum, (f); flagelar 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).

Figure 3. A transmission electron micrograph of a *Leishmania mexicana mexicana* amastigote



Scale bar = 0.5 um. Micrograph courtesy of Dr.L.Tetley.
Key to subcellular structure: Flagellum, (f); flagellar pocket, (fp); kinetoplast, (kp); mitochondria, (m); "megasome", (mg); nucleus, (n); subpellicular microtubules, (spm).

reported.

The plasma membrane in both promastigotes and amastigotes is a normal trilaminar unit membrane 2-4 nm in width. Beneath and associated with the plasma membrane is an array of sub-pellicular microtubules that encompass the whole organism, making it a very tough cell (Dwyer, 1980). At the anterior end of the parasite the plasma membrane is invaginated to form the flagellar pocket from which the flagellum emerges. Secretion and endocytosis by the parasite appears to be limited to the membrane of the flagellar pocket (Chang *et al.*, 1985).

Glycosomes are microbody-like organelles that appear to be unique to trypanosomatids. They were first identified in *Trypanosoma brucei* (Opperdoes and Borst, 1977), and reported since then in *Trypanosoma cruzi* and *Crithidia* species (Taylor *et al.*, 1980) and several *Leishmania* species (Coombs *et al.*, 1982; Hart and Opperdoes, 1983). They are small, membrane-bound organelles associated with several important biochemical pathways such as glycolysis (Opperdoes, 1984; Mottram and Coombs, 1985) and *de novo* purine and pyrimidine biosynthesis (Hassan *et al.*, 1985; Opperdoes, 1988). There appears to be a large variation between trypanosomatids in the abundance of these organelles, probably an indication of their importance to different species and stages. Bloodstream forms of *Trypanosoma brucei* have an average of 240 (Opperdoes *et al.*, 1984), *L.tropica* promastigotes are said to contain 50-100 (Opperdoes, 1987) whilst the amastigotes of *L.mexicana mexicana* were found to have only around 10 (Coombs *et al.*, 1986).

Alexander and Vickerman (1970) first reported the presence of megasomes in *L.m.mexicana* amastigotes. These organelles are variable in shape but frequently large and occupy as much as 15% of the amastigote cell volume (Coombs *et al.*, 1986). They have been shown to

be similar in many respects to lysosomes; structurally they have a similar 10 nm thick membrane and they contain several putative lysosomal enzymes including arylsulphatase and a cysteine proteinase (Pupkis *et al.*, 1986). These organelles are apparently unique to the *L.mexicana* species complex and are found only in the amastigote stage (Pupkis *et al.*, 1986). It has been suggested that they may play an important role in the survival of *L.mexicana* amastigotes within the host (Coombs, 1982; Coombs and Baxter, 1984; section 1.2.2.).

1.1.2. Developmental changes in *Leishmania*

Throughout the lifecycle of *Leishmania* there are several developmental changes that occur. Amastigotes ingested by a sandfly appear to undergo at least one cell division cycle before they transform and develop to promastigotes (Shortt *et al.*, 1926; Hart *et al*, 1981c; Warburg *et al.*, 1986). This transformation is accompanied by an increase in tubulin biosynthesis over a period of two days which appears to correlate well with morphological changes in the flagellum and the cytoskeleton (Fong and Chang, 1981). Amastigotes readily transform to promastigotes *in vitro* and this has provided a useful model system for many experimental studies (see Fong and Chang, 1981; Hart *et al.*, 1981c) as well as diagnosis (Chang and Hendricks, 1985). A variety of morphological changes also occur within the promastigotes as they divide and establish themselves within the sandfly. They move from the midgut or hindgut (depending on species) to eventually give rise to the infective metacyclics. The different forms that occur have been described in detail by Molyneux and Killick-Kendrick (1987). Most of the experimental work on the developmental changes that occur in promastigotes however, has been carried out using promastigotes grown *in vitro*. The biochemical differences between the developmental stages of promastigotes and amastigotes are dealt with in section 1.3..

The change from infective promastigote to the intracellular amastigote is the most important developmental change that occurs in terms of the clinical disease. This change precedes the multiplication of the intracellular amastigote. This stage appears to be adapted specifically for survival in the hostile intracellular environment. There are large changes in morphology (section 1.1.1.) and several metabolic switches (see section 1.3.) when promastigotes transform to amastigotes. Fong and Chang (1981) used changes in the levels of tubulin biosynthesis to follow the time scale of this transformation *in vitro* of *L.m.mexicana*. Surprisingly, based upon these criteria, they concluded that this transformation took 7 days to complete; a process that at the level of the light microscope occurs within 12-24 hours (Alexander, 1975; Ardehali and Khoubyer, 1978; personal observation).

The factors that initiate the promastigote to amastigote transformation have not yet been fully elucidated. Temperature appears to be a very important factor, the change in temperature from the sandfly ($27^{o}C$) to the definitive host ($37^{o}C$) is large. Darling and Blum (1987) showed that raising the temperature of a *L.braziliensis panamensis* promastigote culture from $26^{o}C$ to $35^{o}C$ resulted in amastigote-like forms. A similar effect had been shown previously by various other workers with several other species of *Leishmania* (Hendricks *et al.*, 1978; Hunter *et al.*, 1982; Pan, 1984). This temperature change also induces the synthesis of several heat shock proteins (Hunter *et al.*, 1984; Lawerence and Gero, 1985; Van der Ploeg et al., 1985) which are thought to play a part in the transformation of the parasite (Alcinia and Fresno, 1988).

Although amastigote type forms can be produced axenically from promastigotes in culture (see above) there has been great difficulty in attempting to culture these extracellular forms. Probably the most

succesful has been the growth of *L.m.pifanoi* in cell free media (Pan, 1984) although how closely these are related to the intracellular amastigotes remains unclear (Evans, 1987).

The *in vitro* culture of *Leishmania* species is also temperature dependent. *L.m.mexicana* amastigotes grow best in macrophages at 34-35°C (Biegel *et al.*, 1983) as do *L.tropica* whilst the optimum temperature for *L.donovani* amastigotes is in the range 35-37°C (Berman and Neva, 1981). Personal observations with the species used in this study confirmed that *L.donovani* grew best at 37°C whilst *L.m.mexicana* and *L.major* grew best between 34°C and 35°C. It has been suggested that this temperature sensitivity may account for the site specificity of the different cutaneous and visceral forms of leishmaniasis, presumably a cutaneous site would be several degrees cooler than a visceral site (Biegel *et al.*, 1983).

1.1.3. Taxonomy

The taxonomic position of the genus *Leishmania* is shown in tables 1 and 2. This genus is a member of the family *Trypanosomatidae* which contains parasitic protozoa found in either vertebrate or invertebrate hosts. Monoxenous parasites such as *Crithidia* and digenetic parasites including *Trypanosoma* and *Leishmania* are included in this family (Molyneux and Ashford, 1983).

As yet there is no definitive classification system for the different species of *Leishmania* which infect man. Proper identification of *Leishmania* species is important in the treatment of infected individuals and in understanding how the disease is transmitted, so enabling control programmes to be implemented. Table 2 is based on the WHO Technical Report Series 701, (1984). The classification of *Leishmania* species has been based upon many different factors including morphology, development in the sandfly and

Table 1. Classification of *Leishmania*

Kingdom	Protista
Subkingdom	Protozoa
Phylum	Sarcomastigophora
Subphylum	Mastigophora
Class	Zoomastigophora
Order	Kinetoplastida
Suborder	Trypanosomatina
Family	Trypanosomatidae
Genus	Leishmania

From Molyneux and Ashford (1983).

Table 2. Species of *Leishmania* the diseases they cause and their distribution

SPECIES	DISEASE	DISTRIBUTION
L.braziliensis braziliensis	Cutaneous mucocutaneous	Brazil
L.braziliensis guyanensis	Cutaneous	North Amazon/ Guyanas
L.braziliensis panamensis	Cutaneous	Panama/ Costa Rica
L.peruviana	Cutaneous	Andes
L.mexicana mexicana	Cutaneous	Central America
L.mexicana amazonensis	Cutaneous Diffuse cutaneous	Amazon Basin/ Brasil
L.mexicana pifanoi	Diffuse cutaneous	Venezuela
L.mexicana garnhami	Cutaneous	Venezuela
L.mexicana venezuelensis	Cutaneous	Venezuela
L.aethopica	Cutaneous Diffuse cutaneous	Ethiopia/ Kenya
L.tropica	Cutaneous/ Visceral	Urban areas of Middle East and India
L.major	Cutaneous	Middle East Soviet Asia and Africa
L.donovani donovani	Visceral Mucocutaneous Post kala-azar dermal	China/India/ Kenya/Sudan
L.donovani infantum	Infantile visceral	Central Asia/ Mediterranean/ N.W. Africa/ Middle East
L.donovani chagasi	Infantile visceral Visceral	South/Central America

Adapted from Molyneux and Ashford (1983), WHO (1984) and Chang *et al* (1985).

geographical distribution and the clinical features in human infection (Lainson and Shaw, 1987).

Some controversy surrounds the trypanosomatids found in various lizards. The validity of these parasites as leishmanias has been questioned with the finding that *Leishmania tarentolae*, which infects the Moorish gecko, was apparently identical to *Trypanosoma platydactyli* which also infects geckos (Chance, 1985; Wallbanks *et al.*, 1985). The evidence produced so far lends considerable support for the separation of these lizard parasites from the mammalian leishmanias. Nevertheless, this conclusion has been questioned by Simpson and Holz (1988) who detailed the many similarities between these organisms and the mammalian leishmanias.

1.2. *LEISHMANIA* AND MACROPHAGES

The interaction of *Leishmania* with its host cell, the macrophage, is one of the most interesting aspects of this disease. Macrophages are involved in several areas of the host immune response to invading micro-organisms. These cells have a wide array of antimicrobial defences which include phagocytosis and the concomitant production of oxygen free radicals with subsequent exposure to the degradative enzymes of the lysosomal system. Thus the cell types that are largely responsible for the killing of invading microorganisms are also the host cells for leishmaniasis.

1.2.1. Entry of *Leishmania* into the macrophage

The infection of macrophages is preceded by the inoculation of promastigotes during the bite of a sandfly. Ingested promastigotes attach to macrophages and are subsequently phagocytosed. There is no evidence that chemoattraction of the promastigotes to the potential host cell occurs (Bray, 1983). However, in the same study, promastigotes were shown to activate complement, thereby becoming

chemotactic to macrophages by both the alternative and classical pathways. At high concentrations of complement cytolysis of the promastigotes occurs (Pearson and Steibigel, 1981; Mosser and Edelson; 1984).

Attachment of the promastigotes can occur by either serum-independent or -dependent mechanisms. Serum-independent mechanisms of attachment are thought to be of the ligand-receptor type, similar to the mannose/N-acetylglucosamine pathway of receptor-mediated endocytosis in macrophages. This pathway appears to be important in the retrieval of secreted lysosomal enzymes as well as in the targetting and sequestration of lysosomal enzymes within the cell. The binding of promastigotes to macrophages is inhibited by mannose phosphate and by the plating of macrophages onto a mannan-coated surface (Blackwell *et al.*, 1985; Wilson and Pearson, 1986). This competitive inhibition indicates the importance of the mannose/N-acetylglucosamine receptors in the uptake of promastigotes, although this does not appear to be as important for amastigotes (Saraiva *et al.*, 1987).

The acid phosphatase present on the surface of *L.donovani* promastigotes (Gottlieb and Dwyer, 1981a, b) is a mannose-containing glycoprotein (Glew *et al.*, 1982) and may play a role in the uptake of the parasite via the mannose/N-acetylglucosamine ligand receptor system mentioned above. In his review, Bordier (1987) postulated that the glycans of the 63-68 kDa surface proteinase (gp 63) may bind to the mannose receptors on the surface of the macrophage. A role in the binding of complement to the surface of promastigotes via this molecule has also been proposed (Russell, 1987).

Fibronectin is a large dimeric glycoprotein molecule involved in a wide variety of physiological processes such as wound healing and

regulation of monocyte activity as well as several adhesive functions including cell attachment. This molecule is composed of nine domains responsible for separate functions such as fibrin binding or cell adhesion. The particular domain of this macromolecule that is involved in the cell attachment activity appears to be the Arginine-Glycine-Aspartic acid (RGD) amino acid sequence. This particular sequence is implicated in the binding of proteins such as fibrin and the complement protein fragment C3bi with their respective cell surface receptors. There is an increasing amount of evidence that RGD attachment sites are involved in the adhesion and attachment of *Leishmania* to host macrophages. Fibronectin-mediated attachment of *L.m.amazonensis* promastigotes and amastigotes to human monocytes has been shown and this attachment could be inhibited by anti-fibronectin antibodies (Wyler *et al.*, 1985; Ouaissi, 1988). The use of peptides containing RGD sequences to saturate macrophage fibronectin receptors blocked the uptake of promastigotes whilst unrelated peptides failed to have the same effect (Rizvi *et al.*, 1988). The role of the surface proteinase of *Leishmania* in binding to macrophages is well known (see previous paragraph). The recent molecular cloning and characterization of this gene has predicted the presence of the RGD sequence in this surface proteinase (Button and McMaster, 1988). This could account for the importance of this surface proteinase in the attachment of *Leishmania* to macrophages (reviewed Ouaissi, 1988).

Serum-dependent mechanisms of attachment and phagocytosis of promastigotes are thought to be of two types. The promastigotes adsorb opsonins onto their surface. These are either antibodies or components of complement. Binding to the macrophages then occurs by either the Fc receptors for antibodies or C3b receptors for complement. It has been reported (Chang *et al.*, 1985) that under serum-free conditions complement mediated attachment of the promastigotes can still occur,

presumably as macrophages can secrete complement (Ezekowitz *et al.*, 1984). Alternatively there may be C3b type molecules on the surface of the promastigotes, so enhancing their attachment to and subsequent phagocytosis by the macrophages (Chang *et al.*, 1985).

Leishmanias, promastigotes and amastigotes, attached to macrophages are actively phagocytosed, presumably aided by the host-parasite surface compatability mentioned above (Chang, 1985). Unlike some other intracellular parasites, for example *Plasmodium*, the leishmanias do not actively invade cells. However, some promastigotes are known to infect non-phagocytic cells (Chang, 1978) by what appears to be induced phagocytosis. These promastigotes reside in and transform in phagolysosomes which do not fuse with secondary lysosomes and Chang (1978) has suggested that this may be a survival strategy that allows the initial transformation of the promastigote to amastigote in a less hostile environment.

1.2.2. Survival of *Leishmania* in macrophages

The mechanism behind the survival of *Leishmania* in the macrophage has been the subject of much conjecture. There are many proposed mechanisms although most are not mutually exclusive. It is possible that a variety of survival mechanisms are employed and that these vary between species and stages of *Leishmania*. It is important to understand how the parasite is adapted to survive as a fuller understanding of this mechanism may lead to the development of rational new drug therapies.

Before or during phagocytosis of the parasites the macrophage respiratory burst may be stimulated. The parasites are then potentially exposed to a vast array of microbicidal mechanisms from the macrophage and these may result in parasite death. The respiratory burst involves an increased oxygen utilisation attributable to the

production of toxic oxygen metabolites, such as superoxide anion and subsequently hydrogen peroxide, and the concomitant stimulation of the hexose monophosphate shunt (Buchmuller and Mauel, 1981; Murray, 1982). Hydrogen peroxide appears to be the most important oxygen intermediate for the control of *Leishmania* growth in macrophages. However, the species and stage of *Leishmaia* and source of macrophages does also play an important role in the size of the oxidative burst stimulated (Bray and Alexander, 1987). The precise role of lymphokines, such as gamma interferon and macrophage activation factor, in the control of *Leishmania* infections by the hosts immune system remains unclear (see review by Hughes,1988).

Both amastigotes and promastigotes stimulate this response by macrophages although it is generally accepted that amastigotes stimulate a smaller response and not surprisingly amastigotes are more infective than promastigotes (Channon *et al.*, 1984; Murray, 1981). Metacyclics stimulate a respiratory burst which is intermediate between amastigotes and non-infective promastigotes in magnitude (Mallinson and Coombs, in press).

Acid phosphatase at the surface of leishmanias may also play a role in negating the macrophage's respiratory burst. It has been reported that the enzyme purified from *L.donovani* promastigotes interfered with the production of superoxide anions by neutrophils and macrophages (Remaley *et al.*, 1984). Saha *et al.*, (1985) showed that *Leishmania* surface acid phosphatase is also resistant to inactivation by oxygen metabolites. Surprisingly, however, it seems that there is little correlation between the presence of surface acid phosphatase and infectivity. Such a connection would be expected if the enzyme was crucial to survival. There is also a secreted acid phosphatase in promastigotes of *L.donovani* (Bates and Dwyer, 1987) and some evidence for both a surface and excreted acid phosphatase in the amastigote

(Dwyer and Gottlieb, 1983). It has been suggested that these enzymes play a role in avoiding the host's defence mechanisms. It is possible that this secreted enzyme interferes in the regulation of cellular activities by altering the levels of host phosphorylated compounds. The precise roles, if any, of leishmanial acid phosphatases in parasite survival are yet to be fully understood although there is enough evidence to indicate that they are important.

So, avoidance of exposure to oxygen metabolites may be part of the survival mechanism. However, some cells that do stimulate a respiratory burst manage to survive. The mechanism behind this survival against oxidant damage by free radicals, is not yet fully understood. Superoxide dismutase and possibly catalase are thought to play a role in the detoxification of these radicals by the parasite (Murray, 1981; Hadaris and Bonventre, 1982; Locksley and Klebanoff, 1983). Indeed it has been suggested that differences between mammalian and trypanosomatid superoxide dismutase may provide a potential target for chemotherapy. The role of gluathione in free radical scavenging is well documented in prokaryotes and eukaryotes (Meister and Anderson, 1983) and recently differences between this and the equivalent in tryanosomatids (trypanothione) have been highlighted and may prove useful as a chemotherapeutic target (see section 1.5.1.). The role of trypanothione in the survival of *Leishmania* is as yet unclear.

Once phagoytosed the parasites are enclosed in the parasitophorous vacuole. Several workers have reported that lysosomal fusion with the parasitophorous vacuole occurs (Alexander and Vickerman, 1975; Chang and Dwyer, 1976, 1978; Lewis and Peters, 1977) thus exposing the parasite to the microbicidal mechanisms of the lysosomal system. Other intracellular parasites have direct strategies for avoiding the consequences of these microbicidal activities. *Trypanosoma cruzi* escapes from the phagolysosome and resides in the

cytoplasm whilst *Toxoplasma gondii* and *Mycobacterium tuberculosis* prevent lysosomal fusion (Blackwell and Alexander, 1983). However *Coxiella burnetii* is in a similar position to that of *Leishmania* in that they are both directly exposed to the lysosomal system and yet manage to survive and replicate. However, Alexander (1981) showed that inhibition of lysosomal fusion with the parasitophorous vacuole of macrophages infected with *L.m.mexicana* stimulated amastigote growth, suggesting that the lysosomal system does inhibit the intracellular parasites. So, how do the parasites manage to survive in this potentially lethal enviroment?

Several mechanisms have been proposed to account for the survival of the leishmanias within the lysosomal system. One is that some species of *Leishmania*, particularly of the *L.mexicana* complex, may survive in the vacuole by secreting amines and so raise the lysosomal pH (Coombs, 1982). Other effects of amine secretion would include interference with receptor recycling and the packaging of lysosomal enzymes by the golgi apparatus (Tietze *et al.*, 1980). It has been postulated that the high levels of proteinase activity present in the amastigotes of the *L.mexicana* complex could be responsible for the breakdown of proteins and peptides to eventually yield amines, the secretion of which into the parasitophorous vacuole would alter the lysosomal pH and hence lysosomal function (Coombs, 1982). This theory is attractive in that it would also account for the characteristically large parasitophorous vacuoles present with the *L.mexicana* complex. However Coombs and Sanderson (1985) found no evidence of higher levels of amine secretion by amastigotes than promastigotes of *L.m.mexicana* and published work on the pH of the lysosomal system of macrophages infected with either *L.donovani* or *L.m.amazonensis* indicates that there are no differences between infected and uninfected cells (Chang, 1980).

Previous work has indicated that *L.donovani* is adapted to life at an acidic pH, similar to that reported for the lysosomal system (Mukkada *et al.*, 1985), inferring that the pH of the parasitophorous vacuole is not modified by the parasite. *Coxiella burnetii*, the etiologic agent of Q fever is, like *Leishmania*, an obligate intracellular parasite which remains within the phagolysosomal system. It, like *L.donovani*, appears to be biochemically adapted to living in an acidic enviroment. The use of lysosomotrophic compounds (see section 1.5.2.2.) like methylamine and ammonium chloride to change the pH of the lysosomal system inhibited the growth and survival of this intracellular parasite (Hackstadt and Williams, 1981).

One proposed survival mechanism investigated by several groups involves so called excreted factors. These glycoconjugates are composed of a series of components, each of which may have its own specialised function, and appear in all the leishmanias and are produced by both promastigotes and amastigotes (Hernandez, 1983; Handman *et al.*, 1987). They are known to inhibit lysosomal degradation of protein (Rodriguez *et al.*, 1983), erythrocytes (Eilam *et al.*, 1985) and to allow the survival of some *Leishmania* species in macrophages that are normally leishmanicidal (Handman and Greenblatt, 1977). El-On *et al.* (1980) reported that whilst an excreted factor from *L.donovani* did not inhibit several lysosomal enzymes, such as acid phosphatase, it did cause a large inhibition of β-galactosidase. The large negative charges on these carbohydrate-rich molecules may be involved in the inactivation of the lysosomal hydrolases.

Other proposed survival mechanisms include an enzyme-resistant cell surface and Chang (1983) has presented evidence that the surface antigenic glycoproteins of *Leishmania* are resistant to lysosomal enzymes. Sub-lethal doses of tunicamycin (an inhibitor of N-linked protein glycosylation) render promastigotes of *L.donovani* and

L.m.amazonensis susceptible to intracellular killing (Kink and Chang, 1987; Nolan and Farrell, 1985). This indicates that glycosylated proteins may play a role in the intracellular survival of leishmanias, whether this involves surface antigens or parasite enzymes remains to be clarified. The involvement of the surface proteinase, gp63, in survival is, as yet, unclear (see section 1.3.5.)

1.3. BIOCHEMISTRY OF *LEISHMANIA*

There are various reasons for studying the biochemistry of these parasites. They can act as an interesting model system of cell development as they readily transform *in vitro*. They are involved in a complicated form of intracellular parasitism and an understanding of the biochemistry behind the survival mechanism could lead to the development of ways of interfering with it. Biochemical differences between the host and the parasite are also potential targets for chemotherapeutic attack (see section 1.5.1.).

Leishmania amastigotes are difficult to obtain in sufficient quantities for biochemical studies, consequently most of the work has been carried out on the promastigote stage. In most cases these studies have probably involved a mixture containing metacyclics and multiplicative forms. Current evidence suggests that there are several differences between promastigotes and amastigotes of *Leishmania*, and between the two forms of promastigote (Chang, 1985; Coombs, 1986). However, as there is a general lack of information on the amastigote stage, most of the work discussed here, except where stated otherwise, refers to promastigotes in general.

1.3.1. Energy metabolism

The energy metabolism of *Leishmania* has been quite extensively studied and has been reviewed by Mukkada (1985) and Glew *et al.* (1988). Enzymes of the glycolytic and TCA cycle are present in both

the amastigote and promastigote and their respiration is cyanide sensitive. Promastigotes contain enzymes that allow them to utilise either carbohydrates or amino acids as energy substrates. With some species, amino acids appear to be used mainly during the earlier stages of promastigote growth *in vitro* whilst glucose tends to be utilised more during the stationary phase of growth (Marr and Berens, 1977; Cazzulo *et al.*, 1985). Indeed, Mukkada (1985) suggested that *Leishmania* promastigotes utilise amino acids rather than carbohydrates as their respiratory substrate. It has also been suggested however that these findings reflect the length of time that the promastigotes have been maintained in continuous culture *in vitro*. Hart and Coombs (1982) reported that low sub-passage promastigotes of *L.m.mexicana* preferentially utilised glucose rather than amino acids whilst the reverse was true for high sub-passage promastigotes.

A comparison of some of the enzymes involved in carbohydrate and amino acid metabolism in amastigotes and promastigotes of *L.donovani* by Meade *et al.* (1984) showed that there are quantitative rather than qualitative differences between the stages. However, the promastigotes and amastigotes of *L.m.mexicana* do appear to differ considerably. The amastigotes, unlike promastigotes, utilise fatty acids in preference to glucose for their energy metabolism. There are also higher levels of enzymes responsible for the B-oxidation of fatty acids in the amastigote than in the promastigote (Hart *et al.*, 1981b; Hart and Coombs, 1982). This may reflect differences in the respective environments of the different stages: the sandfly gut may be rich in amino acids and/or carbohydrates whilst the lysosomal enviroment may have a plentiful supply of fatty acids.

1.3.2. Surface membrane

The surface membrane of both stages of *Leishmania* is the primary site at which the parasite interacts with its hosts. The surface membrane is involved in a large number of processes including: uptake and transport of essential nutrients, secretion, protection, recognition phenomena and membrane biosynthesis. An understanding of the events occurring at this parasite/host interface should give an insight into the survival mechanisms employed by the leishmanias.

A wide variety of enzymatic activities have been identified, but as yet their roles in the parasite have to be fully elucidated. Three phosphomonoesterase activities have been shown to be present on the external surface of promastigotes (Dwyer and Gottlieb, 1985). There is a non-specific acid phosphatase (see section 1.2.2.) as well as distinct 5'- and 3'-nucleotidases (Gottlieb and Dwyer, 1981a, 1982; Dwyer and Gottlieb, 1984). These nucleotidases may play a role in fulfilling the purine requirements of the *Leishmania* (see section 1.3.3.). The 3'- activity is higher than the 5'- activity in promastigotes of *L.m.mexicana* and *L.donovani* (Dwyer and Gottlieb, 1981; Hassan and Coombs, 1987). It has been suggested that the 3'-nucleotidase acts as a nuclease to hydrolyse exogenous nucleic acids and that the resulting nucleotides are further catabolised by the 5'-nucleotidase to produce nucleosides which can be readily taken up by the cell. Equivalent 3'-nucleotidases are not found in mammalian cells and so may prove useful in diagnosis or as a target for chemotherapy.

Several other enzyme activities associated with the surface membrane of *L.donovani* promastigotes have been reported, as reviewed by Dwyer and Gottlieb (1985). An ATPase is associated with the internal face of the surface membrane. Normally plasma membrane ATPases act as part of an ion transport system, however this parasite ATPase does not appear to be a Na^+/K^+-stimulated enzyme but rather is

thought to be responsible for proton pumping. Adenylate cyclase is also associated with the surface membrane and may be involved in the regulation of cyclic AMP levels which are generally involved in cellular control and signal recognition processes. Three phospholipases (lipolytic enzymes), which have been implicated in the pathology caused by trypanosomatid infections, are also associated with the surface membrane.

There is only a limited amount of information on the transport of exogenous materials across the parasite surface membrane. Carrier-mediated processes are indicated as being involved in the uptake of several nutrients, notably the amino acids methionine and proline (Simon and Mukkada, 1977; Law and Mukkada, 1979), hexose sugars (Schaefer *et al.*, 1974; Zilberstein and Dwyer, 1985) and alpha-aminoisobutyric acid (Lepley and Mukkada, 1983). Purines and nucleosides are also taken up actively, however the rates of uptake of the nucleosides is much higher than those of the free bases (Hansen *et al.*, 1982; Aronow *et al.*, 1987).

The finding of a proteinase (gp63) on the surface of promastigotes and amastigotes has stimulated much interest in its biological function. Its role in parasite attachment (see section 1.2.1.) and survival (see section 1.3.5.) are discussed elsewhere.

1.3.3. Purine and pyrimidine metabolism

Purine and pyrimidine nucleotides can be synthesised *de novo* in mammalian cells from simple precursors (glycine, formate, CO_2, glutamine and aspartate). Alternatively, bases and nucleosides can be 'salvaged' from exogenous sources or they may be the products of DNA and RNA breakdown within the cell. These can be converted to the appropriate nucleotides which are then used in the synthesis of DNA and RNA.

There have been extensive reviews recently on the purine and pyrimidine metabolism of parasitic protozoa (Hassan and Coombs, 1988) and the leishmanias in particular (Marr and Berens, 1985). Like most parasitic protozoa, the leishmanias appear to be unable to synthesise the purine ring *de novo* and so are reliant on exogenous sources for DNA and RNA synthesis. However they do appear to be able to synthesise *de novo* at least part of their pyrimidine requirements. *Leishmania* purine metabolism has been investigated extensively and several differences from the mammalian system have been identified and offer opportunities for chemotherapeutic attack (see section 1.5.1.). Work on *L.m.mexicana* amastigotes and promastigotes has shown only quantitative differences between the stages in the enzymes involved in these pathways. This is in contrast to the situation in *L.donovani* for which differences have been reported. Notably, promastigotes contain adenine aminohydrolase whilst amastigotes contain adenosine deaminase.

1.3.4. Polyamines.

Polyamines (spermine, spermidine and putrescine) are present in almost all cells, and appear to play an important role in cellular phenomena such as replication and differentiation. Polyamines have been detected in several species of *Leishmania*, both promastigotes and amastigotes (Bachrach *et al.*, 1979; Morrow *et al.*, 1980; Coombs and Sanderson, 1985). Polyamine levels have been found to be higher in promastigotes than amastigotes and the relative levels of each polyamine do vary with species and stage of *Leishmania*. The biosynthesis of polyamines is proving a good are for drug attack, particularly the key enzyme ornithine decarboxylase. This is inhibited by difluoromethylornithine (DFMO) which has promising activity as an antileishmanial drug (Bacchi, 1981; Henderson and Fairlamb, 1987).

1.3.5. Proteinases

Proteinases are degredative enzymes that play a role in the hydrolysis of proteins into peptides and subsequently amino acids for nutritional purposes. Proteinases have also been implicated in a wide range of other cellular processes that include post-translational modification of proteins, the rapid turnover of cellular proteins which can allow rapid adaptation to changes in the environment and in the pathogenicity of various microrganisms (North, 1982).

Proteinases may be classified in several ways; on the basis of their pH range, their ability to hydrolyse specific substrates or on their similarity to other well-characterised proteinases. These methods of classification all have drawbacks. Many proteinases are active over a wide pH range and the pH optima of a proteinase will often differ with a different substrate. Proteinases can often act on several substrates, and the classification of a proteinase by the comparison of a few properties can be misleading. The most satisfactory classification scheme is that of Hartley (1960) based on the catalytic mechanisms of the different proteinases. This is summarised in table 3. Four main types of proteinase can be distinguished on the basis of their sensitivity to various inibitors (see table 3). There is some overlap in sensitivity to certain inhibitors, notably many serine and cysteine proteinases are both inhibited by antipain, chymostatin and leupeptin.

Conclusions concerning the classification of a proteinase do not always relate directly to their physiological role as physiological substrates are not always used and indeed are frequently not known. pH dependence may indicate the localisation of the proteinase, but again considerable differences in pH optima are observed with different substrates and so results using artificial substrates can be misleading.

Table 3. Classification of proteinases

Type	Specific inhibitors: characteristic of enzyme type	Other inhibitors	Activators
Aspartic (EC 3.4.23)	Pepstatin S-PI (acetyl pepstatin) N-Diazoacetyl norleucine methyl ester N-Diazoacetyl-N-'-2,4-dinitro-phenylethylenediamine Epoxy (p-nitrophenoxy) propane		
Metal (EC 3.4.24)	Chelating agents EDTA, ethylene glycol-bis-(B-aminoethyl ether)-N,N-tetra-acetic acid o-phenanthroline Phosphoramidon (not acid metalloproteinases)		
Serine (EC 3.4.21)	PMSF DIFP	TLCK TPCK Leupeptin Antipain Chymostatin	
Cysteine (EC 3.4.22)	Iodoacetamide, Heavy metals N-Ethyl maleimide	p-Chloromercuri-benxoate TLCK TPCK Antipain Chymostatin	Reducing agents DDT EDTA

Adapted from North (1983).

The roles of proteinases have been investigated mostly using mammalian and bacterial systems but in the past few years there has been a considerable increase in the number of studies on proteinases of parasites including, amongst others, *Entamoeba histolytica* (Luaces and Barrett, 1988), *Trypanosoma cruzi* (Cazzulo *et al.*, 1988), *Trypanosoma brucei* (Lonsdale-Eccles and Grab, 1987) as well as schistosomes (McKerrow and Doenhoff, 1988). Several proteinases have been identified and partially characterised in *Trypanosoma cruzi* (see Cazzulo, 1984) and recently a cysteine proteinase has been purified from the epimastigotes (Cazzulo *et al.*, 1989) and appears to be localised in the parasites lysosomes (Bontempi *et al.*, 1989). In many respects this is similar to the situation in African trypanosomes where there also appear to be several cysteine proteinases (Steiger *et al.*, 1979; North *et al.*, 1983) some of which appear to be localised in lysosomes (Lonsdale-Eccles and Grab, 1987). Several different species of African trypanosomes have been shown to possess a similar activiy of molecular weight 28-31 kDa although no proteinase activity could be detected in *T.vivax* (Lonsdale-Eccles and Mpimbza, 1986).

There appears to be a degree of developmental regulation in the proteinases of the African trypanosomes, there are lower activities in the procyclic forms than in either the metacyclics or the bloodstream forms (North *et al.*, 1983; Lonsdale-Eccles and Mpimbaza, 1986). However Pamer *et al.* (1989) have shown that there is an increase in the activity of a 28 kDa cysteine proteinase as the trypanosomes differentiate from long slender forms into short stumpy forms, and that this activity is greater than that found in the procyclics.

Interest has been greatly stimulated recently by the findings that the major surface antigen of *Leishmania* is a proteinase (Etges *et al.*, 1986). The molecular weight of this proteinase has been variously reported as varying between 63 kDa (Etges *et al.*, 1986) and 68 kDa

(Fong and Chang, 1982). Similar enzymes appear to be present in all species (Bouvier *et al.*, 1987) and the proteinase has been shown by Lockwood *et al.* (1987), using gelatin SDS-PAGE, to be present in promastigotes and amastigotes of *L.donovani*, *L.major* and *L.m.mexicana*. The proteinase does not appear to be of the serine type (Etges *et al.*, 1986) but there is some evidence that it may be a metalloproteinase (Pupkis and Coombs, 1984). This has been recently confirmed and shown to be a zinc metalloproteinase (Bouvier *et al.*, 1987). Jahnig and Etges (1988) determined the secondary structure of this surface proteinase and showed that it appears to be a novel kind of membrane-anchored proteinase.

The role of this surface proteinase is yet to be elucidated although it has been proposed that it plays a role in protecting the parasite from microbicidal enzymes in the sandfly gut (Bordier, 1987). However the findings that it is more abundant on the surface of virulent than avirulent promastigotes of *L.m.amazonensis* (Chaudhuri and Chang, 1987) and that it appears to be more abundant on the surface of the more infective stationary phase promastigotes than log phase promastigotes of *L.braziliensis* (Kweider *et al.*, 1987) argues that it may play a role in protecting the parasite in the macrophage. Recently it has been shown that the enzyme incorporated into liposomes prevents breakdown of protein inside the vesicle by macrophages (Chaudhuri *et al.*, 1989). This strongly supports the theory that the enzyme aids survival by protecting the parasites from microbicidal attack.

Pupkis aand Coombs (1984) partially purified and characterised two distinct proteinase activities from amastigotes of *L.m.mexicana*. The first had a molecular weight of approximately 67 kDa, with some of the characteristics of a metalloproteinase and was also present in promastigotes of *L.m.mexicana*. It seems likely that this activity

included the metalloproteinase discussed above. A lower molecular weight cysteine proteinase was also purified from the amastigotes of *L.m.mexicana*. This was shown to be stage and species specific, being found in only the amastigotes of *L.mexicana* species and not in any other stage of either *L.donovani* or *L.major* (Pupkis *et al.*, 1986). This low molecular weight cysteine proteinase was also shown to be localised in lysosomal-like organelles termed megasomes (see section 1.1.1.). Coombs and Baxter (1984) indicated the importance of this proteinase when they showed that cysteine proteinase inhibitors had antileishmnial activity against amastigotes of *L.m.mexicana in vitro*.

Studies by Lockwood *et al* (1987) and North *et al* (1988) using gelatin SDS-PAGE have indicated that a large degree of developmental regulation of proteinases occurs in different species of *Leishmania*. There appear to be two distinct groups of proteinases. Firstly, a group of high molecular weight enzymes apparently present, albeit in different isoenzymes, in all species and stages investigated. This group includes the major surface proteinase discussed above. At least some of this group appear to be particulate (Coombs, 1982). North *et al* (1988) have reported that the proteinases of this group do not appear to fit readily into any of the four well-defined classes of proteinases. Secondly, a lower molecular weight group, characteristic of the *L.mexicana* complex, that consists of what appear to be multiple isoenzymes. They also showed that stationary-phase promastigotes of *L.m.mexicana* possess lower molecular weight proteinases, similar to those found in the amastigote but at much lower activity and with slightly different mobilities on gels, whilst log phase promastigotes do not. This suggests that the synthesis of these proteinases may be a prerequisite for the successful transformation to the amastigote and survival in macrophages (see section 1.2.2.). Developmental changes were also found with the the other species investigated. The

appearance of a proteinase in stationary phase promastigotes of *L.donovani* that is not present in the log phase cells or the amastigotes is particularly interesting but as yet unexplained.

1.4. CONTROL OF LEISHMANIASIS

Potentially leishmaniasis can be controlled at several points, although the interruption and control of the disease at a single point has proved difficult to achieve. At present the only feasible approach to control would encompass intervention at several points in the lifecycle although for several of the diseases this is simply not practical. This could include vector and reservoir control, immunisation and treatment of infected individuals. Unlike malaria there are no chemoprophylactics available which has not helped in the prevention of the disease.

1.4.1. Immunisation

Although cutaneous leishmaniasis due to *L.major* confers a solid immunity to infected individuals (Marsden, 1984; Greenblatt, 1985) and that live vaccine trials in the U.S.S.R., Iran and Israel have given some success (Greenblatt, 1985), there is as yet no good antileishmanial vaccine generally available for most of the diseases. There is extensive immunological crossreactivity between different species but they do not generate crossprotection.

There have been several problems associated with the use of live vaccines, chronic lesions being reported with some *L.major*-vaccinated individuals. The risks associated with using live vaccines with more virulent species have not been acceptable.

Crude parasite-extract vaccines have been used in preliminary trials in Brasil (Antunes *et al.*, 1986) and appear so far to have had few side effects. The Special Programme for Research and Training in Tropical Diseases (TDR) are currently planning phase I trials using

killed vaccines similar to those tested in Brasil (Modabber,1986).

The immunogenic major surface glycoprotein (see sections 1.2.1. and 1.3.5.) provides one hope of a molecular vaccine. A similar surface antigen appears to be present in all species of *Leishmania* (Chang and Chang, 1986; Etges *et al.*, 1985) and so a succesful vaccine could have broad spectrum activity. It has been reported that this molecule incorporated into liposomes was effective against cutaneous leishmaniasis in a mouse model (Alexander and Russel, 1988).

1.4.2. Control of vectors and reservoir hosts

Control of sandfly populations is difficult . At best it is feasible only with the adult stages as the immature stages are normally more difficult to find. Even adult populations have proved difficult to control largely due to the variability and inaccessability of the breeding and resting sites. There is no set pattern for controlling every sandfly colony, each requires it's own specific control strategy.

Malaria control campaigns using DDT to control mosquitoes have, as a side-effect, frequently resulted also in the reduction of leishmaniasis. This has been particularly the case in areas where transmission is peridomestic (Marinkelle, 1980). The destruction of sandfly breeding sites, intentionally or not, has provided some success in the USSR, China and Malta in reducing leishmaniasis (Ward, 1985).

Normally reservoir hosts, such as rodents and dogs, are found in the vicinity of the affected population and measures to control the disease usually involves eradication of all the potential reservoirs or the selection and elimination of infected animals. These measures accompanied by insecticide spraying have proved successful in a few cases notably in the USSR (Molyneux and Ashford, 1983).

1.4.3. Chemotherapy

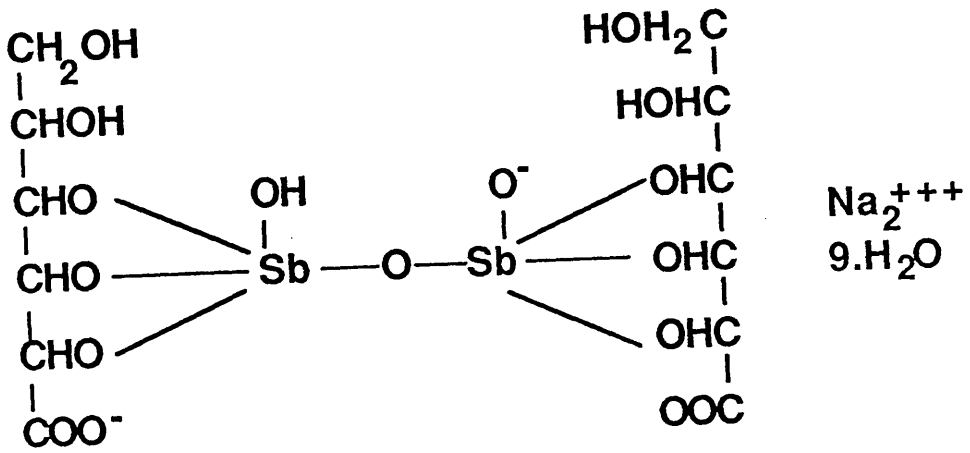
The range of drugs available for the treatment of infected individuals has changed little over the last 35 years, The pentavalent antimonials, sodium stibogluconate (Pentostam) and methylglucamine antimonate (Glucantime) are still the drugs recommended for initial systemic therapy (Berman, 1988; Marsden and Jones, 1985). These drugs are composed of pentavalent antimony complexed to carbohydrates. The classical structures of Pentostam and Glucantime are shown in figure 4, although the precise structure of these complexes are unknown (Berman, 1988).

The mechanism of the antileishmanial action of the antimonials is unknown. Recently work by Berman *et al* (1987) has shown that Pentostam inhibits the glycolytic pathway and fatty acid oxidation in *L.mexicana* amastigotes. Berman and Grogl (1988) suggest that the specific binding of Pentostam to polypeptides involved in the glycolytic and oxidative pathways may contribute to the cytotoxic effect of the drug. They also raise the possibility that Pentostam may interact with the parasite nucleic acids or with low molecular weight parasite components.

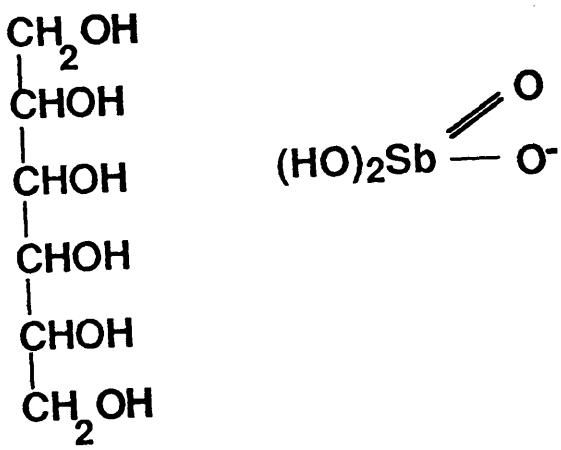
There are several problems associated with the use of the pentavalent antimonials, these include toxicity and the rapid excretion of the drug. Rees *et al.* (1980) reported that within 6 hours of intramuscular administration, 57-84% of the drug was excreted. This rate of excretion could rapidly result in subtherapeutic levels of the drug in the plasma. Recently the pharmokinetics and toxicity of Pentostam have been reassessed (WHO Technical Report Series, 1984, The Leishmanias) and this has shown that more frequent higher doses are better tolerated than was first thought and the implementation of these findings has considerably reduced the failure rates of treatment.

Figure 4. Structures of clinically used antimonials

Sodium stibogluconate



Meglumine antimoniate



10-25% of all initial treatments using the standard regimen are unsuccessful (Berman, 1983), and treatment is usually continued with either the pentavalent antimonials or one of the second line drugs. These are Amphotericin B, which is the preferred drug for Latin American cutaneous leishmaniasis and Pentamidine, which is used mainly in the second line treatment of visceral leishmaniasis (Bryceson, 1987).

There is a wide variability in the susceptibility of different species, and indeed isolates, to antimonials. For instance whilst visceral leishmaniasis is usually responsive to antimonial therapy and cure rates of 70-100% have been reported, relapsing and diffuse cutaneous forms of the disease often prove difficult to treat, due apparently to defective immunity (Marsden and Jones, 1985).

Little work has been carried out on the problemm of drug resistance in *Leishmania* species. Some isolates, from patients who have recieved several courses of treatment with meglumine antimonate, have been shown to be less sensitive to treatment with the same compound (Ercoli, 1964, 1966). Variations in susceptibility to pentavalent antimonials in patients have also been shown to reflect this in infected macrophages *in vitro* (Berman *et al.*, 1982).

There are drawbacks involving all the drugs used. Treatment is parental, time consuming often involving hospitalisation and hence expensive, and is often toxic with symptoms ranging from malaise to heart dysfunction and even death. Patients infected with kala-azar may exhibit signs similar to drug toxicity, such as liver, renal and bone marrow dysfunction, so making therapy difficult to manage (Marsden and Jones, 1985). For these reasons, not all forms of leishmaniasis are routinely treated. For instance with some Old World cutaneous species and the *L.mexicana* complex the skin granulomas produced will heal naturally and so patients with these may not be treated, unless for

cosmetic reasons.

There is a pressing need for new therapies which will overcome the problems associated with current treatments. At present several new formulations have reached phase I trials, including several purine analogues, WR 60206 (an 8-aminoquinoline) and some antidepressant drugs (TDR report). Others are at a more preliminary stage but show promise in experimental models, for example DFMO. However it seems unlikely that effective new treatments will be available in the near future.

1.5. THE SEARCH FOR NEW TREATMENTS

1.5.1. Biochemical strategies

There are several approaches that can be followed in attempts to develop, on a rational basis, new forms of treatment for leishmaniasis. Biochemical strategies, at their simplest, usually involve selecting differences at the enzyme level between host and parasite and designing compounds to specifically inhibit the parasite enzyme system. Unfortunately, relatively little is known about the biochemistry of the amastigotes of *Leishmania* and so only a few possible targets have been recognised. Rather more information is available on the promastigotes and this has been used to identify other possibilities.

There are several areas of leishmanial metabolism that are being investigated with a view to drug development. The ones most relevant to this study are summarised here. Most parasitic protozoa so far studied, including the leishmanias, are incapable of synthesising purines *de novo*, and in this way differ from most other organisms (see section 1.3.3.). Thus the parasites rely on the salvage of preformed purines from their hosts and several of the enzymes involved have been shown to differ from their isofunctional mammalian counterparts

(Hassan and Coombs, 1988). This has led to the discovery that several purine and purine nucleoside analogues, including allopurinol and formycin B, have potent antileishmanial activity (Nelson *et al.*, 1979; Carson and Chang, 1981). These commpounds are believed to be metabolised to nucleotide analogues by the parasites and prove toxic by either inhibiting the purine salvage pathway or by their ultimate incorporation into nucleic acids (Nelson *et al.*, 1979; Rainey and Santi, 1983; Marr, 1983). Initial trials using allopurinol against antimony-resistant cases of visceral leishmaniasis (Kager *et al.*, 1981) showed some success. Clinical trials of the more active ribonucleoside analogues have also proved promising and are being followed up (Modabber, 1986).

There is another approach to the treatment of leishmaniasis which also involves the parasite's requirements for preformed purines. It has been shown that promastigotes of *L.donovani* possess two nucleoside transport mechanisms both of which are refactory to nitrobenzoylthioinosine (NBMPR) and dipyridamole (Aronow *et al.*, 1987). These compounds are potent inhibitors of nucleoside transport into mammalian cells (Aronow *et al.*, 1985). Tubercidin, a cytotoxic analogue of adenosine, has already been used succesfully in combination with NBMPR against experimental infections the extracellular parasites *Trypanosoma gambiense* (Ogbunude and Ikediobi, 1982) as well as *Schistosoma mansoni* and *S.japonicum* (El Kouni *et al.*, 1983, 1985). The toxicity of tubercidin towards mammalian cells is well documented as is the use of NBMPR to lower tubercidin's toxicity to mammalian cells *in vitro* and *in vivo* (Cass and Paterson, 1977; Lynch *et al.*, 1978; Paterson *et al.*, 1979). But is such an approach relavent to intracellular parasites?

It has been shown recently that *Plasmodium falciparum* exerts a stage-specific effect on the permeability of infected erythrocytes to

nucleosides. This allows cytotoxic nucleosides, such as tubercidin, to be targetted towards parasitised cells whilst using inhibitors of nucleoside transport to protect non-infected cells (Gero *et al.*, 1988). It seemed possible that similar use of tubercidin in conjunction with these inhibitors may be succesful against leishmaniasis. This approach would work if macrophages undergo changes in their permeability to nucleosides upon infection with leishmanias such that inhibitors would protect uninfected cells from the effects of the tubercidin whilst allowing the parasitised cells to be targetted and killed.

Even if such changes do not occur, however, it could still be possible to use these combinations, providing that the tubercidin can access the parasitophorous vacuole despite the inhibitory activities of NBMPR and dipyridamole. This could be achieved for instance by pinocytosis. Then, providing that the amastigote has a similar nucleoside transport mechanism to that reported for the promastigote, the parasites would be killed whilst the host cells were unaffected. This approach is one pursued in this study.

Inhibitors of dihydrofolate reductase (an enzyme involved in tetrahydrofolate production and so indirectly in DNA synthesis), such as the antimalarial pyrimethamine, have been found to have activity against a variety of parasites such as *Pneumocystis carinii* and *Toxoplasma gondii*. (Roth, 1983). The finding that the dihydrofolate reductase inhibitor cycloguanil pamoate was of some value in the treatment of cutaneous leishmaniasis demonstrated the potential of this area in the search for new anti-leishmanials (McCormack, 1981). This idea was reinforced by the discovery that the dihydrofolate reductase activity of *Crithidia fasiculata* and *L.tropica* is mediated by a bifunctional protein that also has thymidylate synthetase activity (Ferone and Roland, 1980). This differs from the situation in

mammals in which two separate enzymes are present. Several novel 2,4-diaminopyridamines have recently been shown to have good antileishmanial activity *in vitro* against both amastigotes and promastigotes (Hunter and Coombs, 1987; Scott *et al.*, 1987).

A most interesting recent discovery has been that the roles played by glutathione in most prokaryotic and eukaryotic cells, including the scavenging of free radicals and peroxide metabolism, are carried out in trypanosomatids by a glutathione-spermidine complex given the name of trypanothione (see Henderson and Fairlamb, 1987). It has been suggested that trypanothione plays a role in protecting the parasites from oxidative damage by the host, and it has been postulated that trypanocidal drugs which produce free radicals may be potentially synergistic with inhibitors of trypanothione synthesis. This is an approach which is being vigorously pursued, especially with the use of DFMO as the inhibitor of polyamine metabolism.

1.5.2. Drug targetting

The targetting of drugs in a highly specific manner has many theoretical advantages. All drugs are potentially hazardous and the doses used are normally a compromise between the constraints of drug toxicity and the therapeutic index of the drug. Often a drug is only needed in one specific area of the body or one cell type and yet the drug frequently becomes diluted in the body fluids, large amounts may be degraded, the drug may be excreted rapidly or be concentrated in cells that do not require treatment. Efficient targetting of a drug can considerably improve the efficacy of the compound and overcome many of the problems mentioned. Ideally this results in the use of lower concentrations of the drug whilst improving the therapeutic index. There are several approaches to the physical targetting of drugs. Some of these are discussed below, with particular attention

given to the methods relevant to intracellular parasites.

Many types of site-specific drug delivery systems have been proposed and these were reviewed by Poznansky and Juliano (1984). These included the use of soluble carriers, such as monoclonal antibodies and plasma proteins, that can be directed to particular sites. Several microparticulate carrier systems have also been proposed and these are discussed below. The ideal drug carrier should meet several criteria. It should be non-toxic, bio-degradable, non-immunogenic, have a high drug load yet still give a controlled release of the drug, be cheap to manufacture and, of course, give the desired selective drug delivery.

Site-specific drug delivery can occur at three levels. A particular organ may be targetted, or a particular cell type and subcellular targetting of a specific cell compartment can occur. One example which would involve all three levels is the targetting of the lysosomal compartment of phagocytic cells of the liver.

There are also two types of targetting that can occur, passive and active. Passive targetting refers to the natural distribution of the carrier. Possibly the best example of this is the clearance of colloidal material from the circulation by cells of the reticuloendothelial system (RES) located in the sinusoids of the spleen, liver and bone marrow. Active targetting on the other hand involves altering the normal distribution of the carrier so that other specific cells, tissues or organs are targetted. An example of this is the use of antibody-linked drugs (Poznansky and Juliano, 1984).

1.5.2.1. Pro-drugs

Pro-drugs are compounds composed of two parts, the drug itself a "carrier" to which it is linked. The whole pro-drug lacks any appreciable biological activity except those properties that allow the

compound to be targetted. Once the pro-drug has been successfully targetted, thereby increasing the specificity of the drug, the pro-drug is cleaved so releasing the carrier molecule and the toxic form of the drug at the required site. There are many types of possible carrier, including many of those mentioned in subsequent sections. The increased specificity of pro-drugs over the drug themselves can be due either to specific targetting but can also be due to the cleavage of the pro-drug within the parasites environment, perhaps even by the parasite itself.

With reference to *Leishmania*, two examples of this approach have been suggested. Hart (1986) proposed that drugs entrapped in low density lipoproteins may become concentrated in the parasitophorous vacuole of *Leishmania* infected cells. This could occur because macrophages possess specific receptors for low density lipoproteins. Once within the parasitophorous vauole the enzymes of the lysosomal system would release the entrapped drug which could then affect the amastigote. A second suggestion (Coombs, 1986) was that the use of a carefully chosen peptide link between the carrier and the drug could be cleaved by parasite specific proteinases and not by host enzymes. This could lead to the activation and release of the drug by the parasite and not the host.

1.5.2.2. Lysosomotropic drugs.

Intracellular parasites are in a unique position as the host's membrane intervenes between drugs in the body fluids and the parasite itself. In many cases this makes chemotherapy more difficult than against extracellular parasites. However there are possible ways of exploiting the parasites intracellular existence. For instance parasites may change their host cell in a way which allows infected cells to be targetted, e.g. tubercidin and *Plasmodium* (see section

1.5.1.) or the parasites may be dependent on an aspect of the intracellular environment which can be modified by drugs e.g. the acid pH in a phagolysosome. Many compounds become concentrated in lysosomes, i.e. they are lysosomotropic and parasites that live in lysosomal systems are ideal targets for such lysosomotropic formulations. Such compounds are of particular interest in leishmanias.

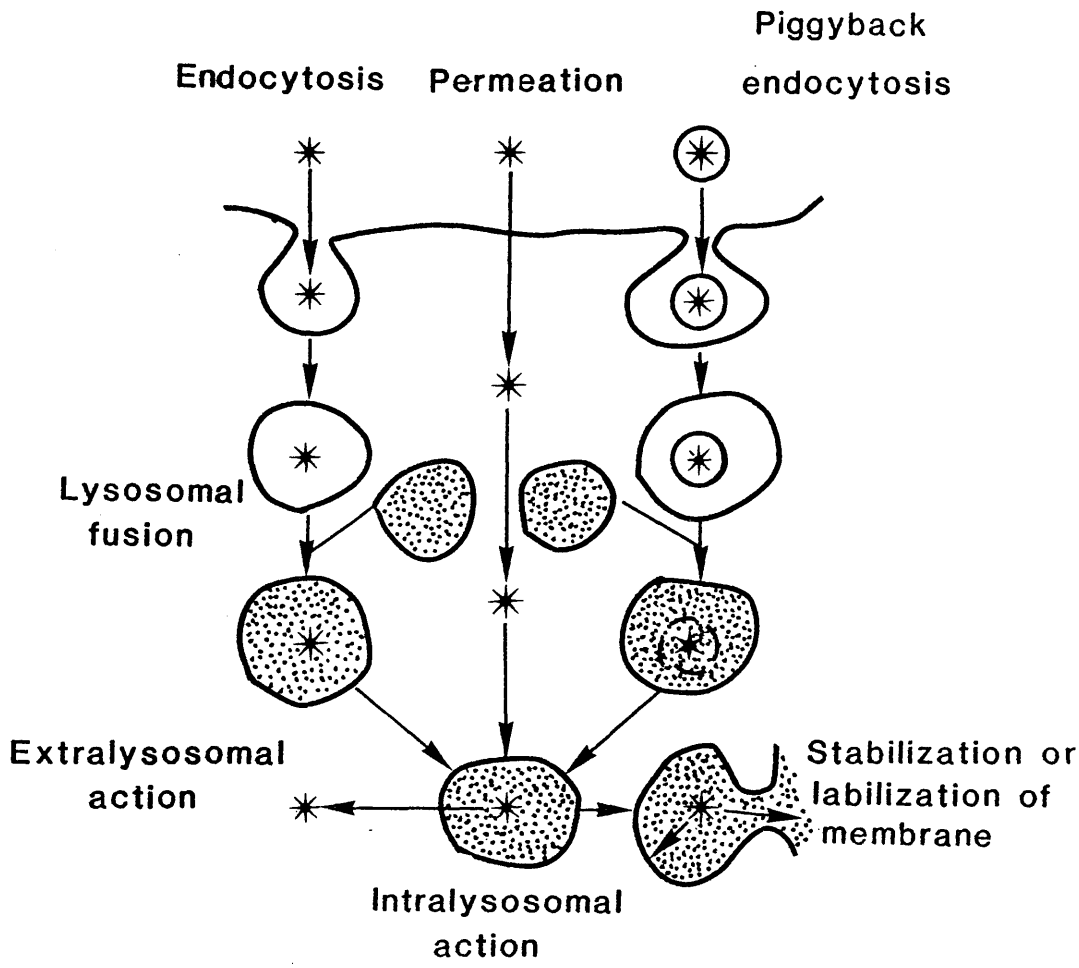
Lysosomotropism, as defined by de Duve *et al* (1974), includes "all substances that are taken up selectively into lysosomes, irrespective of their chemical nature or mechanism of uptake". This property can be conferred on almost any substance by coupling to an appropriate carrier. This method can, in theory, target compounds not just to lysosomes but also to specific cell types. This area of drug targetting offers a large range of possibilities in the treatment of not just intracellular parasites but also leukemia and cancer.

There are several classes of lysosomotropism which are summarised in figure 5. Endocytosis is the main route of entry of compounds into the lysosomal system. A variety of molecules can be taken up in this manner, including small molecules, some groups of macromolecules, insoluble particles and parasites. Endocytosis can be highly specific, different materials may be taken up at different rates and these rates may vary between cell types. Dextran sulphate is one compound that is endocytosed and has proven useful in the investigation of the lysosomal system (see below).

Piggyback endocytosis refers to the uptake of an endocytizable complex which on being processed by the lysosomal system releases the active compound. Probably the best example of this is the use of liposomes to deliver drugs to cells of the RES (see section 1.5.3.).

Several substances are known to accumulate against their concentration gradient by permeation and this will be dealt with more

Figure 5. Mechanisms of entry and mode of action of lysosomotrophic agents



Adapted from de Duve *et al* (1974)

fully below. Lysosomotrophic compounds that access the lysosomal compartment by permeation include the antimalarial chloroquine, many weak bases such as methylamine and ammonium chloride and certain amino acid esters such as L-leucine methyl ester (leuOMe). The rates of uptake of these substances indicate that the intralysosomal accumulation occurs by permeation coupled to a trapping mechaniam (de Duve *et al.*, 1974). Intralysosomal trapping by protonation is the most likely mechanism for the majority of substances in this category. In exceptional circumstances other mechanisms may be involved.

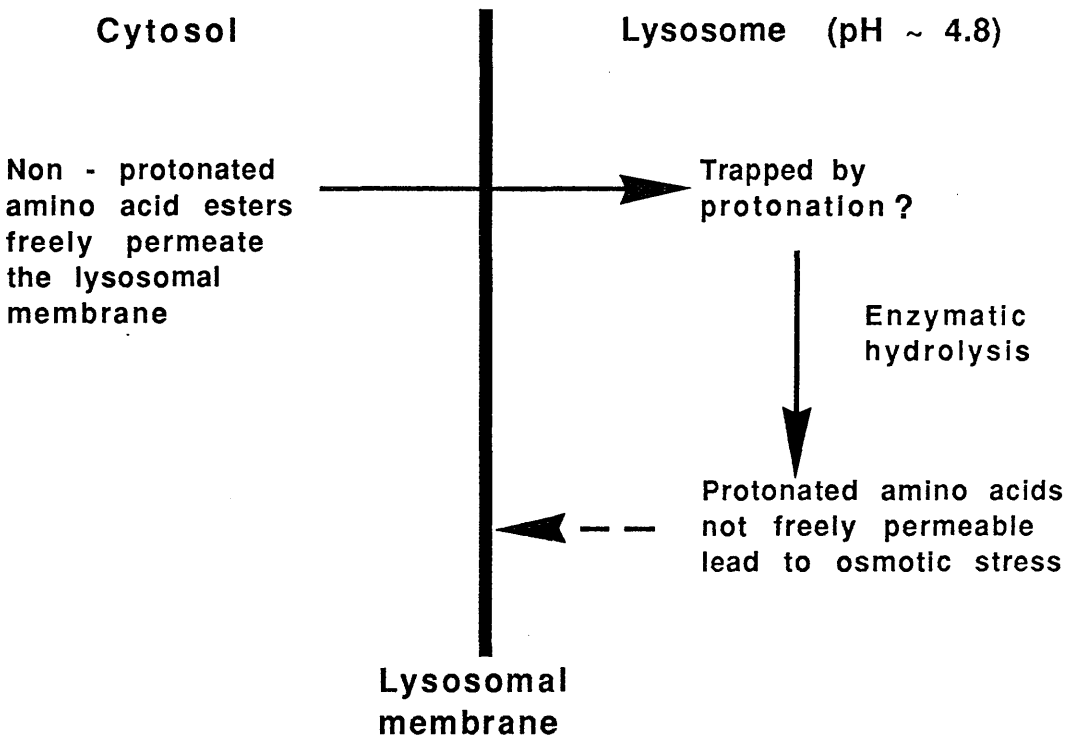
Trapping by protonation relies on the different properties of ionized and unionized compounds. Many weak bases and acids will readily cross biological membranes in their unionized state but will not cross the same membrane when ionized. If the membrane separates two regions of different pH, for example a lysosomal membrane, then acids will accumulate on the basic side (the cytosol) whilst the bases will accumulate on the acidic side (the lysosome). This is thought to be the mechanism behind the lysosomotrophic properties of methylamine and ammonium chloride.

The lysosomotropism of amino acid esters is less straightforward. Goldman and Kaplan (1973) showed that the L-stereoisomers of certain amino acid methyl esters caused disruption of isolated lysosomes when added at concentrations of 0.1 to 10 mM whilst the D-stereoisomers were much less effective. They also showed using paper electrophoresis, that the potent esters were readily hydrolysed by lysed lysosomal preparations. They suggest that the amino acid esters enter the lysosomes by passive diffusion whereupon they are enzymatically hydrolysed. Thus a concentration gradient is maintained and influx of the ester continues. Because of their high polarity, the liberated amino acids cannot easily permeate back out of the lysosome and so they will accumulate within the lysosomal system. The

continuation of this process eventually leads to osmotic lysis of the lysosome (see figure 6). Results presented by Reeves (1979) strongly supported this hypothesis and the use of the proteinase inhibitor TPCK indicated that proteinases may be involved in the hydrolysis of the amino acid methyl esters. Reeves (1979) suggested that more than one enzyme may be involved in hydrolysing the compounds and that an extralysosomal esterase may also be hydrolysing these esters.

From studying the effects of lysosomotropic compounds upon *Leishmania* amastigotes survival in macrophages, Rabinovitch *et al.* (1986) reported that, several L-amino acid esters notably L-leucine methyl ester (leuOMe) had potent leishmanicidal activity. Initially they postulated that the amino acid esters accumulated in the parasitophorous vacuole at such high concentrations that the intracellular parasite was damaged. However they did not rule out the possibility that the amino acid esters may be concentrated and hydrolysed within the amastigote lysosomes and that this led to damage of the parasite. Subsequently they (Rabinovitch *et al.*, 1987) investigated the effect of different amino acid esters on isolated amastigotes of *L.m.amazonensis*. They showed, using compounds known to raise the intracellular pH, that an acidified compartment appeared to be involved in the destruction of the parasites. They also observed that the sensitivity of isolated amastigotes was similar to that of intracellular parasites. Alfieri *et al.* (1987) showed that the destruction of *L.m.amazonensis* amastigotes by leuOMe could be prevented by other amino acid esters. This protective activity occured with both leishmanicidal and non-leishmanicidal esters. Overall this work was compatable with the hypothesis that the antileishmanial activity of the amino acid esters was a reflection of their lysosomotropism and is dependent upon their hydrolysis by parasite

Figure 6. The mechanism of lysosomal disruption by amino acid esters



enzymes.

Lysosomal function can be manipulated in several ways using lysosomotrophic compounds. For instance, weak bases such as methylamine and ammonium chloride have been shown to raise lysosomal pH over short exposure times (Ohkuma and Poole, 1978) whilst dextran sulphate decreases lysosomal pH (Kielan and Cohn, 1982). These compounds can also exert a variety of other effects on the lysosomal system, however, there are some uncertanties as to the full effects and modes of action of these compounds, especially over long exposure periods.

1.5.3. The reticuloendothelial system in drug delivery.

Cells of the reticuloendothelial system are highly phagocytic and consequently are potential host cells for *Leishmania*. Several workers have shown that if colloids are injected intravenously then the phagocytic cells of the liver, spleen and bone marrow will rapidly remove the colloid from the circulation (Saba, 1970; Poste, 1983). Colloid particles have been used for many years to study the RES and it is only relatively recently that the potential of these colloidal systems for the delivery of drugs was realised.

Liposomes are vesicles composed of phospholipids. They behave as colloids in being rapidly phagocytosed by cells of the RES. Hydrophobic drugs, such as amphotericin B, can be incorporated into the membrane of the liposomes whilst hydrophilic compounds, for example sodium stibogluconate, can be entrapped in the aqueous compartment. Gregoriadis and Ryman (1972) used liposomes 10 years after their initial discovery to deliver enzymes to the RES. Subsequently erythrocytes (Ihler *et al.*, 1974) and microspheres (Kramer, 1974) have been tested as vehicles for the delivery of drugs to the RES.

Several diseases are localised in the RES of the liver, spleen and bone marrow and this makes them ideal candidates for the use of colloidal-mediated drug therapy. By far the greatest amount of work has been carried out using liposomes and several diseases of the RES have already been treated in experimental models. These include systemic fungal infections in cancer patients (Lopez-Bernstein *et al.*, 1985), *Candidia* infections (Lopez-Bernstein *et al.*, 1984) and bacterial infections (reviewed Ostro, 1986).

Liposomes provide a good delivery system in many respects. There appears to be little if any toxicity associated with liposomes although they are weakly immunogenic. The composition of liposomes can be modified by inclusion of other lipids and so their properties altered to suit the intended purpose. They can adsorb to almost any type of cell and having done so may release the drug in a controlled manner through their lipid membrane. Liposomes can also exchange appropriate lipids with the plasma membrane and if drugs could be conjugated to an exchange lipid then potentially it can enter the targetted cell. Endocytosed liposomes are degraded within the lysosomal compartment and release their drug there. What happens to the drug then depends on many factors but a water soluble drug, like sodium stibogluconate, may diffuse down its concentration gradient and out of the cell.

Despite these positive points liposomes have several drawbacks. It is difficult to target liposomes to cells outside the circulation. Liposomes are too large to pass through the capillaries of most organs and may be trapped by mechanical filtration. The size of the particles will determine to some degree the area in which they are trapped. For instance, particles greater than 7 μm in diameter will be trapped mainly in the lungs, particles greater than 100 nm will be ingested mainly by the liver and spleen whilst only particles with a diameter

of less than 100 nm have any chance of reaching extravascular sites (Illum and Davis, 1982). Also, the cells of the RES will rapidly remove liposomes from the circulation, no matter what their size. This makes cells of the RES ideal targets for passive vectoring, but also makes targetting of other cells very difficult. Liposomes have appeared to be weakly immunogenic which may be a positive point if they are to be used to present antigens as part of a vaccine but creates several difficulties for their use as a drug delivery system.

Several workers have used liposome-encapsulated drugs successfully in the treatment of experimental visceral leishmaniasis (Black *et al.*, 1977; Alving *et al.*, 1978; New *et al.*, 1978). Berman *et al.* (1986) also used pentamidine in human red cell ghosts in the successful treatment of experimental visceral leishmaniasis. It has also been shown that the efficacy of various other drugs is increased significantly when they are used in liposomal form. This increase may be due to several factors. The most likely is that direct targetting is occuring at all three levels mentioned above. The drug is being targetted to the appropriate organs giving higher levels of antimony in the spleen and liver than is achieved when the free drug is used. The drug is also being targetted to the potential host cells, the macrophages. Work by Heath *et al.* (1984) also showed that liposomes are not only targetted to the macrophage lysosomal system but may also end up in the parasitophorous vacuole. This was a little surprising as the general finding of other workers was that whilst endocytosis of large objects by *Leishmania* infected cells does occur they do not readily gain access to the phagolysosome (Dedet *et al.*, 1982; personal observation).

Another way in which liposomes can increase drug efficacy is that they can help to prevent rapid excretion of the drug. Encapsulated sodium stibogluconate, trapped in the liver or spleen (these organs

could act as drug depots), is released at a slow rate giving an extended plasma half life. This may allow therapeutic levels to be maintained for a longer period of time without necessarily increasing the drug dose (Rao *et al.*, 1983).

These encouraging results have been obtained using experimental models of visceral leishmaniasis with the parasite loads in the liver of infected animals being assessed. Unortunately, if predictably, the use of liposomes does not appear to be as effective against cutaneous disease although there is an increase in the efficacy of Pentostam when used in liposomal form (New *et al.*, 1983). This lower efficacy with cutaneous disease was not surprising as most of the liposomes would have been removed by cells of the RES in the liver, spleen and bone marrow. Presumably the liver depot effect, which extends the half life of the drug in the plasma may account for the small increase in efficacy. Alternatively, these workers suggest that uptake of these liposomes by a circulating blood component, presumably monocytes, that subsequently become activated in the vasculature surrounding the lesion and migrate as macrophages to the site of infection. There they would be protected from parasitism by the liposomal Pentostam and so be free to help control the infection.

In the years immediately preceding this project, another vesicular system in many ways similar to liposomes had been developed. This system involves synthetic non-ionic surfactants, rather than the biological amphiphiles used for liposomes. The vesicles are called niosomes (Vanderberghe *et al.*, 1978). Baillie *et al.* (1985) showed that niosomes have similar properties to liposomes in that they are osmotically active, can form small unilaminar and large multilaminar vesicles and show differing degrees of permeability depending on the cholesterol content of the bilayer. These vesicles can act as a delivery system for methotrexate (Azim *et al.*, 1984), with potential

applications in cancer chemotherapy . In a preliminary study Baillie *et al.* (1986) showed that they may also have potential in the chemotherapy of visceral leishmaniasis.

Microspheres, prepared using various macromolecules as the matrix material, are also attractive as carriers for delivering drugs to the RES (Poznansky and Juliano, 1984). Microspheres differ from liposomes and niosomes in that they are composed of a matrix material to which the delivery load is attached. Tomlinson (1983) lists several types of possible matrix material ranging from lipoproteins and albumin to polyacrylamide. Polyacryl starch microparticles, prepared by radical polymerisation of acryloylated starch and so composed in part of a natural biodegradable macromolecule, have been shown to be capable of delivering enzymes to the RES (Artursson *et al.*, 1984). These have been studied *in vitro* as carriers of low molecular weight drugs (Laakso *et al.*, 1987b). This system is an ideal candidate for use in the treatment of visceral leishmaniasis.

1.6. THE AIMS OF THIS PROJECT.

The overall aim of this project was to investigate several new potential strategies for the chemotherapy of leishmaniasis. It was expected that these investigations would also improve our understanding of how leishmanias interact with their host cell, the macrophage.

The initial part of the project involved an investigation of the usefulness of two relatively new carrier systems, niosomes and starch microparticles, in the treatment of experimental leishmaniasis. Their efficacy was compared to that of liposomes.

Another approach followed was to use the cytotoxic nucleoside tubercidin, in conjunction with inhibitors of mammalian nucleoside transport, to investigate whether or not *Leishmania* changes the

nucleoside permeability characteristics of infected cells. Such changes could be exploited in chemotherapy but might also give an insight into part of the survival mechanism of parasites in the parasitophorous vacuole.

It was with the same aim that studies involving the manipulation of the environment of the parasitophorous vacuole were pursued . This involved testing the affects of manipulating the phagolysosomal environment using compounds thought to modify lysosomal pH. The effect of treatment upon survival and growth of the amastigote was investigated. The role of pH on the uptake and incorporation of leucine and adenosine into different species and stages of the parasites were also determined. A greater knowledge of the conditions prevailing within the parasitoporous vacuole with different species could lead to new strategies in the chemotherapy of leishmaniasis.

A major part of this project was to characterise the antileishmanial activity of amino acid esters and to gain a better understanding of how this activity is mediated. This involved determining the spectrum of activity of the compounds and investigating the parasite properties essential to the compounds toxicity. This led to a study of the proteinases of *L.m.mexicana* amastigotes and their role in drug activation and the possibilities they provide for further drug development.

2.0 MATERIALS AND METHODS

2.1. PARASITES: CULTIVATION AND MAINTENANCE

2.1.1. Amastigotes

Leishmania mexicana mexicana (MNYC/BZ/62/M379) amastigotes were maintained by sub-passage in female CBA mice (Zoology Department, University of Glasgow). Amastigotes were isolated from an infected mouse and washed twice in PSGEMKA buffer by centrifugation (see section 2.2.1.). The cell density was assessed using an Improved Neubauer Haemocytometer. Approximately 3 x 10^7 amastigotes in 0.2 ml were injected subcutaneously into a shaved rump of each mouse to initiate new infections. The area surrounding the site of inoculation was subsequently shaved at regular intervals. Subcutaneous lesions became apparent by 2 months after inoculation of parasites and developed into large lesions within 3 to 5 months. These lesions were routinely used as the source of amastigotes for both transformation to promastigotes (see section 2.1.2.) and for experimental work.

Leishmania donovani (MHOM/ET/67/L82) amastigotes were maintained by sub-passage in female Golden Syrian hamsters (Bantin and Kingman Ltd., Grimsden, Hull, England.). Amastigotes were released by homogenising the spleen from an infected hamster using a glass-glass hand tissue grinder. The spleen in approximately 25 ml of PSGEMKA was homogenised untill all but connective tissue was disrupted. The resulting suspension was then washed twice, by centrifugation and resuspension in PSGEMKA buffer at 4°C, before being used to infect 4-10 week old hamsters by intraperitoneal inoculation of 2-4 x 10^8 amastigotes in 0.4 ml per animal. Infected animals were killed within 2-5 months, the spleen excised and used as a source of amastigotes for experimental work or for the transformation to promastigotes (section 2.3.1.). The condition of the hamsters was a good indicator of the progress of the infection: hamsters remained healthy until a few days before death caused by the parasites. Hamsters were routinely used at

the first signs of ill health to obtain the maximal yield of parasites.

Leishmania major (HMOM/SA/83/RKK2) infections were maintained by the subcutaneous inoculation into the footpads of male or female Balb/c mice (Zoology Department, University of Glasgow) of approximately 5 x 10^6 promastigotes suspended in 0.05 ml of Hanks' balanced salt solution (HBSS). These promastigotes were the infective metacyclic forms obtained by peanut lectin agglutination (see section 2.2.2.). Within 4 - 8 weeks lesions were apparent, and parasites were harvested from them before they ulcerated. The lesions were excised and used to obtain small numbers of amastigotes for transformation to promastigotes (see section 2.1.2.). When larger numbers of amastigotes were required, Golden Syrian hamsters were infected subcutaneously in the footpad with aproximately 1 X 10^7 metacyclic-enriched promastigotes in 0.1 ml HBSS. The resulting lesions were used 4-5 weeks later for the purification of amastigotes (see section 2.2.1.).

2.1.2. Promastigotes

Promastigotes of each species were obtained by the transformation *in vitro* of amastigotes obtained from an infected animal. A small piece of infected tissue was aseptically removed and placed in 5 ml of transformation medium: HOMEM medium (Berens *et al.*, 1976) supplemented with 20% (v/v) heat-inactivated foetal calf serum (HIFCS) and antibacterials (25 µg gentamycin sulphate ml^{-1}, 1 mg streptomycin sulphate ml^{-1} and 1000 units benzylpenicillin ml^{-1}). The infected tissue was then incubated at 25°C for 48-96 hours during which transformation and replication of promastigotes occurred. Promastigotes were sub-passaged and cultured in HOMEM medium with 10% HIFCS and 25 µg gentamycin sulphate ml^{-1} at 25°C. Cultures were normally initiated at a density of 1 x 10^5 promastigotes ml^{-1} and sub-

passaged upon reaching early to mid-logarithmic phase of growth (density of approximately 10^6 ml^{-1}). For some experimental work, batch cultures of up to 300 ml were grown in 500 ml tissue culture flasks (Labtek). For some experiments, stationary phase promastigotes were required. This involved harvesting parasites after they had been in culture for longer than 7 days and were at a density of greater than 1 x 10^7 viable promastigotes ml^{-1}.

2.2. HARVESTING PARASITES

2.2.1. Amastigotes

Amastigotes used for the *in vitro* infection of peritoneal exudate cells (PECs, see section 2.3.2.) were obtained from the appropriate tissue of infected animals. The infected tissue was excised aseptically and placed in a sterile dounce hand-held homogeniser with 10-15 ml of RPMI 1640 supplemented with 10% (v/v) HIFCS (see section 2.3.1.). The amastigotes were released by several strokes of the homogeniser pestle and were then harvested by centrifugation (2100 x g for 10 minutes) at 4°C and washed twice in RPMI medium before being used to infect the PECs.

Purified amastigotes for use in biochemical studies were isolated according to Hart *et al.* (1981) with slight modification. The infected tissue was gently homogenised, at room temperature, between two pieces of gauze mesh in approximately 50 ml of PSGEMKA buffer (20 mM sodium phosphate buffer, pH 7.3, 104 mM NaCl, 10 mM $MgCl_2$, 10 mM KCl, 0.5 mM D-glucose, 1 mM ethylenediaminetetraacetic acid [EDTA] 0.02% [w/v] bovine serum albumin). The suspension was then filtered through a N° 1 Whatman filter paper to remove large debris. Fresh buffer was added to the tissue remaining which was further homogenised and the process repeated untill approximately 400 ml of buffer had been used. The preparation at this stage was called the crude homogenate. Saponin was

then added to a final concentration of 0.5% (w/v) to lyse contaminating erythrocytes and the amastigotes freed from debris by three up and down strokes of a Potter homogeniser at a low speed (2000 rpm). The amastigotes were pelleted by centrifugation as above, washed three times and resuspended in PSGEMKA at a cell density of 1 x 10^8 ml^{-1}. The suspension was then passed through an ion exchange column to remove remaining host cells. Such columns were composed of a mixture containing 0.1 g of Sigmacell type 50 and 0.1 g of alpha cellulose which were layered on top of 2.5 g CM-Sephadex C25. All components were pre-swollen overnight in 0.02 M phosphate buffered saline (PBS), pH 7.3, before being resuspended in PSGEMKA. The columns were prepared in 50 ml syringe barrels which were then pre-washed with approximately 50 ml of PSGEMKA to give an even packing. The amastigote suspension was applied at a flow rate of approximately 5 ml per minute per column and the eluted amastigotes pooled. These were washed twice by centrifugation and resuspended in 25 ml of PSGEMKA and finally washed in 25 ml of 0.25 M sucrose. The parasites were finally pelleted in small capped tubes (eppendorfs) at approximately 5 x 10^9 amastigotes per eppendorf by a few minutes centrifugation at 10000 x g. They were then either used immediately or stored at -70°C. The yield of purified amastigotes varied between preperations but for *L. m. mexicana* was approximately 1 x 10^{10} amastigotes for a single large lesion, for *L.donovani* 5 x 10^9 amastigotes from a single spleen and for *L.major* 3 x 10^9 from 4 lesions. The level of host cell contamination was checked at the end of the preparation and routinely gave less than 0.1 % cell/cell contamination.

For electron microscopy studies amastigotes were only partially purified, the crude homogenate (prepared as above) being washed three times, by centrifugation and resuspension, in PSGEMKA buffer.

2.2.2. Promastigotes

Promastigotes were harvested by centrifugation in an MSE Chilspin centrifuge at 2100 x g for 10 minutes at 4°C. The pellets obtained were then resuspended in HBSS, pH 7.5, washed twice by centrifugation and resuspension in HBSS and finally resuspended in 0.25 M sucrose before either being used immediately or pelleted and stored at -70°C. Promastigotes at different phases of *in vitro* culture were harvested at appropriate times; normally 2-3 days after inoculation of the culture for logarithmic phase cells (normally a density of approximately 5 x 10^6 ml^{-1}) and beyond 7 days for stationary phase cells (approximately 2 x 10^7 ml^{-1}). *Leishmania major* metacyclics were isolated from stationary phase cultures by the method of Sacks *et al.* (1985). The promastigotes were harvested and washed three times in HBSS before being resuspended in HBSS containing 100 ug peanut agglutinin (PNA) ml^{-1} to a density of 4-5 x 10^8 promastigotes ml^{-1}. Such suspensions were incubated for 30 minutes at room temperature to allow agglutination to occur. To separate those cells agglutinated by the PNA (PNA positive) from those remaining free (PNA negative cells), the cell suspension was carefully layered over a 50/50 mixture of HIFCS and HBSS and incubated for a further 30 minutes to allow the agglutinated cells to sediment. The PNA negative cells left above the interface were then removed and harvested as normal. A small amount of the supernatant was left above the interface as this often contained smaller aggregates of agglutinated parasites. A typical yield from a 200 ml stationary phase culture was approximately 5 x 10^8 purified metacyclic promastigotes. The purity of the preparation was assessed microscopically for clumps of PNA positive cells.

2.2.3. Preparation of parasite homogenates

Pellets of purified parasites were lysed either by resuspension

in 0.25 M sucrose containing 2.5% (v/v) Triton X100 or by a cycle of freezing and thawing three times using liquid nitrogen and a water bath at 37°C. Fractionation of *L.m.mexicana* amastigotes into soluble and insoluble fractions for the purification of the low molecular weight proteinases (see section 2.10.1.), was achieved by centrifugation at 200000 x g at 4°C for 1 hour in an MSE Prepspin 50.

2.3. MACROPHAGE-PARASITE INTERACTIONS *IN VITRO*

2.3.1. Macrophage maintenance

The medium used for macrophage culture was RPMI 1640, pH 7.2-7.3, supplemented with 10% (v/v) HIFCS, 50 μg benzylpenicillin ml^{-1}, 50 μg streptomycin sulphate ml^{-1}, 25 μg gentamycin sulphate ml^{-1}, 2 mM L-glutamine, 25 mM HEPES and 20 mM sodium hydrogen carbonate. The medium was adjusted to the appropriate pH using 1 M sodium hydroxide.

Mouse resident peritoneal exudate cells (PECs) were obtained from male Balb/c mice by peritoneal lavage using 5 ml of complete medium (RPMI 1640, with all the supplements) per mouse . The number of mice used in each experiment varied between 3 and 14, depending upon the number of PECs required. The lavages from the mice were pooled and diluted with complete medium to give a cell density of 2.5 x 10^5 PECs ml^{-1}. Eight-chamber Lab-Tek tissue culture slides were used for the maintenance of the PECs: 1 x 10^5 PECs in 0.4 ml were plated into each chamber. The cells were allowed to adhere overnight by incubation at either 34°C or 37°C, depending on the experiment, with a gas phase of 95% air, 5% CO_2. Cells to be infected with *L.donovani* were incubated at 37°C whilst those to be infected with either *L.major* or *L.m.mexicana* were incubated at 34°C. Non-adherent cells were removed the next day by vigorous washing with complete medium and the adherent cells remaining were then used. These cells were mainly macrophges but other cell types were present and such preparations will be referred

to as PECs for the remainder of this thesis.

2.3.2. Infection of PECs

In calculating the ratios of parasites to PECs for experiments, the initial number of PECs plated out was taken to be the number of PECs present after washing, despite the removal of non-adherent cells. PECs were infected by adding appropriate numbers of *L.donovani* (amastigotes), *L.major* (amastigotes) or *L.m.mexicana* (amastigotes or promastigotes) in the complete RPMI medium used for PEC culture. The number of parasites used varied depending upon the time used for the infective period (4-24 hours) and the species and stage of the parasite used. When amastigotes were used the ratio was always between 1 PEC: 2-3 amastigotes whilst with promastigotes of *L.m.mexicana* the ratio varied between 1:3 for 24 hour exposures and 1:5 for a 4 hour exposure period. At the end of the exposure period, the free parasites were removed by washing with RPMI medium and 0.4 ml of fresh RPMI medium added to each well. The cells were incubated at 34°C or 37°C as appropriate and at the end of the experimental periods the PECs were either fixed in methanol and stained with 10% Giemsa's stain for 10 minutes, or alternatively were fixed in glutaraldehyde for electron microscopy (see section 2.8)

2.4. DETERMINING ANTILEISHMANIAL DRUG ACTIVITY

2.4.1. *In vivo* models

The activity of potential antileishmanial agents was determined using several *in vivo* experimental model systems. In these studies using the visceral leishmaniasis model, 2×10^7 *L.donovani* amastigotes isolated from hamster spleens and resuspended in 0.2 ml PSGEMKA were inoculated intravenously, via the tail vein, into each female Balb/c mouse (Department of Zoology, University of Glasgow). On day 7 post-infection, the appropriate treatment regimen was started and on day 14

the animals were sacrificed. Impression smears of the first prominent lobe of the liver were made, air dried and fixed in methanol before being stained for 20 minutes in 10% Giemsa's stain. The number of amastigotes per 100 infected cell nuclei were counted in duplicate smears for each experimental animal. The results for each treated animal were compared with the mean of the pooled results of an untreated control group.

A similar procedure was used to test different treatments against Balb/c mice infected with amastigotes of *L.m.mexicana*. Mice were infected via the tail vein with amastigotes and the parasite load in the liver two weeks later was determined. Several inocula of amastigotes were tested to ascertain the most useful course of infection for drug treatment experiments, and mice were sacrificed over a 4 week period to determine the parasite load of the liver at different times. The most suitable inoculum was found to be 8×10^7 amastigotes which gave a mean parasite load of 128 parasites per 100 host cell nuclei 2 weeks post-infection. There was a high level of variability in this model which made it only of limited use.

Activity against *L.m.mexicana* was also determined using sub-cutaneous inoculation of parasites which resulted in the growth of a cutaneous lesion. An inoculum of either 3×10^6 or 3×10^7 amastigotes in 0.2 ml was injected into the shaven rump of female CBA mice (Department of Zoology, University of Glasgow) and the course of infection monitored by measuring the size of the resultant cutaneous lesions. In studying different forms of therapy, treatment was commenced 7 days post-infection.

Several routes of administration were investigated for treating animals. Water soluble drugs and suspensions of carrier formulations were given either intravenously (i.v.), via the tail vein, or intraperitoneally (i.p.). Other drugs were suspended in

0.375% (w/v) gum tragacanth and given either i.p. or orally.

2.4.2. *In vitro* models

2.4.2.1. Amastigotes *in vitro*

In vitro investigations using PECs infectd with *Leishmania* species were carried out using standard conditions (see section 2.3.). Compounds under investigation were added during preparation of the standard medium. Drugs were dissolved in water wherever possible but several were dissolved in dimethyl sulfoxide (DMSO) or ethanol, as indicated. In such cases controls were caried out to ensure that the final concentrations of these solvents had no effect on the PECs or the parasites. Where experimental procedures involved culture for longer than 48 hours, the medium was replenished at this point and the incubation continued for up to a further 72 hours before the experiment was terminated. Cells were then fixed and stained (section 2.3.2.) and the number of amastigotes per 100 infected cells and the % of cells infected determined by microscopic observation. The results for treated cells are expressed as either a % of the mean of the controls or as a % reduction compared to the controls, so allowing the degree of antileishmanial activity to be quantified.

2.4.2.2. Promastigotes *in vitro*

The activity of a compound against promastigotes, of all three species used in this study, was assessed by monitoring its effect on promastigote growth or using the MTT assay (see section 2.4.2.3.). In the former case, promastigotes were inoculated, to a density of 1×10^5 cells ml^{-1}, into 5 ml of medium (see section 2.1.2.) containing the appropriate concentration of the compound being tested. The cell density of each culture was measured daily for a period of seven days using an Improved Neubauer Haemocytometer. Stock solutions of the compound being tested were normally filter sterilised (0.22 um pore

size filter) and diluted to give the necessary concentration so that the drugs were added as 0.1 ml volumes at the start of the experiment in all cases. 0.1 ml distilled, deionised water was added to control cultures.

2.4.2.3. *In vitro* viability assay

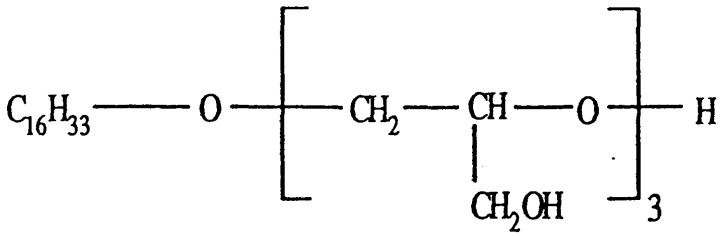
The effects of different compounds on the viability of cultured promastigotes and isolated amastigotes was measured using a method based on the metabolism of a tetrazolium salt, MTT(3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), by living cells (Mossman, 1983). Briefly, parasites were suspended at approximately 1 x 10^8 ml^{-1} (amastigotes) or 2 x 10^7 ml^{-1} (promastigotes). The cells were then washed free from the drug and incubated with MTT at 450 ug ml^{-1} (in distilled, deionised water) for 3 hours at the appropriate temperature. The blue formazan product, produced by the action of various dehydrogenases reducing the MTT, was quantified by dissolving it in 0.5 ml acid (0.04 M HCl) isopropanol and measuring the absorbance at 570 nm. The results obtained are expressed as the reduction of MTT by the treated parasites as a percentage of that by untreated parasites. The absorbance change in control cultures was normally in the range 0.8 - 1.5.

2.5.. LIPOSOME AND NIOSOME PREPARATION

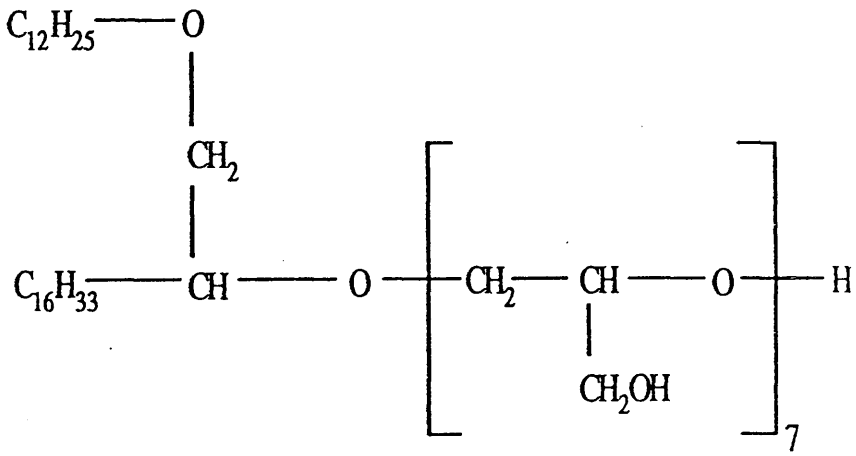
2.5.1. Niosome preparation

The preparation of niosomes containing sodium stibogluconate was carried out using the ether injection method described by Baillie *et al* (1985). Three surfactants (see fig. 7) were used in the preparation of the niosomes, in which 450 µl of surfactant or surfactant-ash free cholesterol (CHOL) or surfactant-CHOL-dicetyl phosphate (DCP) mixture dissolved in diethyl ether was injected slowly into 5 ml of 300 mg sodium stibogluconate ml^{-1} aqueous solution at 60°C. Both niosomes and

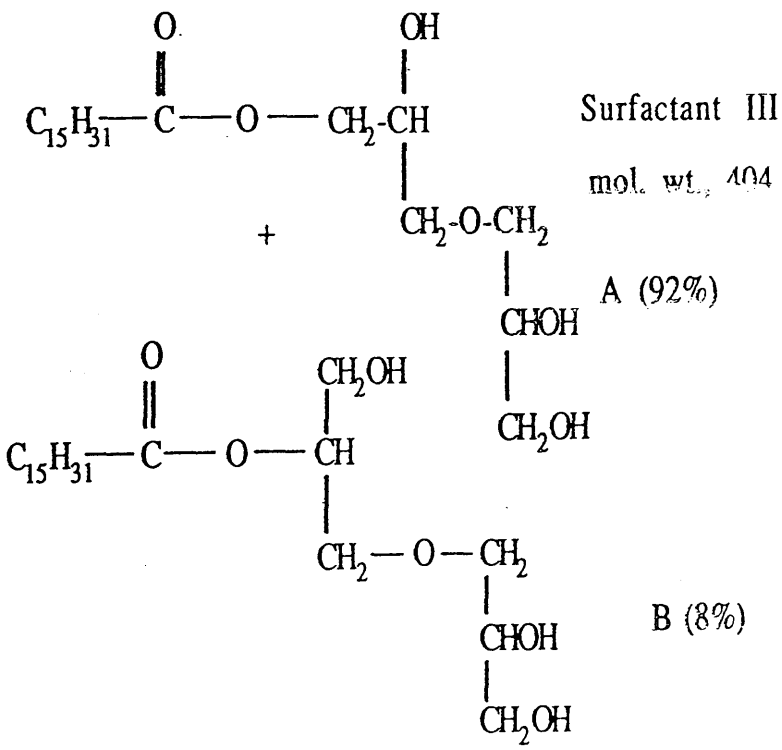
Figure 7. Structure of non-ionic surfactants



Surfactant I, mol wt., 473



Surfactant II, mol wt., 972



Surfactant III

mol. wt., 404

A (92%)

B (8%)

liposomes (see section 2.5.2.) were either 'drug-loaded' and contained sodium stibogluconate or were 'empty' and contained 30mM glucose solution.

2.5.2. Liposome preparation

The peparation of liposomes containing sodium stibogluconate was carried out by hydrating, under a N_2 atmosphere, 114 µmol dipalimotyl (DPPC)-CHOL or DPPC-CHOL-DCP mixture with 8 ml aqueous 300 mg sodium stibogluconate ml^{-1} at 45-50°C for 2 hours with gentle agitation.

2.5.3. Final vesicle preparation

The mean diameter of the niosomes was 333-381 ± 80 nm whilst the liposomes were much larger at 860 ± 50 nm. Unentrapped drug was removed from the vesicle suspensions by dialysis against a 300 mM glucose solution. the dialysed vesicles were adjusted to the required drug concentration by dilution with 300 mM glucose.

2.5.4. Determination of drug content of vesicles

The drug concentration in the vesicles was measured by estimating the antimony content by flame atomic spectrometry. a Pye Unicam SP90 Atomic absorptiometer was used to determine the antimony content of vesicle preparations which had been disrupted in propanol. The antimony standard was a solution of $SbCl_3$ in dilute HCl and the sensitivity of the method 1 µg Sb ml^{-1}.

2.6. PREPARATION OF STARCH MICROPARTICLES

2.6.1. Synthesis of microparticles

The procedure followed was that described by Laasko *et al* (1986) and the microparticles were supplied by these workers. Purified acryloylated starch (0.12 acrylic groups/glucose residue) and N-acryloyl-1,6-diaminohexane (ADH) were dissolved in 5 ml of 0.2 M sodium phosphate buffer, pH 8.5, containing 10^{-3} M EDTA. After

addition of 0.08 M ammonium peroxydisulphate, the aqueous phase was emulsified in 300 ml chloroform-toluene (1:4) with 32 uM Pluronic F68. The monomer present in the the water-oil emulsion was polymerised by the addition of 0.1 ml N,N,N',N'-tetramethyl-ethylenediamine to give the ADH polyacryl starch microparticles. N_2 was bubbled through the system throughout the procedure. The microparticle composition was characterised using the D-T-C nomenclature of Edman *et al* (1980). D denotes the acrylolated starch concentration (g 100 ml^{-1}), T is the total acrylol groups (g 100 ml^{-1}) and C the proportion (% w/w of acrylic monomer) of cross-linker present. The D-T-C ratio of the ADH polyacryl starch microparticles was 10-1.5-0, whilst for 'empty' microparticles it was 10-0.5-0.

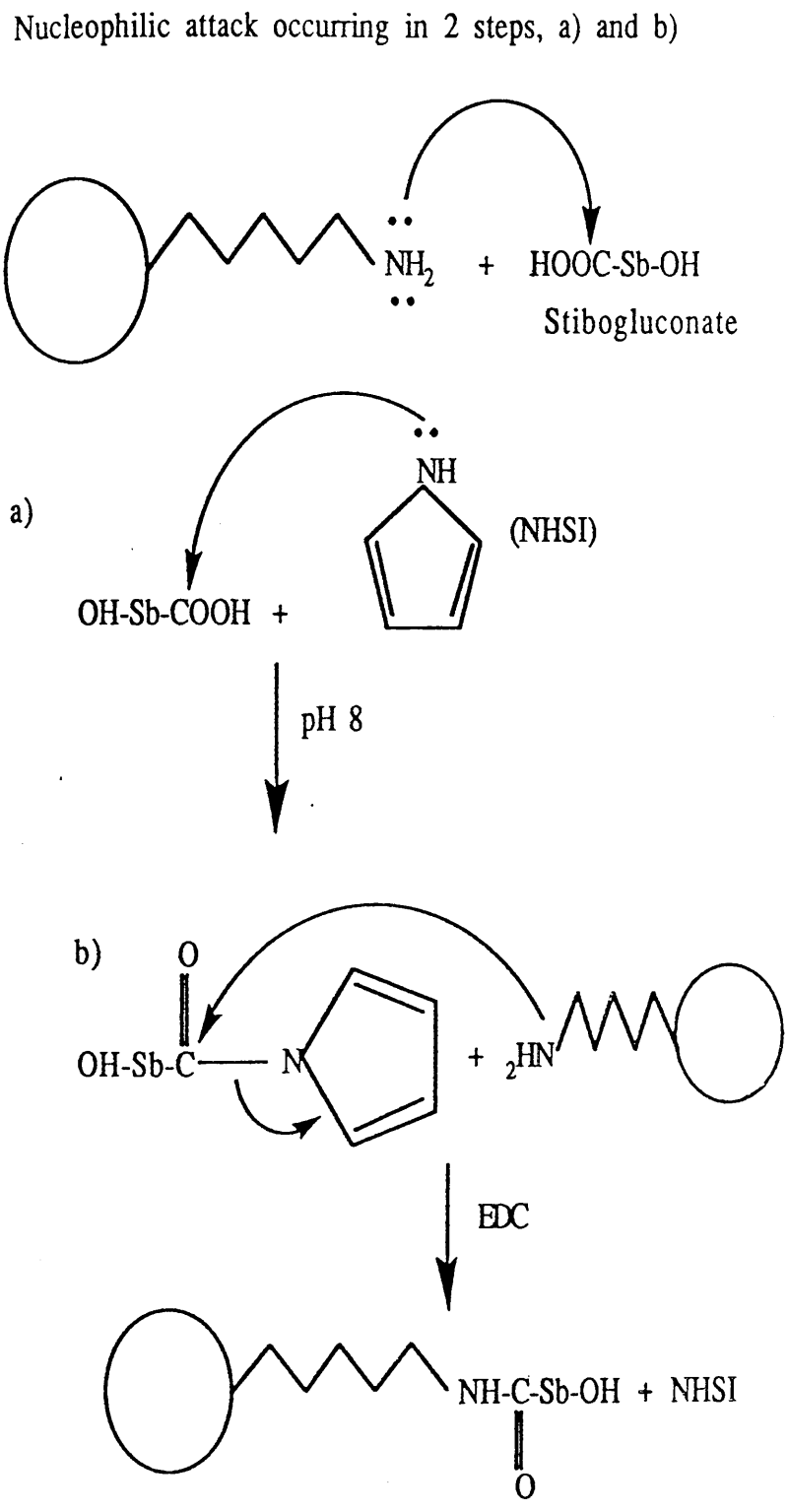
2.6.2. Coupling of sodium stibogluconate to the ADH microparticles

Sodium stibogluconate was coupled via a cationic ligand to the ADH-polacryl starch micropaticles (see fig. 8) by mixing 50 mg ADH microparticles with 200 mg sodium stibogluconate in 10 ml of 1 M $NaHCO_3$ buffer, pH 8.0. Five ml of the same buffer containing 100 mg N-hydroxysuccinamide and 250 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were added and the whole suspension stirred overnight at room temperature. Unreacted drug and the coupling agents were removed by centrifigual (200 x g for 10 minutes) washing in saline. The antimony content of the microparticles was determined as previously described.

2.7 MEASUREMENT OF UPTAKE AND INCORPORATION OF NUCLEOTIDES AND NUCLEOSIDES BY *LEISHMANIA*

The uptake and incorporation of tritiated (^3H) leucine and adenosine (Amersham) by different species and stages was measured essentially using the methodology described by Mukkada *et al* (1985). Parasites were suspended, to give an approximate protein concentration

Figure 8. Coupling of sodium stibogluconate to starch microparticles



of 25 μg ml^{-1}, in 0.1 M McIlvanes buffer (pH 3-7) containing 5.2 g NaCl and 0.5 g KCl per litre, with 10 mM glucose as an energy source. These were incubated at either 25°C (promastigotes), 34°C (*L. m. mexicana* amastigotes) or 37°C (*L. donovani*), as appropriate, for 10 minutes before the addition of either ^{3}H leucine or ^{3}H adenosine to give a final concentration of 0.4 mM and a specific activity of 1 μCi μmol^{-1}. At various points during the incubation of up to two hours, 1 ml samples were removed and the cells washed twice by centrifugation and resuspension in 0.1 M sodium phosphate buffer, pH 7.3. The suspensions of cells were then collected by filtration using glass-microfiber filters (1.2 um porosity, Whatman GF/C) and vacuum and the cells immediately washed either with 10 ml of ice cold 10% TCA followed by 4 ml 95% ethanol if incorporation into macromolecules was being estimated, or alternatively with 14 ml of ice cold phosphate buffer, pH 7.3, if total uptake by the cells was being estimated. The filters were then air dried for 10 minutes an transferred to vials containing 15 ml Ecoscint scintillation fluid (National Diagnostics). The vials were agitated for 30 seconds with a whirlymixer before the amount of radioactivity was determined using an Isocap 1300 Liquid Scintillation System (N.C.D.). Counting efficiencies were determined using the external standard channels ratio method and was approximately 30%.

Several different procedures were tested to ensure that background radioactivity was minimal; these involved varying the number of washes and the volume of buffer or TCA used. The procedure described above proved to be the most consistent. Time courses were carried out to ensure that uptake and incorporation was linear over the incubation periods used. Promastigotes were examined microscopically to ensure that they were viable over the incubation periods used.

2.8. ELECTRON MICROSCOPY

Infected PECs were released with a rubber policeman from the bottom of tissue culture flasks, after the removal of the medium, and fixed by irrigation with 10 ml of 2.5% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.4, at 33°C. During the 40 minutes fixation, the temperature was allowed to drop to 20°C after which the fixed suspension of released, infected PECs was centrifuged at 1500 x g for 15 minutes at room temperature. The fixed cells were washed twice in 0.1 M potassium phosphate buffer containing 2% (w/v) sucrose and post-fixed in 1% buffered, pH 7.4, osmium tetroxide for 1 hour. Following removal of osmium with 3 changes of distilled water, the cells were encapsulated in 1% Seaplaque agarose at 40°C and subsequently processed as small (< 1.0 mm^3) blocks through 0.5% aqueous uranyl acetate for 30 minutes, dehydrated in a series of alcohols and finally embedded via propylene oxide in araldite.

After two days polymerisation at 60°C, 350 nm or 60 nm sections were mounted on 300 mesh grids, stained with 2% methanolic uranyl acetate for 5 minutes and examined or further stainedwith lead citrate for 5 minutes before examination. Exposures were recorded on 70 mm Kodak MP11 roll film using a Zeiss transmission electron microscope 902 incorporating an electron energy spectrometer for contrast enhancement and thick section imaging.

2.9. DETECTION OF ESTERASES OF *LEISHMANIA*

2.9.1. The use of starch gel electrophoresis to detect esterase activity

Starch gels were prepared using the method described by Tait *et al* (1984). Gels were made by heating 250 ml of an 8% (w/v) Connaught hydrolysed starch solution, made up in the gel buffer (0.1 M Tris-HCl, pH 7.5), until the solution was clear. This was then degassed, using a

vacuum pump, poured into a setting mould and left for an hour at 4°C to set. Samples were prepared in homogenisation buffer (10 mM Tris-HCl, pH 7.5, 1mM DTT, 1 mM EDTA, 2% Triton X100), agitated with a whirlymixer and left for 10 minutes at room temperature before being centrifuged at 10,000 x g for 15 minutes. Small pieces of Whatman 3MM paper (10 x 3 mm) were then soaked in the clear supernatant and applied to the gel in cuts made with a razor blade. The samples were then subjected to electrophoresis, using 1 M Tris-borate, pH 7.5, as the running buffer, at 8 V cm^{-1} for 5 hours with cooling. Esterase activity on the gels was detected using 4-methyl umbilliferyl propionate (Harris and Hopkinson, 1976). Ten mg of the substrate was dissolved in a few drops of acetone and then mixed with 100 ml of 0.1 M phosphate buffer, pH 6.5. This was then applied to the gel on a filter paper overlay for approximately 5 minutes. The overlay was then removed and the gel viewed under a long wave UV lamp for fluorescent bands. This visualises the fluorescent hydrolysis product 4-methyl umbilliferone.

2.9.2.The detection of phenol as an assay for measuring the hydrolysis of L-leucine benzyl ester

The method of Pilz (1974) describing the use of phenylacetate as a substrate for the measurement of arylesterase activity was used after modification. This assay is based on the hydrolysis product phenol reacting with 4-aminoantipyrine, with potassium ferricyanide, $K_3(Fe[CN_6])$, as a catalyst, to give a condensation product that is extracted with n-pentanol and can be determined colorometrically. It was thought that a similar method could be used to measure the hydrolysis of leucine benzyl ester.

It was reported that the reaction mixture should be maintained at pH 7.5-8.6 to ensure that the reaction of phenol and 4-aminoantipyrine

was not reversible (Pilz and Johann, 1966). To enable assays to be performed at lower pH the method was modified. Low concentration buffers (1 mM) at lower pHs were used in the incubations for the esterase reactions themselves. The subsequent addition of 4-aminoantipyrine and $K_3(Fe[CN_6])$ was added giving final concentrations of 14 mM and 35 mM respectively. The mixture was then allowed to stand at room temperature for 15 minutes before the addition of 5 ml of n-pentanol and rapid agitation using a whirlymixer. This mixture was then allowed to stand for a further 10 minutes. The upper organic phase was then removed and centrifuged at 10000 x g for 2 minutes and the resulting clear solution used for colorimetric measurements. The absorbances of the samples were read against a blank which contained no substrate or sample. The difference between the absorbances of the sample and the control was used to calculate the amount of the condensation product formed, and thereby the amount of phenol produced. Standard concentrations of phenol were used to calibrate the assay and check that it was sufficiently sensitive to detect low concentrations of phenol at different pHs. The extinction coefficient of the condensation product at 465 nm was taken as 21.7 cm^2 μmol^{-1} (Pilz, 1974).

2.10. PURIFICATION OF AMASTIGOTE PROTEINASES

2.10.1. Chromatogenic assays of proteinase activity

Chromatogenic fractions were assayed for proteinase activity using the nitroanilide substrate N-benzoyl-proline-phenylalanine-arginine nitroanilide (BzPPANan). The subsrate was used at a final concentration of 0.075 mM in a 110 µl reaction volume containing 10 mM DTT and 100 ul of each fraction. The change in absorbance at 405 nm over 10 minutes incubation at room teperature was measured on a Titertek Multiscan MCC/340. To determine the specific activities of

samples towards BzPPANan at 37°C, assays were carried out in 0.15 M sodium phosphate buffer, pH 6.0, containing 0.1 mM BzPPANan and 1 mM DTT in a total reaction volume of 1.2 ml. The reaction mixture was pre-incubated at 37°C for 10 minutes before the addition of the BzPPANan and DTT to initiate the reaction. The release of the 4-nitroanilide caused by the hydrolysis of BzPPANan was monitored on a Perkin-Elmer Lambda 5 spectrometer, at 405 nm. the molar absorbtion coefficient of 4-nitroanilide was taken as 9500 1 $mol^{-1}cm^{-1}$ (Pupkis and Coombs 1984).

2.10.2. Gel filtration

Lysates of *L.m.mexicana* amastigotes were produced by either a cycle of freezing and thawing or by the use of Triton X100 (see section 2.2.3.). Approximately 1 x 10^{10} amastigotes were lysed in 1.2 ml volumes. These were then fractionated into soluble and insoluble fractions by centrifugation at 240000 x g for one hour at 4°C in a Prepspin 50 ultracentrifuge. One ml of the resulting supernatant was then applied to a gel filtration column (1 cm x 1 cm) of Superose 12 previously equilibrated with 0.15 M sodium phosphate bufer, pH 6.0, and fractionated using fast protein liquid chromatography (Pharmacia). Fractions were eluted with the equilibration buffer at room temperature using a flow rate of 1 ml per minute. One ml fractions were collected at 4°C using a Pharmacia Frac 100 fraction collector. The protein concentration of eluted fractions was monitored by measuring the absorbance at 280 nm with a Pharmacia Single Path Monitor Optical Unit, UV-1, spectrophotometer.

2.10.3. Ion exchange chromatography

A column of DEAE-cellulose (HR5/5 mono Q, 1 ml volume, Pharmacia) was used as a second step in the purification of the proteinases. Fractions from gel filtration containing the lower molecular weight

proteinases were concentrated (see section 2.10.4) and applied in either 1 ml or 2 ml volumes to the mono Q column which had been previously equilibrated with 0.025 M sodium phosphate buffer, pH 6.0. Unbound protein was eluted with 5 ml of equilibration buffer. Elution was then continued using a salt gradient of 0-0.35 M sodium chloride in sodium phosphate buffer, pH 6.0, to elute bound proteins. Normally the gradient was commenced at fraction 6 and rose to 0.35 M sodium chloride by fraction 25. At this stage the sodium chloride concentration was increased to 1 M and maintained such that fractions 26-30 contained any proteins still bound to the column at 0.35 M sodium chloride. Fractions were collected as for gel filtration and assayed for proteinase activity as described previously (see section 2.10.1.). Protein elution was followed at 280 nm as described above.

On one occassion the salt gradient concentration was altered such that the gradient commenced as above but between fractions 20 and 30 was shallower. Between fractions 20 and 30 the gradient changed from 0.25 M NaCl to 0.35 M NaCl, whereupon the salt concentration was raised to 1 M and maintained such that fractions 31-35 contained any proteins still bound to the column at 0.35 M NaCl.

2.10.4. Pooling and concentration of low molecular weight proteinases

Proteinase activity in the chromatographic fractions was measured as described previously (see section 2.9.1.) and the fractions containing the low molecular weight proteinases identified using gelatin SDS-PAGE (see section 2.12.3). These fractions were then pooled and concentrated under nitrogen pressure, on ice, using an Amicon Ultrafiltration Cell. A PM 10 membrane filter was used, allowing molecules with an approximate molecular weight of less than 10 kDa to pass through the membrane whilst retaining those of higher molecular weight. The pooled and concentrated preparation of low

molecular weight proteinases i.e. those recovered from ion exchange chromatography, were termed the purified proteinases and were used to characterise these enzymes.

2.11. PROTEIN ESTIMATION

Protein estimations were carried out using the method of Sedmak and Grossberg (1977) using bovine serum albumin as standard. This method was shown to be appropriate for protein concentrations in the range 5-40 μg ml^{-1}. A standard curve was determined for each fresh batch of reagents used.

2.12. POLYACRYLAMIDE GEL ELECTROPHORESIS

2.12.1. Preparation of samples for polyacrylamide gel electrophoresis

Parasite homogenates were prepared using detergent lysis as described in section 2.2.3. and diluted 1:1 with 2 x sample buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol; Laemmli, 1970). Chromatographic fractions were also diluted with the sample buffer at a ratio of 1 : 1, but occasionally (as specified) at a ratio of 2 : 1. Samples were only boiled where stated. In some cases, samples were pre-incubated with inhibitors for 30 minutes at room temperature before the addition of sample buffer.

2.12.2. Preparation and running of gels

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins and estimate their molecular weight. Gels were prepared and run as described by Hames and Rickwood (1981). The % of acrylamide used in the separating gel was either 5, 7.5 or 12.5% (w/v) but was always 5% (w/v) in the stacking gel. Gels were normally run overnight at 8 mA per gel, except where mini-gels were used. These were run for approximately 45 minutes at 100 volts

per gel. Acrylamide gels were used to identify the molecular weights of proteins which correlated with proteinase activity and to determine the purity of samples after different chromatographic procedures. Protein bands were detected by using either Coomassie Blue staining (0.05% [w/v] PAGE blue 83, 25% [v/v] isopropanol and 10% [v/v] acetic acid) for three hours before destaining in 10% (v/v) acetic acid or by the use of silver staining as described by Oakley *et al* (1980).

2.12.3. Gelatin SDS-PAGE

Proteinase activity was detected and analysed using gelatin SDS-PAGE (Lockwood *et al.*, 1987). These gels contained 0.2% (w/v) gelatin which was copolymerised into the separating gel. After electrophoresis, the gels were incubated in 2.5% (v/v) Triton X100 for 30 minutes (mini-gels) or 1 hour (large gels) to remove SDS. The gels were then incubated in 0.1 M acetic acid/sodium acetate buffer, pH 5.5, with 1 mM DTT at 37^{o}C during which time the proteinases hydrolysed the gelatin present in the gel. This incubation lasted up to 4 hours depending on the level of proteinase activity present. After incubation, the gels were stained with Coomassie Blue as described above, and then destained with 10% (v/v) acetic acid. Clear bands indicated the position of proteinases.

2.12.4. Determination of molecular weights from gel electrophoresis

The molecular weight of individual proteins and proteinases were determined by comparing their electrophoretic mobility with that of standard proteins, either high or low molecular weight standards (Sigma Chemical Co. Ltd.).

2.13. CHARACTERISATION OF THE *L.M.MEXICANA* AMASTIGOTE LOW MOLECULAR WEIGHT PROTEINASES

2.13.1. The effect of inhibitors and competing substrates

Enzyme samples were pre-incubated with inhibitors for either 30 or 60 minutes at room temperature before the addition of the substrate and DTT whereupon the activity of the sample towards the BzPPANan was determined and compared with a control. The assay was as described in section 2.10.1. to determine specific activities. Several proteinase inhibitors were used to characterise the type of proteolytic activities being studied.

Several amino acid esters and amino acid amides were used to investigate their possible effect on the hydrolysis of BzPPANan by the purified proteinases. The purified enzymes and the amino acid derivatives were added together to the reaction mixture, which already contained the DTT and the BzPPANan and the effect on the hydrolysis of the BzPPANan compared to a control. The assay used was as described in section 2.10.1. to determine specific activities. Some of these compounds were dissolved in ethanol or DMSO and appropriate controls were carried out to compensate for any potential effects of these solvents.

2.13.2. Determination of the effect of pH on enzyme activity

The pH optimum of the activity of the purified enzymes towards BzPPANan, was calculated using the assay for determination of the specific activities (see section 2.10.1.). Two buffers were used, 0.1 M citric acid (pH 3-6) and a 0.1 M sodium phosphate (pH 6-8). These buffers replaced the 0.15 M phosphate buffer used in the standard assay. These buffer systems were also used in determining the pH optimum for the hydrolysis of leucine methyl ester by the purified enzymes (see section 2.13.3).

2.13.3. Activity towards amino acid esters and amino acid amides detected using paper electrophoresis

Paper electrophoresis was used to separate the products of the

hydrolysis of amino acid esters and amino acid amides from the parent compounds. The reaction mixture was a total volume of 0.3 ml containing 0.15 M sodium phosphate buffer, pH 6.0, 1 mm DTT, the appropriate homogenate or purified enzyme and normally 20 mM of the ester or amide. The reaction mixture was incubated for three hours at 37°C and applied immediately, in either 10 or 40 ul volumes, to strips of Whatman 3 MM paper (3 cm x 25 cm) for electrophoresis. The hydrolysis products of the amino acid esters and amino acid amides were then separated by paper electrophoresis, using an electrophoresis constant power supply (Pharmacia, ECPS 3000/150). Electrophoresis was carried out at 8V cm^{-1} for 3 hours in an electrophoresis tank using a pyridine acetate buffer, pH 3.5 (glacial acetic acid:pyridine:water/10:1:89, by volume). This buffer was also used to pre-soak the paper before sample application, a step which appeared to help in the separation of the various hydrolysis products. After electrophoresis the paper was air dried and stained by immersion in a solution of 0.2% (w/v) ninhydrin in 75% (v/v) acetone. In some experiments, after drying to develop the colour, the stain was fixed by dipping the paper in a $Cu(NO_3)_2$, 0.02% HNO_3 in acetone: ethanol [2:1]) (Goldman and Kaplan, 1973). Alternatively, the reaction products were quantified by eluting them with 10 ml of 0.4% (w/v) cadmium acetate prepared in a water-glacial acetic acid-ethanol (1:5:4 by volume) solution for 30 minutes and measuring the absorbance at 505 nm of the eluted solution. This method was used to generate a standard curve with L-leucine.

2.13.4. Detection of glycoproteins on gels

The procedure used was essentially that of Zacharius *et al* (1969). After electrophoresis, the gel was immersed in 12.5% (w/v) TCA for 30 minutes, then rapidly rinsed with distilled, deionised water

and immersed in a solution of 1% periodic acid in 3% acetic acid for a further 50 minutes. The gel was then washed a minimum of 6 times by immersion in distilled, deionised water with motion before being immersed in 50 ml of fuchsin-sulphite stain (Sigma, UK), in the dark, for 50 minutes. Freshly prepared 0.5% (w/v) metabisulphite was used for 3 rinses, of 10 minutes each, before being replaced with distilled, deionised water to remove the excess stain. Pink bands appear at the site of glycosylated proteins. Fetuin and ovalbumin (Sigma, UK) were used as glycoprotein standards.

2.13.5. Studies using endoglycosidase treatment

The method used was modified from ref. 20 ul of purified enzyme (0.01 units see 3.8.5.) was added to 40 ul of incubation buffer comprising 150 mM $NaPO_4$, 75 mM EDTA, 1.5% Nonidet P40 and 1.5% mercaptoethanol at pH 6.1. Leupeptin was also included to give a final concentration of 100 ug ml^{-1} and 2 ul of Endoglycosidase F was added (Sigma, UK). The reaction mixture was then incubated at 37°C for 8 hours before duplicates of the sample were run on SDS-PAGE to detect any changes in the banding pattern of the proteinases.

2.14. STATISTICAL ANALYSIS OF DATA

One-way and two-way analysis of variance tests in conjunction with the Student-Newman-Keuls procedure were used to analyse the significance of data. The F value, on which the significance is based is given with the degrees of freedom in subscript. The probability (P) was taken as significant when $P<0.05$.

2.15 MATERIALS

Sodium stibogluconate (Pentostam) equivalent to 0.32 mg Sb ml^{-1} was a gift from the Wellcome Foundation, UK. The three non-ionic surfactants (see fig 7) were a gift from L'Oreal, France. These are

the vesicle-forming non-ionic surfactants as synthesised by Vanlerberghe *et al* (1978). In each case the number of units in the hydrophilic portion is a number average value. Surfactant 3 is not ether-linked, and contains the isomers in the proportion A:B, 92:8.

Soluble starch (maltodextrin, molecular weight = 5000) was a gift from Dr Lars Svensson, Stadex AB, Malmo, Sweden. ADH was synthesised essentially as described by Stahl *et al* (1978). Acrylic acid glycicidyl ester, N-t-butyloxycarbonyl-1, and 6-diaminohexane were obtained from Fluka AG, Switzerland, 1-ethyl-3-(3-dimethylminopropyl) carbodiimide from Sigma, UK, and N-hydroxysuccinamide and N,N,N',N'-tetramethylethylene diamine from Merck, FRG.

BzPPANan, was prepared as a stock solution of 1 mM in water and dissolved by heating to 90°C for sveral minutes. It was stored at -20°C. No loss of stability was noted over the periods of time it was stored. The proteinase inhibitors pepstatin (1 mg ml^{-1}), N-tosyl-L-phenylalanine chloromethyl (TPCK, 10mM), and chymostatin (1 mg ml^{-1}) were all dissolved in DMSO whilst phenylmethylsulfonyl fluoride (PMSF, 10 mM) was dissolved in ethanol. All the other proteinase inhibitors leupeptin, trans-epoxysuccinyl L-leucylamido-(4-guanidino)-butane (E64) and antipain (1 mg ml^{-1}), N-p-tosyl-1-lysine chloromethyl ketone (TLCK), iodoacetic acid (IAA) and phenanthroline (10 mM) were dissolved in water. Stock solutions were stored at -20°C except PMSF which was made up freshly each time it was used. All chemicals were obtained from Sigma unless otherwise stated.

The principal components of the growth culture medium for *Leishmania* promastigotes i.e. foetal calf serum, MEM Eagle Suspension powder, MEM (50x) amino acids, MEM (50x) amino acids solution and MEM (100x) non-essential amino acids solution were obtained from Gibco-Biocult, Scotland. RPMI 1640 and Lab-Tek 8-chamber tissue culture slides for PEC culture were obtained from Flow laboratories, England.

3.0 RESULTS

3.1. THE EFFICACY OF CARRIER SYSTEMS IN THE CHEMOTHERAPY OF EXPERIMENTAL VISCERAL LEISHMANIASIS

The first aim of this project was to test two novel carrier systems, niosomes and microparticles, in a direct comparison with the well characterised liposomal system, for the delivery of sodium stibogluconate to treat experimental leishmaniasis.

3.1.1. The use of free sodium stibogluconate against *L.donovani* in mice

In order to compare the efficacy of carrier systems for sodium stibogluconate with that of free sodium stibogluconate it was necessary to evaluate the effect of the free drug in the experimental animal model of leishmaniasis. The infection and treatment regimen was as described earlier (see section 2.4.1.), drug administration was by tail vein inoculation of 0.2 ml on days 7 and 8 post-infection. The data in table 4 and figure 9 show the effect of the free drug (sodium stibogluconate concentration expressed in terms of Sb) on the parasite burden in the liver.

The estimated dose required to give 50% suppression of parasite numbers in the liver (ED_{50}) was approximately 300 ug antimony per mouse (see fig. 9). Complete clearance of parasites from the liver was achieved with a total dose of 2 mg antimony per mouse. This is equivalent to 100 mg antimony per kg. This differs considerably from the figure of approximately 1000 mg kg^{-1} previously reported (Black *et al.*, 1977; Alving *et al.*, 1978).

3.1.2. The efficacy of vesicular systems containing sodium stibogluconate against *L.donovani* in mice

The infection and treatment regimen that had been used to measure the efficacy of free stibogluconate (see sections 2.4.1 and 3.1.1.) was also used to test the different vesicular systems. This allowed a

Table 4. The efficacy of free sodium stibogluconate against experimental visceral leishmaniasis.

Sb dose (μg/mouse)	% suppression
66	10.4 ± 7.7(5)
200	35.6 ± 16.3 (5)
400	65.6 ± 5.8 (13)
660	72.2 ± 7.9 (11)
800	91.0 ± 3.7 (16)
1320	97.6 ± 0.8 (30)
1600	90.7 ± 4.3 (24)
2000	100 n.a. (20)

Mice were infected on day 1 with 2 x 10^7 amastigotes of *L.donovani* and treated on days 7 and 8 via the tail vein with free sodium stibogluconate solution. The total dose of drug (expressed as antimony) given per mouse is shown. Liver parasite burdens were determined on day 14 and % suppression calculated for individual drug treated animals relative to the mean parasite burden for an appropriate untreated control group. The mean ± standard error of mean are given and the number of animals is shown in parentheses.

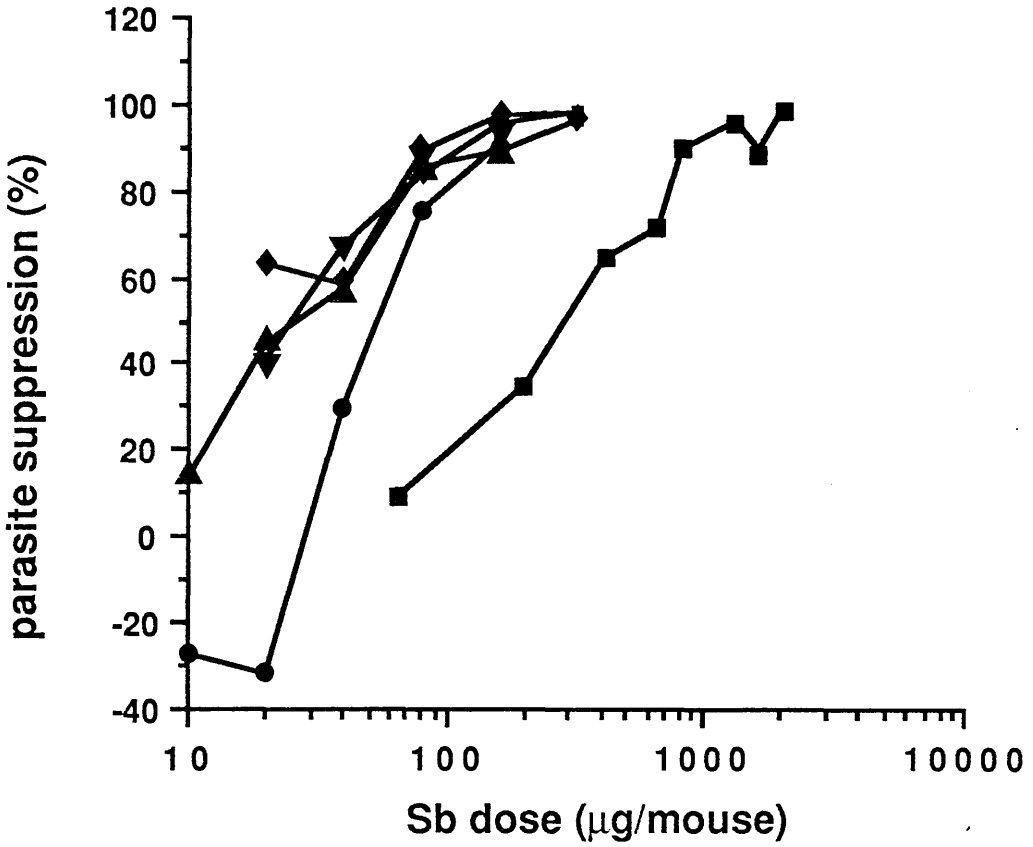
Table 5. The effect of vesicle composition on the efficacy of vesicular sodium stibogluconate on experimental visceral leishmaniasis

Sb dose (μg/mouse)	Amphiphile	Reduction in liver parasite burden, % suppression, and type of vesicular carrier used. Vesicle composition			
		100%	+CHOL 30%	+CHOL 50%	+CHOL 20%, DCP 10%
	Surfactant I				
10		n.d.	−27·4 ± 6·6 (4)	n.d.	14·2 ± 8·8 (5)
20		24·5 ± 7·1 (10)	−31·9 ± 10·4 (4)	38·7 ± 7·6 (6)	5·3 ± 6·1 (8)
40		69·5 ± 1·9 (8)	30·2 ± 5·3 (4)	79·1 ± 5·8 (8)	26·5 ± 11·5 (8)
80		92·9 ± 2·1 (9)	76·5 ± 2·3 (5)	88·1 ± 3·4 (3)	65·2 ± 1·6 (7)
160		96·5 ± 1·4 (7)	91·4 ± 2·7 (4)	96·8 ± 0·9 (7)	84·5 ± 1·9 (8)
320		99·1 ± 0·3 (10)	n.d.	99·2 ± 0·2 (9)	97·6 ± 0·6 (7)
	Surfactant II				
20		20·2 ± 14·3 (4)	41·0 ± 5·3 (7)	53·2 ± 7·7 (9)	n.d.
40		7·2 ± 7·2 (4)	68·0 ± 4·0 (9)	77·8 ± 4·3 (6)	
80		37·2 n.a. (2)	83·9 ± 3·8 (8)	91·0 ± 1·6 (8)	
160		53·7 ± 13·3 (4)	96·5 ± 1·1 (8)	96·6 ± 0·9 (9)	
320		95·4 ± 0·9 (4)	99·2 ± 0·1 (9)	99·2 ± 0·1 (9)	
640		99·0 ± 0 (4)	n.d.	n.d.	
	Surfactant III				
10		n.d.	13·9 ± 14·2 (6)	n.d.	n.d.
20			45·0 ± 11·7 (15)		
40			57·6 ± 10·4 (12)		
80			86·6 ± 3·5 (12)		
160			93·1 ± 1·9 (12)		
320			97·4 ± 0·8 (10)		
	Phospholipid				
8		n.d.	n.d.	n.d.	1·2 ± 11·9 (4)
10			n.d.		4·2 ± 7·6 (6)
16			n.d.		0 ± 10·5 (4)
20			63·4 ± 6·2 (4)		18·1 ± 5·1 (3)
32			n.d.		33·3 ± 3·7 (4)
40			59·1 ± 11·4 (5)		90·1 ± 2·7 (5)
64			n.d.		69·1 ± 6·3 (5)
80			89·7 ± 1·8 (5)		89·9 ± 1·4 (5)
160			98·0 ± 0·3 (4)		90·6 ± 4·1 (4)
320			99·3 ± 0·4 (5)		n.d.

n.a. = not applicable, n.d. = not determined.

Mice were infected on day 1 with 2 x 10^7 amastigotes of *L.donovani* and treated on days 7 and 8 via the tail vein with sodium stibogluconate in several vesicular forms. Vesicles comprised amphiphile, non-ionic surfactant or phospholipid, with and without admixture of cholesterol (CHOL) and dicetyl phosphate (DCP). Molar composition is shown. The total dose of drug (expressed as antimony) given per mouse is shown. Liver parasite burdens were determined on day 14 and % suppression calculated for individual drug treated animals relative to the mean parasite burden for an appropriate untreated control group. For any treatment, the mean % suppression ± standard error of the mean, with the number of animals in parenthesis, is given.

Figure 9. The efficacy of free and vesicular sodium stibogluconate on experimental visceral leishmaniasis



Mice were infected on day 1 with 2 x 10^7 amastigotes of *L.donovani* and treated on days 7 and 8 via the tail vein with free sodium stibogluconate solution (■) or a vesicular form of the drug; liposomes (◆), niosomes composed of surfactant I (●), II (▼) or III (▲). Vesicles comprised amphiphile, non-ionic surfactant or phospholipid, with the admixture of 30 mol % cholesterol. The total dose of drug (expressed as antimony) given per mouse is shown. Liver parasite burdens were determined on day 14 and % suppression calculated for individual drug treated animals relative to the mean parasite burden for an appropriate untreated control group. The figures are means ± standard errors of mean.

direct comparison of the results achieved with the vesicular systems to those with the free drug.

Various types of vesicles were used to deliver the drug. They differed in the type of surfactant (either phospholipid or non-ionic surfactant, see figure 7), the % of cholesterol, and the inclusion or not of DCP. The results are summarised in table 5 and figure 9. They show that the use of vesicles to entrap the sodium stibogluconate improved the efficacy of the drug in this experimental model. There appeared to be little difference between the efficacy of liposomes and niosomes as vehicles for the drug.

There were some differences between the efficacy of the different types of niosomes tested. For example, it is evident that niosomes composed of surfactant I with 30 mol % cholesterol were less effective, particularly at low dosage, than niosomes with the same cholesterol content but prepared from surfactant II or III. Based on the ED_{50}s for the niosomes and the free drug (300 μg per mouse), the increased efficacy of these formulations were a 6-fold increase for surfactant I niosomes (ED_{50}=50 μg Sb), a 12.5 fold increase for surfactant II niosomes (ED_{50}=24 μg Sb) and a 15 fold increase for surfactant III niosomes (ED_{50}=20 μg Sb). The basis for these between vesicle differences is not known. At higher doses of drug (160-320 μg Sb per mouse), all non-ionic surfactant vesicle formulations were equally effective.

At antimony doses of 10 and 20 μg per mouse, infected mice treated with niosomes composed of surfactant I and 30 mol % cholesterol showed an increase in their parasite burden. This was not observed with any other form of treatment and has not been investigated further. These results may simply be a product of the natural variation inherent in this type of experiment.

The administration of vesicles which contained 300 mM glucose to

infected mice also resulted in parasite suppression as high as 42% (see table 6). The 'empty' niosomes could be ranked in the order surfactant I > surfactant II > surfactant III in terms of inhibitory activity. The 'empty' liposomes used in these investigations were as active as empty surfactant III niosomes against the liver parasites. The mechanism behind this anti-parasitic activity of the empty vesicles was not investigated further. There did not appear to be any toxicity to mice with the vesicle preparations tested.

3.1.3. Microparticles as a delivery system for sodium stibogluconate

Two batches of drug-loaded microparticles were used, one containing 8.4 μg Sb/mg beads and the other 3.3 μg Sb/mg beads. Each preparation was diluted with HBSS or concentrated by centrifugation to give the required Sb concentration before treatment. Doses of microparticulate stibogluconate used were equivalent to 12, 9, 6, 3 and 1.5 μg Sb per mouse.

The procedure for the treatment of infected mice was the same as that used for the vesicular preparations (see section 2.4.1. and 3.1.1.), except that one group was treated only on day 7. The experiment was repeated twice and the results are shown in figure 10. The control, undosed liver burdens were different in the 2 experiments with means of 197 and 352 amastigotes per 100 host cell nuclei. When expressed in terms of % suppression, relative to the control, the two microparticulate preparations had similar activities, indicating the importance of dose rather than the efficiency of bead loading with drug.

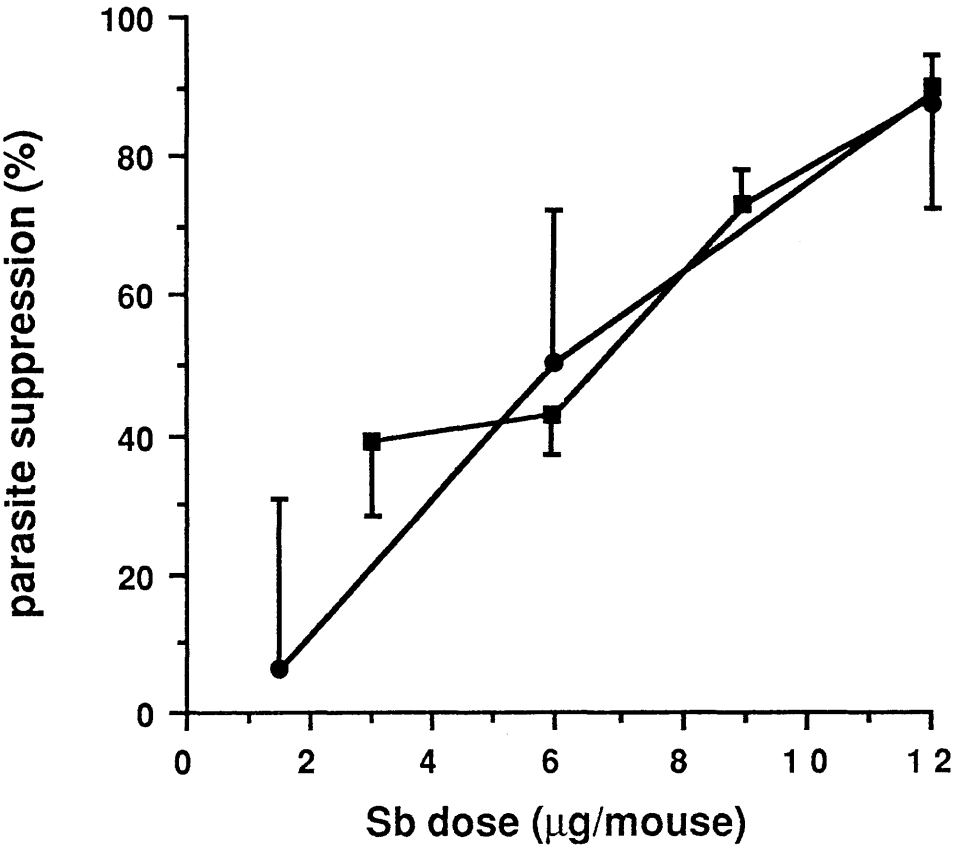
The ED_{50} was found to be approximately 6 μg microparticulate antimony per mouse. Total suppression of the parasites in the liver was not achieved in these experiments but the results can be extrapolated to give an indication of the dose needed to clear the

Table 6. The effect of empty vesicles on experimental visceral leishmaniasis

Vesicle type	Dose level	% suppression
Surfactant I	high	41.6 ± 4.9 (17)
	low	30.5 ± 5.1 (8)
Surfactant II	high	39.1 ± 4.1 (13)
	low	11.3 ± 10.4 (11)
Surfactant III	high	14.4 ± 9.5 (7)
	low	n.d.
Phospholipid	high	17.6 ± 9.4 (18)
	low	8.1 ± 4.9 (12)

Mice were infected on day 1 with 2 x 10^7 amastigotes of *L.donovani* and treated on days 7 and 8 via the tail vein with a suspension of vesicles containing 300 mM glucose. In all cases, non-ionic surfactant vesicles comprised 70% amphiphile and 30% cholesterol, and liposomes 70% phospholipid, 20% cholesterol and 10% DCP (molar compositions). Vesicles were given at two dose levels, high and low, equivalent to the amount of vesicular material administered to achieve a total dose of 320 and 160 ug Sb per mouse in niosomal or liposomal form. Liver parasite burdens were determined on day 14 and % suppression calculated for individual drug treated animals relative to the mean parasite burden for an appropriate untreated control group. For any treatment, the mean % suppression ± standard error of the mean, with number of animals in parenthesis is gven.

Figure 10. The efficacy of microparticulate sodium stibogluconate on experimental visceral leishmaniasis



Mice were infected on day 1 with 2 x 10^7 amastigotes of *L.donovani* and treated on days 7 and 8 via the tail vein with microparticulate sodium stibogluconate at either 8.4 µg Sb/mg beads (●) or 3.3 µg Sb/mg beads (■). The total dose of drug (expressed as antimony) given per mouse is shown. Liver parasite burdens were determined on day 14 and % suppression calculated for individual drug treated animals relative to the mean parasite burden for an appropriate untreated control group. The mean parasite suppressions ± standard deviations are shown.

liver: 14 μg Sb per mouse. A comparison with the results found with the free drug (see section 3.1.1.) shows that the carrier-bound drug appears to be 100 x as potent as the free drug.

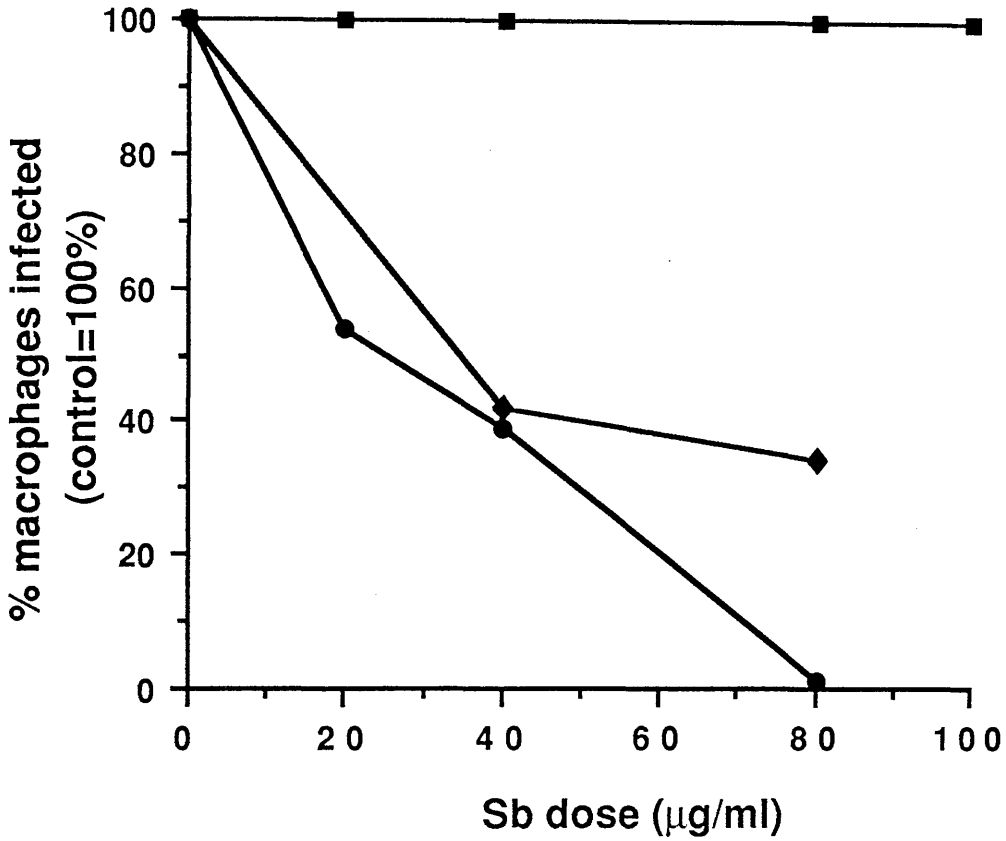
Unlike the situation with the vesicular systems, neither 'empty' polyacryl starch microparticles or ADH-polyacryl starch microparticles without drug attached appeared to affect the parasite burden of the liver at the doses used here. It would appear then that the microparticles had no inherent antiparasitic activity *in vivo* and that the parasite suppression reported here is a function of the drug content of the microparticles. No toxic effects were observed with drug loaded microparticles.

3.1.4. Activity of carrier systems for sodium stibogluconate against *L.donovani* and *L.m.mexicana* in PECs

Experimental procedures were as described in section 2.4.2.1., with promastigotes of *L.m.mexicana* and amastigotes of *L.donovani* used to initiate infection of the PECs.

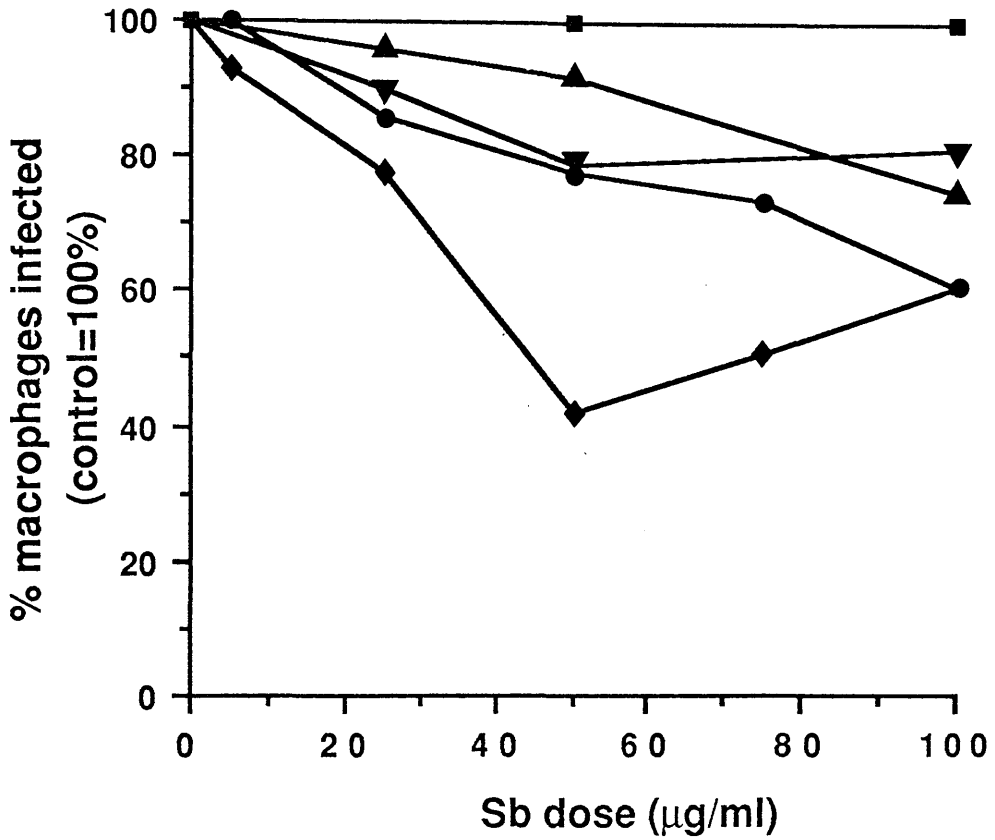
Both types of vesicular carrier increased sodium stibogluconate efficacy against *L. donovani* and *L. m. mexicana* in PECs. The effect was most pronounced against the latter species, which was unaffected by a 24 hour exposure to the free drug at concentrations of up to 1 mg Sb ml^{-1}. A 24 hour exposure to liposomal (DPPC:CHOL, 70:30 mol ratio) and niosomal (surfactant I:CHOL, 70:30 mol ratio) stibogluconate of *L.m.mexicana* infected PECs led to a large reduction in the number of infected cells (see fig. 11). The liposomal drug at 80 μg Sb ml^{-1} gave total clearance of the parasites whilst the niosomal drug (80 μgSb ml^{-1}) gave a 61% reduction in the number of infected cells. A two hour exposure to the vesicular drug resulted in fewer PECs being cleared of parasites (see fig. 12), indicating that activity of the carrier-mediated form of the drug was time-dependent. Stibogluconate-loaded

Figure 11. The effect of 24 hour exposure to free and vesicular sodi stibogluconate on PECs infected with *L.m.mexicana*



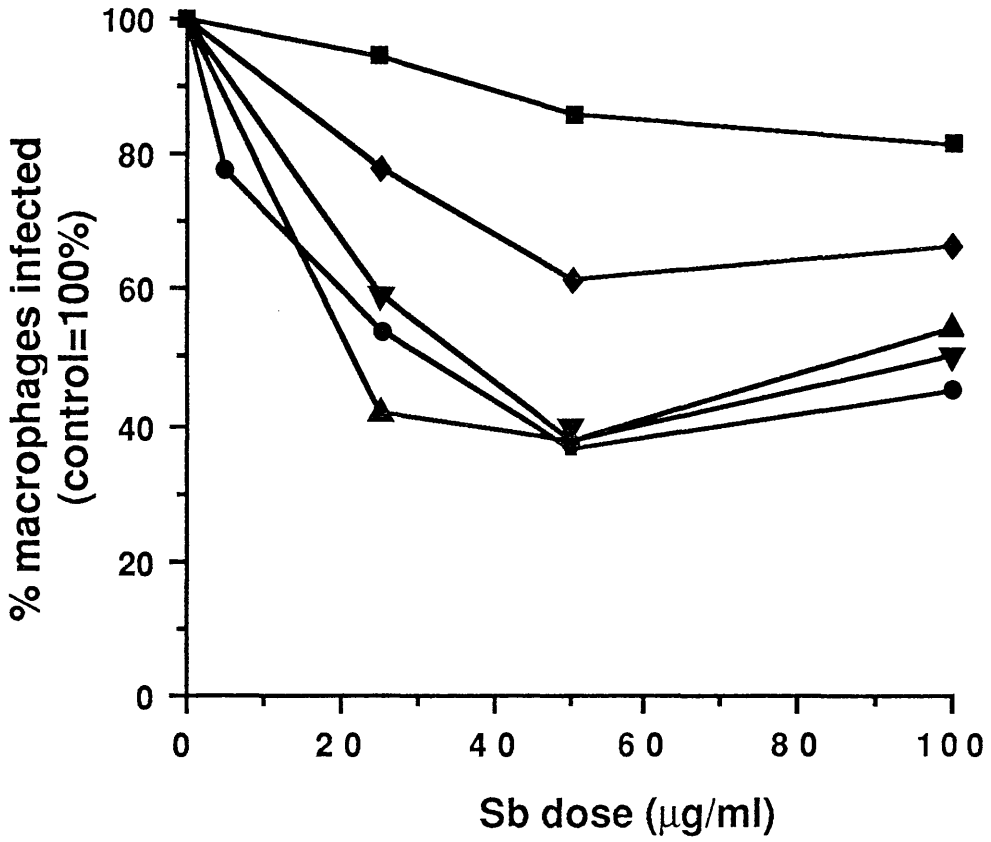
PECs were exposed for 24 hours to *L.m.mexicana* stationary phase promastigotes before a 24 hour incubation with either free stibogluconate (■), or drug encapsulated in niosomes composed of surfactant I and 30% cholesterol (◆) or liposomes composed of 70% phospholipid, 20% cholesterol, 10% DCP (●). The drug dose is expressed in terms of antimony content. Cells were washed thoroughly after the exposure period, the medium replenished and the cells incubated for a further 24 hours before they were fixed, stained and the number of infected PECs assessed. The results for the free sodium stibogluconate are from 3 experiments, carried out in duplicate, whilst the vesicular results from a single experiment carried out in duplicate. In the control, 80% of the cells were infected.

Figure 12. The effect of 2 hour exposure to free and vesicular sodium stibogluconate on PECs infected with *L.m.mexicana*



PECs were exposed for 24 hours to *L.m.mexicana* stationary phase promastigotes before a two hour incubation with free stibogluconate (■), or drug encapsulated in niosomes with 30% cholesterol composed of surfactant I (●), surfactant II (▼), surfactant III (▲) or liposomes composed of 70% phospholipid, 20% cholesterol, 10% DCP (◆). The drug dose is expressed in terms of antimony content. Cells were washed thoroughly after the exposure period, the medium replenished and the cells incubated for a further 46 hours before they were fixed, stained and the number of infected PECs assessed. The results for the free sodium stibogluconate are from 3 experiments, carried out in duplicate, whilst the vesicular results are from single experiments carried out in duplicate except for the niosomes comprised of surfactant I which are from two experiments. In these experiments 64-84% of the control cells were infected.

Figure 13. The effect of 2 hour exposure to free and vesicular sodium stibogluconate on PECs infected with *L.donovani*



PECs were exposed for 24 hours to *L.donovani* amastigotes before a 2 hour incubation with either free stibogluconate (■), or drug encapsulated in niosomes with 30% cholesterol composed of surfactant I (●), surfactant II (▼), surfactant III (▲) or liposomes composed of 70% phospholipid, 20% cholesterol, 10% DCP (◆). The drug dose is expressed in terms of antimony content. Cells were washed thoroughly after the exposure period, the medium replenished and the cells incubated for a further 46 hours before they were fixed, stained and the number of infected PECs assessed. The results for the free sodium stibogluconate are from 2 experiments, carried out in duplicate, whilst the vesicular results are from single experiments carried out in duplicate. In these experiments the % of cells infected in the controls were between 96% and 100%.

niosomes prepared from surfactants II and III, when used in a two hour exposure, showed similar activity to surfactant I niosomes (see fig. 12).

Leishmania donovani amastigotes in PECs were found to be more sensitive than those of *L.m.mexicana* to free sodium stibogluconate. Only two hour treatments were tested against *L.donovani*-infected PECs, but these experiments involved all the different vesicular systems (30% mol CHOL) and free drug (see fig 13). Free stibogluconate at 100 μg Sb ml^{-1} resulted in an 18% reduction in the number of infected cells, whilst at the same concentration the liposomal preparation used gave a 53% reduction and the three types of niosomes gave reductions of the order of 60%. Thus the relative resistance of *L.m.mexicana* to treatment by sodium stibogluconate *in vitro* appears to be overcome to an extent by using these carrier systems such that its susceptibility is similar to that of *L.donovani*.

The use of empty vesicles for a two hour exposure period had no effect on parasite viability. However with treatment periods greater than four hours, significant suppression of both species was observed with empty niosomes. Toxicity to the PECs was also observed at higher concentrations of niosomes and liposomes with longer exposure times although the degree of toxicity did vary between batches of vesicles.

3.2. THE POTENTIAL OF TUBERCIDIN IN CONJUNCTION WITH INHIBITORS OF NUCLEOSIDE TRANSPORT AS ANTILEISHMANIAL AGENTS

The use of tubercidin with inhibitors of nucleoside transport to treat *Leishmania* infected cells was carried out as a potential therapeutic strategy and to investigate the possibility that the nuleoside permeability characteristics of potential host cells are altered when infected with *Leishmania*.

3.2.1. Activity against promastigotes of *L.donovani* and *L.m.mexicana*

The effect on promastigote growth of tubercidin, dipyridamole and nitrobenzylthioinosine (NBMPR) on their own and combinations of tubercidin with NBMPR and dipyridamole were determined, as described in section 2.4.2.2. with an exposure time of 48 hours.

NBMPR and dipyridamole had no apparent effect on growth at concentrations up to 50 μM. Tubercidin however had a marked effect against *L.donovani* and *L.m.mexicana* with ED_{50}s of approximately 0.1 μM and 0.2 μM respectively. With both species, concentrations of tubercidin above 1 μM resulted in the death of > 99% of the promastigotes. The use of combinations of NBMPR and dipyridamole at 50 μM in conjunction with tubercidin at concentrations from 0.05 to 50 μM showed that neither NBMPR or dipyridamole protected the promastigotes from the toxicity of tubercidin.

3.2.2. Activity against amastigotes of *L.donovani* and *L.m.mexicana* in PECs

A series of experiments similar to those carried out with the promastigotes were performed using amastigote-initiated infections of PECs *in vitro*, as described in section 2.4.2.1..

Uninfected PECs treated with tubercidin alone were all killed by the end of the two days exposure at all concentrations of tubercidin tested (1-10 μM). Similar results were found with infected cells; all were killed by tubercidin itself when used in the range 0.1-5 μM. The inhibitors NBMPR and dipyridamole on their own, at concentrations as high as 20 μM, had no effect on uninfected cells. These inhibitors at concentrations of either 10 or 20 μM, fully protected uninfected PECs from tubercidin at concentrations up to and including 2.5 μM. Some protection was still evident with 5μM tubercidin, but marked toxicity was observed particularly with the 10 μM inhbitor concentrations.

The results of treating infected PECs with different combinations of tubercidin and inhibitors are given in table 7. The protective activity of NBMPR and dipyridamole towards PECs infected with amastigotes of *Leishmania* appeared to be the same as for uninfected cells. No difference was noted between the susceptibility of PECs infected with the species tested here. There did not appear to be an increase in the percentage of cells that were uninfected as would be expected if infected cells were being targetted, nor a decrease in the number of amastigotes per 100 infected cells. Thus it appears tht the parasite did not affect the characteristics of nucleoside transport into the PECS, nor was the parasite directly exposed to tubercidin.

3.2.3. Efficacy against *L.donovani* and *L.m.mexicana* in mice

Drug testing *in vivo* using the two visceral models was carried out as described in section 2.4.1. with several routes of drug administration being tested.

The results (table 8) show that a single tubercidin dose of 5 mg kg^{-1} i.p. resulted in death of 4 out of 5 mice, although the surviving mouse had an 83% reduction in its parasite burden. All mice survived treatment with tubercidin at 1 mg kg^{-1} i.p. for 5 days but this regimen did not reduce the parasite burden. Attempts to lessen the toxicity by oral administration of the drug proved successful, with only 30% of the mice dying after 3 daily doses of 5 mg kg^{-1}, However, there was no affect on the parasite load. The co-administration of NBMPR with tubercidin in i.p. treatment alleviated the toxicity to mice of the tubercidin, although the complete protection previously reported (Ogbunude and Ikediobi, 1982) was not apparent. 50% of the mice died after being subjected to a combination of 5 mg kg^{-1} tubercidin and 25 mg kg^{-1} NBMPR on 4 consecutive days. This treatment did not reduce the parasite burden in the surviving mice.

Table 7. The effect on *Leishmania* infected PECs of tubercidin in combination with nucleoside transport inhibitors

L.m.mexicana	Number of amastigotes per 100 infected cells (% of control)		
Tubercidin (μM)	Dipyridamole (10 μM)	Dipyridamole (20 μM)	NBMPR (20 μM)
1.25	85 ± 10	98 ± 26	91 ± 24
2.5	118 ± 6	116 ± 18	106 ± 6
5.0	96 ± 14	95 ± 30	-

L. donovani			
Tubercidin (μM)		Dipyridamole (20 μM)	NBMPR (20 μM)
1.25	-	92 ± 7	90 ± 6
2.5	-	81 ± 13	104 ± 35
5.0	-	94 ± 2	86 ± 10

PECs were exposed for 24 hours to amastigotes of the appropriate species and washed thoroughly before treatment commenced. Infected PECs were treated for 48 hours then fixed, stained and the parasite load assessed. The number of amastigotes per 100 infected cells varied between 1000-1200 for *L.m.mexicana* and 1400-2000 for *L.donovani* controls; the infection rates were between 91% and 97% for *L.m.mexicana* and 95% and 100% for *L.donovani*. All experiments were done a minimum of 4 times, the figures given are the means ± the standard deviations.

Table 8. The efficacy against *Leishmania* in mice of tubercidin alone and in combination with the nucleoside transport inhibitor NBMPR

	Parasite load in liver (number of parasites per 100 host cell nuclei)
L.donovani infections.	
1) Control	178 ± 41 (n=5)
Treated, 1 mg tubercidin kg^{-1}, i.p., 5 days.	184 ± 34 (n=5)
2) Control	227 ± 64 (n=5)
Treated, 5 mg tubercidin kg^{-1}, i.p., 1 day.	39 (n=5, 4 dead)
3) Control	194 ± 67 (n=6)
Treated, 5 mg tubercidin kg^{-1}, 25 mg NBMPR kg^{-1} i.p., 4 days.	283 ± 33 (n=6, 3 dead)
L.m.mexicana infections.	
4) Control	115 ± 113 (n=6)
Treated, 5 mg tubercidin kg^{-1}, orally, 3 days.	142 ± 89 (n=10, 3 dead)

Balb/c mice were infected with amastigotes of either *L.donovani* (2×10^7) or *L.m.mexicana* (3×10^8) on day 0 and treatment commenced on day 7 with the mice sacrificed on day 14 and the parasite load of the liver assessed. For administration, the drugs were suspended in 0.375% gumtragacanth. The results are the means ± standard deviations from single experiments, the number of animals in each group are given in parenthesis.

3.3. THE EFFECT OF MANIPULATING THE PHAGOLYSOSOMAL ENVIRONMENT UPON PARASITE GROWTH *IN VITRO*

In an attempt to improve our knowledge of the phagolyosomal environment and the role that it plays in the parasite-macrophage interaction, a series of experiments were designed involving the manipulation of the lysosomal environment of infected cells. Methylamine, ammonium chloride and dextran sulphate were used as they are known to affect vacuolar pH, although they do also affect other lysosomal functions (see section 1.5.2.2.).

3.3.1. The effect of methylamine, ammonium chloride and dextran sulphate upon growth of promastigotes

Promastigotes of *L.m.mexicana* and *L.donovani* were incubated at 25°C in the presence of 10-50 mM concentrations of dextran sulphate, methylamine or ammonium chloride in complete HOMEM medium (see section 2.4.2.2.). After 48 hours incubation, the cultures were assessed and compared to a control. Growth of *L.m.mexicana* was unaffected by the ammonium chloride at the concentrations tested whereas an ED_{50} of approximately 30 mM was found with the methylamine. In contrast, *L.donovani* was greatly affected by both methylamine and ammonium chloride with an ED_{50} of approximately 10 mM for both of these compounds. When methylamine was used at 50 mM promastigotes were not detectable after 48 hours. Dextran sulphate did not affect the multiplication of either of the species at the concentrations tested.

3.3.2. The effect of methylamine, ammonium chloride and dextran sulphate upon the growth of *L.donovani* and *L.m.mexicana* amastigotes in PECs

PECs were infected with amastigotes of either *L.donovani* or *L.m.mexicana* and incubated for up to 5 days under standard conditions, except for the addition of either methylamine, ammonium chloride or

dextran sulphate. At appropriate time points during treatment, cells were fixed, stained and the parasite load assessed as described in section 2.4.2.1.. One way and two way analysis of variance was used to analyse the differences between the observed results.

The results of treatment of infected PECs with methylamine and ammonium chloride are given in table 9. PECs infected with *L.m.mexicana* and treated with either compound were unaffected over the first 72 hours whereupon there was a slight but not significant increase in the number of amastigotes compared to the controls. However, the *L.donovani* infection was suppressed for the whole of the period by methylamine treatment whereas ammonium chloride treatment affected the infection only in the later stages. Results from a typical experiment with *L.donovani*-infected PECs treated with methylamine or ammonium chloride are shown in figure 14. These results indicate that the suppression achieved with methylamine was mainly due to an inhibition of growth of the amastigotes although it appears that some amastigotes were killed.

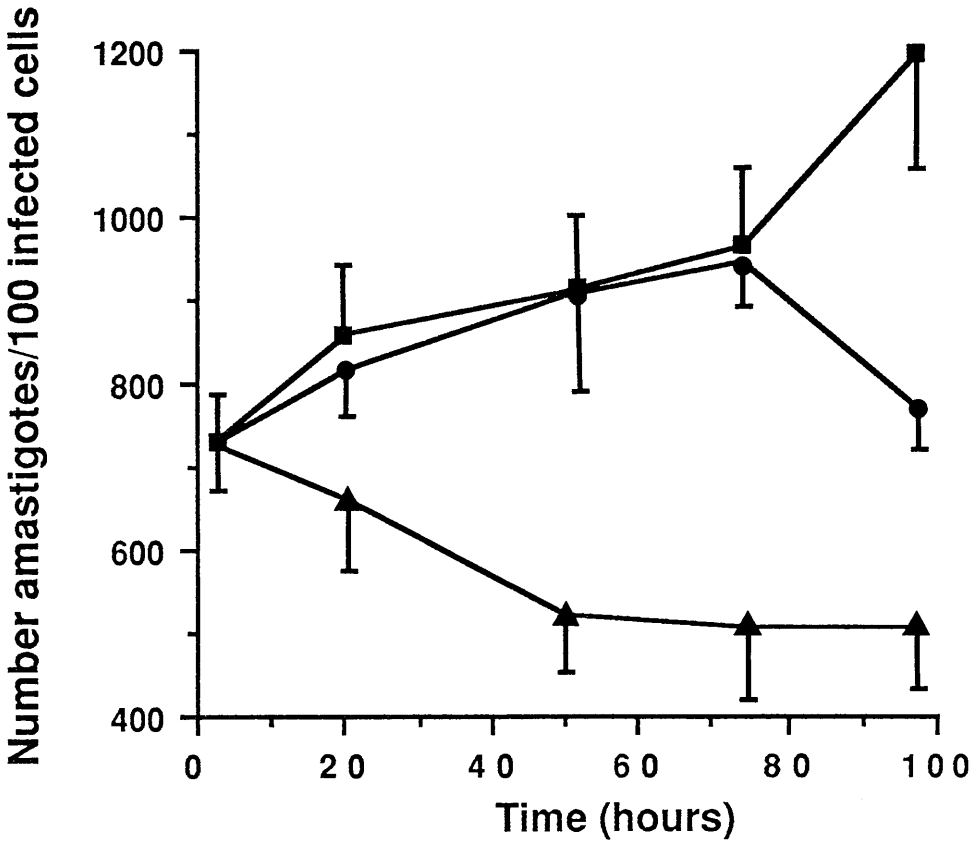
The results of exposure of *L.m.mexicana* infected PECs for 5 days to methylamine or ammonium chloride at various concentrations are given in table 10. No effects were apparent with the compounds at 5mM or 10mM in agreement with the results in table 9. However, treatment with either 20 mM ammonium chloride or 20 mM methylamine did inhibit the infection. In similar experiments *L.donovani* growth was affected by both ammonium chloride and methylamine at all concentrations tested. There was no significant difference between the effects of 10 mM and 20 mM ammonium chloride, although at both concentrations the effect was significantly greater than the affect at 5 mM. Methylamine at both 5 mM and 10 mM significantly reduced the number of amastigotes present and there was also a concentration-dependent effect with the reduction in parasite load being greater with 10 mM methylamine than

Table 9. The effect of methylamine and ammonium chloride on the number of amastigotes of *L.m.mexicana* and *L.donovani* in PECs

	Time post-infection (hours)				
	24	48	72	96	120
L.donovani	Number of amastigotes in PECs as a % of the control				
Methylamine (10 mM)	65 ± 7 (n=6)	56 ±15 (n=6)	75 ± 15 (n=4)	41 ± 9 (n=2)	24 ± 0.5 (n=2)
NH_4Cl (10 mM)	103 ± 9 (n=6)	79 ± 16 (n=6)	104 ± 45 (n=4)	63 ± 8 (n=2)	54 ± 7 (n=6)
L.m.mexicana					
Methylamine (10 mM)	95 ± 17 (n=8)	92 ± 31 (n=10)	116 ± 27 (n=8)	133 ± 47 (n=4)	106 ± 36 (n=8)
NH_4Cl (10 mM)	90 ± 38 (n=7)	103 ± 28 (n=10)	110 ± 21 (n=8)	126 ± 31 (n=4)	115 ± 44 (n=9)

PECs were exposed for 24 hours to either *L.donovani* or *L.m.mexicana* amastigotes and then washed thoroughly before the addition of fresh medium containing either 10 mM ammonium chloride or methylamine. At different time points the cultures were fixed, stained and assessed for the effect of treatment on the infections. Cultures maintained beyond 48 hours treatment had their medium replenished as appropriate. Results are the number of amastigotes per 100 infected cells expressed as a % of the untreated controls. They are given as means ± standard deviation and the number of experiments given in parenthesis. Experiments were carried out in duplicate. For *L.donovani*-infected controls 89%-100% of PECs were infected at the different time points with 700-4000 amastigotes per 100 infected cells, whilst with *L.m.mexicana* 78%-100% of PECs were infected at the different time points with 900-1400 amastigotes per 100 infected cells. Statistically, for *L.m.mexicana* infected PECs there was no difference between treated and untreated cells ($F_{2,99}=0.50$), whilst for *L.donovani* there was a significant difference between treated and untreated cells ($F_{2,41}=2.64$, $P<0.001$).

Figure 14. The effect of methylamine and ammonum chloride on the course of *L.donovani* growth in PECs



PECs were exposed for 24 hours to *L.donovani* amastigotes at a ratio of 1 : 2, and then washed thoroughly before the addition of fresh medium alone (■) or containing either 10 mM methylamine (▲) or 10 mM ammonium chloride (●). At different time points thereafter the cultures were fixed, stained and assessed for the effect of treatment on the infection. Results are expressed as the number of amastigotes per 100 infected cells. These are the results from a single experiment carried out in duplicate. The figures given are the means with the ranges shown. Cultures maintained beyond 48 hours treatment had their medium replenished at this point.

Table 10. The effect of 5 day exposures to methylamine and ammonium chloride on the number of amastigotes of *L.m.mexicana* and *L.donovani* in PECs at different concentrations

L.donovani infections:

Ammonium chloride. Number of amastigotes in experimental PECs as a % of the numbers in the control.

Control	5 mM	10 mM	20 mM
100 + 18	76 + 10	54 + 7	58 + 12

Methylamine:

Control	5 mM	10 mM
100 + 32	51 + 17	24 + 0.5

L.m.mexicana infections.

Ammonium chloride:

Control	5 mM	10 mM	20 mM
100 + 18	125 + 33	115 + 44	79 + 30

Methylamine:

Control	5 mM	10 mM	20 mM
100 + 6	114 + 23	106 + 36	81 + 23

PECs were exposed for 24 hours to amastigotes of the appropriate species before the addition of fresh medium containing either methylamine or ammonium chloride. The medium was replenished 48 hours after treatment commenced. After 5 days treatment the cultures were fixed, stained and assessed for the effect of treatment on the infections. The results are expressed as a % of the mean of the controls. Experiments were carried out 6-9 times, in duplicate, except for the 10 mM methylamine treatment of *L.donovani* which was carried out twice. For *L.donovani* infected controls 89%-100% of PECs were infected with 1100-4000 amastigotes per 100 infected cells, whilst with *L.m.mexicana* 78%-100% of PECs were infected with 900-1400 amastigotes per 100 infected cells. Statistically, *L.m.mexicana* was inhibited by treatment with 20 mM methylamine ($F_{3,28}=3.28$, $P<0.05$) and 20 mM ammonium chloride ($F_{3,28}=4.12$, $P<0.05$) but not at lower concentrations. However, with *L.donovani* there were differences between infected controls and treated groups at all levels, ($F_{2,41}=22.64$, $P<0.001$). Using 10 and 20 mM ammonium chloride results were comparable but differed from the 5 mM treatment ($F_{3,20}=19.29$, $P<0.01$). Treatment with 5 mM and 10 mM methylamine of *L.donovani* infections differed from each other ($F_{2,14}=10.75$, $P<0.001$).

Table 11. The effect of dextran sulphate on *L.m.mexicana* and *L.donovani* in PECs

Number of cells infected as % of control.

L.donovani	Dextran sulphate concentration ($\mu g\ ml^{-1}$)		
Control	6.25	12.5	25
100 ± 4	78 ± 13	54 ± 23	52 ± 2
(n=4)	(n=4)	(n=4)	(n=2)

L.m.mexicana		
Control	6.25	12.5
100 ± 4	95 ± 8	62 ± 28
(n=6)	(n=4)	(n=2)

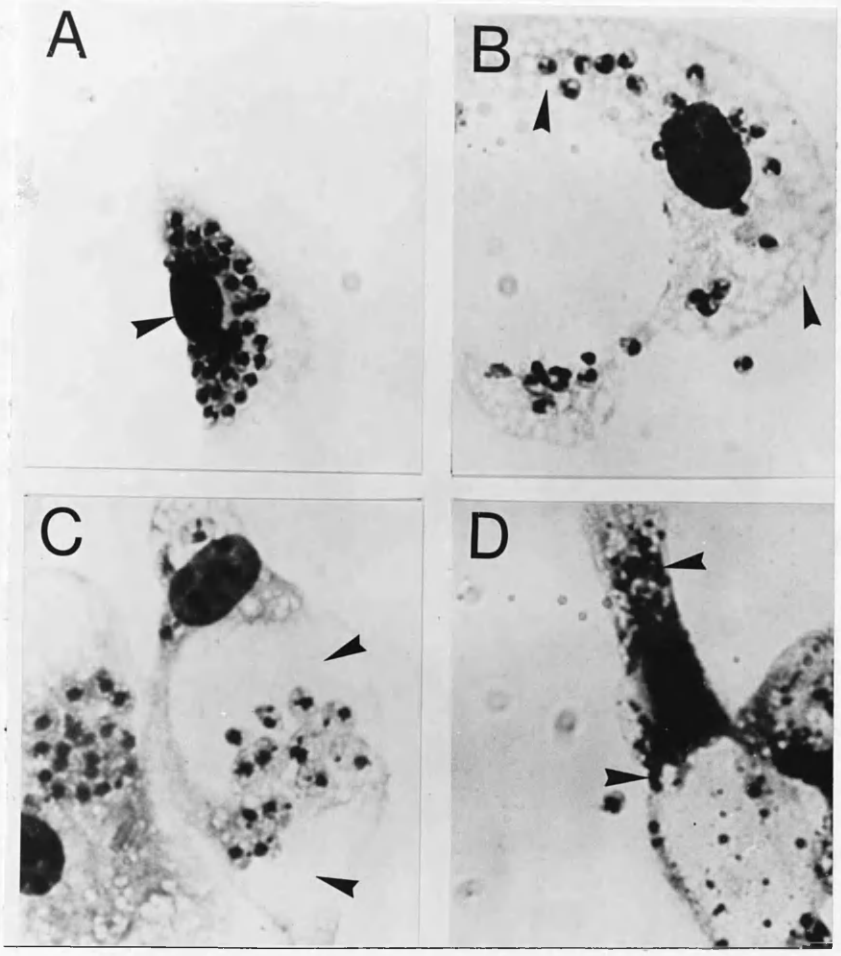
Number of amastigotes per 100 infected cells, % of control.

L.donovani	Dextran sulphate concentration ($\mu g\ ml^{-1}$)		
Control	6.25	12.5	25
100 ± 10	64 ± 9	58 ± 16	42 ± 16
(n=4)	(n=4)	(n=4)	(n=2)

L.m.mexicana		
Control	6.25	12.5
100 ± 20	96 ± 18	68 ± 29
(n=6)	(n=4)	(n=2)

PECs were exposed for 24 hours to amastigotes of the appropriate species before the addition of fresh medium containing dextran sulphate. After 48 hours treatment the cultures were fixed, stained and assessed for the effect of treatment on the infections. The results are expressed as a % of the mean of the controls, with the number of experiments, carried out in duplicate, in parenthesis. For *L.donovani* infected controls 90%-100% of PECs were infected with 1300-2200 amastigotes per 100 infected cells, whilst with *L.m.mexicana* 95%-100% of PECs were infected with 900-1400 amastigotes per 100 infected cells.

Figure 15. The effect on *Leishmania* infected PECs of treatment with methylamine and dextran sulphate



A: *L.donovani* infected PEC, no treatment, infected for 5 days. Densely stained PEC nucleus is arrowed, note the 'tight' parasitophorous vacuoles. B: *L.donovani* infected PEC, treated with 10 mM methylamine for 48 hours. PEC is highly vacuolated, arrowed. The parasitophorous vacuoles appear to be less 'tight'. C: *L.m.mexicana* infected PEC, no treatment, infected for 5 days. The charecteristically large parasitophorous vacuoles are arrowed. D: *L.m.mexicana* infected PEC, treated with 12.5 $\mu g\ ml^{-1}$ dextran sulphate for 48 hours. Densely staining granules are arrowed. All these cells were fixed in methanol and stained in 10% giemsa.

with 5 mM.

Dextran sulphate affected the growth of both species, resulting in a reduction in the % of macrophages infected and in the number of amastigotes per 100 infected cells (see table 11). Again, *L.donovani* appeared to be affected more than *L.m.mexicana*. It was interesting to note that infected PECs treated with dextran sulphate were often filled with densely staining granules, possibly the remains of dead parasites. This was not observed in uninfected cells treated with dextran sulphate and appeared more often in cells infected with *L.m.mexicana* than with *L.donovani* (see fig. 15).

Treatment of either infected or uninfected cells with these lysosomotrophic compounds caused extensive changes in the morphology of the lysosomes. The large differences between uninfected cells treated with methylamine and an untreated control group are shown in figure 15. Interestingly *L. donovani*-infected cells treated with methylamine were morphologically quite similar to untreated *L. m. mexicana*-infected cells. The characteristic 'tight' parasitophorous vacuole of *L.donovani* became somewhat similar in appearance to the 'looser' vacuole in which *L.m.mexicana* characteristically resides. This affect was more pronounced in cells treated with methylamine than ammonium chloride and only at concentrations of 10 mM and greater. The onset of this change in morphology after treatment was rapid.

3.4. THE EFFECT OF pH ON THE UPTAKE AND INCORPORATION OF LEUCINE AND ADENOSINE BY DIFFERENT SPECIES AND STAGES OF *LEISHMANIA*

The results in section 3.3. raised the possibility that *L.donovani* and *L.m.mexicana* may be adapted to living at different pHs. One method of further investigation was to look at the optimal pH for the uptake of leucine and adenosine in different species and stages of the parasites. This could give an indication of the pH to which these

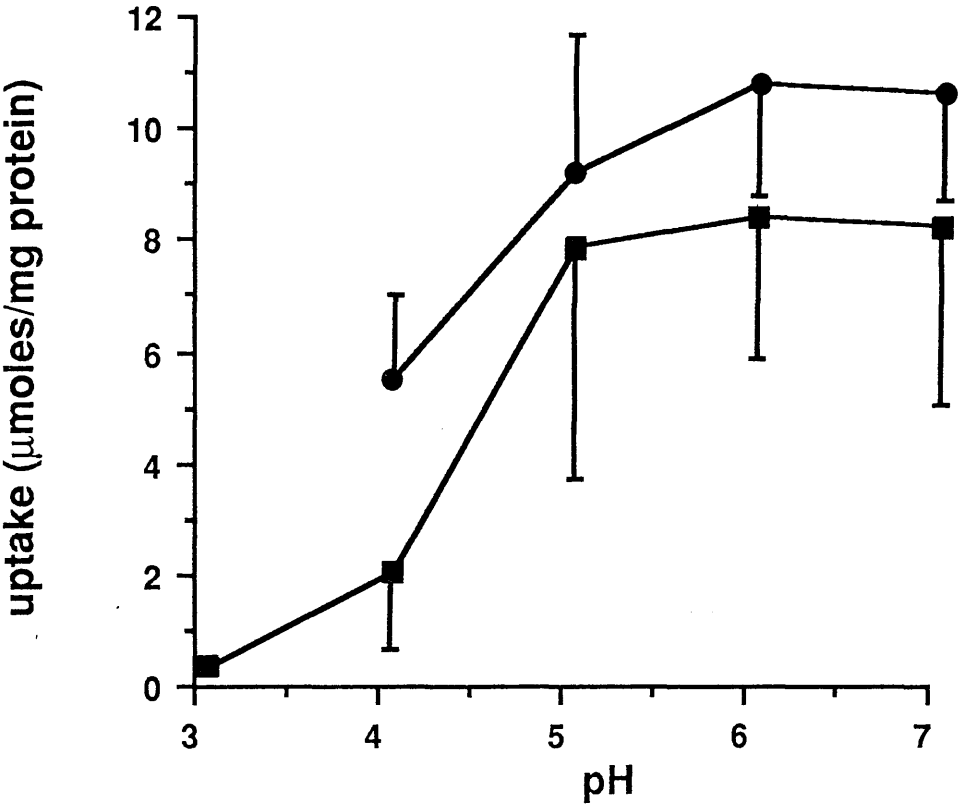
parasites are adapted. Here, representative results of a preliminary study are presented.

The uptake of leucine and adenosine by stationary phase promastigotes of *L.donovani* and *L.m.mexicana* (see figs. 16 and 17) was greatest around pH 6 or 7 whilst the greatest uptake by *L.major* metacyclics was at pHs 5 and 6, uptake at pH 7 was much less. The pH of the growth medium (HOMEM) was also measured at the time the cells were harvested and varied between 5.3 and 6.8 with the majority being in the 6.3 range. This represented a change in the pH of the growth medium from 7.3 at the start of the cultures to 6.3, presumably caused by the excretion of acid end products by the promastigotes. Thus from these preliminary results it appears that the pH optimum for uptake of adenosine by metacyclics of *L.major* is below the measured pH of the medium and also lower than that of multiplicative promastigotes.

The results for amastigotes of *L.donovani* and *L.m.mexicana* are given in figure 18. There was a similar effect of pH on the incorporation of adenosine as reported by Mukkada et al. (1985) for uridine and thymidine incorporation by *L.donovani* amastigotes i.e. a sharp peak at pH 5. However the results for *L.m.mexicana* do not show as sharp a peak as for *L.donovani*, with a relatively small difference between the incorporation at pHs 5 and 6.

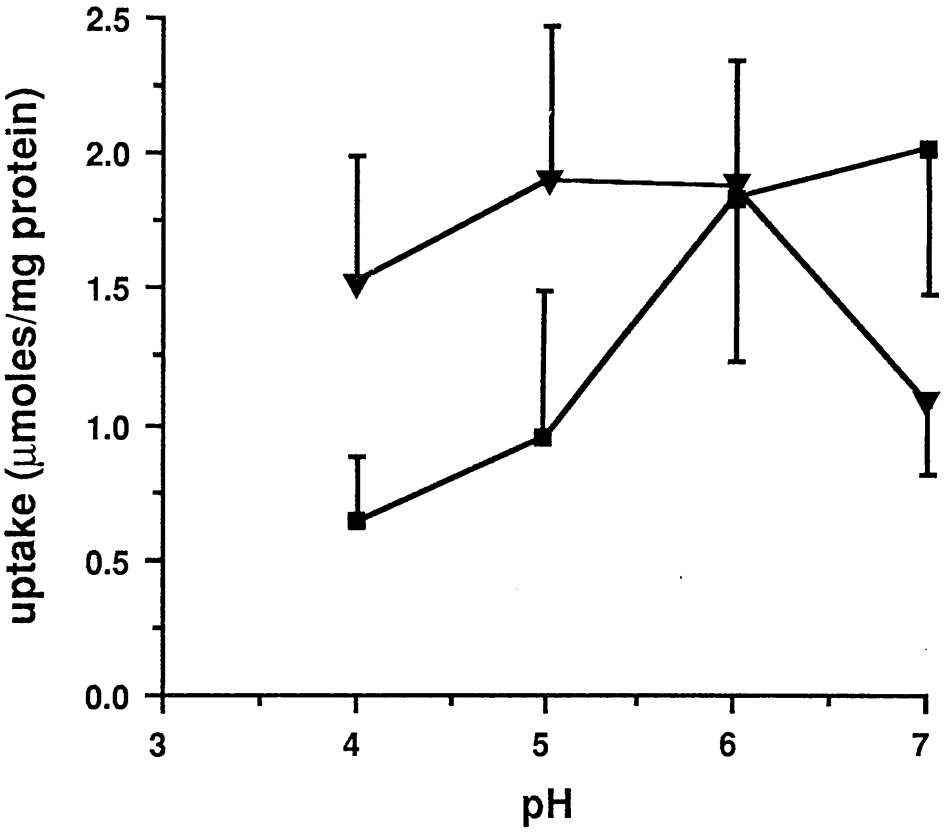
The results from this and the previous section (section 3.3.) raised the question as to whether or not the pH of the parasitophorous vacuole differs between species of *Leishmania*. Attempts were made to measure the pH of the parasitophorous vacuole of cells infected with *L.m.mexicana* and uninfected cells. This was done using a fluorescent probe (FITC labelled dextran) which is endocytosed and accumulates in lysosomes. The fluoresence spectrum of this compound changes in the pH range 3-8 and by using light at 620 nm and 480 nm a ratio can be determined and from this the lysosomal pH can be estimated using a

Figure 16. The uptake of H^3-leucine at different pHs by *Leishmania* promastigotes



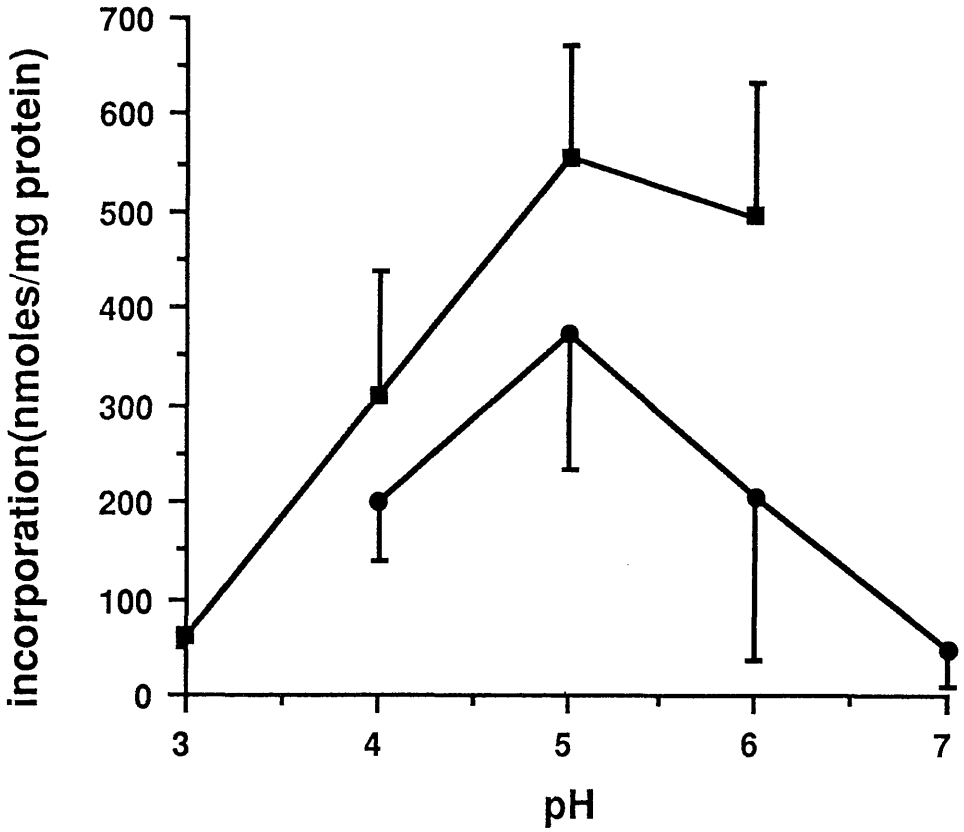
The uptake of H^3-leucine by stationary phase promastigotes of *L.m.mexicana* (■) and *L. donovani* (●) over a 2 hour period was measured at different pHs. The cells were washed twice with the incubation buffer by centrifugation, collected by filtration using glass microfiber filters and washed thoroughly with ice cold HBSS. The filters were air dried and transferred to scintillation vials containing Ecoscint scintillation fluid and the radioactivity counted. These are the means from 4-5 experiments carried out in duplicate.

Figure 17. The uptake of H^3-adenosine at different pHs by *Leishmania* promastigotes



The uptake of H^3-adenosine by stationary phase promastigotes of *L.m.mexicana* (■) and *L.major* metacyclics (▼) over a 2 hour period was measured at different pHs. The cells were washed twice with the incubation buffer by centrifugation, collected by filtration using glass microfiber filters and washed thoroughly with ice cold HBSS. The filters were air dried and transferred to scintillation vials containing Ecoscint scintillation fluid and the radioactivity counted. These are the means from 4 experiments (*L.m.mexicana*) and 2 experiments (*L.major*) carried out in duplicate.

Figure 18. The incorporation of H^3-adenosine into *Leishmania* amastigotes



The incorporation of H^3-adenosine into amastigotes of *L.m.mexicana* (■) and *L.donovani* (●) over a 2 hour period was measured at different pHs. The cells were washed twice with the incubation buffer by centrifugation, resuspended in 5% TCA and collected by filtration using glass microfiber filters and washed thoroughly with 10 ml ice cold TCA. The filters containing the TCA-insoluble material were then washed with 4 ml of 95% ethanol, air dried and transferred to scintillation vials containing Ecoscint scintillation fluid and the radioactivity counted. These are the means from 2 experiments done in duplicate.

calibration curve (Ohkuma and Poole, 1978). Initial results suggested that the lysosomal pH of infected and uninfected PECs is different, however the equipment was not sufficiently sensitive or consistent to allow quantification of the intial results any further.

3.5. THE ANTILEISHMANIAL ACTIVITY OF AMINO ACID ESTERS AND AMINO ACID AMIDES

One of the aims of this project was to characterise the antileishmanial activity of the amino acid ester L-leucine methyl ester (Rabinovitch *et al.* 1986) and to investigate the potential antileishmanial activity of other similar compounds. This was carried out by testing these compounds against different species, both amastigotes and promastigotes, in a variety of models.

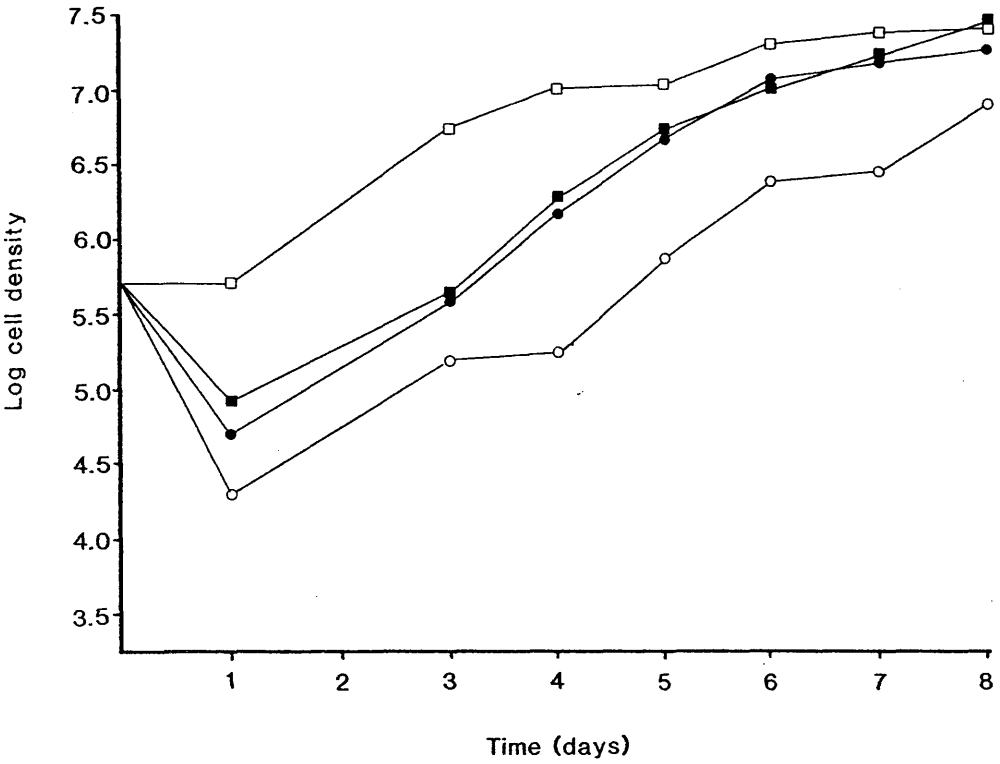
3.5.1. Activity against promastigotes of *L.donovani*, *L.major* and *L.m.mexicana*

L-leucine methyl ester (leuOMe) and L-tryptophanamide both partially inhibited the growth of promastigotes of all 3 species tested. With leuOMe the effect was transient (see fig. 19), even when the compound was present initially at 10 mM. With L-tryptophanamide at 10 mM, many promastigotes were killed during the first 24 hours and there was no subsequent cell multiplication, although not all parasites were killed even by 8 days (see fig. 20). A concentration-dependent effect was evident and promastigotes treated with concentrations < 5 mM of either compound were much less effective. Similar results were achieved with the other two species (data not shown).

3.5.2.1. Activity against amastigotes of *L.donovani*, *L.m.mexicana* and *L.major* in PECs

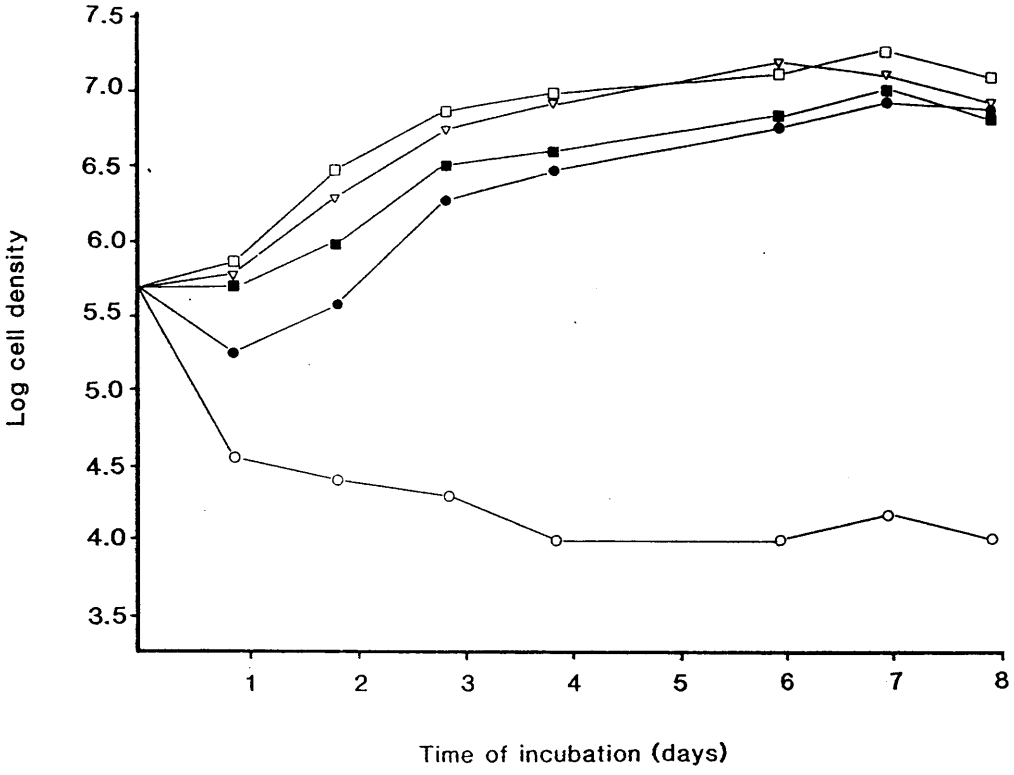
Experiments to determine the activities of the above compounds

Figure 19. The effect of L-leucine methyl ester on the growth of *L.m.mexicana* promastigotes *in vitro*



Promastigotes of *L.m.mexicana*, at an initial density of 5 x 10^5 ml^{-1}, were incubated in HOMEM medium in the presence of different concentrations of leuOMe: 10 mM(○), 5mM(●), 2mM(■), control (□) and the growth of the promastigotes monitored over an 8 day period. These results are the means of duplicate counts.

Figure 20. The effect of L-tryptophanamide on the growth of *L.m.mexicana* promastigotes *in vitro*



Promastigotes of *L.m.mexicana*, at an initial density of 5 x 10^5 ml^{-1}, were incubated in HOMEM medium in the presence of different concentrations of tryptophanamide: 10mM(○), 5mM(●), 2mM(■), 0.5mM (▽), control(□) and the growth of the promastigotes monitored over an 8 day period. These results are the means of duplicate counts.

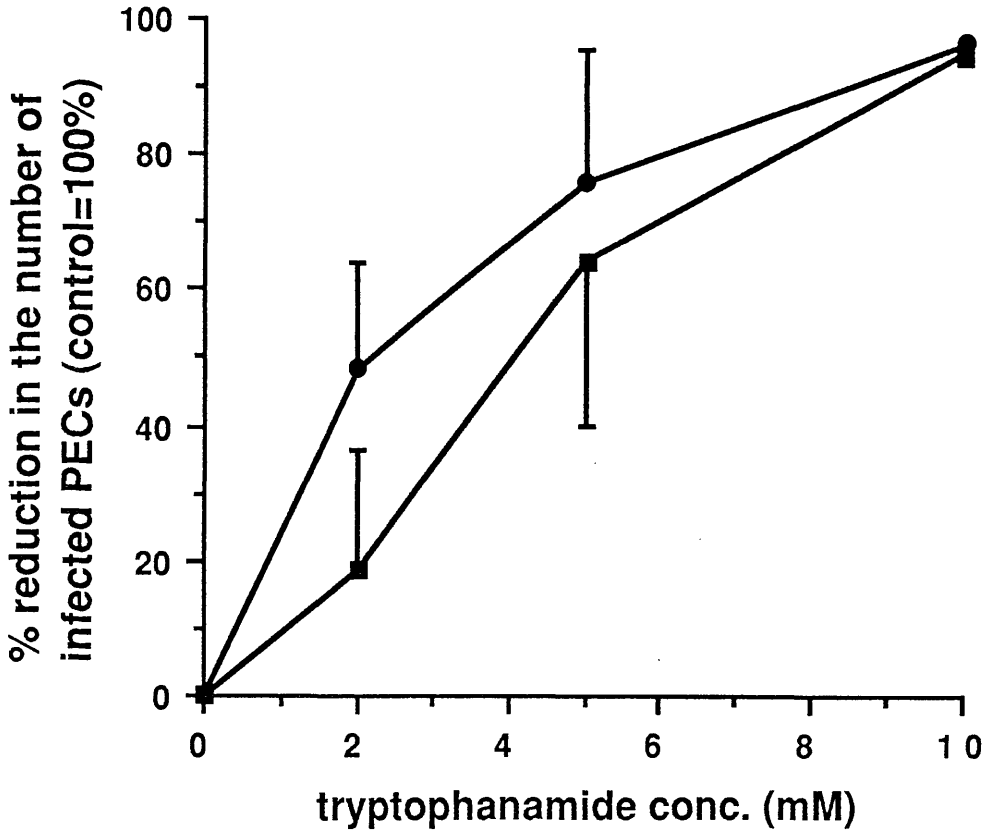
against amastigotes in PECs *in vitro* were carried out as described in section 2.4.2.1.. Treatment with leuOMe was generally for 1 hour whilst exposure to the amides was normally for a longer period. The PECs were fixed, stained and assessed for parasite load 48 hours post-infection.

With a 1 hour exposure to 2 mM leuOMe, the numbers of PECs infected with *L.donovani* and *L.major* were unaffected in the experiments carried out (n=5 and 2 respectively), whereas with similar exposure there was a 99% reduction in the number of PECs infected with *L.m.mexicana*. These results are in accordance with those of Rabinovitch *et al.* (1986) for amastigote initiated infections of *L.m.amazonensis* in PECs.

The antileishmanial activity of L-tryptophanamide differed from that of leuOMe in that both *L. donovani* and *L.m.mexicana* were susceptible. Exposure of PECs infected with either species to concentrations of 2, 5 and 10 mM tryptophanamide for 2 hours resulted in marked reductions in the number of infected PECs (see fig. 21). *Leishmania donovani* appeared to be slightly more susceptible than *L.m.mexicana*. Thus when *L.donovani*-infected PECs were treated with 2 mM tryptophanamide for 1, 2 or 24 hours there was a reduction in the number of infected macrophages of 0.5%, 48% and 93%, respectively (see fig. 22). Under the same conditions the reductions achieved with *L.m.mexicana* promastigote initiated infections were 0%, 18% and 44%, respectively. Clearly with both species, the effect produced was related to exposure time. Toxicity of tryptophanamide towards PECs was noted at the higher concentrations tested, especially with exposure times of 4 hours and greater. This was evident in the altered morphology of treated cells, relative to the controls, and with longer exposures the death of all the cells.

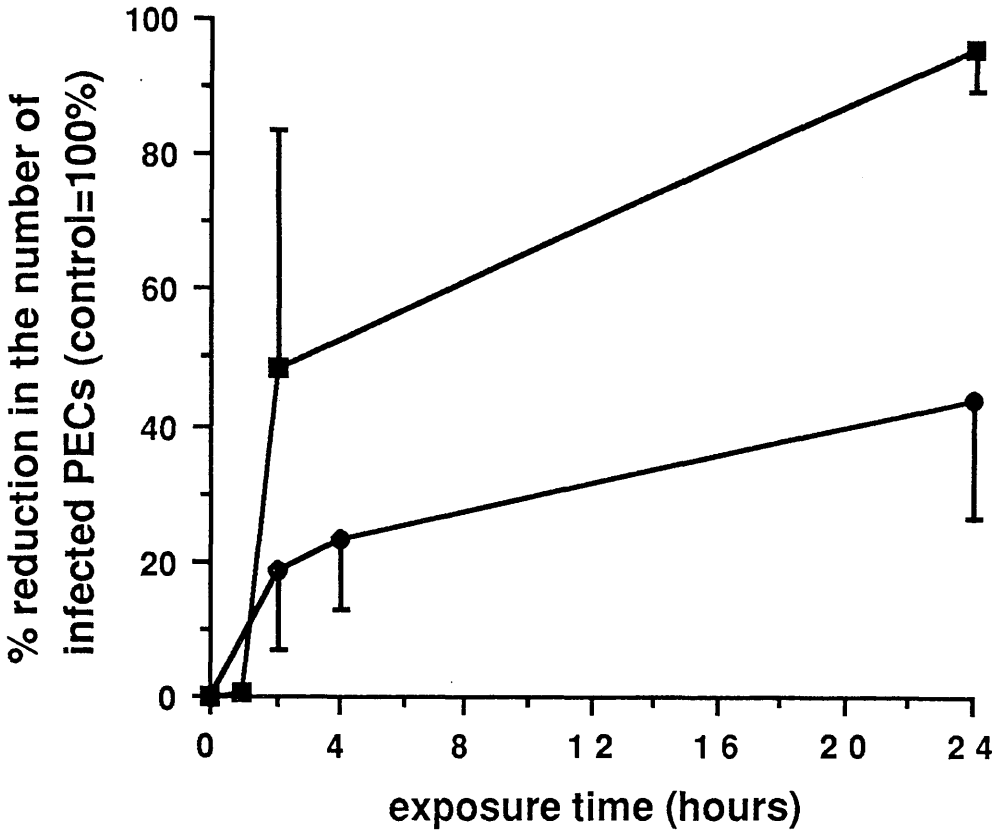
L-arginamide was also tested against *L.m.mexicana* promastigote-

Figure 21. The effect of L-tryptophanamide concentration on *L.m.mexicana* and *L.donovani* infections of PECs



PECs were exposed for 24 hours to either *L.donovani* amastigotes or *L.m.mexicana* stationary phase promastigotes, washed and then exposed to different concentrations of tryptophanamide for 2 hours. Cells were washed thoroughly after the exposure period, the medium replenished and the incubation continued for a further 46 hours before the PECs were fixed, stained and the number of infected PECs assessed. The results for *L.donovani* (●) and *L.m.mexicana* (■) are the means ± the standard deviations from 3 experiments done in duplicate. The infection rates of the controls for *L.donovani* were between 97% and 100% and for *L.m.mexicana* 65% to 87%; these controls are expressed as 100%.

Figure 22. The time dependent effect of L-tryptophanamide on *L.m.mexicana*- and *L.donovani*-infected PECs



PECs were exposed for 24 hours to either *L.donovani* amastigotes or *L.m.mexicana* stationary phase promastigotes, washed and then exposed for different lengths of time to 2 mM tryptophanamide. Cells were washed thoroughly after the exposure period, the medium replenished and the incubation continued untill 48 hours after the initial exposure to drug and the PECs fixed, stained and the number of infected PECs assessed. The results for *L.donovani* (■) and *L.m.mexicana* (●) are the means ± the standard deviations from 3 experiments done in duplicate. The infection rates of the controls for *L.donovani* were between 97% and 100% and for *L.m.mexicana* 65% to 87%; these controls are expressed as 100%.

initiated infections of PECs for exposure times of up to 5 days at 3.5 and 7 mM. There were no effects on the parasite load.

3.5.2.2. Activity of L-leucine methyl ester against *L.m.mexicana* promastigote initiated infections of PECs

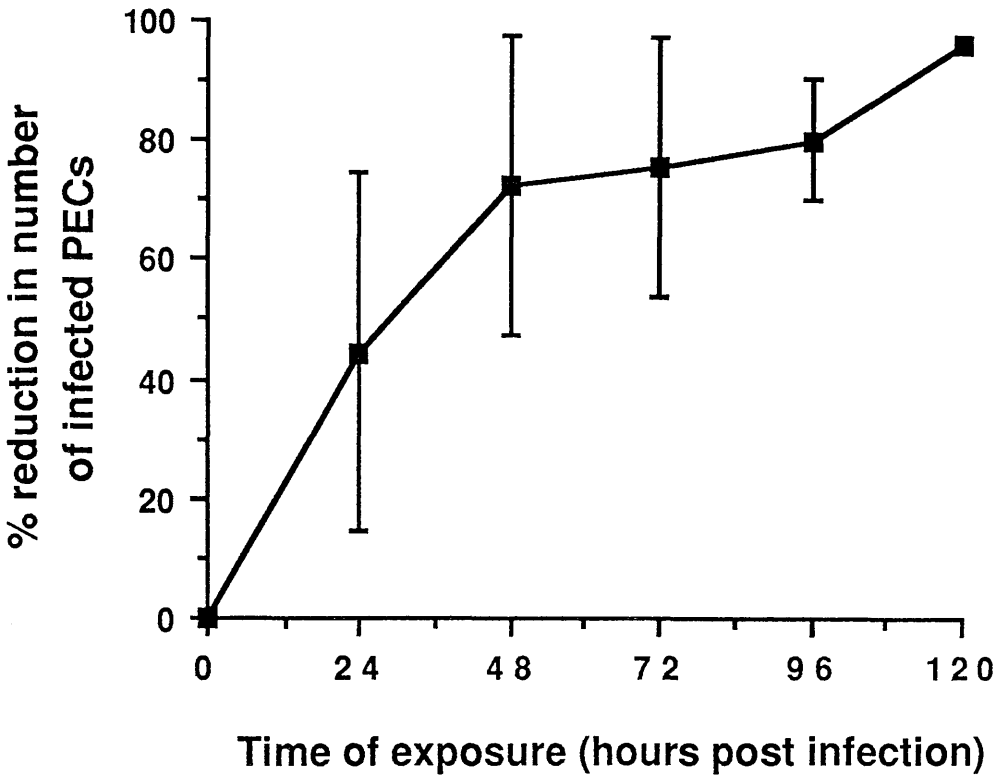
PECs infected with *L.m.mexicana* by exposure to stationary phase promastigotes did not respond in the same way to leuOMe as PECs initially infected with amastigotes. Promastigote initiated infections were relatively resistant to leuOMe for the first 24 hours whereupon their susceptibility increased, in a time dependent manner, over a period of 5 days post-infection (see fig. 23). It was found that only after this period were all the amastigotes arising from promastigote initiated infections consistently killed by leuOMe. A similar protocol was used against amastigote-initiated infections of *L.donovani* to see if there was a time dependent onset of leuOMe susceptibility. The compound had no apparent effect at any time.

3.5.3. Use of the tetrazolium salt (MTT) reduction assay to assess the effects of compounds on the viability of isolated parasites

The effects of a 1 hour exposure of isolated parasites to either leuOMe or tryptophanamide was assessed using the procedure involving the tetrazolium salt MTT, as described in section 2.4.2.3..

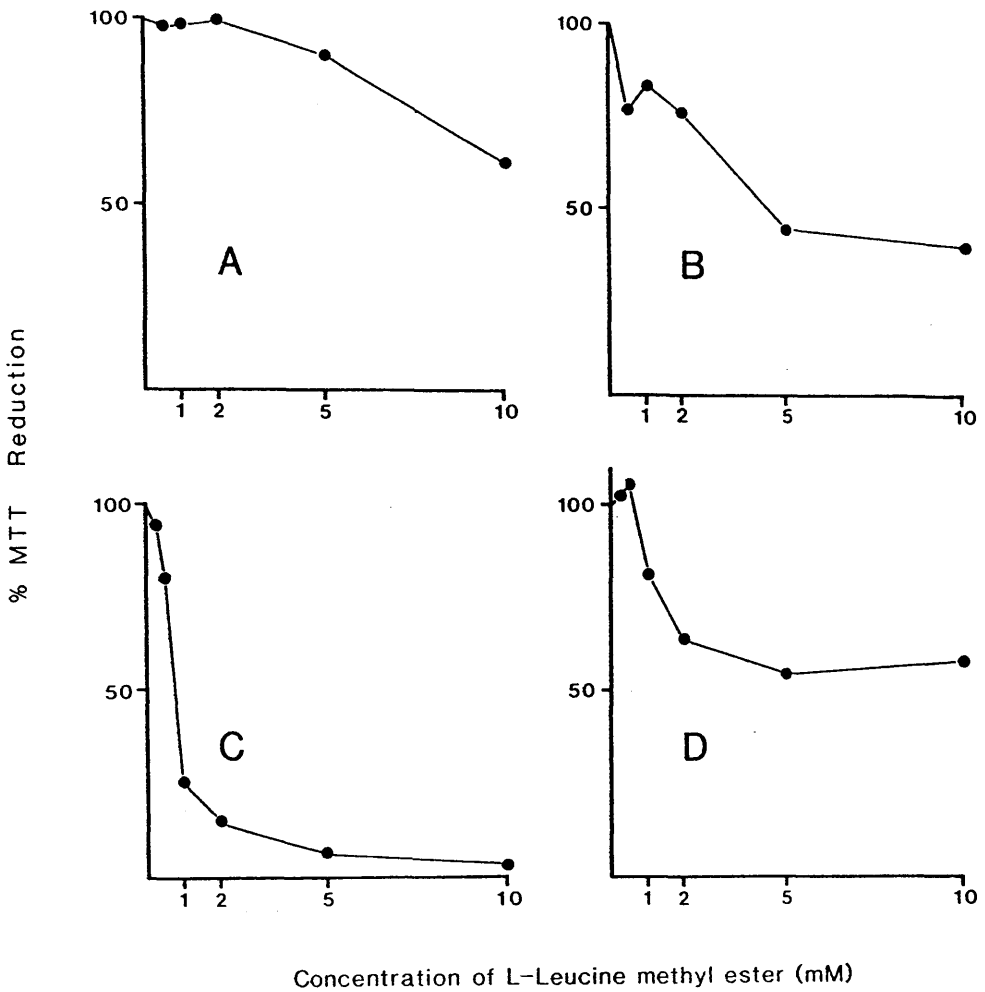
Amastigotes and promastigotes of *L.m.mexicana* and *L.donovani* were all affected by leuOMe, as measured by their reduction of MTT (see fig. 24). The amastigotes of *L.m.mexicana*, however, were the most susceptible to leuOMe. Exposure of the parasites to tryptophanamide also resulted in diminished MTT reduction (fig. 25) With this compound however, both forms of the two species were affected similarly and large effects were observed only when concentrations were 5 mM or more. These results concur with the general findings in section 3.5.2.1. that amastigotes of *L.m.mexicana* are less susceptible to

Figure 23. The effect of L-leucine methyl ester on *L.m.mexicana* promastigote-initiated infections of PECs



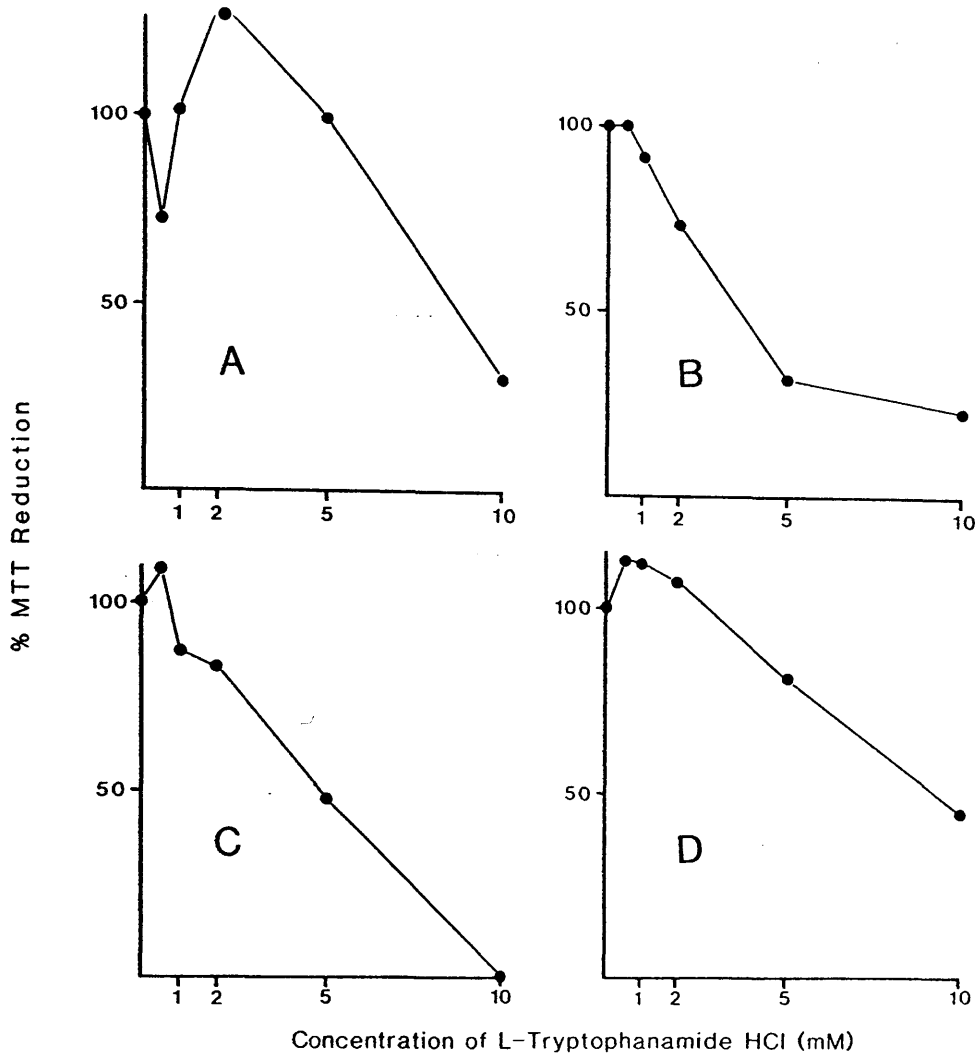
PECs were exposed for 24 hours to stationary phase *L.m.mexicana* promastigotes before being exposed to 2 mM leuOMe at different time points for 1 hour. After the exposure period the cells were washed thoroughly, the medium replenished and the incubation continued. PECs were fixed, stained and assessed for the number of infected PECs at 5 days post-infection. The results are the means ± standard deviations from 5 experiments, done in duplicate. The infection rates varied between 65% and 84%. The results are expressed as the % reduction in the number of infected PECs with the controls taken as 100%.

Figure 24. The effect of L-leucine methyl ester on the viability of different species and stages of *Leishmania*, as measured by their ability to reduce MTT



Isolated parasites were suspended at a cell density of approximately 1 x 10^8 ml^{-1} (amastigotes) or 2 x 10^7 ml^{-1} (promastigotes) in complete HOMEM medium and exposed to different concentrations of tryptophanamide for 1 hour at 25°C (A, *L.m.mexicana*; B, *L.donovani* promastigotes) or either 35°C (C, *L.m.mexicana* amastigotes), 37°C (D, *L.donovani* amastigotes). The cells were then washed free of the drug and incubated at the appropriate temperature with MTT at 450 µg ml^{-1} for 3 hours. The formazan product produced by the reduction of the MTT was then quantified by dissolving it in acid isopropanol and measuring the absorbance at 570 nm. The results obtained are expressed as the reduction of MTT by the treated parasites as a % of that by untreated parasites. Each of these results are the means from 3 experiments.

Figure 25. The effect of L-tryptophanamide on the viability of different species and stages of *Leishmania*, as measured by their ability to reduce MTT



Isolated parasites were suspended at a cell density of approximately 1 x 10^8 ml^{-1} (amastigotes) or 2 x 10^7 ml^{-1} (promastigotes) in complete HOMEM medium and exposed to different concentrations of tryptophanamide for 1 hour at 25°C (A, *L.m.mexicana*; B, *L.donovani* promastigotes) or either 35°C (C, *L.m.mexicana* amastigotes), 37°C (D, *L.donovani* amastigotes). The cells were then washed free of the drug and incubated at the appropriate temperature with MTT at 450 ug ml^{-1} for 3 hours. The formazan product produced by the reduction of the MTT was then quantified by dissolving it in acid isopropanol and measuring the absorbance at 570 nm. The results obtained are expressed as the reduction of MTT by the treated parasites as a % of that by untreated parasites. Each of these results are the means from 3 experiments.

tryptophanamide than leuOMe.

3.5.4. The effect of L-leucine methyl ester against *L.donovani* and *L.m.mexicana* in mice

Leucine methyl ester was tested against *L.donovani* using the standard mouse model (see section 2.4.1.). The dose regimen was 1 mg per mouse, intravenously, on days 7 and 8. Activity against *L.m.mexicana* was assessed using the 2 experimental mouse models of leishmaniasis (section 2.4.1.). The effects on the liver parasite load of leuOMe, at 2 mg per mouse administered subcutaneously on days 7-11, were determined. Also the efficacy agaist *L.m.mexicana* growing subcutaneously in mice was tested using a dose regimen of 5 mg per mouse given subcutaneously on days 7-11 post-infection.

There was no detectable effect on the parasite load using any of the regimens described above. Intravenous administration of leuOMe at dose levels above 1 mg per mouse resulted in severe toxicity to the mice.

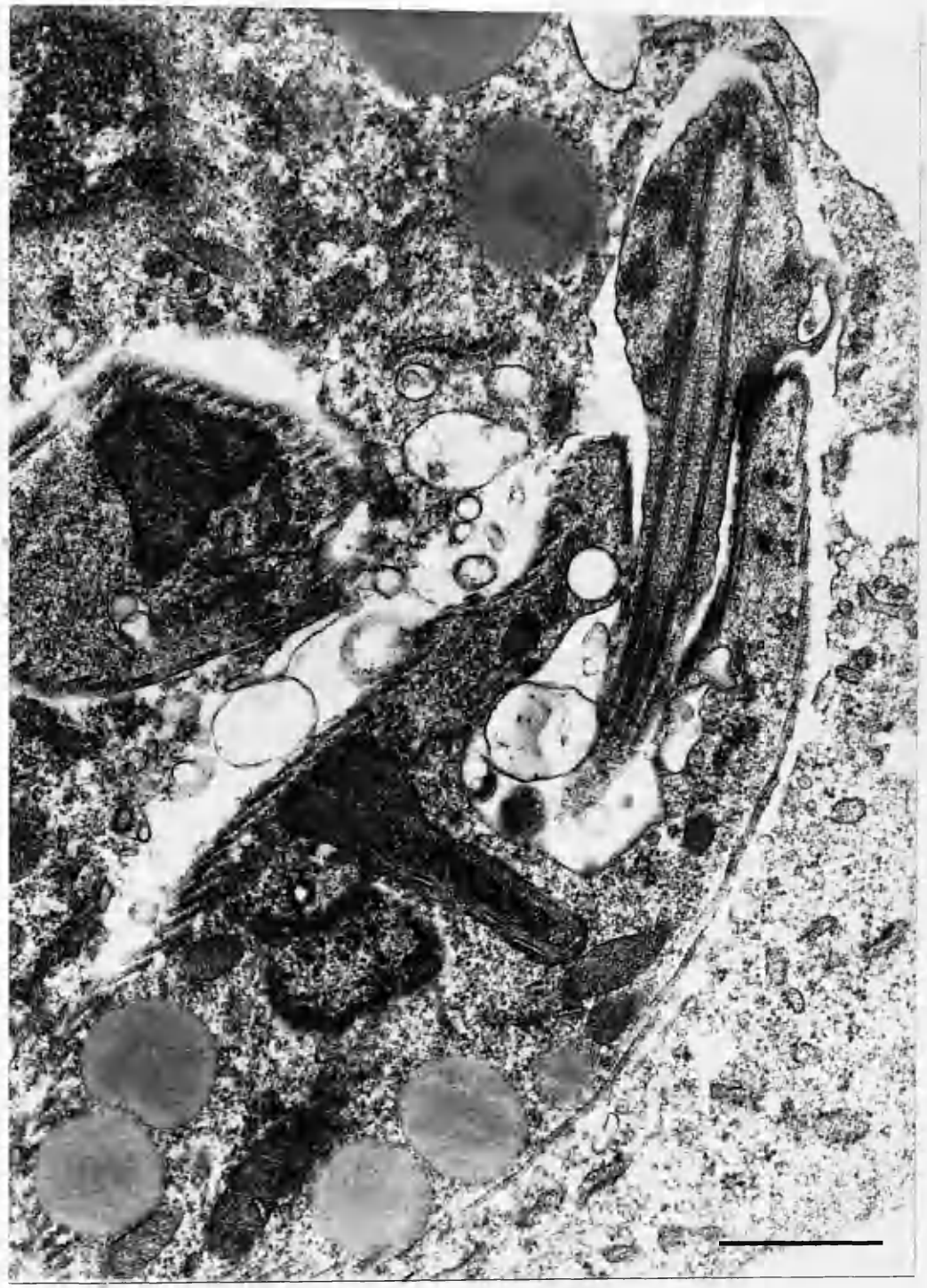
3.6. THE APPEARANCE OF MEGASOMES DURING THE TRANSFORMATION *IN VITRO* OF *L.M.MEXICANA* FROM PROMASTIGOTE TO AMASTIGOTE

In an attempt to correlate the presence of megasomes with the onset of the antileishmanial activity of leuOMe in promastigote initiated infections (see section 3.5.2.2.) the transformation from promastigote to amastigote was investigated using transmission electron microscopy.

PECs were cultured and exposed to stationary phase promastigotes of *L.m.mexicana* for 4 hours as described in section 2.3.2.. Subsequently they were harvested, at 4, 23, 47 and 73 hours post-infection, and processed for electron microscopy (see section 2.8.).

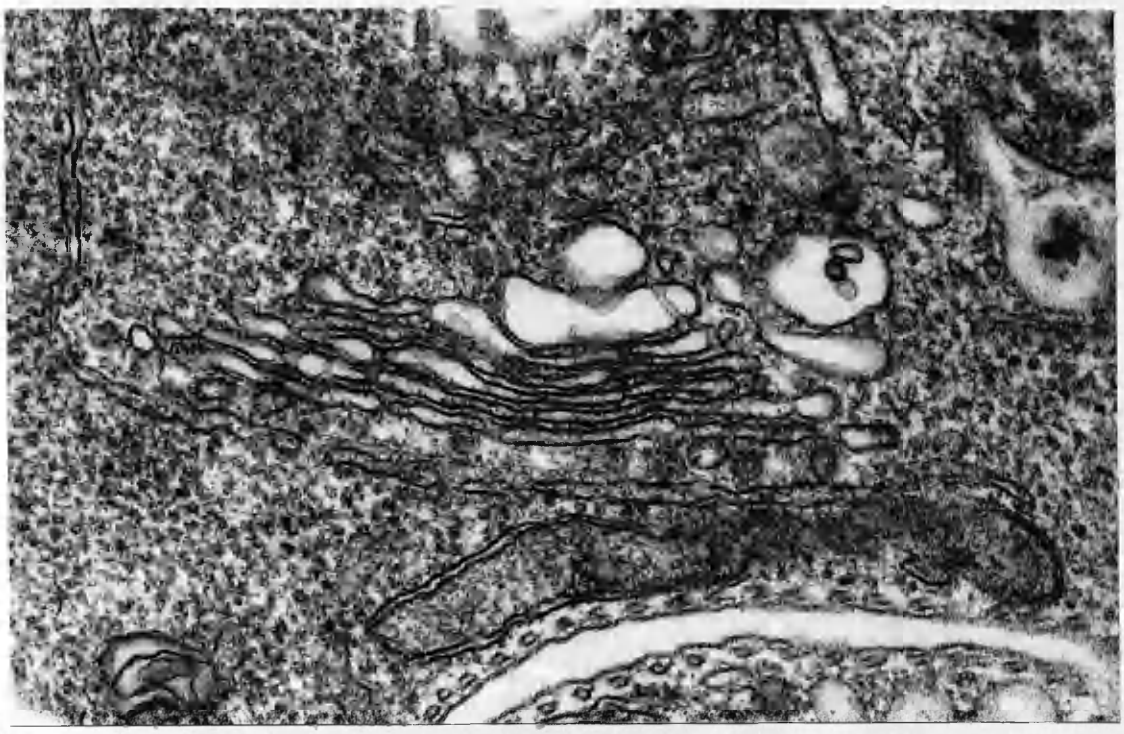
There was a time-dependent increase in the number of megasomes apparent in the sections. None were seen in the samples taken at 4

Figure 26. Transmission electron micrograph of a transforming parasite 23 hours after infection of a PEC *in vitro*



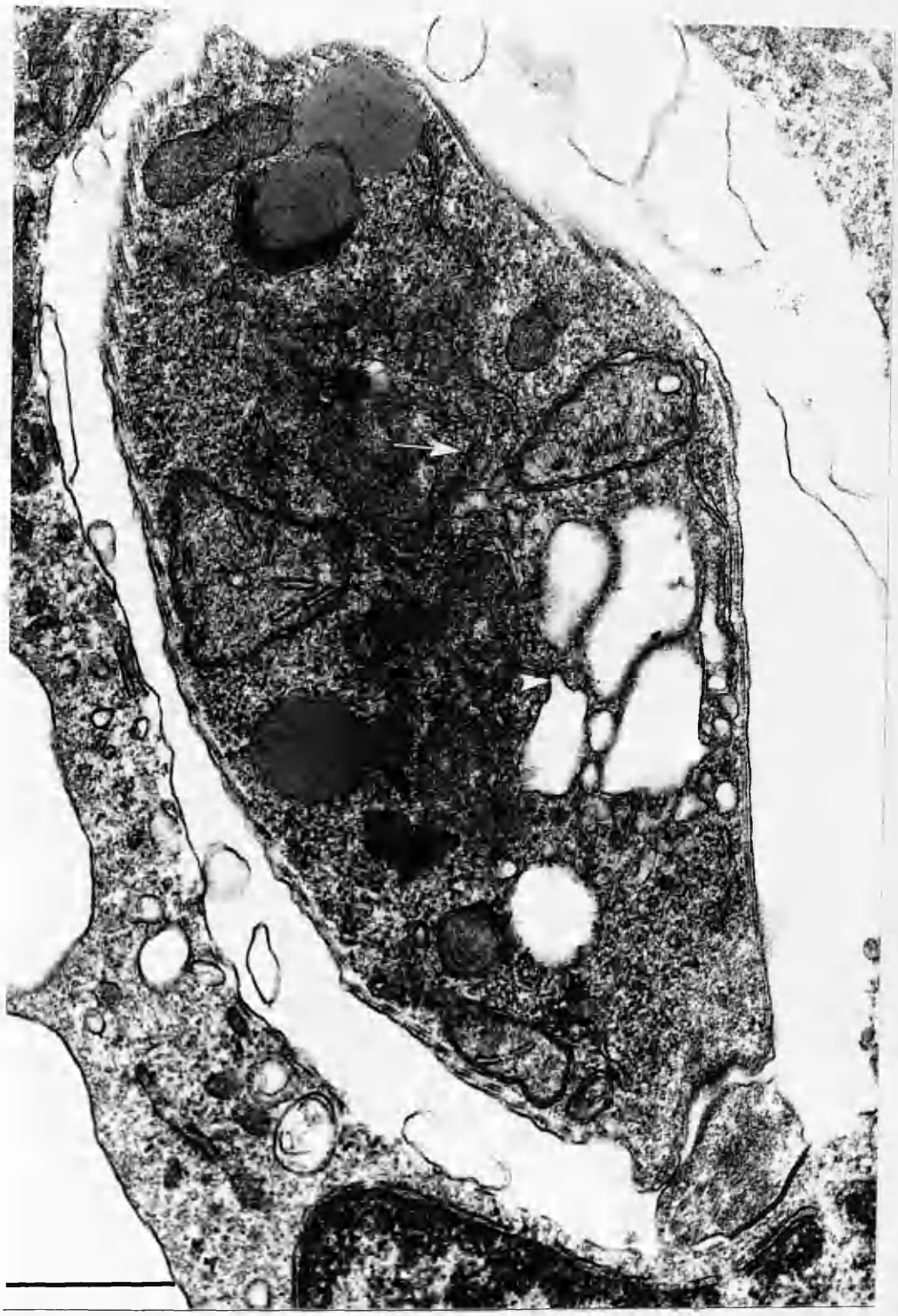
The extracellular flagellum is being resorbed: the posterior end of the flagellate contains abundant lipid globules; an elongate megasome is visible alongside the kinetoplast. Scale bar = 500 nm.

Figure 27. Transmission electron micrograph of a transforming parasite 23 hours after infection of a PEC *in vitro*



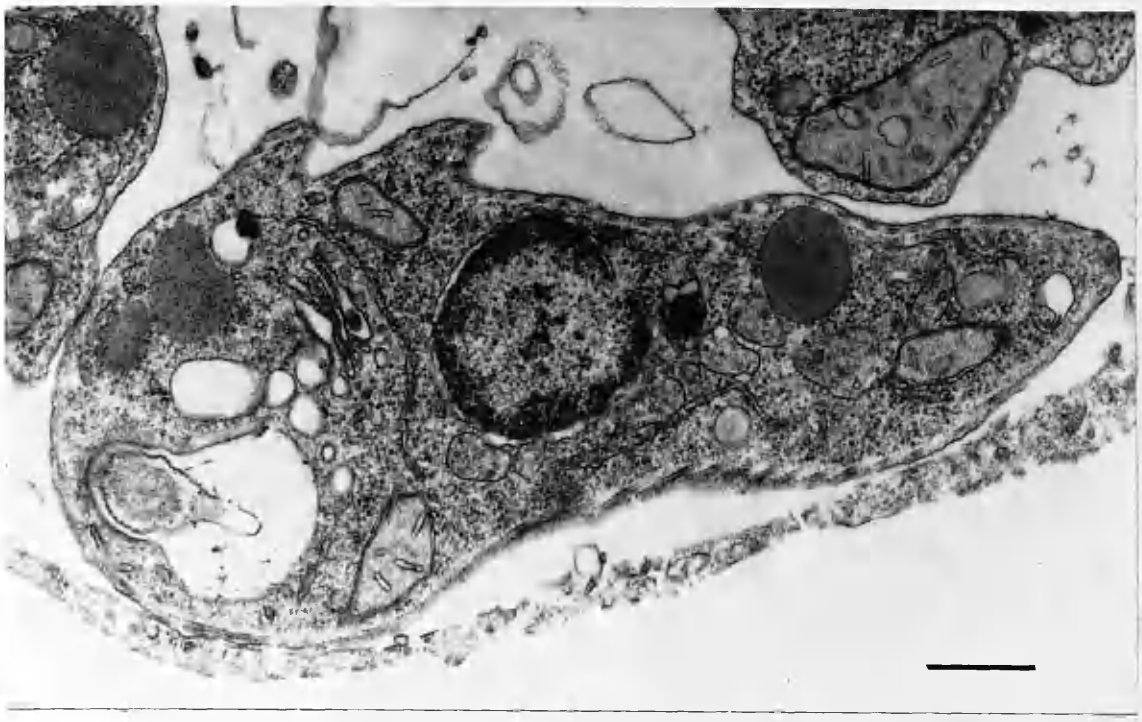
Detail of the golgi apparatus of a transforming parasite showing subtending endoplasmic reticulum at a cis face and swollen clear saccules at the trans-face.

Figure 28. Transmission electron micrograph of a transforming parasite 23 hours after infection of a PEC *in vitro*



Megasomes and swollen clear saccules lie alongside one another in the trans-golgi region. Megasomes are continuous within the trans-golgi network (arrow); clathrin-coated vesicles (arrowhead) are associated with the clear saccules but not the megasomes. Scale bar = 500 nm.

Figure 29. Transmission electron micrograph of a transforming parasite 23 hours after infection of a PEC *in vitro*



A chain of megasomes extends from the golgi apparatus to the posterior end of the cell. Lipid droplets are also present in the posterior region. Smooth clear vesicles of the trans-golgi region abut onto the flagellar pocket. Scale bar = 500 nm.

hours post-infection. By 23 hours, however, the megasomes were present in substantial numbers in the trans-golgi region, although the parasite still retained an extracellular flagellum (fig. 26). The trans-golgi region contained swollen, empty looking sacs and a network of smooth-membraned tubules (fig. 27). The megasomes appeared to arise among the latter as irregular bloated tubule segments (fig. 28) and to extend from the prenuclear golgi region into the posterior part of the cell where they assumed their more characteristc shape (fig. 29). At times subsequent to 23 hours, the transforming parasites had megasomes resembling those of fully transformed amastigotes (see fig. 3). No attempt was made to quantify the numbers of megasomes at the different time points.

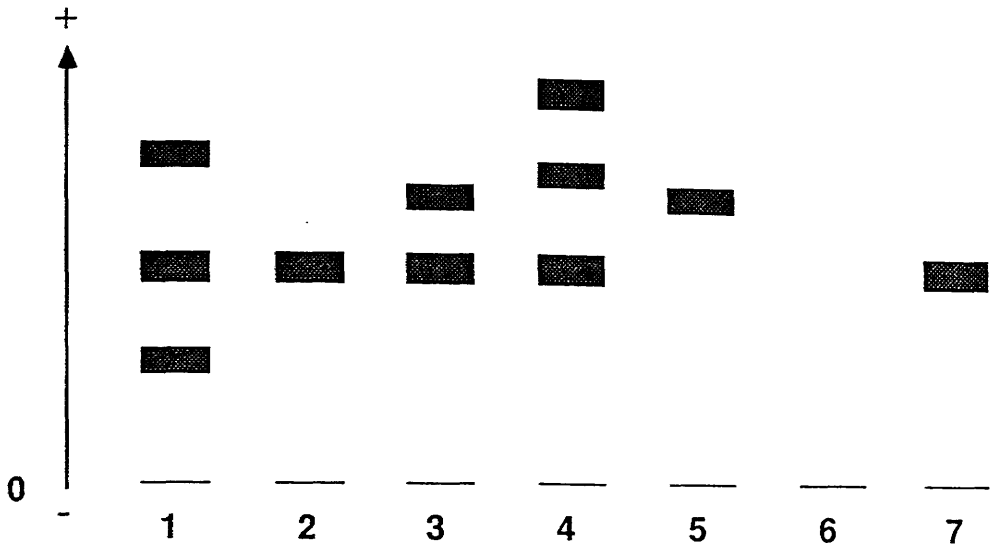
The timescale of the appearance of the megasomes correlates well with the onset of activity of leuOMe against promastigote-initiated infections (see section 3.5.2.2.). This would suggest that the appearance of these organelles is related to the antileishmanial activity of leuOMe.

3.7. ESTERASES OF *LEISHMANIA*

3.7.1. The use of starch gel electrophoresis to detect esterases of different species and stages of *Leishmania*

In an attempt to identify the enzymes involved in the hydrolysis of amino acid esters by *L.m.mexicana* amastigotes the esterase isoenzyme patterns of different species and stages of *Leishmania* were investigated using starch gel electrophoresis (see section 2.9.1.) to separate the isoenzymes and 4-methyl umbilliferone as substrate. The results are summarised in figure 30. Clear differences between the banding patterns of different species and stages were found. There were, however, several bands in common; one present in promastigotes of both *L.m.mexicana* and *L.donovani* and also the metacyclics of

Figure 30. Esterase banding patterns of different species and stages of *Leishmania*



Samples of *Leishmania* species were run for 5 hours at 8 V cm^{-1}, with cooling, on an 8% starch gel and esterase activity visualised by incubating the gel with 4-methyl umbiliferyl propionate and viewing under an ultraviolet light source. Some samples had leupeptin present (10 µg ml^{-1}) whilst being prepared for electrophoresis. The results presented are a summary of those obtained in the many experiments conducted. Sample 1: *L.major* metacyclics + leupeptin. 2: *L.major* metacyclics - leupeptin. 3: *L.m.mexicana* promastigotes - leupeptin. 4: *L.donovani* promastigotes - leupeptin. 5: *L.m.mexicana* amastigotes + leupeptin. 6: *L.m.mexicana* - leupeptin. 7: *L.donovani* amastigotes - leupeptin.

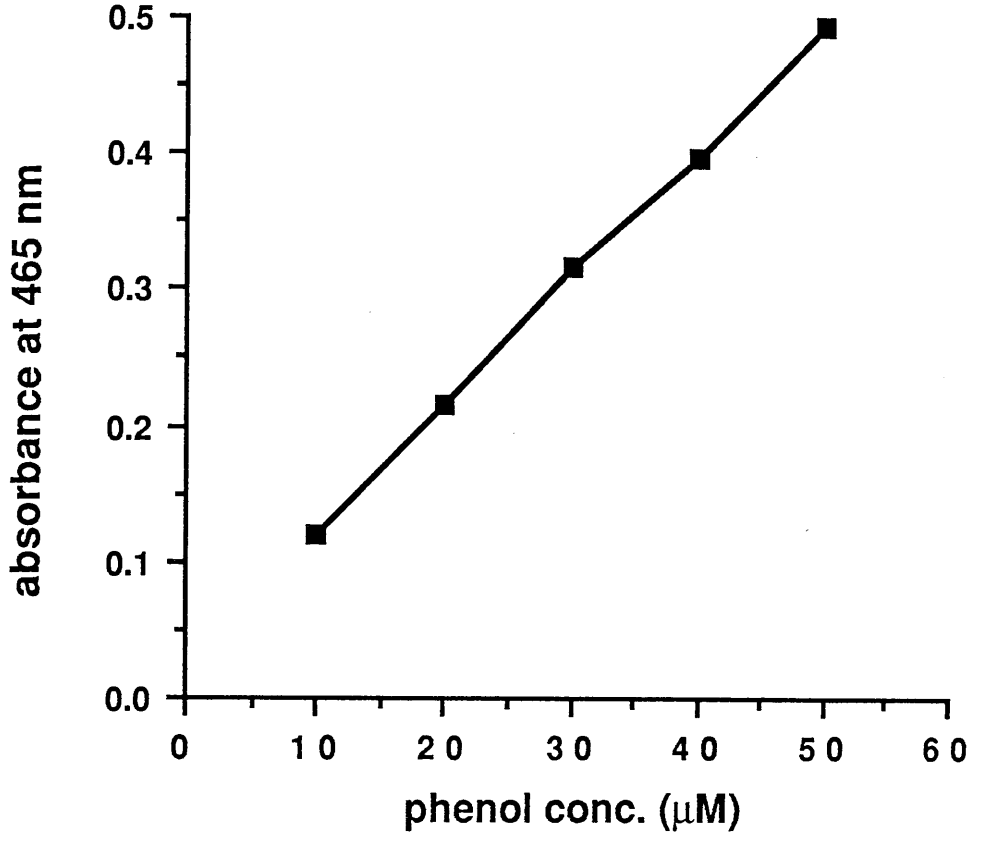
L.major and the amastigotes of *L. donovani*, and another present in both amastigotes and promastigotes of *L.m.mexicana*.

Interestingly the addition of the proteinase inhibitor leupeptin resulted in the appearance of 2 extra bands in the *L.major* metacyclic samples and the appearance of a single band in the *L.m.mexicana* amastigotes whereas none was detected in samples without leupeptin. This finding with the amastigotes is not particularly surprising given their high proteinase activity (Pupkis and Coombs, 1984 and the results given in section 3.8.3.1.). Nevertheless there was no indication that this esterase activity was greater in amastigotes of *L.m.mexicana* than in promastigotes, whereas activity towards leuOMe is. Therefore the possible role of this isoenzyme in the hydrolysis of amino acid esters was not investigated further.

3.7.2. The detection of phenol as a means of following the hydrolysis of L-leucine benzyl ester

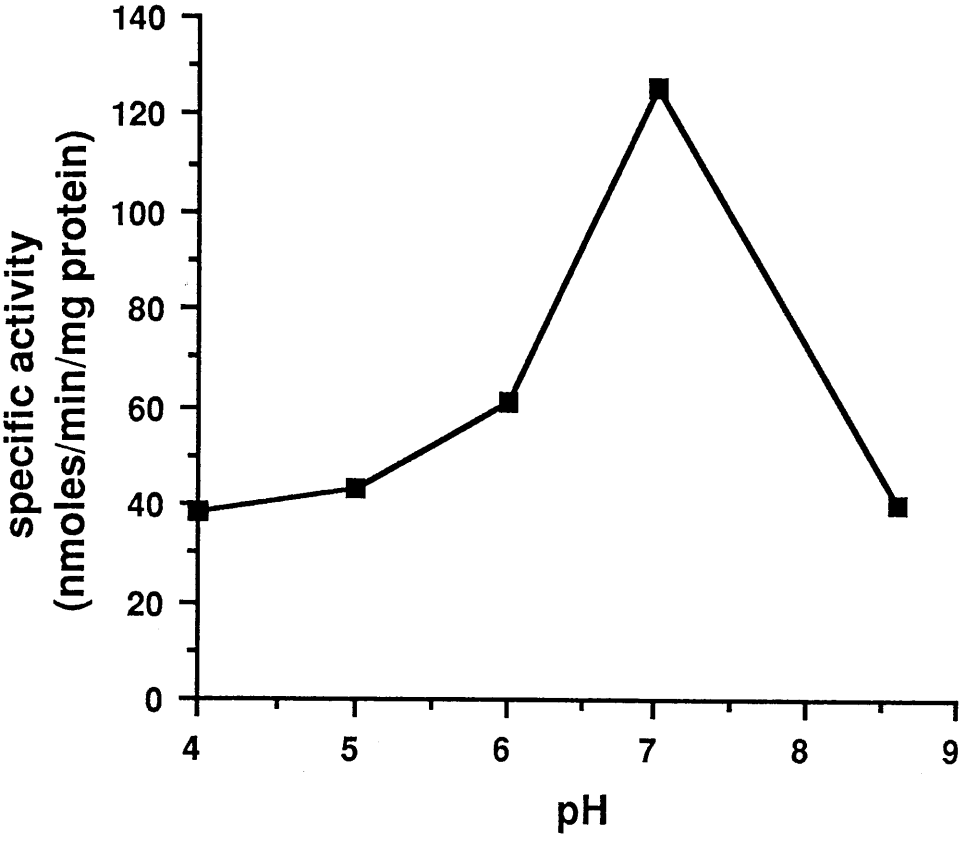
In order to characterise the activity of the amino acid esters it was necessary to develop an assay to quantify the products of amino acid ester hydrolysis. The assay system described in section 2.9.2. was satisfactory for detection of phenol in the concentration range 10 uM-50 uM in buffers at pHs from 4-8.6 (see fig. 31). However, it was unable to detect the hydrolysis of leucine benzyl ester by any samples used (*L. m. mexicana* amastigote lysates and the purified low molecular weight proteinases [see section 3.8.]). To check the assay system, horse serum, which is known to contain arylesterases, was used with phenylacetate as substrate to determine the specific activities of arylesterase at different pHs. The results obtained are shown in figure 32. The greatest activity was at pH 7, as would be expected for a serum enzyme, and so these results showed the potential use of the assay. Subsequent work with the purified low molecular weight

Figure 31. Detection of different concentrations of phenol at different pHs



Phenol was made up at concentrations from 10 μM to 50 μM in 2.5 ml of 1 mM McIlvanes buffer at different pHs. 0.5 ml of 4-aminoantipyrine (98.5 M) and 0.5 ml of $K_3(Fe[CN_6])$ (0.243 M) dissolved in 1 M Veronal buffer, pH 8.6, were added. The condensation product was extracted with 5 ml of n-pentanol and the absorbance at 465 nm read against a blank. The absorbance of the control, in the range 0.8 -0.9, was then subtracted from that of the sample to give the absorbance due to the phenol.

Figure 32. The effect of pH on arylesterase activity of horse serum



Five µl of horse serum was added to 2.5 ml of the appropriate buffer (either 1 mM McIlvanes buffer at pHs 4,5,6 and 7 or 1 M Veronal buffer, pH 8.6) which contained the arylesterase substrate phenylacetate, 1.33 mM. After 30 minutes incubation at 37°C 0.5 ml of 4-aminoantipyrine (98.5 M) and 0.5 ml of $K_3(Fe[CN_6])$ (0.243 M) dissolved in 1 M Veronal buffer, pH 8.6, were added. The condensation product was extracted with 5 ml of n-pentanol and the absorbance at 465 nm read against a blank. The absorbance of the control, in the range 0.8-0.9, was then subtracted from that of the sample to give the absorbance due to the phenol. Using the standard curve phenol concentration was estimated and the specific activities calculated. These are the results from a single experiment done in triplicate.

proteinases of *L. m. mexicana* showed them to be sensitive to DMSO (see section 3.8.3.1), the solvent in which leucine benzyl ester was dissolved in attempts to detect its hydrolysis using this assay. No further attempts were made to measure the hydrolysis of leucine benzyl ester in this manner or to measure the levels of esterase activity in *Leishmania*.

3.8. THE POTENTIAL OF *L.M.MEXICANA* CYSTEINE PROTEINASES AS ACTIVATORS OF PRO-DRUGS

The findings in the previous sections indicated that enzymes in the amastigotes of *L. m. mexicana*, probably localised within the megasomes, were responsible for the hydrolysis of leuOMe which would lead to osmotic lysis of the amastigotes. A personal communication from Dr. Rabinovitch, now published (Alfieri et al., 1988) showed that several inhibitors of cysteine and serine proteinases inhibited the antileishmanial activity of several amino acid esters including leuOMe. This, in combination with the previous evidence indicated that the low molecular weight cysteine proteinases, unique to the amastigotes of *L.mexicana* and purified by Pupkis and Coombs (1984), were possibly involved in the hydrolysis of leuOMe. The role of these enzymes in the antileishmanial activity of leuOMe was investigated.

3.8.1. Purification of amastigote proteinases

Partial purification of the low molecular weight proteinases from amastigotes of *L.m.mexicana* was carried out using gel filtration followed by ion exchange chroatography. Proteinase activity was detected using the nitroanilide substrate BzPPANan. Initially two methods of cell lysis were compared to determine which was the most satisfactory in releasing the proteinase activity into the soluble fraction (see table 12). The results clearly show that detergent lysis was more efficient than the freeze and thaw method tested, especially

Table 12. Comparison of freezing and thawing and the use of detergents as methods of solubilising the amastigote enzymes responsible for the hydrolysis of L-leucine methyl ester and BzPPANan

	Hydrolysis of leuOMe		Hydrolysis of BzPPANan	
	Triton X100	Freeze-thaw	Triton X100	Freeze-thaw
Lysate	100%	100%	100%	100%
Pellet	9%	54%	5%	32%
Supernatant	82%	41%	110%	97%

The distribution of the different hydrolysing activities towards leuOMe and BzPPANan was measured using the paper electrophoresis method for leuOMe and the spectrophotometric assay for BzPPANan. Lysates were prepared in 0.25 M sucrose and then either frozen and thawed 3 times using liquid nitrogen and a water bath at 37°C or detergent lysis using Triton X100 at a final concentration of 0.25%. These results, from a single experiment, show the activity recovered in each fraction expressed as a % of the activity in lysates.

for the enzymes responsible for the hydrolysis of leuOMe. Consequently this method of cell lysis was used in the majority of subsequent experiments and the freezing-thawing method was used only where stated.

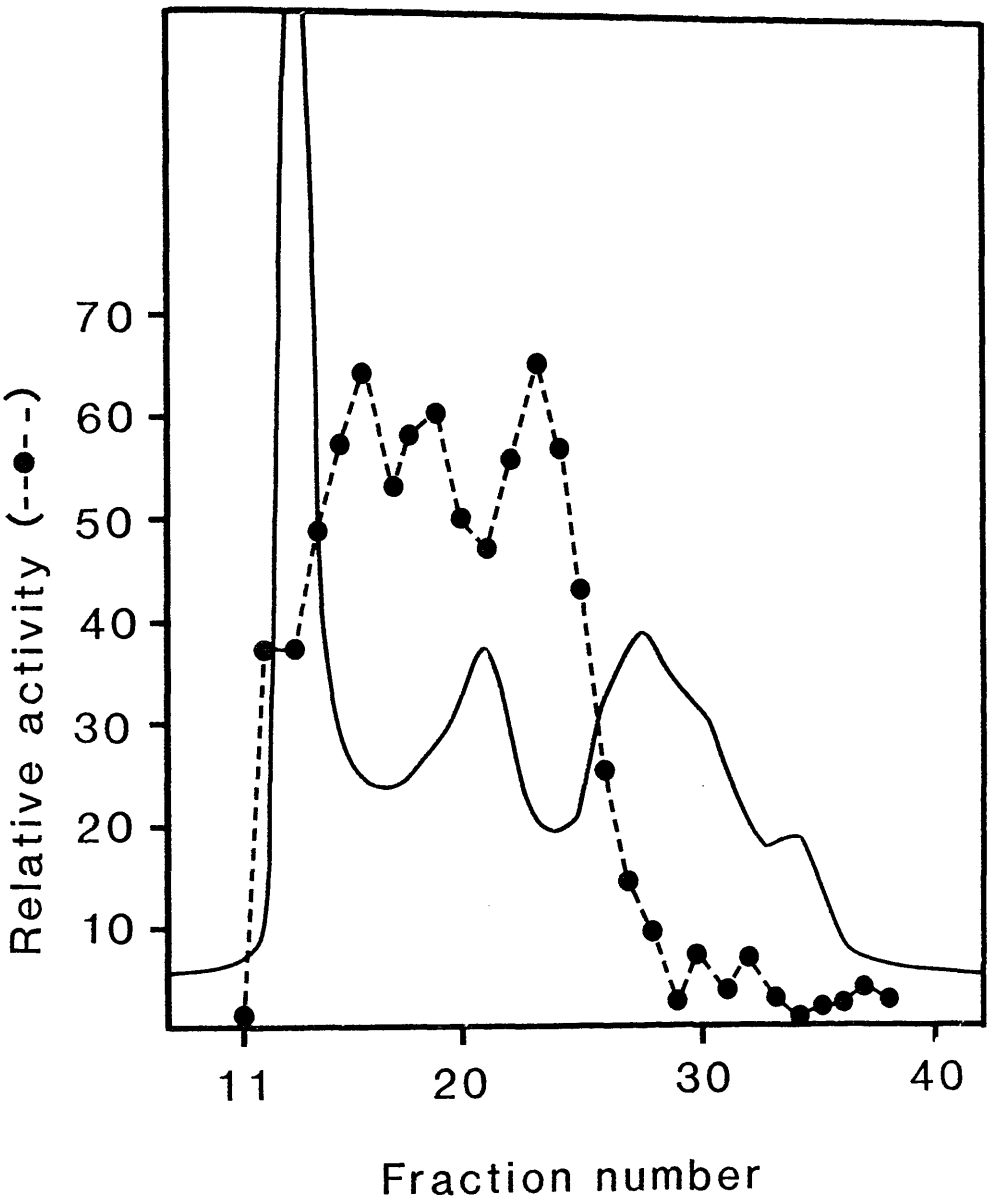
3.8.1.1. Gel filtration

Gel filtration was carried out as described in section 2.10.2.. The procedure used was based on the method described by Pupkis and Coombs (1984), except that FPLC equipment was used and homogenates were normally produced by detergent lysis instead of freezing and thawing.

A typical elution profile is shown in figure 33. Pupkis and Coombs (1984) reported that two main, but distinct peaks of proteinase activity were eluted from gel filtration when using the soluble fraction from homogenates obtained by freezing and thawing. Using the soluble fraction from homogenates obtained with the detergent Triton X100 several peaks were eluted (see fig. 33). However, when using the soluble fraction from amastigotes lysed by freezing and thawing, two well separated peaks of proteinase activity were eluted as shown in figure 34. These results suggest that additional proteinases may be released by detergent lysis compared to freezing and thawing and that the structure of some of the proteinases is modified by the lysis method used.

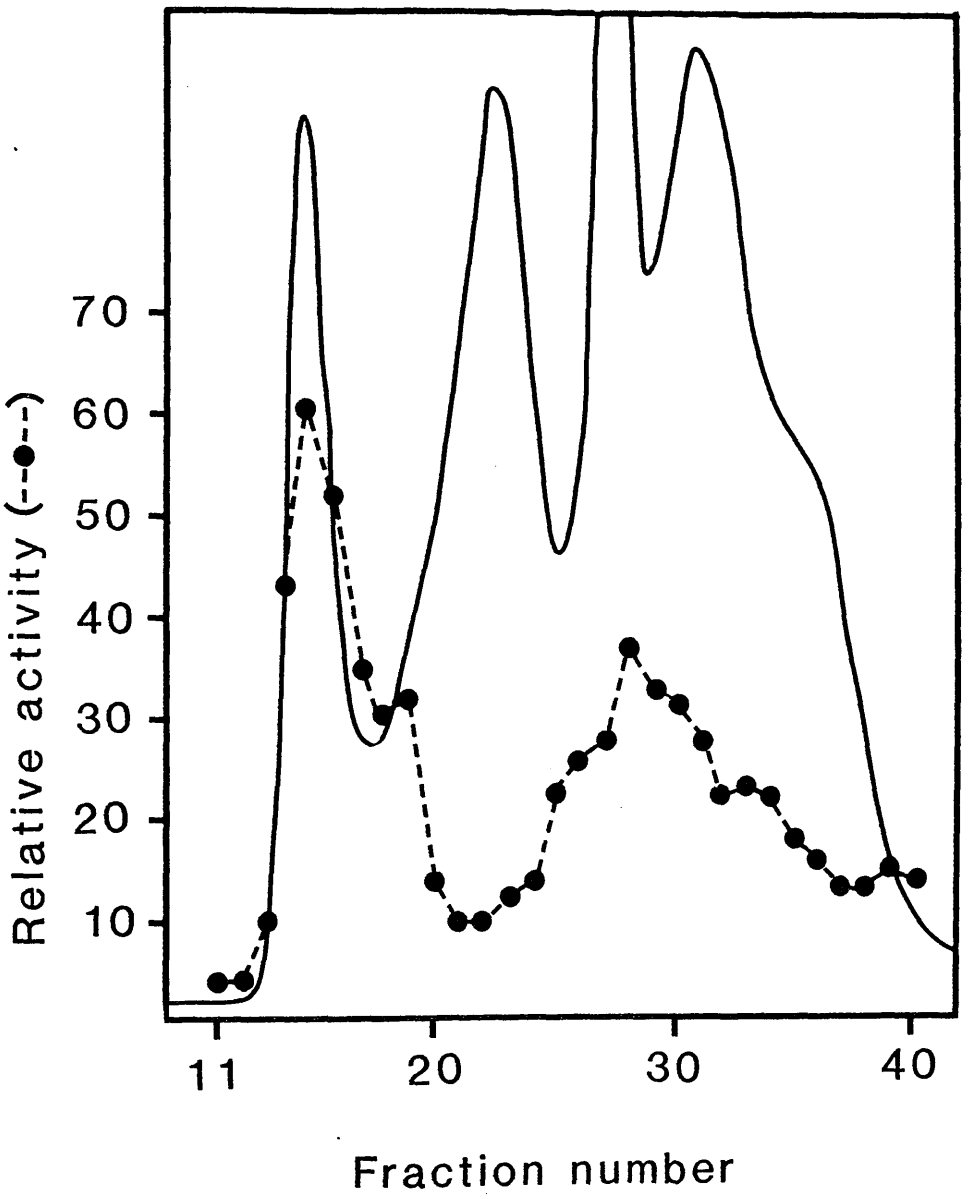
The use of gelatin SDS-PAGE to detect the proteinase activity clearly demonstrated the distribution of enzymes in the fractions. The use of 5% acrylamide gels shows the presence of at least 7 high molecular weight proteinases (see fig. 35) which would normally appear as 3-4 bands on a 7.5% acrylamide gel (see fig. 36). However the low molecular weight proteinases, which normally appear as 7-8 bands on a 7.5% acrylamide gel appear as a single band on a 5% gel. It is clear

Figure 33. Gel filtration of *L.m.mexicana* amastigote proteinases



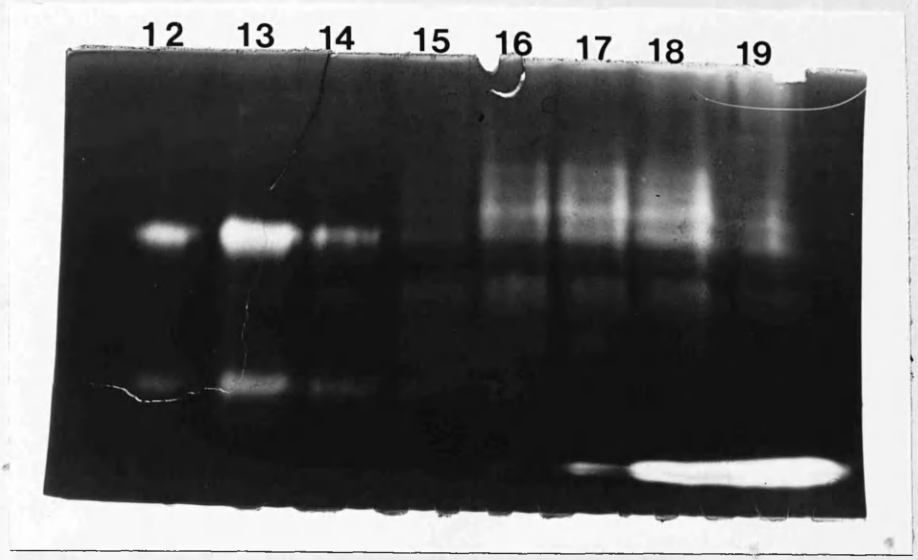
The supernatant from Triton X100 lysed cells was applied to a gel filtration column of Superose 12. The protein profile, measured as A_{280}-(—) and the distribution of proteinase activity towards BzPPANan (-●-) in the eluant of the column is shown. The AUFS is 2.0.

Figure 34. Gel filtration of *L.m.mexicana* amastigote proteinases



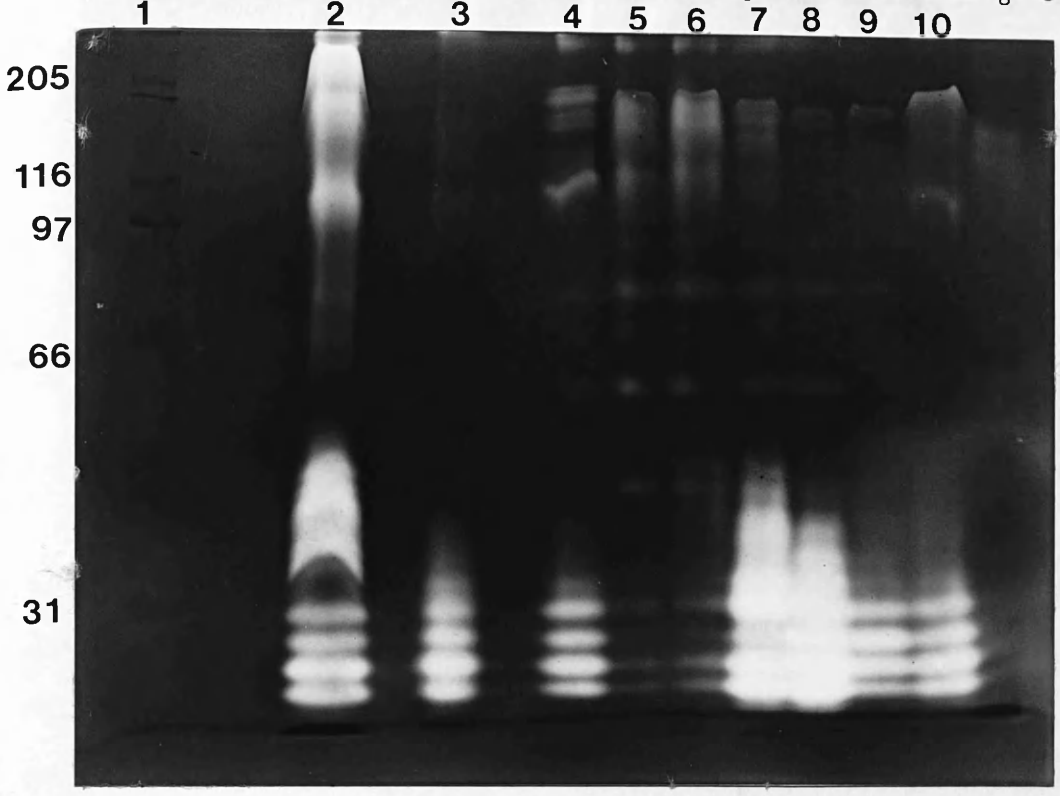
The supernatant from freeze-thawed cells was applied to a gel filtration column of Superose 12. The protein profile, measured as A_{280} (—) and the distribution of proteinase activity towards BzPPANan (-●-) in the eluant of the column is shown. The AUFS is 0.5.

Figure 35. The distribution of high molecular weight proteinases of *L.m.mexicana* amastigotes, in fractions from gel filtration



Samples (25 µl) from fractions 12-19 of the gel filtration eluant, from a Triton X100 lysed sample, were run on a 5% acrylamide gelatin SDS-PAGE mini-gel and the activity developed, after washing in 2.5% (v/v) Triton X100 for 30 minutes, by incubation in 0.1 M acetate buffer at 37°C, pH 5.5. for 30 minutes in the presence of 1 mM DTT. The numbers in each lane correspond to the fraction number. Approximate molecular weights in kDa are given on the right hand side.

Figure 36. The proteinases of *L.m.mexicana* amastigotes, separated by gel filtration and detected on 7.5% acrylamide gelatin SDS-PAGE gels



Samples (50 µl) from alternate fractions 17-27 (lanes 5-10) of the gel filtration eluant, the pooled fractions containing low molecular weight proteinases from gel fitration (50 µl, lane 4) from a Triton X100 lysed sample (5 µl, lane 2) and the pooled proteinase activity from ion exchange (75 µl, lane 3), were run on a 7.5% acrylamide gelatin SDS-PAGE gel and the activity developed, after washing in 2.5% (v/v) Triton X100 for 1 hour, by incubation in 0.1 M acetate buffer at 37°C, pH 5.5. for 1 hour in the presence of 1 mM DTT. Molecular weight markers were run in lane 1 and approximate molecular weights, in kDa, are given.

from these results that gel filtration gave a partial but in no way complete separation of the high molecular weight proteinases from the low molecular weight proteinases, as is clearly seen in figures 35 and 36. Typically fractions 17-27 contained the majority of the low molecular weight proteinases and these fractions were pooled before further processing.

This gel filtration step resulted in a 57% recovery of proteinase activity in fractions 17-27 and on average a six-fold increase in specific activity, compared to the homogenate (see table 13).

3.8.1.2. Ion exchange chromatography

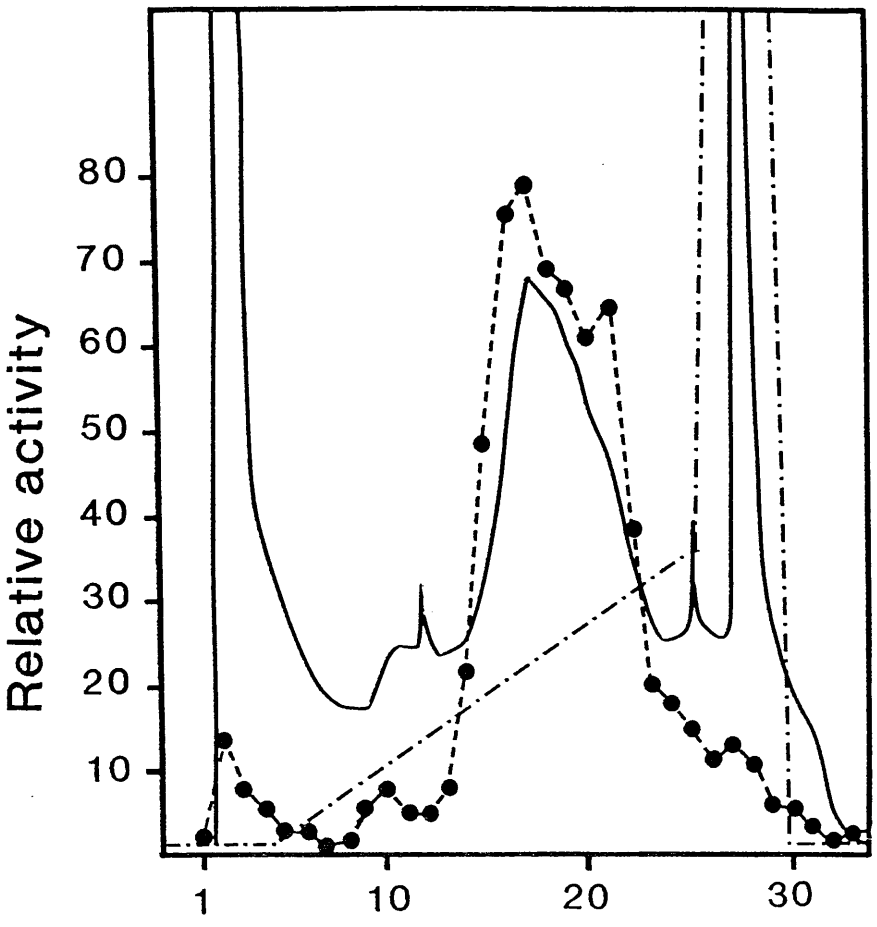
Ion exchange chromatography was carried out as described in section 2.10.3.. A typical elution profile obtained using the pooled and concentrated (see section 2.9.4.) proteinases from gel filtration fractions 17-27 is shown in figure 37. The proteinase activity detected using BzPPANan correlated with a large protein peak and the use of gelatin SDS-PAGE to detect the proteinase activity (see fig. 38) confirmed that this peak contained at least 7 low molecular weight proteinases, which appeared to have been seperated from the remaining high molecular weight proteinases. Silver staining of proteins separated by SDS-PAGE showed that there were several protein bands which correlated with the proteinase activity detected on gelatin SDS-PAGE (see fig. 39). By comparing the relative mobilities of these proteins and those of several low molecular weight standards, approximate molecular weights of the proteinases were calculated. Those of the main enzymes were 25,400., 26,300., 27,850., 29,850., 31,250., and 33,900 kDa (see fig. 38). Several other proteins were detected in the sample indicating that the enzymes were only partially purified. An attempt was made to investigate if these proteinases could be separated from each other by altering the salt gradient at

Table 13. Data from the purification of amastigote low molecular weight proteinases by gel filtration and ion exchange

Stage	% Recovery	Specific activity
Lysate	100	176 ± 35
Pellet	5 ± 2	26 ± 8
Supernatant	103 ± 14	534 ± 185
Gel filtration	57 ± 17	930 ± 362
Ion exchange	35 ± 18	1511 ± 348

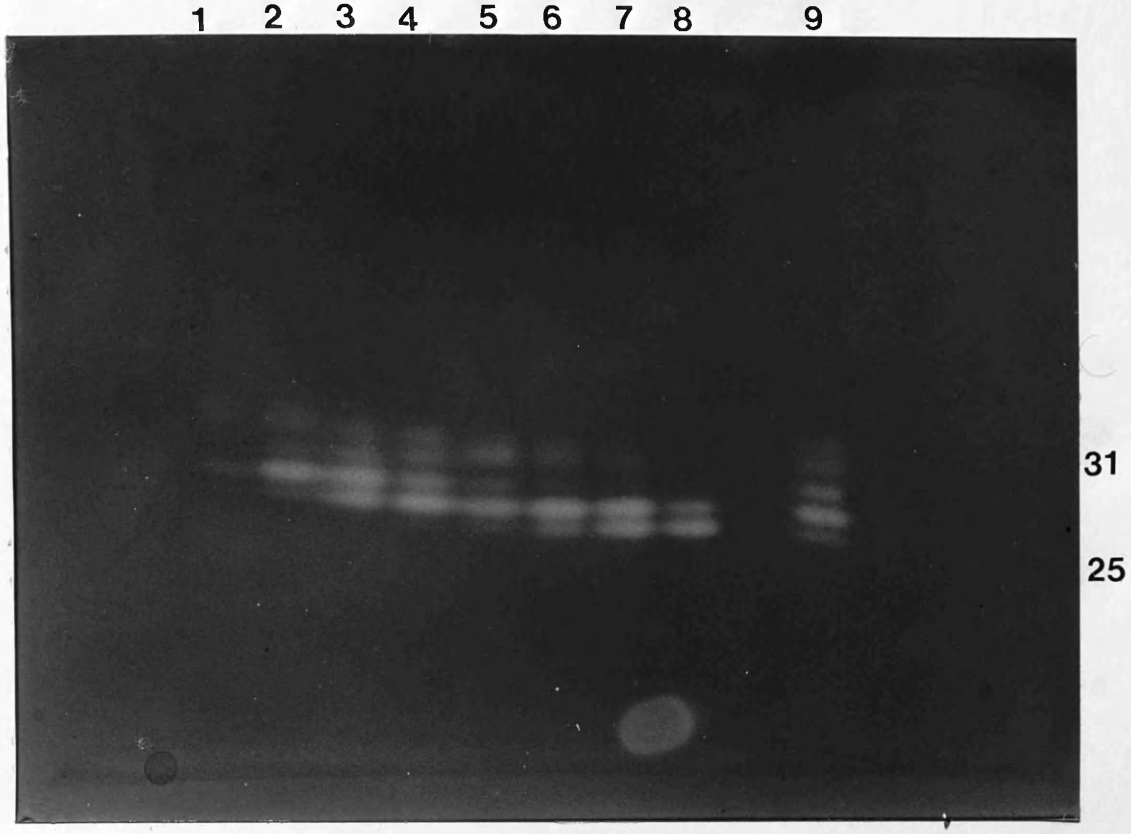
These are the mean results of 7-9 full purifications carried out on separate occasions. The lysates were obtained using Triton X100 and the pellet and supernatant fractions obtained by centrifugation at 200000 x g for 1 hour. Fractions 17-27 from gel filtration were pooled and concentrated using ultrafiltration and fractions in the range 14-24 from ion exchange were pooled and if necessary concentrated using ultrafiltration. The loss of activity during ultrafiltration was not included when calculating the % recovery. The activity towards BzPPANan is in nmoles min^{-1} mg $protein^{-1}$.

Figure 37. Ion exchange chromatography of *L.m.mexicana* amastigote low molecular weight proteinases



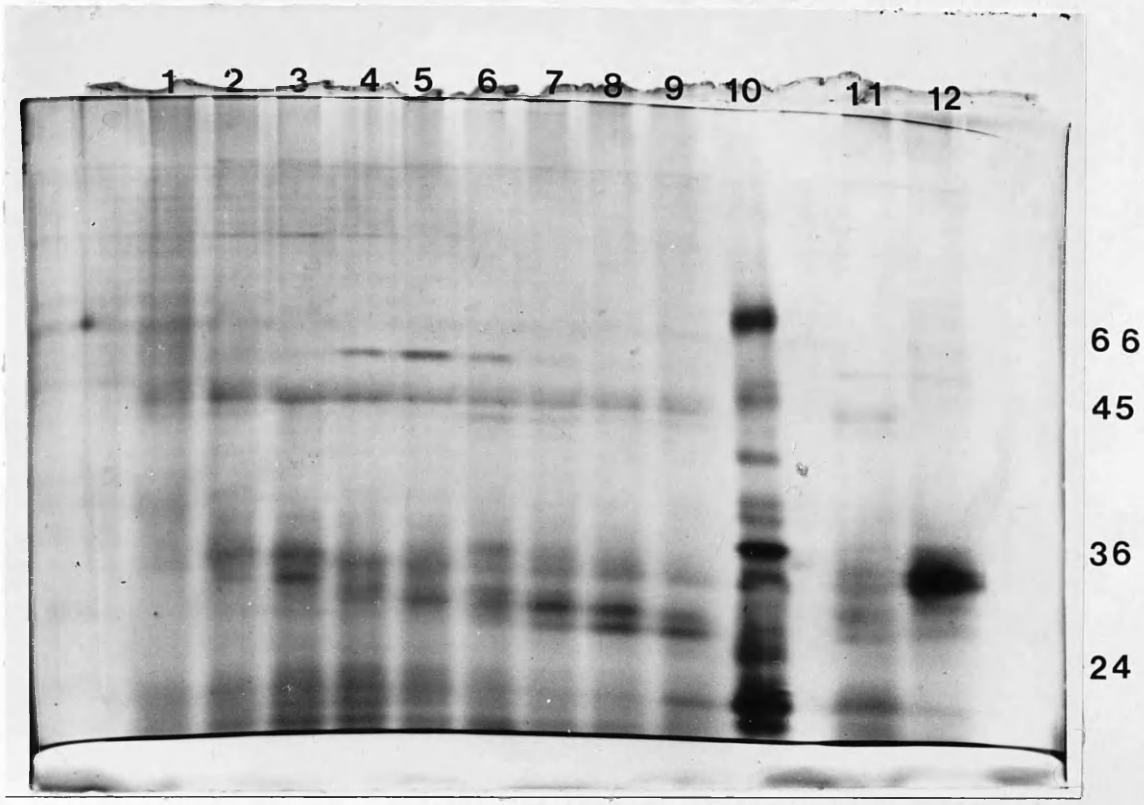
Ion exchange chromatography of the low molecular weight proteinases, separated by gel filtration, was carried out using a mono Q column. The protein profile, Measured as A_{280} (—), the distribution of proteinase activity towards BzPPANan (-●-) and the NaCl gradient (0-1 M,·—·) in the eluant from the column is shown. The AUFS is 0.2.

Figure 38. The distribution of low molecular weight proteinases of *L.m.mexicana* amastigotes, separated by ion exchange chromatography and detected using 10% acrylamide gelatin SDS-PAGE gels



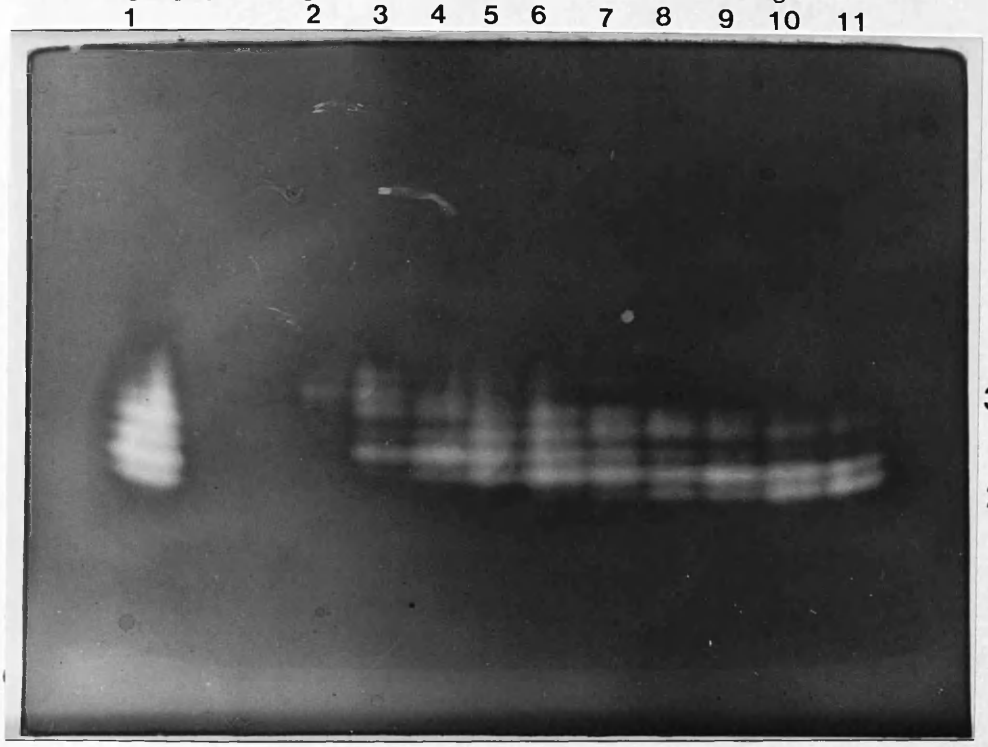
Samples (75 µl) from fractions 15-22 (lanes 1-8) and the pooled fractions (lane 9) of the ion exchange eluant, were run on a 10% acrylamide gelatin SDS-PAGE gel and the activity developed, after washing in 2.5% (v/v) Triton X100 for 1 hour, by incubation in 0.1 M acetate buffer at 37°C, pH 5.5. for 1 hour in the presence of 1 mM DTT. Approximate molecular weights in kDa are given on the right hand side.

Figure 39. The protein bands detected using silver staining, in the fractions of the eluant from the ion exchange chromatography containing proteinase activity



Samples (75 µl) from fractions 14-22 (lanes 1-9) of the ion exchange eluant, were run on a 10% acrylamide SDS-PAGE gel and silver stained to detect the protein banding patterns. Lane 10 shows low molecular weight markers whilst samples of the pooled fractions were run in lanes 11 and 12; that run in lane 12 had been boiled for 2 minutes. Approximate molecular weights in kDa are given on the right hand side.

Figure 40. The distribution of low molecular weight proteinases of *L.m.mexicana* amastigotes in fractions separated by ion exchange chromatography using a shallower than usual salt gradient



Samples (75 µl) from fractions 15-24 (lanes 2-11) and the pooled fractions 15-24 of the ion exchange eluant, were run on a 10% acrylamide gelatin SDS-PAGE gel and the activity developed, after washing in 2.5% (v/v) Triton X100 for 1 hour, by incubation in 0.1 M acetate buffer at 37°C, pH 5.5. for 1 hour in the presence of 1 mM DTT. Approximate molecular weights, in kDa, are given on the right hand side.

the point where these proteinases are normally eluted (see section 2.10.3.). Analysis of the proteinases in the fractions by gelatin SDS-PAGE showed that a slightly better separation was achieved (see fig. 40); although whether this could lead to the purification of individual isoenzymes remains an open question.

The fractions eluted using the standard ion-exchange chromatography procedure and which contained proteinase activity varied slightly between experiments but were always within the range 14-24. It was these pooled fractions, concentrated as necessary, that were used as the purified proteinase preperation for subsequent studies including enzyme characterization. The overall enzyme recovery from the ion exchange chromatography step was 35%. The average specific activity was 1511 nmol min^{-1} mg $protein^{-1}$, an approximate 8.5 increase compared to the homogenate (see table 13).

3.8.2. The effect of different detection methods and boiling on the protein banding patterns obtained for the purified proteinases preparation

The results reported by Pupkis and Coombs (1984) showed a single protein in the purified proteinase preperation which was obtained using very similar procedures to those reported here. They used comassie blue to stain for protein, however, which is a much less sensitive protein detection method than silver staining. When comassie blue staining was used to detect the proteins in the purified enzyme preparation which had not been boiled, separated by SDS-PAGE, no protein bands could be detected. In contrast, boiling of the sample before electrophoresis led to the detection of a faint single band at 31 kDa. When the same sample was silver stained, the banding pattern shown in figure 39 was observed. On this 10% acrylamide gel, two bands, corresponding with two of the proteinase bands, become much

more prominent. This would probably account for the detection of a single band on 7.5% acrylamide gels using comassie blue as found here and reported by Pupkis and Coombs (1984). The explanation for these changes was not investigated further.

3.8.3. Characterization of the amastigote low molecular weight proteinases

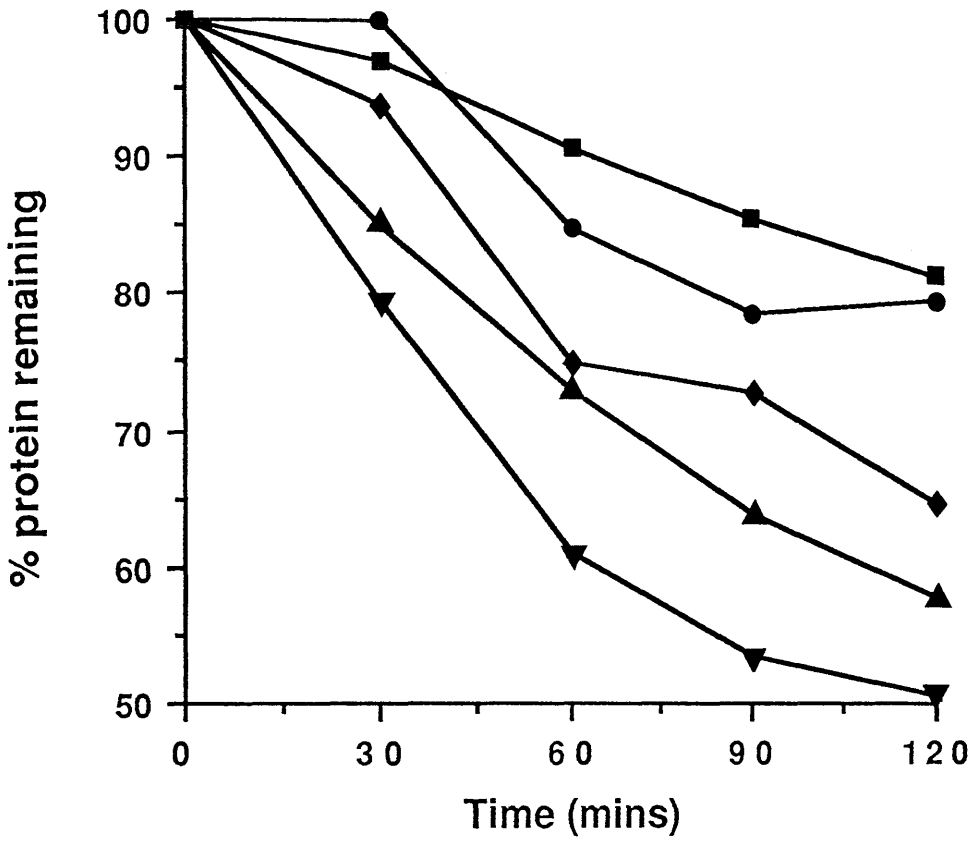
3.8.3.1. Sensitivity to inhibitors

The hydrolysis of *L.m.mexicana* amastigote proteins in detergent-lysed homogenates was investigated by measuring the amount of detectable protein, using the method of Sedmak and Grossberg (see section 2.11). This was carried out to quantify the wholesale protein breakdown first reported by Pupkis and Coombs (1984, 1986) and to investigate the enzymes involved in this auto-hydrolysis. The effect of several proteinase inhibitors on this degredation was also studied. Homogenates were prepared as described in section 2.2.3., the appropriate inhibitor added and incubation at room temperature instigated. Protein content of samples, taken every 30 minutes for 2 hours, was estimated. The specific activity of different samples towards BzPPANan were also determined at several time points.

The results, presented in figure 41, show that proteolysis occurred rapidly. An approximate half life of 90 minutes can be calculated from this data. Several proteinase inhibitors considerably reduced the rate of proteolysis with leupeptin having the greatest effect of those tested. The specific activities of samples towards BzPPANan increased concomitantly with proteolysis occurring, as is shown in table 14. This would indicate that the proteinases which account for the BzPPANan activity are being hydrolysed at a slow rate if at all compared to the other proteins in the sample.

The proteinase inhibitors chymostatin, leupeptin, antipain and

Figure 41. The effect of proteinase inhibitors on the auto-degredation of Triton X100-lysed *L.m.mexicana* amastigotes



The rates of proteolysis in homogenates of *L.m.mexicana* amastigotes in the absence (▼) or presence of (at 100 µg ml^{-1}) E64 (▲), antipain (◆), chymostatin (●) and leupeptin (■), was monitored by taking 10 µl aliquots every 30 minutes for 120 minutes and measuring the amount of protein. These are the results from a single experiment.

Table 14. The effect of proteinase inhibitors on proteolysis in *L.m.mexicana* amastigote homogenates

		Activity towards BzPPANan ($nmol^{-1}$ min^{-1} mg $protein^{-1}$)			
Time	Lysate	E64	Antipain	Leupeptin	Chymostatin
0	163	-	-	-	-
30	207	7	0	6	10
60	252	8	2	11	14
90	312	-	-	-	-
120	293	13	2	11	14

Lysates were pre-incubated with the inhibitors (100µg ml^{-1}) for 30 minutes at room temperature whilst the lysate without inhibitor was kept on ice. The experiment involving measurements of the specific activities towards BzPPANan commenced at time 0. Two 10 µl aliquots were taken from the samples at the different time points, one was used to measure the amount of protein in the sample and the other to determine the activity of the sample towards BzPPANan.

E64 all totally inhibited the purified enzymes if used at 25 μg ml^{-1} with a 30 minute pre-incubation at 37°C. Other inhibitors were also tested against the purified enzymes, with a 1 hour pre-incubation at room temperature with the sample before measuring its activity towards BzPPANan. The results are given in table 15. These inhibitor studies confirmed that the purified enzymes were of the cysteine proteinase type. This is consistent with the inhibition of the activity by TPCK, TLCK and IAA (see section 2.13.1.). The inhibitors phenanthroline, pepstatin and EDTA (which inhibit metal, aspartic and serine proteinases, respectively) had no effect on the hydrolysis of the substrate by the purified sample. The effect of DMSO and ethanol on the hydrolysis of BzPPANan by the purified enzymes was also quantified, a 32% inhibition of activity was achieved with a 5% DMSO concentration and a 46% inhibition with 10% ethanol under the above conditions.

The sensitivity to inhibition of the amastigote low molecular weight proteinases by the specific inhibitor of cysteine proteinases, E64, was also investigated using gelatin gels. Samples of amastigote homogenates were incubated with leupeptin, antipain, chymostatin or E64 (all at 100 μg ml^{-1}) for 30 minutes at room temperature and then were subjected to electrophoresis using a gelatin 7.5% acrylamide gel under normal conditions and the proteinase activity developed without the presence of inhibitors in either the Triton X100 solution or the acetate buffer. The results are shown in figure 42. Only E64 resulted in inhibition of the low molecular weight proteinases, none of the other inhibitors used had any observable effect. The high molecular weight proteinases appeared unaffected by any of the inhibitors.

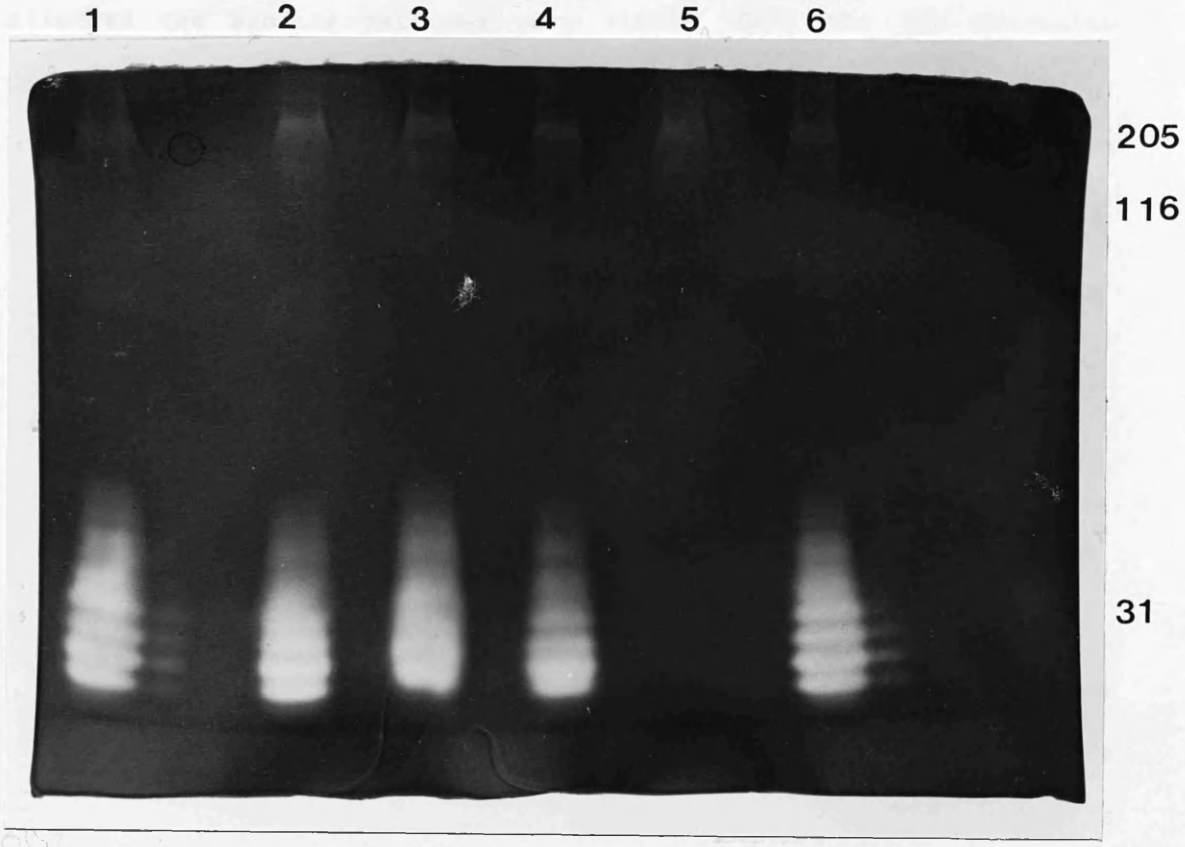
This procedure was also used to investigate the inhibitor sensitivity of proteinases of other stages and species. The use of 5%

Table 15. Sensitivity of the amastigote low molecular weight proteinases to inhibitors

Inhibitor	Specificity	% Inhibition
Phenanthroline	metal/slight cysteine	0
Pepstatin	aspartite	0
TPCK	cysteine/serine	87 ± 6
TLCK	cysteine/serine	96 ± 3
EDTA	metal	0
IAA	cysteine	79 ± 12

Samples of the purified amastigote low molecular weight proteinases were incubated for 1 hour at room temperature with the appropriate inhibitor. All the inhibitors were at a final concentration of 1 mM except pepstatin which was at 100 μg ml^{-1}. After pre-incubation the rates of hydrolysis of BzPPANan by the different samples at 37°C were determined, and compared to that of a control. Each inhibitor was tested a minimum of two times and the results given are the means ± standard deviations or ranges.

Figure 42. The effect of proteinase inhibitors on the detection of *L.m.mexicana* proteinases on gelatin SDS-PAGE gels



Lysates of *L.m.mexicana* amastigotes were pre-incubated with the inhibitors (leupeptin, lane 2; antipain, lane 3; chymostatin, lane 4; E64, lane 5) for 30 minutes, at room temperature, and 20 µl samples were then run on a 10% acrylamide gelatin SDS-PAGE gel and compared to a control homogenate (lanes 1 and 6). The proteinase activity was developed, after washing in 2.5% (v/v) Triton X100 for 1 hour, by incubation in 0.1 M acetate buffer at 37°C, pH 5.5. for 1 hour in the presence of 1 mM DTT. Approximate molecular weights, in kDa, are given on the right hand side.

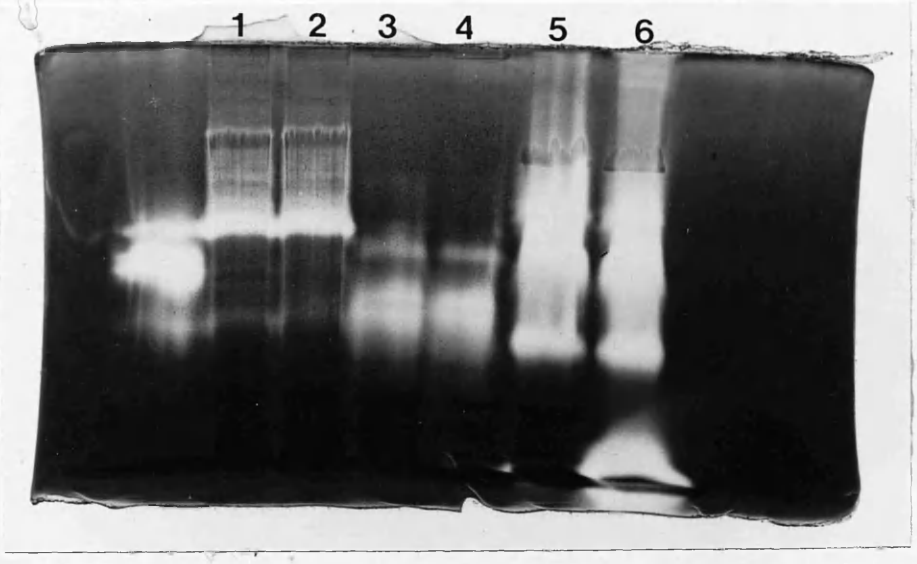
acrylamide gels allowed reasonable separation of the proteinases of *L.donovani*, *L.major* and *L.m.mexicana*, as shown in figure 43. Pre-incubation of the samples with E64 for 30 minutes at room temperature affected the banding patterns very little. Only the low molecular weight cysteine proteinases present in amastigotes of *L.m.mexicana* were clearly inhibited. If in addition to pre-incubation E64 was included in the post-electrophoresis incubation media partial inhibition of some of the higher molecular weight proteinase bands was obvious. Because of the expense of E64 only very small volumes of incubation media were used, this appeared to affect the development of the bands of proteinase activity giving poor definition and results that were not worth photographing.

3.8.3.2. Substrate competition studies

The effects of various amino acid esters and amino acid amides upon the hydrolysis of BzPPANan by the purified enzymes were determined in an attempt to characterize the substrate specificities of these enzymes and to investigate the reported protective activity of certain amino acid esters and amino acid amides against the anti-leishmanial activity of other amino acids and amides. The conditions used were as described in section 2.9.5., the enzyme sample was added to the buffer containing the appropriate ester or amide wherupon the substrate (BzPPANan) was immediately added, allowing direct competition between the BzPPANan and the amides or esters.

The results of these studies are given in table 16. Those amides or esters tested which are derivatives of basic amino acids, i.e. arginine and lysine, were relatively poor competitors. Derivatives of aliphatic or aromatic amino acids were more effective competitors, for example leucine and tryptophan (see fig. 44). There was little detectable difference in the % inhibition caused by methyl esters and

Figure 43. The effect of E64 on the detection of proteinases of different species of *Leishmania* amastigotes



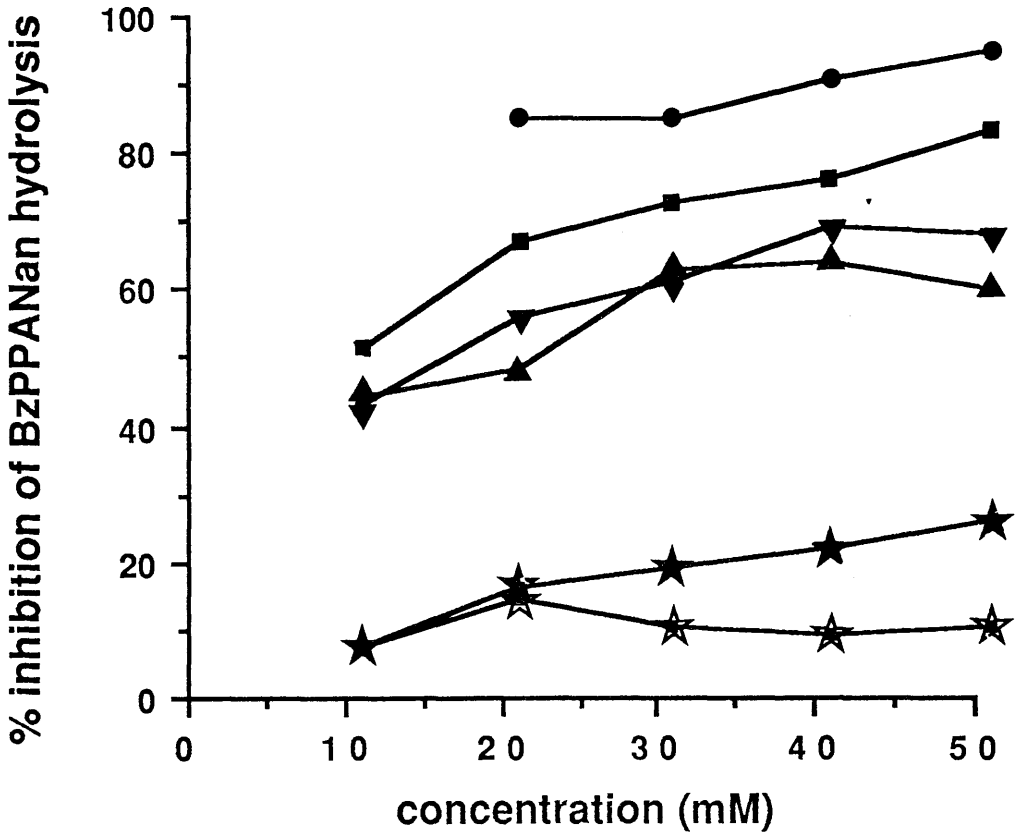
Lysates of *L.donovani*, *L.major* and *L.m.mexicana* amastigotes with 100 µg E64 ml^{-1} (lanes 1, 3 and 5, respectively) or without (lanes 2, 4 and 6, respectively) were pre-incubated for 30 minutes at room temperature before being run on a 5% acrylamide gelatin SDS-PAGE mini gel. The proteinase activity was developed, after washing in 2.5% (v/v) Triton X100 for 30 minutes, by incubation in 0.1 M acetate buffer at 37°C, pH 5.5. for 30 minutes in the presence of 1 mM DTT. Approximate molecular weights, in kDa, are given on the right hand side.

Table 16. The effect of amino acid esters and amino acid amides on the hydrolysis of BzPPANan by the amastigote low molecular weight proteinases

Amino acid ester or amide	Concentration of esters and amides (mM) 50	40	30	20	10
Leucine methyl ester	67 ± 10	67 ± 10	59 ± 8	54 ± 6	41 ± 8
Leucinamide	64 ± 6	58 ± 9	54 ± 8	42 ± 14	34 ± 9
Leucine benzyl ester	93	89	83	83	-
Glycine benzyl ester	81 ± 2	74 ± 1	70 ± 1	65 ± 1	49 ± 1
Tryptophan methyl ester	69 ± 8	67 ± 8	55 ± 8	48 ± 4	29 ± 8
Tryptophanamide	68 ± 8	64 ± 9	60 ± 10	53 ± 8	40 ± 9
Isoleucinamide	58	62	61	46	42
Isoleucine methyl ester	61 ± 7	59 ± 9	51 ± 5	43 ± 9	25 ± 1
Arginamide	24 ± 17	20 ± 6	17 ± 7	14 ± 1	35 ± 4
Arginine methyl ester	15 ± 6	21 ± 8	12 ± 12	8 ± 11	6 ± 4
Lysine methyl ester	8 ± 4	7 ± 8	8 ± 8	12 ± 4	5 ± 4

These studies were carried out by comparing the rates of hydrolysis of BzPPANan by the purified enzymes in the presence and absence of different concentrations of esters and amides. Most experiments were carried out 3 times, those for which no standard deviation is given were carried out once or twice. The results are means and standard deviations expressed as the % inhibition of BzPPANan hydrolysis compared to that of the control over the same time period. For leucine benzyl ester the controls took into account the effect of the DMSO that the compound was initially dissolved in.

Figure 44. The effect of different amino acid esters and amides on the hydrolysis of BzPPANan by the *L.m.mexicana* purified proteinases



Direct competition studies using arginamide (★), lysine methyl ester (★), leucine methyl ester (▼), isoleucinamide (▲), glycine benzyl ester (■), and leucine benzyl ester (●) were carried out by comparing the rates of hydrolysis of BzPPANan by the purified enzymes in the presence and absence of different concentrations of these compounds. The results are expressed as the % inhibition of BZPPANan hydrolysis compared to that of a control over the same time period and is based on the data presented in table 15.

amides of the same amino acid. The benzyl ester of leucine, however, was a considerably better competitor than leuOMe or leucinamide.

3.8.4. Hydrolysis of amino acid esters and amino acid amides by *Leishmania* extracts

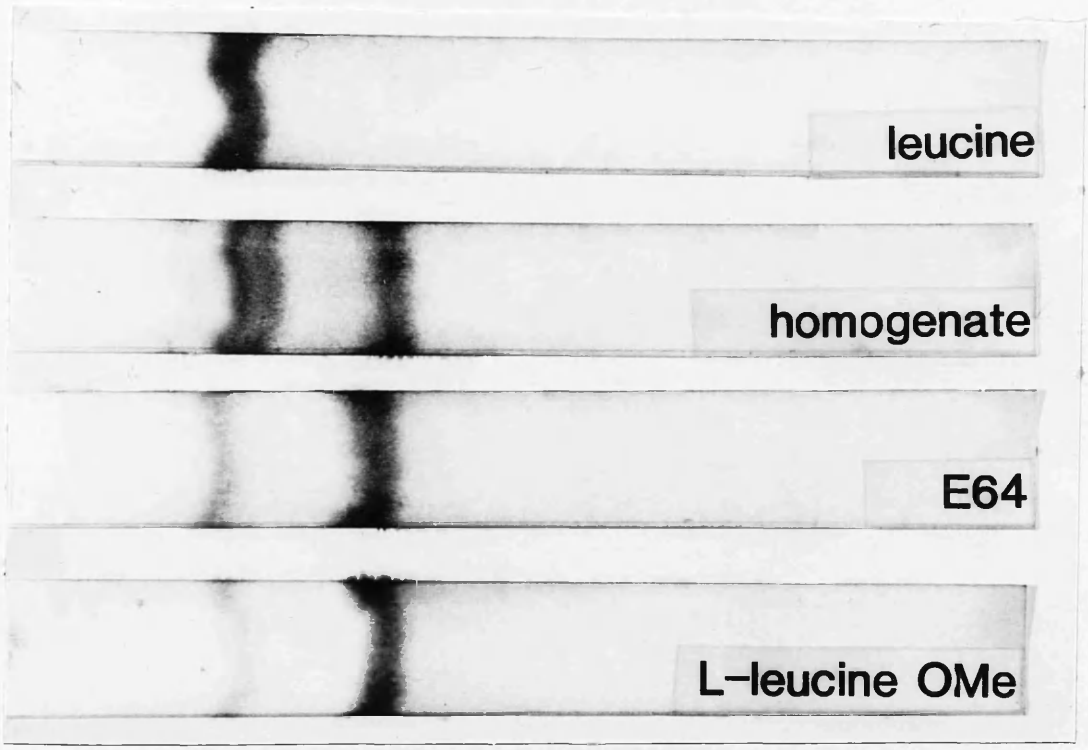
3.8.4.1. Kinetics of hydrolysis of L-leucine methyl ester by the purified proteinases of *L.m.mexicana*

The hydrolysis of leuOMe by different species and stages of *Leishmania* was followed using paper electrophoresis to separate the products from hydrolysis of the amino acid ester. Figure 45 shows typical results. L-leucine methyl ester and leucine were clearly separated, also the dipeptide leucyl-leucine was distinguishable from leucine itself. Use of a range of concentrations of leucine showed that the method could be used quantitatively (see fig. 46) by eluting the leucine after electrophoresis. A standard curve was used to quantify the hydrolysis products of leuOMe separated by paper electrophoresis. The lower limit of detection was taken as 1.25 mM; i.e. 0.375 μmoles 0.3 ml^{-1}.

To optimise the conditions for the measurement of the hydrolysis of leuOMe, the length of time over which the reaction was linear and the pH optimum for the reaction had to be ascertained. To standardise the amount of enzyme being used, the amount of sample that would cause a change in absorbance of 0.1 per minute by hydrolysing BzPPANan, under the standard conditions described in section 2.10.1., was used. This, was for convenience, defined as 1 unit of enzyme activity.

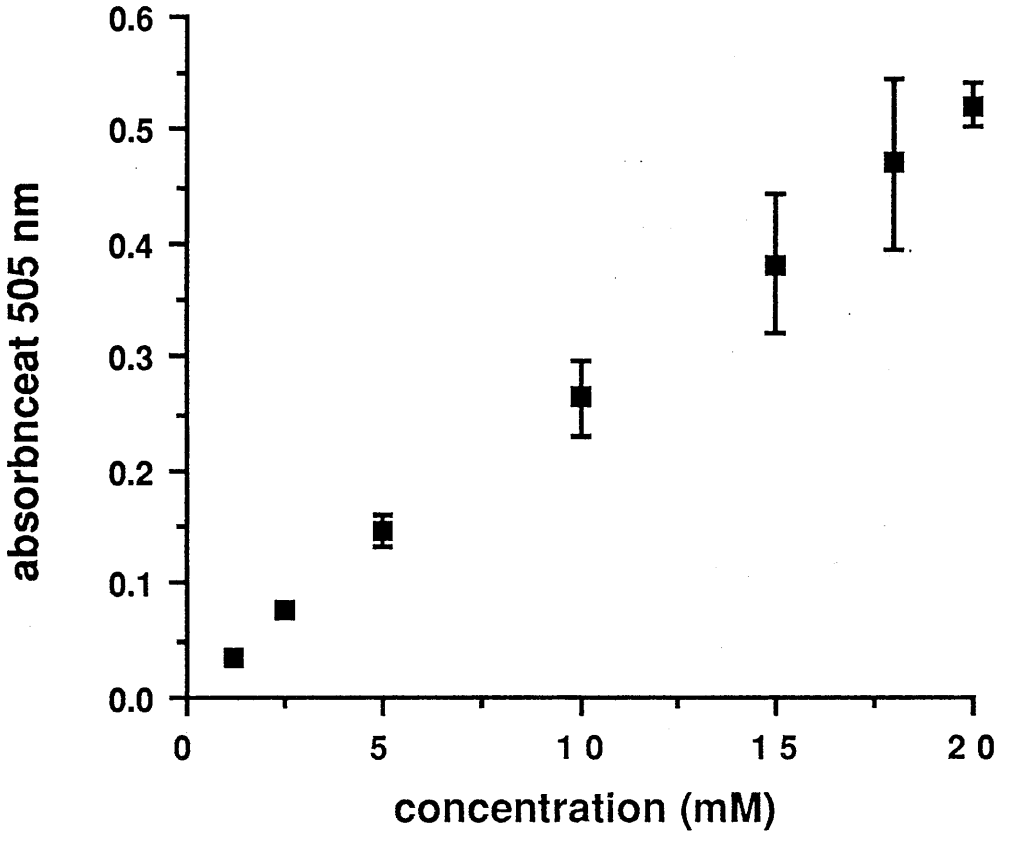
The course of hydrolysis was followed using a purified enzyme sample under the conditions described previously (see section 2.13.3.) over a three hour period at pH 6.0. The results (see table 17) indicate that the reaction under these conditions and over this incubation period was approximately linear. Consequently a three hour incubation under these conditions was used as the standard for all

Figure 45. Separation and detection of the hydrolysis products of leucine methyl ester



Samples of homogenates of *L.m.mexicana* amastigotes, produced by Triton X100 lysis, were incubated in 0.15 M phosphate buffer, pH 6.0, with 20 mM leuOMe in the presence of 1 mM DTT with or without 100 µg E64 ml^{-1} for 3 hours at 37°C. Controls of 20 mM leucine and 20 mM leuOMe were also incubated without any lysates. 40 µl aliquots were then applied to paper strips and the hydrolysis products separated by electrophoresis at 8 V cm^{-1} for 3 hours. The hydrolysis products were visualised by immersion in 0.2% ninhydrin in 75% acetone.

Figure 46. Standard curve for the detection of L-leucine by paper electrophoresis



Concentrations of leucine from 1.25 mM to 20 mM were made up in 0.15 M phosphate buffer, pH 6.0, and 40 µl aliquots were then applied to paper strips which were subjected to electrophoresis at 8 V cm^{-1} for 3 hours. The hydrolysis products were visualised by immersion in 0.2% ninhydrin in 75% acetone. The area that contained the leucine was eluted for 30 minutes and the absorbance measured at 505 nm. These results are the means ± standard deviations from 3-4 experiments.

Table 17. Hydrolysis of L-leucine methyl ester over 3 hours by the *L.m.mexicana* amastigote low molecular weight purified enzymes

Time	Absorbance	Activity towards leuOMe (nmoles min^{-1} mg protein)
30	(N.D.)	-
60	(N.D.)	-
90	0.042 ± 0.006	1383 ± 192
120	0.056 ± 0.008	1372 ± 196
150	0.065 ± 0.002	1284 ± 49
180	0.079 ± 0.004	1290 ± 65

Hydrolysis of leuOMe by the purified amastigote enzymes was followed over a 3 hour incubation at 37°C. The same enzyme activity towards BzPPANan (1 unit) was used in each case and incubated with 20 mM leuOMe and 1 mM DTT in a total volume of 0.3 ml in 0.15 M sodium phosphate buffer, pH 6.0. Hydrolysis products were separated using paper electrophoresis and quantified by elution. Paper electrophoresis was performed immediately at the appropriate time points. The lower limits of detection of leucine was taken as 1.25 µM (0.375 µmoles 0.3 ml^{-1}) and levels of leucine below this were taken as not detectable (N.D.). The figures are the means ± standard deviations or ranges from 2-3 experiments.

subsequent work.

The pH optimum for the purified enzymes with BzPPANan as substrate was between pH 7-8, however with leuOMe as the substrate there appeared to be a sharp peak of activity at pH 6.0. (see table 18). This differs from that found for BzPPANan but agrees with that reported by Pupkis and Coombs (1984) for the pH optimum of the activity of these cysteine proteinases towards azocaesin.

Using these standard conditions, the specific activities of different samples towards leuOMe were determined. A comparison of the activities towards leuOMe of the purified sample and homogenate shows an approximate 10-fold increase in specific activity, from 97 to 1290 μmoles min^{-1} mg $protein^{-1}$. This is similar to the increase in specific activity towards BzPPNan on purification of the low molecular weight proteinases (see table 13).

3.8.4.2. Hydrolysis of various amino acid esters and amino acid amides by different species and stages of *Leishmania* as well as the purified enzymes from *L.m.mexicana* amastigotes

The activities of different forms and species of *Leishmania* towards leuOMe and BzPPANan were measured using Triton X100 solubilised homogenates. This was carried out to determine if there was a correlation between the species and stage specificity of the antileishmanial activity of leuOMe (see section 3.5.) and the ability of these same species and stages to hydrolyse leuOMe and BzPPANan. The results are given in table 19. They demonstrated that the promastigotes of all species investigated had lower activities towards leuOMe and BzPPANan than the amastigotes of the same species. Of the amastigotes tested, *L.m.mexicana* had by far the highest activity towards leuOMe.

The activities of lysates of amastigotes of *L.donovani*, *L.major* and *L.m.mexicana* activities towards BzPPANan were inhibited 92%, 96%

Table 18. pH optima for the hydrolysis of BzPPANan and L-leucine methyl ester by the amastigote purified proteinases

pH	Activity towards leuOMe (nmoles min^{-1} mg $protein^{-1}$)	Activity towards BzPPANan
3	N.D.	59 ± 14
4	N.D.	250 ± 132
5	N.D.	403 ± 25
6	1354 ± 364	783 + 44
7	624 ± 263	1347 ± 15
8	829 ± 329	1448 ± 188

Hydrolysis of leuOMe by the purified amastigote enzymes was followed over a 3 hour incubation at 37°C. The same enzyme activity towards BzPPANan (1 unit) was used in each case and incubated with 20 mM leuOMe and 1 mM DTT in a total volume of 0.3 ml in the appropriate buffer. Hydrolysis products were separated using paper electrophoresis and quantified by elution. The lower limits of detection of leucine was taken as 1.25 mM (0.375 μmoles 0.3 ml^{-1}) and levels of leucine below this were taken as not detectable (N.D.). The specific activity towards BzPPANan was calculated by monitoring the change in absorbance at 505 nm of a sample in 1.2 ml buffer containing 1 mM DTT and 0.1 mM BzPPANan. The figures are the means ± standard deviations or ranges from 2-3 experiments. The buffers used were citric acid buffer (0.1 M) between pHs 3-6 and sodium phosphate buffer (0.1 M) between pHs 6-8.

Table 19. Rates of hydrolysis of L-leucine methyl ester and BzPPANan by homogenates of different species and stages of *Leishmania*

Species/stage	Activity towards leuOMe	Activity towards BzPPANan
L.major promastigotes	7.6 ± 4.5	12 ± 7
L.major amastigotes	41.6 ± 9.6	170 ± 30
L.donovani promastigotes	9.4	14 ± 7
L.donovani amastigotes	32.7 ± 10	69 ± 6
L.m.mexicana promastigotes	8.8	14 ± 7
L.m.mexicana amastigotes	97.2	175 ± 35

The activities of Triton X100 lysates towards leuOMe was measured over a 3 hour incubation at 37°C. The same enzyme activity towards BzPPANan (1 unit) was used in each case and incubated with 20 mM leuOMe and 1 mM DTT in a total volume of 0.3 ml in 0.15 M sodium phosphate buffer, pH 6.0. Hydrolysis products were separated using paper electrophoresis and quantified by elution. The specific activity towards BzPPANan was calculated by monitoring the change in absorbance at 505 nm of a sample in 1.2 ml phosphate buffer, pH 6.0, containing 1 mM DTT and 0.1 mM BzPPANan. The figures are the means ± standard deviations from 3-4 determinations. Those without standard deviations are from 2 determinations. The promastigotes used are all from stationary phase cultures and the *L.major* promastigotes were obtained using PNA agglutination to give a population of metacyclics.

and 94% respectively, by the specific inhibitor of cysteine proteinases, E64 (100 μg ml^{-1}). This would indcate that there are cysteine proteinases present in both *L.major* and *L.donovani* which are responsible for the majority of these samples activity against BzPPANan; but that other enzymes are also hydrolysing the BzPPANan in the amastigotes of all three species.

The effect of inhibitors on the hydrolysis of leuOMe by lysates of amastigotes of *L.donovani* and *L.m.mexicana* as well as the purified enzymes from *L.m.mexicana* amastigotes were studied. The results are shown in table 20. The addition of E64 to *L.m.mexicana* amastigote homogenates inhibited the hydrolysis of leuOMe by 82%, while its use with the purified enzyme gave total inhibition. The proteinase inhibitors antipain and chymostatin also totally inhibited the activity of the purified enzyme while they gave a 91% and 62% inhibition, respectively, of *L.donovani*'s activity towards leuOMe. The absence of DTT resulted in a reduction of greater than 30% for the *L.donovani* and *L.m.mexicana* amastigote lysates and the purified enzymes.

Paper electrophoresis was also used to look qualitatively at the hydrolysis of a range of amino acid esters and amino acid amides by lysates of *L.donovani* and *L.m.mexicana* amastigotes as well as the purified proteinases of *L.m.mexicana* amastigotes. The results of these studies are summarised in table 21. All of the compounds tested with *L.m.mexicana* amastigote lysates were hydrolysed to some extent, isoleucine methyl ester being the poorest substrate. With *L.donovani* lysates there was extensive hydrolyis of some of the compounds tested, for example leucine methyl ester and leucinamide. However many of the other compounds were hydrolysed poorly compared with their hydrolysis by *L.m.mexicana*. Using the purified amastigote proteinases, only leuOMe and leucinamide were hydrolysed detectably, although these were

Table 20. The effect of inhibitors and DTT on the hydrolysis of L-leucine methyl ester by homogenates of *L.donovani* and *L.m.mexicana* amastigotes and the low molecular weight proteinases purified from *L.m.mexicana* amastigotes

	% Inhibition		
Inhibitor	*L.m.mexicana*	*L.donovani*	Purified enzyme
E64	82 ± 11	-	100
Chymostatin	-	62 ± 15	100
Antipain	-	91 ± 9	100
No DTT	56 ± 25	35 ± 11	34 ± 12

Hydrolysis of leuOMe was measured over a 3 hour incubation at 37°C. The same enzyme activity towards BzPPANan (1 unit) was used in each case and incubated with 20 mM leuOMe and 1 mM DTT in a total volume of 0.3 ml in 0.15 M sodium phosphate buffer, pH 6.0. Hydrolysis products were separated using paper electrophoresis and quantified by elution. The Triton X100 lysates and the purified enzymes were pre-incubated for 30 minutes with the different inhibitors at 100 $\mu g\ ml^{-1}$ at room temperature in a volume of 0.27 ml before the addition of leuOMe to give a final volume of 0.3 ml. The effect of the inhibitors or the exclusion of DTT was compared to a control under standard conditions. The results are means from two experiments ± the ranges, except with E64, which was carried out 3 times and the standard deviations are given. All of the results show the % inhibition compared with the control under standard conditions.

Table 21. Qualitative assessment of the extent of hydrolysis of different amino acid esters and amino acid amides by homogenates of *L.m.mexicana* and *L.donovani* amastigotes and the low molecular weight proteinases purified from *L.m.mexicana* amastigotes

Compound	*L.m.mexicana*	*L.donovani*	Purified enzyme
Leucine methyl ester	++++	+++	++++
Leucinamide	+++	+++	+++
Tryptophanmethyl ester	++	+++	-
Tryptophanamide	+++	++	-
Tyrosine methyl ester	+++	+-	-
Arginine methyl ester	++	++	-
Arginamide	++	+	-
Lysine methyl ester	++	++	-
Isoleucine methyl ester	++	-	-
Isoleucinamide	+	+	-
Glycine benzyl ester	++	+-	-

Hydrolysis of the different amino acid esters and amides by Triton X100 amastigote lysates or the purified enzymes was measured using a 3 hour incubation at 37°C. The same enzyme activity towards BzPPANan (1 unit) was used in each case and incubated with 20 mM leuOMe and 1 mM DTT in a total volume of 0.3 ml in 0.15 M sodium phosphate buffer, pH 6.0. Hydrolysis products were separated using paper electrophoresis and visualised by immersion in 0.2% ninhydrin in 75% acetone. Controls were carried out to estimate the amount of free amino acids in each sample; this tended to be minimal under these conditions. The extent of ester or amide hydrolysis was scored qualitatively in comparison to the hydrolysis products produced with leuOMe (++++), (-) represents no more hydrolysis products than found in the control. These are the results from 3 determinations.

broken down at a comparable rate as with amastigote lysates.

3.8.5. Studies on the relationship between the low molecular weight cysteine proteinases of *L.m.mexicana* amastigotes

Schiff's stain (see section 2.12) was used in an attempt to detect glycosylated proteins on SDS-PAGE gels. When the preperation of purified *L.m.mexicana* amastigote proteinases was used several faint pink bands were observed in a similar position to where the low molecular weight proteinases were detected using gelatin gels. This indicated that these proteinases are probably glycosylated. Similar bands were also detected when lysates of *L.m.mexicana* amastigotes were used as were two higher molecular weight bands of approximately 60 and 80 kDa. Fetuin and ovalbumin were used as positive controls, several intense bands occured with both. The nature of the staining did not lend itself to photographing.

Endoglycosidase F (see section 2.13.5.) was used in an attempt to deglycosylate the low molecular weight proteinases to determine if the multiple isoenzymes were caused by differing glycosylation. Treated and control samples were run on both standard and gelatin 10% acrylamide SDS-PAGE gels to investigate if the banding patterns of the purified proteinases changed after treatment with endoglycosidase F. These were then either silver or Schiff stained or developed for proteinase activity where appropriate. The results suggested that there were no apparent effect of the endoglycosidase F on the purified *L.m.mexicana* proteinases. Unfortunately, only limited amounts of endoglycosidase F were available which prevented the use of positive controls, using for instance, fetuin or ovalbumin.

DISCUSSION

4.1. DRUG DELIVERY SYSTEMS IN THE TREATMENT OF LEISHMANIASIS

4.1.1. Vesicular systems for the delivery of sodium stibogluconate in the treatment of experimental leishmaniasis

The results have shown that the vesicular systems increase the efficacy of sodium stibogluconate approximately 10 fold, as compared to the free drug. The high efficacy of several drugs in liposomal form against visceral leishmaniasis in animal models has been well established (Black *et al.*, 1977; Alving *et al.*, 1978a,b; New *et al.*, 1978; Alving *et al.*, 1980; New *et al.*, 1981a,b; Berman *et al.*, 1986b). This work has been recently reviewed by Alving (1986) and Croft (1988). In this model system the responses to free and carrier forms of sodium stibogluconate have been closely defined and so the relative efficacies of the vesicular formulations have been established. Surprisingly, however, the previously reported several 100 fold increase in drug efficacy brought about by the use of liposomes has not been achieved.

There are considerable differences in the methodologies previously used to investigate the use of liposomes to treat experimental leishmaniasis. Important variables include the size of the infective inoculum and hence challenge to the host, route of administration, volume inoculated, the timing of the treatment in relation to the infection and the strain of parasite and host animal used. These methodological differences may serve to explain, partially at least, the observed differences between these results and those reported by other workers. However, if the activities of the liposomal preparations are compared directly it is clear that these results are similar to those previously reported. Examination of the data of Black *et al.*, (1977), Alving *et al.*, (1978) and New *et al.*, (1978) shows that all of their liposomal preparations were active in the range 1 to 16 mg Sb kg^{-1}. For the 20 gram animals used in this study, this is

equivalent to 20 to 320 μg Sb per mouse and thus is similar to the activities found for the liposomal and niosomal preparations used here. This implies that the efficacies of the different vesicular systems reported in the literature are similar to those used here, but that the free drug used in this study had a higher efficacy than those reported by other workers. Whether this is due to the methodological differences mentioned earlier or to the reported differences between batches of sodium stibogluconate (Berman and Grogl, 1988) needs further investigation.

The effect of vesicle cholesterol content on the efficacy of niosomes appeared to differ between surfacants I and II. Cholesterol condenses the phospholipid bilayers and increases the stability of the vesicles as well as reducing their permeabilty to entrapped solutes (Baillie *et al.*, 1985) and so may give the vesicles longer lives *in vivo*. There was an observed increase in efficacy with cholesterol content with surfactant II in the Sb dose range 10 to 80 μg Sb per mouse. At greater Sb doses there did not appear to be any differences between vesicles with different cholesterol contents, although at these Sb levels large reductions in liver parasite burdens were achieved so that differences between vesicles were harder to detect. There did not appear to be any affect of cholesterol content on the efficacy of vesicles composed of surfactant III vesicles although why these vesicles should differ in this way from the vesicles composed of surfactant I and II is unclear.

The inclusion of the negatively charged ampiphile DCP did not appear to affect the efficacy of the niosomes or liposomes tested. Alving *et al.* (1978b) had shown that liposome charge influenced the efficacy of liposomal meglumine antimonate. In those experiments a range of different types of vesicles was used and although some of the

results were contradictory it was generally found that neutral or negatively-charged liposomes were slightly more effective than positively-charged liposomes. Other workers (Juliano and Stamp, 1975; Tagesson *et al.*, 1977) have observed that negatively-charged vesicles are removed from the systemic circulation at a more rapid rate than neutral or positively-charged vesicles. This would indicate that although the manipulation of vesicle charge may affect the rate of clearance of the vesicles from the circulation it does not have a great affect on the drug effficacy and so has little effect on the drug delivering ability of the vesicles.

One interesting result from the *in vitro* studies that was that the susceptibility of both *L.m.mexicana* and *L.donovani*-infected PECs to the vesicular forms of the drug were broadly similar, whereas their susceptibilities to the free drug differed markedly; *L.donovani* was much more susceptible than *L.m.mexicana* to treatment with free sodium stibogluconate. The reasons for the vesicular treatments overcoming species differences in susceptibility are unknown, although the role of the vesicle itself, for example in causing macrophage activation, cannot be ruled out especially as empty vesicles caused a degree of parasite supression *in vivo*.

The *in vitro* results support those obtained *in vivo* in that no differences between the carrier functions of liposomes and niosomes could be discerned. The antiparasite activity of these formulations *in vitro* is evidence for endocytotic uptake of the entrapped drug into infected macrophages. Such uptake probably explains the effective targetting of these forms of the drug *in vivo*. The pivotal role of the lysosomal environment in the performance of the stibogluconate carrier is suggested by the ratio of antileishmanial activities, vesicular form : free form, observed between the *L.donovani* and *L.m.mexicana* systems. Whether this reflects differences between the conditions in

the respective parasitiphorous vacuoles is open to conjecture. Using neutron activation analysis to follow the distribution of antimony in uninfected mice we have shown (Hunter *et al.*, 1987) that liver antimony levels were 5-6 times higher at 24 hours post-treatment when the drug was given in vesicular form (niosomes or liposomes) than when free drug was used. However in other organs, such as the lungs and spleen, there were also differences in the Sb levels when vesicles were used for drug delivery. There were no detectable antimony levels in the lungs and spleen when the mouse was treated with lipososmal drug whilst considerable levels could be detected when niosomal drug was used. In this study, however, there were large differences in the sizes of the vesicle used; the mean niosome diameter was 333 nm whilst that of the liposomes was 860 nm. Vesicle size does appear to be important in the treatment of sites of infection other than the liver. Carter *et al.* (1988) have shown that liposomes, niosomes and free drug doses used to clear parasites from the liver of Balb/c mice had little effect on the parasites in the spleen and bone marrow. These workers also found that decreasing vesicle size had little affect on the parasites in the other sites. These workers have subsequently reported (Carter *et al.*, 1989) that multiple dosing with < 100 nm vesicles did have an effect on the other sites of infection. However, even multiple dosing with small vesicles did not clear the bone marrow although a degree of suppression was achieved.

The importance of vesicle size for the delivery of drugs to different sites is well known and Scherphof *et al.* (1983) have shown that vesicles larger than 100 nm cannot pass easily through the fenestrations of the liver's sinusoidal epithilium so making it more likely that they are phagocytosed by the Kupffer cells of the liver. Smaller vesicles have a greater likelihood of passing through these

fenestrations and so circulating to the spleen in appreciable numbers.

The data available so far is consistent with passive drug delivery to the infected liver. The similarity of both the efficacy and the tissue distribution data for the liposomes and niosomes, despite the differences in size, is consistent with passive vectoring of the vesicles to the liver. However whilst this passive vectoring is useful for the targetting of the liver it may prove to be a major difficulty if vesicles are to be used to treat other sites of infection. The use of techniques, such as hepatic blockade (Poznansky and Juliano, 1984), to bypass the liver may prove useful in the treatment of these other sites of infection.

4.1.2. The use of polyacryl starch microparticles as a delivery system for sodium stibogluconate in the treatment of experimental visceral leishmaniasis

The results obtained show that the polyacryl starch microparticles increase the efficacy *in vivo* of sodium stibogluconate and that there was a 10-fold and 100-fold increase in efficacy over that of the vesicular and free drug, respectively. Although the antimony tissue levels have not been determined following dosing with the microparticulate form of the drug, it seems reasonable to assume that higher levels of drug are attained in the infected RES using the carrier form of the drug than with the free drug. Laakso *et al.* (1986) have reported that there is a high uptake of this type of particle by the RES after i.v. administration. It seems likely that the microparticles are behaving in a similar manner to the vesicular systems in that they are probably acting as a passive vector and relying on the high phagocytic activity in the liver to remove them from the circulation.

Although the effect of microparticulate sodium stibogluconate in

the *in vitro* PEC system was not investigated Stjarnkvist, *et al.* (1987) have reported that in a similar *in vitro* system microparticulate primaquine was active against *L.donovani* promastigote initiated infections, although less so than the free drug. This indicates that the efficacy of carrier mediated therapy is not just dependent on uptake into the infected cell but also on the release of the drug so that it can affect the parasite.

If the vesicular systems and the polyacryl starch microparticles are both acting as passive vectors for sodium stibogluconate, and as it appears that it is the total Sb dose that is important and not the efficacy of bead loading, then the reason for the approximate 10-fold difference between the efficacy of these systems must be addressed. The pharmacokinetics at the cellular level may have to be considered and, as far as sodium stibogluconate's antileishmanial activity is concerned, the lysosome is the important organelle. Phagocytosis of the drug-loaded carrier exposes it to the hostile lysosomal environment. Destabilisation of the liposome or niosome bilayer in this environment will increase the efflux of drug from the vesicles which, although available for antiparasite activity, will tend to diffuse down the concentration gradient and out of the lysosome. This will limit exposure time of parasite to drug and the lower activity of drug encapsulated in niosomes lacking cholesterol, which would stabilise the vesicles, tends to support this idea. By contrast, microparticulate stibogluconate is covalently bound to the carrier rather than simply entrapped and although polyacryl starch is degraded by lysosomal enzymes, some degradation products of molecular weight $>$ 200 remain in the lysosomal system (Laakso *et al.*, 1986). Particulate material is excreted from the lysosomal compartment by exocytosis, but this is a slow process (Munniksma *et al.*, 1980). Thus even after the degradation of the microparticle some of the drug may remain

covalently linked to the degradation products. This fraction of the total drug, would be less liable than free drug to diffuse from the lysosomal compartment, thus maintaining effective drug concentrations in the lysosomal compartment for longer periods. Laakso *et al.* (1987) have followed the tissue elimination of microparticles of the type used here and observed that total elimination does not occur from mice livers for up to 24 weeks after injection. Whilst this may represent an obstacle to the use of polyacryl starch microparticles clinically, it does suggest that one of the advantages of this delivery system is that it prolongs drug-parasite contact.

One other element of targetting should also be considered. As most of the vesicular and particulate drug is sequestered into the liver, not necessarily into infected cells, the slow release of the drug over time would maintain levels of free drug for a longer period. This liver depot effect may also partially explain the differences in efficacy between the vesicular and microparticulate systems.

The ideal drug delivery system for sodium stibogluconate should correct its innate pharmacokinetic difficiencies (see section 1.4.3.) by optimising its uptake by target tissues and slowly releasing the drug *in situ*, thus maintaining effective concentrations for an appropriate period of time. The results discussed here suggest that solid particles, such as polyacryl microparticles, may match this ideal more closely than the vesicular systems. However, factors such as toxicity, manufacture, storage and cost of different carrier systems must also be considered and although efficacy is a prime consideration the ultimate development of such formulations for clinical use, not just in the treatment of leishmaniasis, will acknowledge all of these factors.

4.2. THE USE OF TUBERCIDIN IN CONJUNCTION WITH INHIBITORS OF NUCLEOSIDE TRANSPORT IN THE TREATMENT OF EXPERIMENTAL LEISHMANIASIS

The lack of activity of either dipyridamole or NBMPR in protecting promastigotes of *L.m.mexicana* or *L.donovani* against the toxicity of tubercidin is in agreement with the findings of Aronow *et al.* (1987), who reported that the two nucleoside transport systems of *L.donovani* promastigotes are not inhibited by either dipyridamole or NBMPR. These findings suggest that similar transport systems are present in the promastigotes of *L.m.mexicana* and supports the rationale behind this approach to the chemotherapy of leishmaniasis.

However infected and non-infected PECs were found to be protected from tubercidin by similar concentrations of the inibitors used (NBMPR and dipyridamole). These levels are similar to those previously reported to protect cells in culture against toxic levels of tubercidin (Paterson *et al.*, 1979; Gero *et al.*, 1988).

If infected cells were being targetted then, there should have been an increase in the percentage of uninfected cells, which was not obvious. There was little evidence for parasite clearance from infected cells, indicating that the tubercidin did not gain access to the parasite, at least at concentrations that would have an affect on the amastigote. Alternatively, the amastigote stage may have a nucleoside transport mechanism which differs from that of the promastigote and is closer to that of mammalian cells. If this is so, the parasite itself would be protected from the toxic effects of tubercidin by dipyridamole and NBMPR. The situation should be clarified by direct determination of the sensitivity of nucleoside uptake by amastigotes to the inhibitors.

The *in vivo* work confirms that NBMPR can be used to lessen the toxicity of tubercidin when treating infected animals; although this form of treatment proved more toxic than previously reported (Ogbunude

and Ikediobi, 1982). However, there was little if any reduction in the parasite burden when this form of treatment was used, in contrast to the 80 % suppression achieved in the single surviving mouse treated with free tubercidin.

These results thus indicate that there do not appear to be changes in the mechanism of nucleoside transport when PECs are infected with leishmanias. Thus, the results of this study indicate that although the toxicity of tubercidin to the host can be overcome by the use of these inhibitors this approach to the chemotherapy of the leishmanias may not be fruitful. The major problem associated with this approach is the delivery of tubercidin, in high enough concentrations to kill the parasites, specifically to the parasitophorous vacuole. The use of drug delivery systems may be one method of overcoming this problem. Further investigations in this area should include the study of the nucleoside transport mechanism of the amastigote to ensure that this stage would be susceptible to this approach.

4.3. ADAPTATION OF *LEISHMANIA* TO ITS ENVIRONMENT

4.3.1. Manipulation of the parasitophorous vacuole of PECs infected with *Leishmania* species by the use of methylamine, ammonium chloride and dextran sulphate

The results reported in section 3.3. suggest that there is a difference in the environment of the parasitophorous vacuole of *L.donovani* and *L.m.mexicana* infected cells. *L.donovani* was found to be more sensitive than *L.m.mexicana* to changes in it's environment induced by methylamine and ammonium chloride than *L.m.mexicana*. However, it is not possible to conclude that this is due to the amastigotes of the two species being adapted to different environments as the extent the effects that methylamine and ammonium chloride are

having on the parasites. These compounds not only raise lysosomal pH (Ohkuma and Poole, 1978) but also ammonium chloride and dextran sulphate, but not methylamine, inhibit lysosomal movements (D'Arcy Hart *et al.*, 1983) and phagosome-lysosome fusion (D'Arcy Hart and Young, 1979; Gordon *et al.*, 1980).

Chang (1980) attempted to measure the pH of the parasitophorous vacuole of both *L.m.mexicana* and *L.donovani* infected cells and reported that he could detect no differences. This appears to contradict the hypothesis being tested here although it is possible that the pH of the vacuole of both *L.m.mexicana* and *L.donovani* is the same but that *L.mexicana* does not require such a low pH. Nevertheless a direct toxic effect on the amastigotes cannot be discounted, especially as the promastigotes of both species are affected by methylamine. However, if this was the case then it would be expected that the amastigotes of both species would be equally affected by methylamine and not by ammonium chloride or dextran sulphate neither of which inhibited promastigote growth. That the lysosomal system itself is affected, by the presence of methylamine, even after five days, was confirmed by the presence of large translucent vacuoles. Whether this is due to the long term accumulation of these lysosomotrophic amines or to a decrease in membrane fluidity remains to be investigated. It has been reported that at 5 mM the effects of ammonium chloride on lysosomal movement and fusion begin to fade after 4-5 hours continual treatment (D'Arcy Hart, *et al.*, 1983). This may account for ammonium chloride not having as great an effect as methylamine on *L. donovani* infections although the effects of long term treatment with methylamine on cell function remain unclear.

Alexander (1981) has reported that the use of poly-D-glutamic acid to inhibit phagosome-lysosome fusion in *L.m.mexicana* infected PECs increased parasite multiplication whilst the use of chloroquine

to enhance fusion reduced growth. This affect was more pronounced when promastigotes rather than amastigotes were used to infect macrophages. However, chloroquine also raises lysosomal pH so the underlying mode of action remains unclear. These results, however, do suggest that the effect of methylamine and ammonium chloride on *L.donovani* in PECs is not based on the inhibition of phagosome-lysosome fusion.

Normally *L.donovani* is enclosed in a tight fitting vacuole but on treatment with methylamine, and to a lesser extent ammonium chloride, these vacuoles become much 'looser' and there is a striking similarity to the characteristically large phagolysosome found with *L.m.mexicana*-infected cells. A similar situation has been observed with the yeast *Saccharomyces cerevisiae* when ingested by PECs (D'Arcy Hart and Young, 1979). The results suggest that inhibitors of phagosome-lysosome fusion evoke loose parasitophorous vacuoles around the yeasts through their effect on the fusion process. If compounds which enhanced phagosome-lysosome fusion were used this effect could be partially reversed. The authors suggest that the complexing of polyanions with calcium could reduce the intracellular levels of free calcium and so perturb the normal control mechanisms of fusion. Whether the large parasitophorous vacuoles found with *L.m.mexicana* infected macrophages are a result of it's survival mechanism and whether this mechanism differs from that of *L.donovnai* is still open to question.

Ammonium ions are also known to inhibit receptor-mediated endocytosis of mannose-glycoconjugates by inhibiting receptor recycling of this ligand which is involved in the function, synthesis, assembly and recirculation of lysosomal componments. Mannose receptor-mediated recognition and entry of glycoproteins have been shown to occur in macrophages infected with *L.m.amazonensis* (Rabinovitch *et al.*, 1985; Shepherd *et al.*, 1983) indicating that this parasite does not interfere with this particular aspect of host cell function. It

would be interesting to investigate this further in other species of *Leishmania* and the effect of ammonium ions on receptor recycling in *Leishmania* infected cells.

Dextran sulphate also appears to have similar affects to those of the amines, especially in inhibiting phagosome-lysosome fusion, but is reported as lowering the intralysosomal pH of treated cells as well as decreasing the membrane fluidity of lysosomal membranes, presumably by interacting with the lipid or polypeptide moieties of the luminal face of the lysosome (Kielian and Cohn, 1982). Unlike the other compounds used in this study dextran sulphate is taken up by endocytosis and is reported as taking 3-5 days to exert its effect (D'Arcy Hart *et al.*, 1983; Kielian and Cohn, 1982). In the experiments involving dextran sulphate the infected PECs were only exposed to this compound for 48 hours so the precise effect of treatment is unclear. However, treatment did reduce the parasite load. Perhaps treatment for longer periods of time would result in a further decrease in parasite numbers. The use of dextran sulphate complexed to either serum lipoproteins or human low density lipoproteins is one method to increase the rate of uptake of the dextran sulphate; such that inhibition of phagolysosome fusion occurs within 4 hours instead of taking 3-4 days (Kielian and Cohn, 1982). The use of lipoproteins to target dextran sulphate to *Leishmania* parasites is an interesting prospect and is similar in some respects to the proposal of Hart (section 1.5.2.1.). The possibility that dextran sulphate is having a direct affect on the parasites, by affecting their membrane fluidity for example, cannot be ruled out. However, the lack of effect of dextran sulphate on promastigotes suggests that this is not the case. It is interesting to speculate that the remains of parasites seen in infected cells treated with dextran sulphate is due to the inhibition of phagosome-lysosome fusion.

These results illustrate the potential for the use of compounds which manipulate the lysosomal environment in the investigation of the basic macrophage-parasite interactions in the phagolysosome. It also indicates the importance of further studies, which are now technically feasible, to look at the role of lysosomal pH and indeed the membrane fluidity of the phagolysosome. Attempts along these lines (see section 3.4) proved unsuccessful; nevertheless this is an approach well worth pursuing.

4.3.2. The effect of pH on the uptake and incorporation of ^{3}H leucine and ^{3}H adenosine by different stages and species of *Leishmania*

The data from the uptake experiments using promastigotes showed several interesting points. For *L.donovani* and *L.m.mexicana* promastigotes, uptake was maximal between pH 6 and 7. In a similar study on *L.donovani* promastigotes, Mukkada *et al.* (1985) found a distinct pH optimum at pH 7. However they were using cells harvested from the middle of the exponential growth phase, whilst the cells used in this study were from stationary phase cultures. There is a considerable difference in the pH of the growth medium at these different stages of the culture and by stationary phase the pH of the medium is between 6 and 7. This could account for the differences in the pH optimum found in this study and that by Mukkada *et al.* (1985) especially if promastigotes become adapted to the pH at which they are growing. This does not appear to be the case with the metacyclics of *L.major*. The pH optimum for nucleoside uptake of these cells was between pH 5 and 6, well below the measured pH of the growth medium. This may represent a preadaptation by these metacyclics to life in the acidic enviroment of the parasitophorous vacuole.

The results for the incorporation of ^{3}H adenosine by amastigotes of *L.donovani* showed a sharp optimum at pH 5, in agreement with the

results of Mukkada *et al.* (1985) for uridine and thymidine. However the results obtained using amastigotes of *L.m.mexicana* did not show as sharp a pH optimum with uptake being similar and high between pH 5 and 6. Although this study was not extensive and the results should be interpreted with caution, the results give some more indications of a difference in the environments of *L.donovani* and *L.m.mexicana*.

This led to attempts to quantify the pH of the parasitophorous vacuole of cells infected with *L.m.mexicana* and compare them with uninfected cells. The approach followed was to use a fluorescence probe (FITC-labelled dextran sulphate) which is endocytosed and accumulates in lysosomes. The fluoresence spectrum of this compound changes in the pH range 3-8 and by using incident light at 620 nm and 480 nm a ratio can be established which allows the pH of the environment to be estimated from a calibration curve (Ohkuma and Poole, 1978). Initial results indicated a difference between normal lysosomal pH and that of the parasitophorous vacuole, however the equipment was not sufficiently sensitive to quantify the preliminary results any further.

Further investigations of the parasitophorous vacuole environment are needed in order to understand fully the relationship between the parasite and its host cell and how the presence of a growing and multiplying intracellular parasite can affect host cell functions. This could give a greater insight into the survival mechanisms used by the leishmanias and may prove useful in the rational development of new chemotherapeutic strategies.

4.4. INVESTIGATIONS INTO THE ANTILEISHMANIAL ACTIVITY OF AMINO ACID ESTERS AND AMINO ACID AMIDES

4.4.1. Antileishmanial activity of L-leucine methyl ester and L-tryptophanamide

The MTT viability assay proved a useful method in this study. It was modified such that the parasites are incubated with MTT for only three hours instead of overnight; the shorter incubation avoids possible interference from other microorganisms and complications produced by promastigotes dividing. L-Tryptophanamide had a similar antileishmanial activity against promastigotes and amastigotes of *L.donovani* and *L.m.mexicana* as measured by the MTT viability assay. At the higher concentrations tested the growth of promastigotes was inhibited. Testing of this compound against *Leishmania* infections of PECs *in vitro* showed that both *L.m.mexicana* and *L.donovani* were susceptible to a similar degree to the activity of this compound. Under the conditions used the activity of this compound was time and concentration dependent. These results are in close agreement with the work of Rabinovitch and Zilberfarb (1988) using *L.m.amazonensis*-infected PECs.

The results for leuOMe showed that this compound was not equally active against all species and stages of *Leishmania*. The activity of leuOMe against *L.m.mexicana* amastigotes, both free and residing in PECs, are very similar to those previously reported (Rabinovitch *et al.*, 1986; Rabinovitch *et al.*, 1987). Investigating the activity against different species and stages of *Leishmania* has given a deeper insight into the antileishmanial activity of leuOMe. It has been confirmed that leuOMe acts directly against the parasite, but the findings that *L.major* and *L.donovani* amastigote infections of PECs are unaffected by leuOMe whereas *L.m.mexicana* is highly susceptible, and that this susceptibility appears only slowly after infection of PECs

with promastigotes, are the most crucial. There is a distinct correlation with the presence of megasomes and the antileishmanial activity of leuOMe. Only the amastigotes of *L.mexicana* possess these lysosomal like organelles (see section 1.1.1.).

Unfortunately, *in vivo* activity of the compound was not detected and as there was considerable toxicity of both of these compounds *in vitro* and *in vivo* it seems unlikely that either could be useful clinically for the treatment of leismaniasis.

The spectrum of antileishmanial activity of L-tryptophanamide differs markedly from that of leuOMe. This suggests that although the amino acid ester and amino acid amide may exert their antileishmanial activity in a similar manner, involving hydrolysis to the amino acid which causes osmotic stress, they are activated by different enzymes. It is probable that the activation process is the key to stage and species specific activity of leuOMe.

4.4.2. The appearance of megasomes during the transformation *in vitro* of *L.m.mexicana* from promastigote to amastigote

Megasomes arise early in the transformation from promastigote to amastigote; they are present by 23 hours, before the loss of the flagellum, and by 48 hours the transforming parasite resembles amastigotes, at least structurally. The finding that the megasomes arise from the trans-golgi-network is in agreement with the proposals of Griffiths and Simons (1986) on the packaging of lysosomal enzymes. This observation further supports the proposal that these organelles are lysosomal in nature. However, no clathrin-coated vesicles believed to be responsible for the transport of enzymes in a mature lysosomal system (Griffiths and Simons, 1986) were observed.

The production of megasomes parallels the acquisition of sensitivity to leuOMe by *L.m.mexicana* promastigotes transforming to

amastigotes in PECs *in vitro* (as discussed above). Interestingly Rabinovitch *et al.* (1987) pointed to the involvement of an acidified compartment within the amastigotes of *L.m.amazonensis* in the hydrolysis of amino acid esters. Recently Antoine *et al* (1988) showed that the megasomes of *L.m.amazonensis* were acidified. This in parallel with my own work clearly points to these unique organelles as the site of ester hydrolysis.

4.4.3. Esterases of *Leishmania*

The use of starch gel electrophoresis to try and identify the enzymes responsible for the hydrolysis of leuOMe showed several isoenzymes in the different species and stages of *Leishmania*. There are several common bands; a shared band between the promastigotes of *L.m.mexicana*, *L.donovani*, the metacyclics of *L.major* and the amastigotes of *L.donovani*; and a shared band between the amastigotes and promastigotes of *L.m.mexicana*. There were also several developmental changes in the transformation from promastigote to amastigote of both *L.m.mexicana* and *L.donovani*. In both species the promastigotes have two and three isoenzymes respectively, whilst the amastigotes have only a single isoenzyme. The role of these enzymes has not been elucidated. It was interesting to note that the inclusion of the proteinase inhibitor leupeptin allowed the detection of several other isoenzymes in the species treated, indicating the importance of proteolysis in these experiments. It would have been interesting to have looked at the banding patterns of all the stages tested with leupeptin included.

The assay developed to measure the amounts of phenol potentially released by the hydrolysis of leucine benzyl ester worked well, being able to detect concentrations as low as 10uM phenol. The problems encountered with attempts to determine phenol production at pHs

outwith the range 7.5-8.6 were overcome by the use in the incubations of buffers with a low molarity. The addition of higher molarity buffers of appropriate pH for the subsequent phenol determination avoided any problems of variable or inappropriate pH. This gave a potentially very useful assay which could measure the rates of hydrolysis of leucine benzl ester by parasite enzymes at a range of pHs. However, no such hydrolysis was observed either when using homogenates of *L.m.mexicana* amastigotes or the purified amastigote low molecular weight proteinases. Subsequent work with these enzymes showed them to be inhibited by high concentrations of DMSO and ethanol, the two solvents that are used to dissolve the leucine benzyl ester. This could explain the lack of activity of the enzymes against the benzyl ester. The assay itself was shown to work well by the use of the arylesterase substrate phenylactate to detect esterase activity in horse serum. This gave satisfactory results.

4.4.4. The exploitation of *L.m.mexicana* cysteine proteinases as activators of pro-drugs

The original procedure of Pupkis and Coombs (1984) for the purification of these amastigote low molecular weight proteinases has been improved by the use of a two step procedure to give a 10 fold purification. The gelatin SDS-PAGE analysis of the proteinases separated by the different steps has confirmed the work of Lockwood *et al.* (1987) showing that there are at least seven of these low molecular weight proteinases. However, the use of silver staining to detect these proteins also revealed the presence of contaminating proteins. The application of further techniques, such as affinity chromatography, should lead to the purification of this group of enzymes and indeed the eventual isolation of each of the individual isoenzymes.

By using the partially purified enzymes and a range of proteinase inhibitors it was confirmed that these enzymes are cysteine proteinases. The previous work of Pupkis and Coombs (1984) demonstrated only one cysteine proteinase. This difference appears to be due to the use of a less sensitive protein detection method coupled with the effect of boiling on the samples. Why two of these bands should become more prominent on boiling is, at present, unknown.

Interestingly, the use of gelatin gels to analyse the chromtographic fractions from gel filtration have revealed a larger number of high molecular weight proteinases than has previously been reported (Lockwood *et al.*, 1987; North *et al.*, 1981). The comparison of freeze-thawed and detergent solubilised samples gave very different results in terms of the proteinase activity profiles, suggesting that several of the proteinases were membrane bound as they did not appear to be released by freeze-thaw but were released by detergent. Coombs (1982) indicated the presence of particulate proteinases in *L.m.mexicana* amastigotes and promastigotes. The relatively high leuOMe hydrolysing activity remaining in the pellet fraction from freeze-thawed cells suggested the possibility that some membrane bound enzymes may be involved in leuOMe hydrolysis. It is possible, however, that the results obtained are due to a failure of freeze-thaw to fully release soluble enzymes from cells that are notoriously hard to fractionate (Dwyer, 1980). This was not investigated further.

The high molecular weight proteinases present in amastigotes of *L.m.mexicana* were easily separated and distinguished from the lower molecular weight proteinases but also they differed from the high molecular weight proteinases found in promastigotes of *L.m.mexicana* (Lockwod *et al.*, 1987). Interestngly North *et al.* (1988) reported that the high molecular weight proteinases found in all species and stages so far investigated do not appear to belong to any of the four well

defined classes found in other organisms (see table 3). Only phenanthroline, normally a metalloproteinase inhibitor, appears to inhibit these enzymes, although there are instances of it acting as an inhibitor of cysteine proteinases. The recent finding that gp63 is a zinc metalloproteinase (see section 1.3.5.) suggests that the other high molecular weight proteinases may also belong to this category.

The extent of proteolysis within homogenates of *L.m.mexicana* amastigotes was quantified to some extent by the measurements of the amount of protein remaining after incubation at room temperature. This confirms the findings of Pupkis and Coombs (1986) that this is a rapid process. The use of various proteinase inhibitors slowed down this process considerably and the use of E64 indicated that other proteinases besides cysteine proteinases were involved in this process.

The high molecular weight proteinases remain interesting as their role in the leishmanias is unclear and little work has been carried out on them in terms of substrate specificity. The effect of the specific cysteine proteinase inhibitor E64 on the hydrolysis of BzPPANan by homogenates of different species and stages of *Leishmania* indicates that at least some of the proteinase activity in all the other species and stages was due to cysteine proteinases. However, attempts to detect cysteine proteinases on gelatin SDS-PAGE gels by first incubating the samples with E64 showed that only the low molecular weight proteinases of *L.m.mexicana* amastigotes were inhibited in this manner. The use of E64 in the buffers used to develop the proteinase activity did show some changes in the banding patterns of the other species and stages used but the results were unclear. Perhaps these proteinases have some cysteine proteinase type characteristics and need the E64 there at all times to be inhibited.

The other proteinase inhibitors used did not inhibit the low molecular weight activities on gelatin gels when only preincubated with the extracts and this affect may be attributable to the reversible binding to these inhibitors to proteinases and the conditions they are subjected to during SDS-PAGE. For example, leupeptin is known to be a reversible inhibitor in contrast to E64 which irreversibly modifies the active thiol group to a thioether form (Shoji-Kasai *et al.*, 1988).

Although the use of paper electrophoresis to separate the hydrolysis products of leuOMe had its limitations, it allowed the analysis of the activity of the partially purified amastigote low molecular weight cysteine proteinases towards leuOMe as well as the relative activities of extracts of promastigotes and amastigotes of other species. It also allowed the qualitative assessment of the activity of *L.donovani* and *L.m.mexicana* amastigote homogenates against a series of other amino acid esters and amides. However, although the assay was shown to work adequately it was of limited use.

The results indicate that the low molecular weight cysteine proteinases are responsible for the majority of the hydrolysis of the leucine methyl ester by amastigotes of *L.m.mexicana* and so account for the toxicity of leuOMe towards these amastigotes. However, there do appear to be other enzymes involved in hydrolysing leuOMe although to a lesser degree. These may account for the lysis in other species of amastigotes and in promastigotes. This correlates with the findings that amastigotes of *L.donovani* and *L.major* both hydrolyse BzPPANan at significant rates;not as rapidly as amastigotes of *L.m.mexicana*, but higher than the promastigotes of any species.

Although the amastigotes of *L.donovani* and *L.major* can hydrolyse leuOMe at a low rate, they are not appreciably affected by this ester. It is interesting to speculate on why this is this case. It appears that a well developed lysosomal system and presumably a high hydrolytic

activity towards the compound is needed for leuOMe to be toxic. This is highlighted by the prevention of murine graft-versus-host disease by a short exposure of the donor bone marrow cells to the dipeptide ester L-Leu-L-LeuOMe (Thiele *et al.*, 1987). Other cells affected by treatment with leuOMe have also had well developed lysosomal systems, for example heart myocytes (Decker and Fuseler, 1984). The lysosomal systems of *L.donovani* and *L.major* are both poorly developed whilst the megasomes, where the low molecular weight cysteine proteinases are located, are very well developed, taking up to 15% of the cell volume (Pupkis *et al.*, 1986). Perhaps for toxicity to be apparent, leuOMe needs to accumulate in a compartment and thereby cause osmotic lysis. Even though there is a suitable compartment, the toxicity of these compounds does rely on their enzymatic hydrolysis. It seems likely that it is a combination of the higher specific activity towards leuOMe found in *L.mexicana* amastigotes than in other species of *Leishmania* and the presence of these enzymes within a well developed lysosomal system that is responsible for the stage and species specificity found.

Alfieri *et al.* (1988) reported that chymostatin proved to be the most effective inhibitor of leuOMe toxicity to *L.m.amazonensis*. Antipain and leupeptin were also effective, whereas pepstatin had no effect. All of the cysteine proteinase inhibitors do inactivate the low molecular enzymes whilst pepstatin, as expected, had no effect. The lack of reported protective activity of E64 with intracellular amastigotes (Alfieri *et al.*, 1988) is not really surprising as this inhibitor is not membrane-permeant (Shoji-Kasi *et al.*, 1988) and so could not access the intracellular amastigotes. However, the use of membrane-permeant analogues of E64 (Shoji-Kasi *et al.*, 1988) should antagonise the antileishmanial activity of leuOMe and indeed could prove useful as antileishmanial agents.

Although the low molecular weight enzymes purified in this study appear to be responsible for the toxicity of leuOMe, their role in the toxicity of other amino acid esters and amino acid amides remains unclear. Certainly they do hydrolyse leucinamide at a comparable rate to leuOMe, but they do not appear to be responsible for the hydrolysis of the other esters and amides tested. Several of these other compounds are toxic to the amastigotes of *L.m.amazonensis* and are hydrolysed by homogenates of *L.donovani* and *L.m. mexicana* amastigotes. The enzymes responsible for this hydrolysis were not characterised in this work but need further investigation. Tryptophanamide is perhaps the most interesting compound as it poccesses a broader spectrum of antileishmanial activity than leuOMe and as such the basis behind its activity may prove useful in the design of antileishmanial drugs for the treatment of all forms of leishmaniasis. Unfortunately, however, it has a lower chemotherapeutic index as measured *in vitro*.

Previous work has shown that several amino acid esters and amino acid amides can also have a protective effect against some of the most toxic amides and esters, notably leuOMe and leucinamide but not tryptophanamide (Alfieri *et al.*, 1987; Rabinovitch and Zilberfarb, 1988). The results of using amino acid esters and amides to inhibit the purified proteinases activity towards BzPPANan showed that several of the protective amides and esters do inhibit these enzymes in a concentration dependent manner. It appears that esters and amides composed of basic amino acids, are poor competitors whilst those composed of either aromatic or aliphatic amino acids are much better competitors.

There are several apparent contradictions in the work of Alfieri *et al.* (1987), and some of the results presented in this study. Some of the esters which are reported as not having any protective activity against leuOMe do appear to be able to inhibit the purified enzymes to

some degree. As this group have been working on *L.mexicana amazonensis* this may be one reason to account for these apparent differences. However, it seems more likely that whilst these amino acid esters and amino acid amides may inhibit the purified enzymes the concentrations necessary to achieve protective activity are not attained in the amastigotes. Whether this is due to them not being hydrolysed witin the parasite and so not being lysosomotrophic or not being able to cross the parasites membranes is unclear. Also some of the toxic amides and esters have a protective effect against leuOMe when they are used at low concentrations. This would indicate that there is a point at which the rates of hydrolysis of these compounds and the efflux of the amino acids produced are equal yet these compounds are still competing sufficiently with the leuOMe to give rise to an antagonism.

Only a few of the potential differences between the multiple isoenzyme forms of these low molecular weight cysteine proteinases were investigated. The results suggest that these enzymes may be glycosylated, although the lack of effect with endoglycosidase F did not support this. The importance of glycosylated proteins in the survival and virulence of *Leishmania* species has already been investigated (Dagger *et al.*, 1984; Nolan and Farrell, 1985) and has recently been highlighted in *L.m.amazonensis* by Kink and Chang (1987).

Whether differences in how these proteinases are glycosylated accounts for their different molecular weights remains to be discovered. Recent work by Koehler and Ho (1988) has shown that similar differences between multiple cysteine proteinases are due to minor differences in the N-terminal sequence. The effect that these differences could have on the cellular processing of these proteinases and on their physiological role remain to be investigated. A possible role in the survival of *L.mexicana* species has been proposed on

several occasions (see section 1.2.2. and 1.3.5.) and further characterisation of their substrate specificity may help in clarifying their physiological roles in the parasite.

Overall these results indicate that the concept of the design and use of pro-drugs in the treatment of leishmaniasis is not unrealistic. *Leishmania* specific pro-drugs (see section 1.5.2.1.) could involve the design of a non-toxic carrier molecule which when hydrolysed by leishmania specific proteinases would result in the release of a toxic drug within the parasite. This approach is reliant on the specific activation of the pro-drug by the parasite and not the host In leuOMe we have a compound which, because it is lysosomotrophic, is targetted to the host as well as the parasite lysosomal system. On hydrolysis by the parasite cysteine proteinases free leucine is released, which, under these circumstances, proves toxic to the parasite; so in effect leuOMe is acting as a pro-drug.

Although the activity of leuOMe appears to rely on the low molecular weight cysteine proteinases, which are specific to just some species of *Leishmania*, there are enzymes in other species capable of hydrolysing this type of compound. This highlights the potential for the design of similar but broader spectrum pro-drugs They need not rely on osmotic stress to kill the parasite, many types of released drug couldbe envisaged e.g. an inhibitor of the parasite's dihydrofolate reductase. It is also possible to envisage pro-drugs which have been designed to be species specific, perhaps relying on the different high molecular weight proteinases present in all species and stages to activate them. In all cases, however, good knowledge of the activating enzymes will be required as a prerequisite to drug design. These studies are simply a step in this direction.

5.0.REFERENCES

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