Studies on the energy metabolism of *Leishmania mexicana mexicana*

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SUMMARY

The metabolism of the mammalian form of Leishmania mexicana mexicana has been investigated by comparing the amastigote activities of a number of enzymes involved in the glycolytic sequence, tricarboxylic acid cycle and associated pathways with those of the promastigote. Initial studies suggested that there are only quantitive differences between the two forms with the greatest of the differences found in the area of CO_2-fixation. Phosphoenolpyruvate carboxykinase (PEP carboxykinase) was detected at much higher activity in amastigotes than promastigotes. This enzyme was found to be ADP specific and to have an absolute requirement for Mn^{2+}. There appeared to be no regulation of the amastigote enzyme in crude homogenates by ATP, GTP, ITP or the end products malate and succinate. Pyruvate carboxylase was undetectable in either parasite form and malic enzyme (carboxylating) was detected only at low activity in promastigotes. Amastigotes had low pyruvate kinase but high malate dehydrogenase activities in comparison to promastigotes. This suggests that in amastigotes the main catabolic route for PEP is to succinate, a major end-product of metabolism, by way of carboxylation and reduction involving malate dehydrogenase and fumarate reductase. In contrast, the high activity of pyruvate kinase in promastigotes suggests that, in this parasite form, PEP is more likely to be converted to pyruvate which either enters the TCA cycle or is transaminated to alanine. Promastigotes had a high NADH-linked glutamate dehydrogenase activity in comparison to amastigotes and may reflect the higher glutamate utilization by the insect form of the parasite. A low NADPH-linked glutamate dehydrogenase activity was found in amastigotes, whereas none was detected in promastigotes. Isocitrate lyase could not be detected in either form, suggesting

that the glyoxylate cycle plays no part in L. m. mexicana metabolism.

The theory that the glycolytic kinases, and in particular phosphofructokinase, are primary targets in the action of pentavalent antimonial drugs against Leishmania was investigated with L. m. mexicana. Pentostam (sodium stibogluconate), active in vivo against Leishmania, had no effect on promastigote growth in vitro even at concentrations of 100μg/ml. In contrast, Triostam, the trivalent analogue of Pentostam, inhibited the growth in vitro of L. m. mexicana promastigotes with an LD_{50} of 20μg/ml and an MLC of 400μg/ml. It was found to be almost as effective on a weight/weight basis as the trivalent arsenical melarsen oxide (LD_{50}, 20μg/ml; MLC, 100μg/ml). The leishmanicidal effect of Triostam, however, could not be correlated with any enzyme inhibitory activity. Neither Pentostam (110μM) nor Triostam (600μM) inhibited hexokinase, phosphofructokinase, pyruvate kinase, malate dehydrogenase or PEP carboxykinase from either amastigote or promastigote. In contrast, melarsen oxide was a potent inhibitor of all leishmanial enzymes tested except hexokinase. The action of Triostam was antagonised by cysteine indicating that the drug's action may involve thiol groups in some way. Triostam, but not Pentostam, inhibited phosphofructokinase of adult Schistosoma mansoni with an I_{50} of 200μM, suggesting that antimonials may act in different ways against schistosomes and Leishmania.

The subcellular organisation of L. m. mexicana promastigotes was investigated using differential and isopycnic centrifugation. Glycosomes and mitochondrial vesicles from culture promastigotes were separated on linear sucrose gradients. Hexokinase, glucose phosphate isomerase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and PEP carboxykinase were recovered largely in association with glycosomes (density; 1.215 g/ml). Phosphoglycerate

kinase and glucose-6-phosphate dehydrogenase had some glycosomal activity but were mostly recovered in the soluble fractions. Pyruvate kinase was totally cytosolic. Malate dehydrogenase showed a broad peak corresponding to that of the mitochondrial marker oligomycin-sensitive ATPase (density; 1.190g/ml). Glutamate dehydrogenase and alanine aminotransferase both showed small mitochondrial peaks, but most of the activities were recovered elsewhere on the gradient and in the soluble fractions. Amastigotes of L. m. mexicana were not successfully fractionated using the technique developed for promastigotes, therefore the subcellular location of enzymes in amastigotes was investigated by following the release of soluble enzymes from digitonin-treated amastigotes. This revealed distinct cytosolic, mitochondrial and glycosomal compartments. The findings give an insight into the organisation of L. m. mexicana promastigote and amastigote energy metabolism.

The importance of PEP carboxykinase and malate dehydrogenase to the metabolism of the amastigote prompted a more detailed investigation of these two enzymes. Isoelectric focusing studies showed that amastigotes possessed particulate malate dehydrogenase isoenzymes apparently absent from promastigotes. The particulate activities of amastigote malate dehydrogenase and PEP carboxykinase were purified to apparent electrophoretic homogeneity by hydrophobic interaction chromatography using Phenyl-Sepharose CL-4B, affinity chromatography using 5' AMP-Sepharose 4B and gel filtration using Sephadex G-100. Malate dehydrogenase was purified 150-fold overall with a final specific activity of 1230 units/mg protein and a recovery of 63%. PEP carboxykinase was purified 132-fold with a final specific activity of 30.3 units/mg protein and a recovery of 20%. Molecular weights determined by gel filtration and SDS gel electrophoresis were

39,800 and 33,300 for malate dehydrogenase and 63,100 and 65,100 for PEP carboxykinase, respectively. Kinetic studies with malate dehydrogenase assayed in the direction of oxaloacetic acid reduction showed a K_m NADH of 41μM and a K_m oxaloacetic acid of 39μM. For malate oxidation there was a K_m malate of 3.6mM and a K_m NAD^+ of 0.79mM. Oxaloacetic acid exhibited substrate inhibition at concentrations greater than 0.83mM and malate was found to be a product inhibitor at high concentrations, however there was no modification of enzyme activity by a number of glycolytic intermediates and cofactors suggesting that malate dehydrogenase is not a major regulatory enzyme in L. m. mexicana. The results show that these L. m. mexicana amastigote enzymes are in several ways similar to their mammalian counterparts, nevertheless their apparent importance and unique subcellular organisation in the parasite make them potential targets for chemotherapeutic attack.

1.0 INTRODUCTION

1.1. LEISHMANIA AND THE LEISHMANIASES

The leishmanias are parasitic protozoa that cause a group of diseases, the leishmaniases, that are widespread throughout the tropics and sub-tropics and affect many millions of people (Chance 1981). The medical, economic and biological importance of the diseases was emphasised by their inclusion in the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases.

1.1.1. Taxonomy of Leishmania

The classification of the genus Leishmania is outlined in table 1. It is a member of the order Kinetoplastida and the family Trypanosomatidae, which also contains the genera Crithidia and Trypanosoma (Honigberg et al. 1964, Kudo 1966). There are eight species of Leishmania known to infect man, and two of the species, L. mexicana and L. braziliensis, are subdivided into subspecies (Molyneux and Ashford 1983) (Tables 2 and 3). In the majority of cases these species cannot be distinguished by their morphology alone and so have been classified according to their geographical locations, clinical manifestations and biochemical characteristics. Isoenzyme electrophoresis and the measurement of the buoyant density of nuclear and kinetoplast DNA are the biochemical techniques generally used to distinguish the species and sub-species. This is the current classification for Leishmania at the time of writing, however to avoid confusion in nomenclature for Leishmania species mentioned in papers that predate this classification, the species names used by the authors in the original papers will be given throughout this thesis.

Table 1. Classification of *Leishmania*

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

From Molyneux and Ashford 1983

Table 2. Species of *Leishmania* that infect man in the Old World

Species[a]	Distribution	Disease	Main Reservoir Host
L. donovani	Africa, Asia	Visceral, "Kala-azar"	Many
L. aethiopica	Ethiopia, Kenya	Dry cutaneous; diffuse, "Oriental sore"	Rock hyrax
L. major	Asia, Africa	Wet cutaneous; rural	Rodents
L. tropica	Europe, Asia North Africa	Dry cutaneous; urban	Man
L. infantum	Mediterranean	Infantile visceral	Dogs, foxes

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a From Molyneux and Ashford 1983

Table 3. Species of *Leishmania* that infect man in the New World

Species[a]	Distribution	Disease	Main Reservoir Host
L. braziliensis braziliensis	Brazil	Mucocutaneous "Espundia"	Rodents
L. b. guyanensis	South America	Cutaneous	Sloths
L.b. panamensis	Panama	Cutaneous	Sloths
L. b. peruviana	South America	Cutaneous	Dogs
L. donovani	South America	Visceral	Dogs
L. chagasi	South America	Visceral	Dogs
L. mexicana mexicana	Central America	Cutaneous "Chiclero ulcer"	Rodents
L. m. amazonensis	Brazil	Cutaneous	Rodents
L. m. pifanoi	Venuzuela	Cutaneous	Unknown

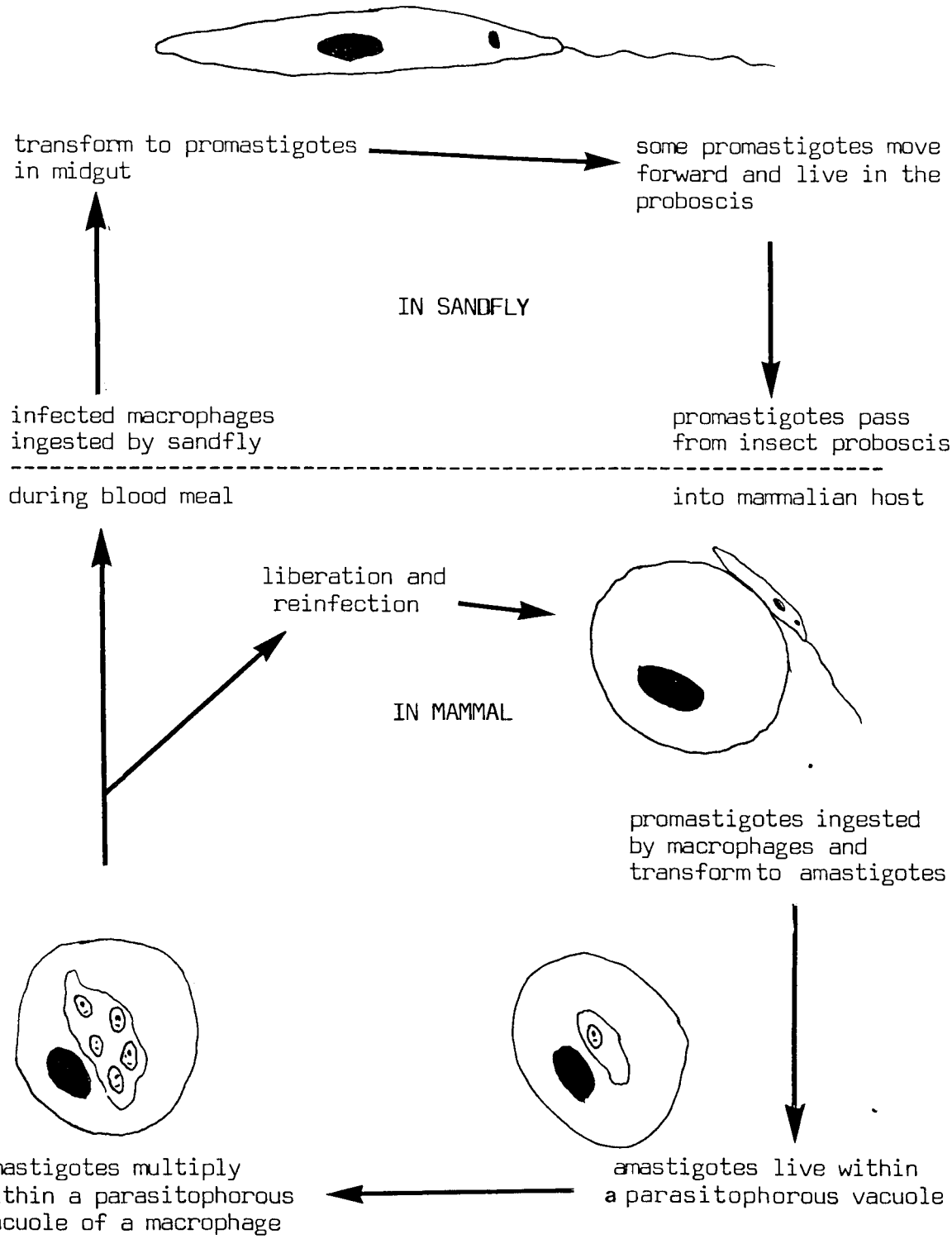
a From Molyneux and Ashford 1983

1.1.2. Life-cycle of Leishmania

There are two major stages in the life-cycle of Leishmania: The promastigote living in the gut of the sandfly vector and the amastigote which resides in a parasitophorous vacuole of a host macrophage (Bray 1974). The promastigote is elongated, approximately 20μm in length, with the kinetoplast and single flagellum at the anterior end, whereas the amastigote is ovoid in shape, with a length of 4-6μm, and a flagellum that hardly protrudes from the flagellar pocket (Vickerman 1976, Vickerman and Preston 1976). A simplified life-cycle of Leishmania is shown in fig. 1.

Only phlebotomine sandflies (Diptera: Psychodidae: Phlebotomine) are known to be insect vectors for Leishmania, there being two genera that infect man; Phlebotomus in the Old World and Lutzomyia in the New World (Williams and Coelho 1978). Following the ingestion of a blood meal from an infected host by female phlebotomine sandflies, the Leishmania amastigotes taken into the midgut transform into promastigotes, usually within 24-36 hours (Strang ways-Dixon and Lainson 1966). In all the mammalian species of Leishmania except the L. braziliensis complex, promastigotes multiply in the abdominal midgut until, after as little as 4-6 days, there is a spread of infection through the thoracic midgut to the oesophagus, pharynx and finally the proboscis ready for infection of a mammal (Strang ways-Dixon and Lainson 1966, Hertig and McConnell 1963, Killick-Kendrick 1979). Several different morphological forms of Leishmania have been recognised as being present in different parts of the sandfly gut (Killick-Kendrick 1979), however the significance of these variations in morphology has not been established. Promastigotes of the L. braziliensis complex have a different pattern of development as they become established in the pylorus and ileum of the sandfly before moving forward with subsequent infection of the proboscis.

Fig 1. A simplified life-cycle of Leishmania



The transformation of amastigote to promastigote has been followed successfully *in vitro* using both light and electron microscopy (Rudzinska *et al.* 1964, Brun and Krassner 1976, Hart *et al.* 1981a). Rudzinska *et al.* (1964) showed with *L. donovani* that there was an increase in the mitochondrial volume which appeared to be formed by the kinetoplast, whereas Brun and Krassner (1976) reported a small decrease in relative mitochondrial volume. It was shown with *L. m. mexicana* that division occurred during the transformation process (Hart *et al.* 1981a). The changes that occur have also been studied biochemically with it being shown in different studies with various species of *Leishmania* that carbohydrates (Simpson 1968), L-proline (Krassner and Flory 1972), and non-esterified fatty acids (Hart *et al.* 1981a) are important nutritional requirements for parasite transformation. In addition, the *in vitro* transformation process has been used to test the effect of inhibitors on amastigotes, giving information on amastigote metabolism as well as its sensitivity to drugs (Hart *et al.* 1981a, Coombs *et al.* 1983).

The transmission of *Leishmania* into the mammalian host occurs during the bite of an infected sandfly when promastigotes are probably inoculated during the probing of the insect. Parasite entry may possibly also occur later if the bite is scratched and the skin broken (Williams 1966, Williams and Coelho 1978). The cell damage caused by penetration of the proboscis at the site of the bite, together with the residual saliva from the sandfly, are thought to attract white cells including mononuclear phagocytes. These latter cells are presumed to take up the promastigote which transforms into the amastigote within the parasitophorous vacuole. The amastigotes divide within the macrophages by binary fission (Vickerman 1980) and reinfect other cells after the progeny are released. The different species of *Leishmania* can infect cells from different organs of the

body, thereby producing the characteristic clinical symptoms of each disease. Leishmania donovani mainly parasitizes the phagocytic cells of the liver and spleen to produce the appropriately named visceral leishmaniasis. In cutaneous leishmaniasis the parasites infect macrophages of the skin, whereas in mucocutaneous leishmaniasis the parasite can spread from the skin to the palate and pharynx.

1.1.3. The interaction of Leishmania with macrophages

1.1.3.1. Entry of Leishmania into macrophages

One of the most fascinating aspects of Leishmania is its ability to survive and multiply within a macrophage - a host cell that specialises in microbicidal activity. The parasite lives in a potentially hostile environment the properties of which include low pH, anionic free radicals, and a large number of digestive enzymes (Silverstein et al. 1977). The first step in the establishment of an infection is parasite-macrophage recognition. In vitro studies have shown that promastigotes bind strongly to macrophages, weakly to lymphocytes and not at all to erythrocytes (Benoliel et al. 1980, Chang 1983). This binding has been shown with L. donovani to occur at multiple sites on both the cell body and the flagellum of the promastigote (Chang 1983), whereas for L. tropica and L. b. braziliensis attachment has been reported to be predominantly via the flagellum (Zenian et al. 1979, Merino et al. 1977). It has been suggested that surface membrane proteins of Leishmania may be involved in forming ligand-receptor complexes on the macrophage surface (Benoliel et al. 1980, Chang 1981, Chang 1983). Macrophages have a system of retrieval and recycling of extracellular acid hydrolases (Stahl et al. 1978, Stahl and Schlessinger 1980) and one such enzyme, acid phosphatase, which is an enzyme usually found in

lysosomes of eukaryotic cells, has been found on the surface of L. donovani promastigotes (Gottlieb and Dwyer 1981). This enzyme may therefore be an important ligand involved in binding and entry of Leishmania into the macrophage (Dwyer and Gottlieb 1983).

Macrophages are naturally phagocytotic in vivo, therefore it is unlikely the Leishmania needs to release substances that would promote entry into the cell as may be the case with Plasmodium lophurae. This distantly related protozoan releases a histidine-rich protein that reacts with the erythrocyte membrane and may help penetration by the merozoite (Kilejian 1976). Phagocytosis is implicated as the mechanism of parasite entry as the uptake of Leishmania by macrophages is inhibited by cytochalasin B, suggesting involvement of actin filaments (Alexander 1975, Zenian et al. 1979, Chang 1983), and iodoacetate, suggesting an energy requirement (Benoliel et al. 1980).

1.1.3.2. Survival of Leishmania within macrophages

Intracellular parasites have different mechanisms of avoiding or resisting the microbicidal activities of the host macrophages. Toxoplasma gondii appears to prevent fusion of the secondary lysosomes thereby protecting itself from proteolytic digestion (Jones and Hirsch 1972) and there is evidence that this is mediated through its active penetration into vacuoles rather than being phagocytosed into normal phagosomes. Trypanosoma cruzi can cross the membrane of the original phagosome and live in the cytoplasmic matrix (Dvorak and Hyde 1973), whilst Mycobacterium lepraemurium forms a physical protective barrier in the form of a thick enveloping capsule (Draper and Rees 1970). Although secondary lysosomes fuse with the parasitophorous vacuoles of Leishmania-infected macrophages, the parasites not only survive but multiply in this environment

(Alexander and Vickerman 1975, Chang and Dwyer 1976, Berman et al. 1979, Brazil 1984).

The ability of the promastigote to transform and differentiate into the amastigote is vital for continued leishmanial survival within the macrophage (Lewis and Peters 1977). New antigens appear on the amastigote surface after transformation and may signify a molecular reorganisation of the plasma membrane which may be important in preferential amastigote survival (Chang and Fong 1983). In addition, the two forms of Leishmania may have differing abilities to survive the oxidative response of phagocytes to invasion. The ability of phagocytes to destroy intracellular parasites is partly dependent on their capacity for generating toxic oxygen metabolites, namely superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\bullet$) and singlet oxygen ($^1O_2$) (Klebanoff et al. 1983, Locksley and Klebanoff 1983). Resident macrophages have a weak respiratory burst and intracellular parasites such as Leishmania can survive within these cells, whereas activated macrophages have both an increased respiratory burst and a more effective antiparasitic activity. Although with L. donovani and L. tropica both promastigotes and amastigotes induce a respiratory burst, the response to promastigotes is far greater than amastigotes (Murray 1981, Murray and Cartelli 1983). This correlates well with the greater susceptibility of the promastigote to the microbial activity of phagocytes. The levels of endogenous scavengers of oxygen metabolites may also play a part in intracellular survival. L. donovani amastigotes have been shown to have a 3-fold higher activity of superoxide dismutase and catalase, and a 14-fold higher glutathione peroxidase activity than the promastigote, which could make the amastigote more resistant to the toxic activity of macrophages (Murray 1982). Catalase and glutathione peroxidase were not detected in L. m. mexicana

amastigotes ,however, and superoxide dismutase was found to have similar activity in both forms of the parasite (Ghafoor and Coombs 1980).

There are a number of other ways that Leishmania may survive macrophage attack. A number of cell surface components from both Leishmania amastigotes and promastigotes have been shown to be resistant to degradation by isolated rat liver lysosomes and these may form a barrier that protects the cell (Chang and Dwyer 1978, Chang and Fong 1983). Another possible mechanism for amastigote survival is the excretion into the parasitophorous vacuole of substances that could antagonise the microbicidal activity of the macrophages. Leishmania m. mexicana amastigotes contain high cysteine proteinase activity which, if released into the parasitophorous vacuole, could protect the parasite by degrading host digestive enzymes (Coombs 1982). There is no evidence as yet however to suggest that there is secretion of proteinases either in vivo or in vitro. It has been reported that the intravacuolar pH does not seem to be affected by the presence of the parasite (Chang 1980), however high concentrations of amines and ammonia are produced, by proteolysis and amino-acid metabolism, and these could inactivate host enzymes by raising the pH of the lysosomal vacuoles (Coombs 1982, Coombs and Sanderson 1984). Leishmania also produce excreted factors that have been shown to inhibit β-galactosidase activity (El-On et al. 1980). These factors have been characterised as glycosylated proteins containing galactose and may play a role in amastigote survival within the parasitoporous vacuole of the macrophage by inhibiting lysosomal enzymes (Zehaui et al. 1983).

1.2. ULTRASTRUCTURE OF *LEISHMANIA*

Trypanosomatids contain a number of subcellular structures that are common to all eukaryotic cells as well as some organelles apparently unique to this group of Protozoa (Vickerman and Preston 1976). A transmission electron micrograph of some *L. m. mexicana* amastigotes showing the major structural features of the cells is given in fig. 2.

The plasma membrane is a typical unit membrane 2-4nm in width, and is associated with a complex array of sub-pellicular microtubules that completely surrounds the organism except for a gap at the point of attachment of the flagellum to the parasite body (Vickerman 1974). These microtubules, which are found in both the vertebrate and invertebrate stages of *Leishmania*, give internal support to the cell and consequently makes the parasite very difficult to lyse without drastic breakage methods such as grinding with abrasive powders or incubating with detergents.

The endomembrane systems of trypanosomatid flagellates have not been well studied, although some information has been derived from electron microscopy studies. The nucleus, which is typically eukaryotic, is surrounded by a nuclear envelope with two membranes both of which contain pores which link the matrix to the cytoplasm. There is an endoplasmic reticulum consisting of flattened, ramifying cisternae and tubules, and a Golgi apparatus with stacks of smooth flattened membranes; neither show striking differences from the generalized eukaryote (Vickerman and Preston 1976). The endoplasmic reticulum is in part rough, with attached ribosomes, and in part smooth, without ribosomes, and is usually concentrated around the nucleus and between the nucleus and flagellar pocket. The Golgi apparatus is usually associated with the flagellar pocket and is attached by a limb to smooth endoplasmic reticulum (Vickerman and

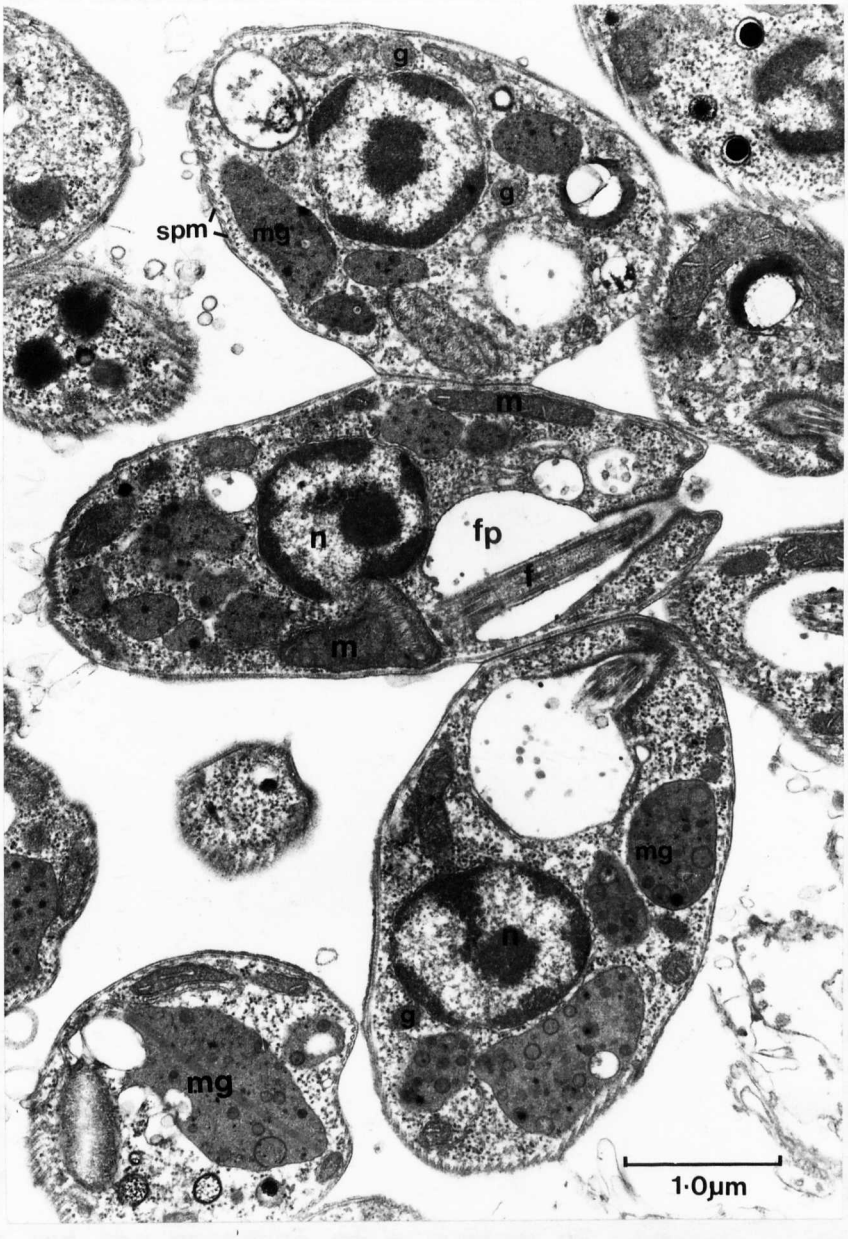


Fig. 2. A transmission electron micrograph of Leishmania mexicana mexicana amastigotes (x 25,000). Key to subcellular structures: Nucleus (n), mitochondrion (m), megasomes (mg), glycosomes (g), subpellicular microtubules (spm), flagellum (f) within the flagellar pocket (fp). Micrograph courtesy of L. Tetley.

Preston 1976).

The characteristic feature of the organisms that gives the order Kinetoplastida its name is the kinetoplast-mitochondrion complex. The kinetoplast, which is found close to the basal body from which the flagellum arises, is located within the unitary mitochondrion which is branched and forms a reticulum of canals running the length of the organism (Simpson 1972, Vickerman and Preston 1976). The kinetoplast is not associated, as its name implies, to the movement of the organism and the reason for its position so close to the basal bodies of the flagellum is unknown. The central part of the concave disk-like kinetoplast capsule contains DNA that is comprised of a gigantic network of catenated circles (See section 1.4.1.).

There is considerable cyclical variation in the kinetoplast-mitochondrion complex during the life-cycle of salivarian trypanosomes, but this does not occur to the same extent in other trypanosomatids such as T. cruzi and Leishmania species (Rudzinska et al. 1964, Vickerman 1974, Vickerman and Preston 1976). In T. brucei long slender bloodstream form, there is a simple unbranched mitochondrion with few tubular cristae. In the subsequent short stumpy bloodstream form there is an increased mitochondrial volume and the presence of a number of tubular cristae. On transformation to the insect procyclic form, there is the formation of a well developed mitochondrion containing plate-like cristae. These large morphological changes are associated with the development of an active TCA cycle and respiratory chain together with a change in the energy metabolism of the parasite (Vickerman 1974, Vickerman and Preston 1976, Bowman and Flynn 1976). In Leishmania, however, cristae are present in the mitochondrion of both amastigotes and promastigotes which, together with the available information on the respiration of the two stages, suggests that there is little

variation in mitochondrial function between the two forms (Vickerman and Preston 1976, Hart et al. 1981c).

Another unique feature of trypanosomatid subcellular structure is the presence of microbody-like organelles known as glycosomes. These were first discovered in bloodstream forms of T. brucei and contain a number of glycolytic enzymes (Opperdoes and Borst 1977, Oduro et al. 1980a) (See section 1.4.3.1.). These organelles have since been found in all trypanosomatids studied including T. cruzi and C. fasciculata (Taylor et al. 1980, Cannata et al. 1982) and L. m. mexicana (Coombs et al. 1982, Tetley et al. 1983). The glycosomes of bloodstream T. brucei have been studied in the most detail, including purification to near homogeneity by differential and isopycnic centrifugation (Opperdoes et al. 1984). The organelles, which are bound by a single membrane, appear to be remarkably homogeneous with an average diameter of 0.27μm and containing a dense core displaying a lamellar structure, but apparently no DNA. From in situ analysis, the number of glycosomes in each T. brucei cell was found to be approximately 230, which represents 4.3% of the total cell volume (Opperdoes et al. 1984). 3-dimensional reconstruction of L. m. mexicana amastigotes from electron micrographs of serial thin sections has revealed that there are only 9 to 10 glycosomes per amastigote (Tetley et al. 1983). This is considerably less than the number found in bloodstream T. brucei and, although Leishmania amastigotes are considerably smaller, the low number of glycosomes may reflect a smaller reliance on glycolysis by the cell. Leishmania m. mexicana amastigotes also contain a number of lysosome-like organelles given the name "Megasomes" (Alexander and Vickerman 1975) which are interestingly not found in promastigotes. They were shown to be concentrated at

the posterior end of the cell away from the flagellar pocket (Tetley et al. 1983). The "Megasomes", which vary considerably in size, are thought to contain a number of lysosomal enzymes and may play a part in the survival of the mammalian form of the parasite within the host macrophage (Coombs 1982).

1.3. CONTROL OF LEISHMANIASIS

The control of leishmaniasis through the interruption of its life-cycle is a long term aim that is proving very difficult to achieve (Chance 1981). The control of the insect vector by spraying with insecticides has faced insurmountable problems in areas such as tropical rain forests and has also provided a major ecological and environmental problem (Lainson and Shaw 1978). Although there are a number of drugs suitable for treating patients with leishmaniasis, there are no chemoprophylactic drugs or effective multivalent vaccines which could be used in the eradication of the disease through interruption of the parasite life-cycle in the mammalian host.

1.3.1. Immunisation

Immunisation has been extremely successful as a method of control and even eradication of many bacterial and viral diseases, but has so far proved almost totally unsuccessful as a method for treating the three major groups of diseases caused by parasitic protozoa; trypanosomiasis, malaria and leishmaniasis. The reasons for this are many fold, but in particular the parasites have evolved complex ways of evading the immune response. In the mammaliam forms of african trypanosomes the plasma membrame is totally covered with a glycoprotein surface coat that is responsible for the way in which the parasite evades the immune response by antigenic variation

(Vickerman and Luckins 1969, Vickerman 1978). The prospect of a vaccine against trypanosomes is still remote, but with increasing knowledge of the molecular biology relating to antigenic variation an effective cocktail of variable surface antigens produced through genetic engineering is a possibility for the future (Diggs 1982, Vickerman and Barry 1982). There are also many problems associated with vaccination against Leishmania, largely due to the parasite evading the immune system by becoming intracellular and living in the parasitophorous vacuole of macrophages, cells of the immune system itself (Mauel and Behin 1981). There has been some success using vaccines of attenuated promastigotes of L. major in the U.S.S.R. and Israel, but due to the differences in host response and the lack of cross-immunity with different species of Leishmania the possibility of a multivalent Leishmania vaccine appears to be very remote (Chance 1981).

1.3.2. Chemotherapy

Chemotherapy is still the only effective form of treatment for leishmaniasis. The drugs of choice are the pentavalent antimonials sodium stibogluconate (Pentostam) and methylglucamine antimoniate (Glucantime) which have been in use for over thirty years (See Steck 1972, 1974). The lack of response to these drugs of Leishmania species from various geographical locations, however, together with the variable host immune responses that occur, make the drugs unsuitable for use in many instances (Peters 1974). Drug resistance to antimonials has not become a major problem in leishmaniasis, although resistance to Pentostam does occasionally occur in a few types of the disease, especially post kala-azar dermal and diffuse cutaneous leishmaniasis (Peters 1974). There is, however, frequent

unresponsiveness to antimonials amongst other types of Leishmania, although this is not apparently attributable to aquired drug resistance (WHO Bulletin 1982). In cases where pentavalent antimonial treatment fails, the drugs pentamidine, allopurinol or amphotericin B are generally used (Steck 1981a). These drugs, however, can be toxic and frequently have only poor activity. Consequently there is an urgent requirement for new antileishmanial agents, not only for treating drug-resistant cases but also to avoid the toxic side effects of those in current clinical use.

1.3.2.1. The search for new drugs

A number of different strategies are employed in the search for new chemotherapeutic agents (Steck 1981b). There is random screening of available chemicals, many of which are produced as part of research into other diseases. Chemotherapeutic studies with Leishmania have been hindered by the lack of effectiveness of many antileishmanial agents against promastigotes growing in vitro (Beverage 1963). Amastigotes have not so far been successfully grown axenically in vitro, however the development of other in vitro methods to assess drug sensitivities against both Leishmania-infected monocyte-derived macrophages (Berman and Wyler 1980) and also amastigotes transforming in vitro (Hart et al. 1981a, Coombs et al. 1983) has allowed screening of new potential drugs against the target form of the parasite, the amastigote. The former method has the advantage that it involves the complete host-parasite system in which the amastigotes multiply, whereas the latter method has the potential advantage that interference from host cells that might process the drug is avoided.

A significant advance in the treatment of visceral leishmaniasis appears to have been achieved with the developement of liposome-

entrapped antileishmanial drugs with which the efficacy of treatment has been increased several hundred fold (Black et al. 1977, New et al. 1978, Alving et al. 1978). The liposomes containing the drugs are taken up by Kupffer cells of the liver and spleen, the cells in which Leishmania resides in visceral infections, and then fuse with the parasitophorous vacuole thus bringing the drug into close proximity with the parasites (Heath et al. 1984). The specific targeting of the drug to cells in which the Leishmania lives, such that it is kept isolated from the general circulation, shows great promise in reducing toxicity and increasing efficacy in the treatment of leishmaniasis.

Another approach in the search for new drugs is target-directed biochemical rationale, that is the selection of an enzyme system only found in the parasite and the design of inhibitors specific for that enzyme system (Steck 1981b, Wang 1984). Unfortunately, there has been little biochemical research on the amastigote of Leishmania, the form that is the causative agent of the disease in man, but initial work has indicated that there are significant differences between the amastigote and promastigote. The unique subcellular organisation of trypanosomatid flagellates, including the arrangement of various glycolytic and associated enzymes in glycosomes, provides several aspects of metabolism that are potential targets for chemotherapy. Biochemical investigations into the organisation and control of pathways associated with the glycosomes and other subcellular organelles could reveal major differences between the parasite and host cells and it is this approach that forms the overall basis for the research carried out in this project.

Compartmentation has been suggested as a major factor in the antitrypanosomal effect of melarsen oxide. Pyruvate kinase has been shown to be the likely target of the drug in vivo (Flynn and Bowman

1974, Bowman and Flynn 1976), although a number of other trypanosomal enzymes were inhibited to a greater extent than pyruvate kinase in crude homogenates (Bowman and Flynn 1976, Fairlamb and Bowman 1977). An explanation is that pyruvate kinase is a cytosolic enzyme, freely available to inhibition by the drug, whereas the other enzymes are located in organelles that may be impermeable to melarsen oxide (Fairlamb 1982).

Polyamine function and biosynthesis have also been investigated as potential chemotherapeutic targets in trypanosomatids for which these compounds appear to be especially important (Bacchi et al. 1980, Bachrach et al. 1981, Bacchi 1981). Polyamine metabolism has been implicated with the activity of a number of cationic trypanocides such as ethidium bromide and antrycide (Bachrach et al. 1979). A specific inhibitor of polyamine biosynthesis is α-difluoromethylornithine (α-DMFO) which is an enzyme-activated irreversible inhibitor of ornithine decarboxylase, one of the key enzymes of polyamine biosynthesis (Bacchi et al. 1980,1982). This drug has potent antitrypanosomal activity against T. brucei in mice (Bacchi et al. 1982), is non-toxic to mammals (Seiler et al. 1978) and may have some future therapeutic potential. Other new analogues of ornithine such as sinefungin also show some promise as antileishmanials (Bachrach et al. 1980).

Ketoconazole is a N-substituted imidazole derivative that was first shown to have antifungal properties but is also active against L. tropica and L. mexicana amastigotes infecting human monocyte-derived macrophages (Berman 1981,1982). Ketoconazole appears to have a similar mechanism of action in both yeast and Leishmania by disturbing sterol biosynthesis and function in membranes (Berman et al. 1984). In studies on L. mexicana promastigotes it was found that the removal of the 14 α-methyl group of lanesterol was

inhibited, thereby preventing the formation of ergosterol, an important sterol of Leishmania membranes. The mode of action of the polyene antibiotic Amphotericin B, which also has both antifungal and antileishmanial properties, is also thought to involve interaction with ergosterol resulting in membrane damage (Kobayashi and Medoff 1977).

All trypanosomatids so far studied have been shown to be deficient in the de novo synthesis of purine nucleotides (Marr et al. 1978, Ceron et al. 1979) (See section 1.4.1.1.) and consequently purine salvage enzymes are essential for parasite growth and survival (Gutteridge and Coombs 1977). Leishmania species possess an unusual purine salvage enzyme, purine nucleoside phosphotransferase, which catalyses phosphorylation of purine nucleosides (Nelson et al. 1979). A number of purine nucleoside analogues such as allopurinol, allopurinol riboside and formycin B have been synthesised (Nelson et al. 1979, Carson and Chang 1981) and shown to have antileishmanial activity in both in vivo and in vitro tests (See Marr and Berens 1983). The drugs are believed to be metabolised to the nucleotides by the parasite and are then leishmanicidal either due to incorporation into nucleic acids or by inhibition of other purine salvage enzymes (Nelson et al. 1979, Rainey and Santi 1983, Marr and Berens 1983). These compounds are relatively non-toxic to mammalian cells which do not possess the nucleoside phosphotransferase that metabolises the nucleoside analogues (Nelson et al. 1979, Wang 1984). Initial clinical trials shows some efficiacy for allopurinol in antimony-resistant cases of Leishmania (Kager et al. 1981), although clinical trials on the more active ribonucleoside analogues are still to be completed (Marr and Berens 1983). The results so far, however, have emphasised the potential of a rational approach to parasite chemotherapy.

1.3.2.2. The mode of action of antileishmanial antimonial drugs

Pentavalent antimonials, such as Pentostam, are the most important group of drugs for treating leishmaniasis (Steck 1981a). There are, however, no reports on the mechanism of action of these antileishmanial antimonials, although it has been suggested that glycolytic kinases, especially phosphofructokinase, may be involved (Gutteridge and Coombs 1977). This idea probably arose from the report that the activity of antischistosomal trivalent antimonials, such as Stibophen, are thought to be mediated through inhibition of phosphofructokinase (Mansour and Bueding 1954, Bueding 1969). Pentostam was shown to be effective against leishmanial amastigotes growing in human monocyte-derived macrophages *in vitro* (Berman and Wyler 1980) and against amastigotes transforming *in vitro* (Coombs *et al.* 1983) but it had no activity against cultured promastigotes (Mattock and Peters 1975, Berman and Wyler 1980, Coombs *et al.* 1983). It has been suggested that pentavalent antimonials, like the pentavalent antitrypanosomal arsenicals, must be reduced to the trivalent form to be active (Goodwin and Page 1943, Crawford 1947, Flynn and Bowman 1974) and the inhibition of transforming amastigotes by Pentostam suggests that prior metabolism by the host cell is not required for activity (Coombs *et al.* 1983). Pre-dosing of host cells growing *in vitro* with antimonials before their infection by amastigotes increased significantly the antileishmanial activity of the drugs and it was suggested that these compounds exert their main effects on the amastigotes, during the brief extracellular phase of their existence in the vertebrate host, when infecting other host cells (Mattock and Peters 1975). Nevertheless, the drugs do exert a significant antileishmanial effect on intracellular amastigotes (Berman and Wyler 1980).

1.4 INTERMEDIARY METABOLISM OF *LEISHMANIA*

Leishmania is similar to many other unicellular parasites in that it multiplies relatively rapidly, approximately once every 12 hours in the case of *L. m. mexicana* promastigotes in culture, and once every 24 hours for amastigotes growing in macrophages *in vitro*. Consequently the parasites are dependent upon the production of large quantities of new cellular materials and the formation of sufficient energy to meet this requirement (Von Brand 1979). The amastigote and promastigote of *Leishmania* have contrasting morphology and live in very different environments, therefore it is to be expected that their metabolism differs to some extent. The biochemistry of the promastigote has been extensively studied, as it can be cultivated easily axenically *in vitro*, but it is only recently, since a procedure was developed to isolate amastigotes essentially free from host cell contamination (Hart *et al.* 1981b), that sufficient material has been available for detailed biochemical study of this stage of the parasite. This development has allowed the comparative studies on the two forms that are essential for the elucidation of both the changes that occur during transformation of the amastigote to promastigote (and vice-versa) and also the relationship between the amastigote and its host cell, the macrophage. It should also be noted that all the biochemical studies performed on the promastigote have been done on those cells grown in culture, from amastigotes transformed *in vitro*, and not on promastigotes isolated from the sandfly, as far too few promastigotes can be isolated from the insect vector to allow extensive biochemical study. It has been generally assumed, however, that the biochemistry of culture promastigotes is similar to that found in the insect gut.

1.4.1. Biosynthetic processes

The basis for the growth and functioning of any cell is the formation of complex molecules from low molecular weight precursors and metabolic intermediates and the subsequent incorporation of these macromolecules into supramolecular assemblies and finally organelles. The metabolic pathways and molecular mechanisms whereby these precursors are converted into macromolecules can vary greatly between species, even though the major classes of macromolecules, nucleic acids, proteins, lipids and polysaccharides all have similar functions in different types of cell. Polysaccharides have two major functions. Some serve as energy stores, e.g. starch and glycogen whilst others, e.g. cellulose, are structural components of the cell. Leishmania apparently contain no polysaccharide energy stores (Gutteridge and Coombs 1977) or complex carbohydrates used in cell structure, therefore no further mention will be made of these macromolecules. Nucleic acid, protein and lipid metabolism, however, are important areas of Leishmania biochemistry as they have some unique features which are the basis for the mode of action of a number of antiparasitic agents (Gutteridge and Coombs 1977, Von Brand 1979).

1.4.1.1. Nucleic acids

Nucleic acids are synthesised from purine and pyrimidine nucleotides. In mammalian cells purines can either be synthesised de novo from glycine, formate, CO_2, glutamine and aspartate or, in certain tissues, salvaged from the bloodstream. Most trypanosomatid protozoa are unable to synthesise the purine ring de novo and rely totally on salvage for their purine requirements (Jaffe and Gutteridge 1974). The one known exception to this is C. oncopelti, which has been shown to incorporate radioactively labelled glycine

into nucleic acid purines (Jaffe and Gutteridge 1974). This organism, however, as unusual as it contains "bipolar bodies", which are thought to be endosymbiotic bacteria that may synthesise the purine ring de novo for the protozoan (Gutteridge and Macadam 1971, Gutteridge and Coombs 1977). Purine metabolism has been studied extensively in promastigotes of Leishmania (Marr et al. 1978, Tuttle and Krenitsky 1980, Lafon et al. 1982), but only recently in amastigotes of L. donovani (Looker et al. 1983) and L. m. mexicana (Hassan and Coombs 1984). The purine salvage pathways of L. donovani have been shown to be similar in both forms of the parasite with the exception of adenosine metabolism (Looker et al. 1983). In amastigotes of L. donovani, adenosine is deaminated to inosine by adenosine deaminase before it is subsequently cleaved to hypoxanthine. In contrast, promastigotes, which do not contain adenosine deaminase, metabolise adenosine to adenine, which is subsequently converted to hypoxanthine, by adenine deaminase (Looker et al. 1983). Adenine deaminase is not found in amastigotes of L. donovani (Looker et al. 1983). This stage specific adenosine metabolism in L. donovani has not been found with L. m. mexicana (Hassan and Coombs 1984). Both amastigotes and promastigotes of L. m. mexicana contain adenosine and adenine deaminases and it has been suggested that adenosine is converted either to AMP by adenosine kinase or to hypoxanthine via adenosine deaminase and inosine nucleosidase. Purine bases are salvaged by purine phosphoribosyltransferases (PRTases), which have been found in high activity in both L. donovani (Looker et al. 1983) and L. m. mexicana (Hassan and Coombs 1984). Both hypoxanthine and guanine are believed to be metabolised by the same enzyme, hypoxanthine-guanine PRTase (Tuttle and Krenitsky 1980, Looker et al.. 1983, Hassan and Coombs

1984) which together with xanthine PRTase is found in glycosomes (Hassan and Coombs 1984). Adenine PRTase is cytosolic. The PRTases of Leishmania have been shown to be very different from mammalian isofunctional enzymes as they have a higher affinity for various purine analogues, such as allopurinol, than the mammalian enzyme (Marr and Berens 1983) (See section 1.3.2.1.). Promastigotes of L. donovani have also been shown to have a unique purine nucleoside phosphotransferase activity that converts allopurinol ribonucleoside to its monophosphate nucleotide (Nelson et al. 1979). Although no physiological substrates or phosphate donors have been discovered for this enzyme (its only activity being with allopurinol ribonucleoside and formycin B), it has great potential as a target for antileishmanial drugs (Looker et al. 1983).

In contrast to purines, pyrimidines can be synthesised de novo by parasitic protozoa (Gutteridge and Coombs 1977, Hammond and Gutteridge 1982). All the enzymes involved in de novo synthesis have been detected in homogenates of L. m. amazonensis promastigotes (Hammond and Gutteridge 1982). The first three enzymes of the pathway, carbomyl phosphate synthase, aspartate carbomyltransferase and dihydro-orotase, are cytosolic in Leishmania. In mammalian cells these three enzymes are thought to be found in a multienzyme complex in the cytosol (Jones 1980), but there is no evidence to suggest that this occurs in Leishmania or any other trypanosomatid. In all eukaryotic cells other than trypanosomatids in which it is found, the fourth enzyme of the pyrimidine biosynthetic pathway, dihydro-orotate dehydrogenase is a particulate mitochondrial enzyme that is linked to the respiratory chain at the ubiquinone level (Miller 1975). In Leishmania, however, the isofunctional enzyme is soluble, utilizes oxygen but is not linked to the respiratory chain (Gero and Coombs 1982, Hammond and Gutteridge 1982). Studies with amastigotes and

promastigotes of L. m. mexicana suggested that the enzyme does not have a hydroxylase mechanism that requires the involvement of an unconjugated reduced pteridine (Gero and Coombs 1982) as had originally been suggested for C. fasciculata and other trypanosomatids (Kidder et al. 1976, Gutteridge et al. 1979). Recent data on the enzyme purified from C. fasciculata and T. brucei supported the previous work on L. m. mexicana and showed that the enzyme exhibited a characteristic flavin electronic spectrum suggesting that dihydroorotate oxidation in trypanosomatids is mediated by flavoprotein oxidases rather than pteridine-linked hydroxylases (Pascal et al. 1983). The last two enzymes of de novo pyrimidine biosynthesis, orotate phosphoribosyl transferase and orotidine-5'-phosphate carboxylase, have also been found to have unusual properties in trypanosomatids in comparison to mammalian enzymes. In mammals the enzymes are cytosolic whereas in the trypanosomatids they have been shown to be particulate (Gero and Coombs 1980, Hammond and Gutteridge, 1980, 1982, 1983) and associated with glycosomes (Hammond et al. 1981, Hammond and Gutteridge 1982).

Pyrimidines may be produced not only by synthesis de novo but also via salvage pathways and a number of the enzymes involved in the salvage of uracil have been detected in L. m. amazonensis (Hammond and Gutteridge 1982).

Another important area of nucleotide metabolism in parasitic protozoa is the production of deoxyribonucleotides for DNA synthesis, either from the corresponding ribonucleotide diphosphates by the little studied ribonucleotide reductase complex, or through salvage of thymine and thymidine (Gutteridge and Coombs 1977). The methylation of the pyrimidine ring in the formation of dTMP from dUMP involves the thymidylate synthase cycle which, together with the

biosynthesis of tetrahydrofolate, is of particular importance as the target for drugs used in the chemotherapy of a number of parasitic diseases, most notably malaria. Thymidylate synthase and dihydrofolate reductase both occur on the same bifunctional protein in protozoa (Garrett et al. 1984), whereas the enzymes from mammalian cells, bacteria and yeast are distinct and readily separable (Blakely 1969).

Little is known about the conversion of nucleotides into nucleic acids in parasitic protozoa although there has been some work on the the DNA polymerases of T. brucei (Dube et al. 1979) and C. fasciculata (Holmes et al. 1984). DNA polymerase A, the predominant DNA polymerase of T. brucei, is similar to the high molecular weight enzyme found in C. fasciculata. Both of these enzymes, however, are different from the mammalian equivalent DNA polymerase, as judged by the inability of specific antisera that cross-reacts with DNA polymerase from different mammalian species to cross-react with the trypanosomatid polymerases (Holmes et al. 1984). A second polymerase, DNA polymerase B, has been isolated from C. fasciculata. It was found to be of lower molecular weight and is more easily classified as a type similar to DNA polymerase β from mammalian cells (Holmes et al. 1984).

In Leishmania, and all other members of the Kinetoplastida, DNA is found in both the nucleus and in association with the Kinetoplast (kDNA). kDNA is found as a giant network composed of thousands of catenated minicircles (0.8-2.5Kb depending on species) and 20-50 identical DNA maxicircles, which in different species range from 20-40Kb. Maxicircles are similar to, but a little larger than, mammalian mitochondrial DNA (Englund 1981), and their major transcripts have been found to be 9S and 12S ribosomal RNAs. As yet, however, no translation products have ben detected (Englund et al.

1982). The minicircles are heterogenous in base sequence and apparently do not have a conventional coding sequence as it appears that they are not transcribed (Challberg and Englund 1980). The function of kDNA is still not understood, although it is likely that the maxicircles are trypanosomatid equivalents to mitochondrial DNA. A number of theories concerning minicircle function have been suggested, including a structural role in the segregation of maxicircles equally to progeny mitochondria (Englund 1981). As well as understanding the function of kDNA, its unique properties may also lead to new chemotherapeutic drugs that interfere with either replication or transcription of this satellite DNA.

1.4.1.2. Proteins

Parasitic protozoa require the same twenty amino acids for protein synthesis as mammalian cells. In mammals ten of the common amino acids cannot be formed de novo and must be obtained from their food, whilst the remainder can either be synthesised de novo from metabolic intermediates or formed from the essential amino acids. The precise amino acid requirements for Leishmania are not known, although studies on L. m. mexicana (Hart and Coombs 1982), L. donovani and L. b. braziliensis (Steiger and Meshnick 1977) give some information for both promastigotes growing in vitro and, in the case of L. m. mexicana, transforming amastigotes. Proline was excreted by promastigotes of L. m. mexicana (Hart and Coombs 1982), suggesting that this species is unlike several other kinetoplastid flagellates including L. donovani, the insect stages of which are reported to use proline as a major energy source (Srivastava and Bowman 1971, Krassner and Flory 1972). The anabolism of proline in L. donovani involves arginine, ornithine and citrulline as precursors

(Krassner and Flory 1972, Camargo et al. 1978) and interestingly both argenine and ornithine were consumed by L. m. mexicana promastigotes (Hart and Coombs 1982).

The apparent importance of amino acid catabolism in the energy metabolism of some Leishmania species has prompted studies on the uptake of amino acids into the parasites. L. tropica promastigotes have been shown to have an active transport system for L-proline (Law and Mukkada 1979) and methionine (Mukkada and Simon 1977). In addition, studies with L. tropica promastigotes with the non-metabolisable analogue of neutral amino acids, α-aminoisobutyric acid, has shown that there is a common transport system, regulated by feedback inhibition and transinhibition, for alanine, cysteine, glycine, methionine, serine and proline (Lepley and Mukkada 1983).

There has been very little work reported on the mechanism by which proteins are synthesised in parasitic protozoa. The first step, the transcription of nuclear DNA to produce mRNA, is catalysed by a DNA-dependent RNA polymerase. Trypanosoma cruzi and T. brucei have been shown to contain unique RNA polymerases which fail to separate into different classes, whereas C. fasciculata and L. m. amazonensis have chromatographically distinct RNA polymerases (Kitchin et al. 1984). Translation of the mRNA then occurs on ribosomes found in the endoplasmic reticulum. Ribosomal structure and the mechanism of protein synthesis have not been investigated in Leishmania, although there has been some work on polypeptide synthesis in Crithidia which suggested that it is more similar to other eukaryotic cells than to prokaryotes (Gutteridge and Coombs. 1977).

1.4.1.3. Lipids

Studies on the lipids of various species of Leishmania have shown that in promastigotes total lipids accounted for 2-15% of the dry weight of the cell and that neutral and polar lipids were 14-55% and 45-86% respectively of the total lipids (Gercken et al. 1976, Beach et al. 1979, Herrmann and Gercken 1980). In L. donovani, the main lipids, phospholipids, sterols and acyl glycerols were found in a ratio of 1:0.37:0.24. In the phospholipid fraction, phosphatidyl choline (57%), phosphatidyl ethanolamine (23%), phosphatidyl inositol (11%) and sphingomyelin (6%) were the major lipids identified (Gercken et al. 1976). In addition, it was found that up to 50% of phosphatidyl ethanolamine existed in the plasmalogen form (Gercken et al. 1976), and that Leishmania were able to synthesise ether lipids from basic metabolites such as glucose and amino acids (Herrmann and Gercken 1980). Recently, the initial steps of alkoxyphospholipid biosynthesis, including plasmalogens, have been discovered in the glycosomes of bloodstream T. brucei (Opperdoes 1984a) and therefore these enzymes may also may be present in glycosomes of Leishmania. Free sterols and sterol esters were also found in L. donovani promastigotes in a ratio of 1:0.3. The main sterols detected were isomers of ergosterol , similar to those found in fungi, and the identification of squalene and lanesterol suggested that sterols were being produced by the route typical of non-photosynthetic organisms such as animals and fungi (Goad et al. 1984). The possible interference by the drug ketoconazole in the metabolism of sterols in Leishmania (See section 1.3.2.1.) makes an understanding of this area of lipid metabolism of prime importance.

1.4.2. Degradation processes

The major energy producing catabolic pathways found in trypanosomatid parasitic protozoa are glycolysis, the tricarboxylic acid cycle (TCA cycle), the respiratory chain, amino acid catabolism and β-oxidation of fatty acids. The enzyme levels and *in vivo* metabolic functioning of the pathways have been characterised for a number of trypanosomatids and have been found to vary considerably between different species and indeed between different developmental stages of the same species (Gutteridge and Coombs 1977, Bowman and Flynn 1976).

1.4.2.1. Substrate utilization and end products of metabolism in *Leishmania*

The major end products of *Leishmania* promastigote energy metabolism are succinate, CO_2, pyruvate and alanine (Steiger and Meshnick 1977, Marr 1980, Hart and Coombs 1982). There is some controversy, however, on the relative importance of glucose and amino acids as substrates for *Leismania* energy metabolism, although the variations found may be related to species differences and the length of time that the promastigotes have been growing in culture. The growth of *L. tropica*, *L. donovani* and *L. braziliensis* was found to be similar in media with and without added glucose (Mukkada *et al.* 1974, Marr and Berens 1977), nevertheless when glucose was added, this utilization was found to be greatest in the late-log phase and stationary phase of growth. Later studies on *L. m. mexicana*, *L. donovani* and *L. braziliensis* also showed substantial glucose utilization throughout promastigote growth (Steiger and Meshnick 1977, Hart and Coombs 1982).

The importance of glucose as an energy substrate in promastigotes has prompted studies on its uptake. In studies with

L. tropica and L. donovani involving the non-metabolisable analogue 2-deoxy-D-glucose, it was demonstrated that glucose was actively accumulated via a carrier-mediated transport system with a high affinity and specificity for D-glucose (Schaefer et al. 1974, Schaefer and Mukkada 1976, Zilberstein and Dwyer 1984). This high affinity for glucose could be important to Leishmania promastigotes at times when the glucose content in the environment of the parasite is low; possibly when the promastigote is taken up into the host macrophage or when the glucose in the insect gut, taken up in the blood meal of the sandfly, has been used (Zilberstein and Dwyer 1984).

The catabolism of amino acids, and in particular proline, has been suggested as a major source of energy in promastigotes of L. donovani and L. tropica (Krassner and Flory 1972, Law and Mukkada 1979). High concentrations of proline and other amino acids have been found in the haemolymph of insects (tsetse flies) (Bursell 1966) and it has been suggested that the catabolism of amino acids by promastigotes of Leishmania may be an adaptation to life in the sandfly. Steiger and Meshnick (1977), however, suggested that amino acids were not important energy sources in L. braziliensis or L. donovani promastigotes and these results are similar to those of Hart and Coombs (1982) who found that both promastigotes and amastigotes of L. m. mexicana consumed some amino acids but also excreted others including alanine, glycine and proline.

Although the majority of work on substrate utilization has involved promastigotes growing in culture, the energy metabolism of L. m. mexicana amastigotes transforming in vitro has also been investigated (Hart and Coombs 1982). The major end-products of

amastigote catabolism, CO_2 and succinate, were the same as for promastigotes, but the utilization of substrates was found to differ significantly between the two forms. Glucose was catabolised by amastigotes at a lower rate than by promastigotes, but, in contrast, the rate of uptake and metabolism of non-esterified fatty acids was 10 fold higher in the mammalian stage (Hart and Coombs 1982). This suggests that fatty acids are important energy substrates for amastigotes, whereas they may only be important as substrates in promastigote anabolism. This ability to utilize fatty acids by the amastigote may be related to environmental conditions as macrophages regulate the level of lipoproteins in the serum (Brown et al. 1979) and may therefore provide a large supply of lipids for amastigote catabolism (Hart and Coombs 1982).

1.4.2.2. Gaseous requirements of Leishmania

Relatively high O_2 tensions (maximum growth at 20% v/v O_2) were found to be essential for continual growth of L. m. mexicana promastigotes in vitro, whereas purified amastigotes transformed successfully in vitro with as low as 0.4% (v/v) O_2 (Hart et al. 1981c, Hart and Coombs 1981). Carbon dioxide levels in the range 0.1-5% had little effect on promastigote growth, but at 5% CO_2 amastigote transformation was greatly stimulated compared with that at 0.1%. This finding may reflect the different environments of the two forms of the parasite as L. m. mexicana amastigotes are found in poorly vascularised cutaneous lesions that could well be expected to have low O_2 tensions. In addition, the host cell, the macrophage, may utilize a high proportion of the available O_2 producing CO_2 as an end product. It has been suggested that the susceptability of amastigote transformation to high CO_2 levels may be the trigger whereby transformation is initiated after ingestion of the amastigote

into the sandfly gut (Hart and Coombs 1981), however there is no evidence that CO_2 levels are higher in the insect gut than the parasitophorous vacuole of macrophages residing in the centre of a poorly vacularised lesion. CO_2 levels are thought to be important to a number of parasites; they may be involved in the hatching of coccidial oocysts, the exsheathment of nematode eggs and larvae and are also of importance to the activity of various CO_2-fixing enzymes involved in the metabolism of many parasitic helminths (Von Brand 1979, Barrett 1981).

1.4.2.3. Catabolism in *Leishmania*

Glycolysis, the TCA cycle and the pentose phosphate pathway (PPP) are the major pathways involved in the catabolism of carbohydrates in higher animals. All three pathways have been shown to be present in many parasitic protozoa, although in some instances the TCA cycle is absent, incomplete or even acts to a certain extent in the reverse direction (Von Brand 1979). In addition, the TCA cycle is a key entry point into general metabolism for the products of protein degradation, through amino acid catabolism, and fatty acid catabolism through β-oxidation. With this area of *Leishmania* biochemistry, as with most other aspects, it is mainly the culture promastigote that has been studied (See Marr 1980), although there has been some recent work on purified amastigotes (Coombs *et al.* 1982, Meade *et al.* 1984).

1.4.2.3.1. Glycolysis and the pentose phosphate pathway

The detection of metabolic intermediates and end-products by the use of radioactively-labelled substrates and the measurement of enzyme activities are classic ways of confirming the presence of an

active metabolic pathway. The production of labelled succinate and pyruvate from radioactive glucose in promastigotes of L. donovani (Chatterjee and Datta 1973), together with the detection of activities of all the glycolytic enzymes from hexokinase to pyruvate kinase in four species of Leishmania (Martin et al. 1976), suggested that the Embden-Meyerhoff pathway is functional in Leishmania promastigotes. The essential enzymes of the pentose phosphate pathway have also been found in four species of Leishmania (Martin et al. 1976).

Many eukaryotic cells which can utilize glucose under both aerobic and anaerobic conditions exhibit a phenomenon known as the Pasteur effect. If oxygen is admitted to an anaerobic suspension of cells that are utilizing glucose at a high rate by fermentation involving glycolysis, the rate of glucose catabolism declines to a small fraction of its anaerobic rate. This effect is mediated by the high [ATP]/[ADP] ratios produced during oxidative phosphorylation inhibiting the rate of glycolysis through inhibition of phosphofructokinase. In mammalian cells phosphofructokinase is an allosteric enzyme that is stimulated by ADP and AMP and inhibited by ATP and citrate and is a key enzyme in the control of glycolysis. Two other enzymes, hexokinase and pyruvate kinase, are also involved in the control of glycolysis but not to the same extent as phosphofructokinase (Krebs 1972). There are at least two kinds of pyruvate kinase found in mammalian cells which are classified according to functional parameters. In cells in which both glycolysis and gluconeogenesis occur (for example, liver cells), pyruvate kinase is allosterically regulated by various heterotrophic modulators, whereas in muscle cells, which are primarily concerned with energy production, the enzyme is not allosterically controlled.

Leishmania promastigotes do not exhibit the Pasteur effect and a

number of workers have shown that there appears to be no such regulation of glycolysis in Leishmania (Berens and Marr 1977a,b, Marr and Berens 1977, Berens et al. 1980, Marr 1980). Partially purified hexokinase from promastigotes of L. donovani and L. braziliensis showed conventional Michaelis-Menten kinetics with respect to its substrates but no inhibition or activation was found with any glycolytic intermediates or phosphorylated nucleotides (Berens et al. 1980). Glucose-6-phosphate, the product of hexokinase, can be metabolised further either via glycolysis or the PPP. The first enzyme of the PPP, glucose-6-phosphate dehydrogenase, also appeared to be uncontrolled (Berens et al. 1980), suggesting that some other mechanism, such as subcellular compartmentation, may be involved in regulating the fate of glucose-6-phosphate. Phosphofructokinase from both L. braziliensis and L. donovani promastigotes was found to be allosterically activated by its substrate fructose-6-phosphate and required AMP for activation, but was unaffected by other modifiers (Berens and Marr 1977a).

Pyruvate kinase from the supernatants of crude homogenates of L. donovani and L. braziliensis was reported to be allosteric with respect to its substrate PEP but to be unaffected by any heterotrophic modifiers except H^+ ions, and was therefore thought to be similar to the mammalian muscle enzyme (Berens and Marr 1977b). Previous studies on crude homogenates of L. tropica promastigotes suggested that pyruvate kinase was activated by fructose-1,6-diphosphate and AMP (Mukkada et al. 1974). Both of these studies, however, were performed on crude extracts that contained substrates and other factors that could affect the function of the enzyme and so have limited usefulness in elucidating regulation mechanisms. A recent preliminary report by Etges and Mukkada (1983) suggested that

purified pyruvate kinase from L. tropica was under both heterotrophic and homotrophic allosteric regulation. The enzyme was reputed to be activated by phosphoenolpyruvate and fructose-1,6-diphosphate and strongly inhibited by ATP, GTP and ITP. Clearly this enzyme could be an important regulatory point in Leishmania metabolism as phosphoenolpyruvate may also be carboxylated to oxaloacetic acid by phosphoenolpyruvate carboxykinase (PEP carboxykinase). These results also bring into question the suggestion that the lack of apparent regulation of glycolysis in Leishmania promastigotes indicates that the aerobic fermentation of carbohydrates is of only secondary importance to these organisms (Berens and Marr 1977a,b, Marr 1980). Control of glycolysis in Leishmania could occur by some mechanism not found in other animal cells and microorganisms, for instance the unique subcellular organisation of trypanosomatid flagellates (See section 1.4.3.).

There is little data on glycolysis and the PPP in amastigotes of Leishmania, although a number of glycolytic enzymes have been detected in amastigotes of L. m. mexicana (Coombs et al. 1982) and more recently all the enzymes of glycolysis and the PPP were demonstrated in purified L. donovani amastigotes (Meade et al. 1984). The activities of the glycolytic enzymes of both L. donovani and L. m. mexicana amastigotes are generally lower than promastigotes with pyruvate kinase especially low in L. m. mexicana amastigotes. This suggests that glycolysis may be less important to the amastigote than the promastigote.

1.4.2.3.2. The tricarboxylic acid cycle, respiratory chain and glyoxylate cycle.

The two main forms of Leishmania have a well-developed mitochondrion with plate-like cristae, utilize oxygen in a cyanide-sensitive

fashion and produce CO_2 as a major end product (Rudzinska et al. 1964, Simpson 1968, Hart et al. 1981a,c, Hart and Coombs 1982). All these facts suggest that Leishmania has a fully functional TCA cycle and indeed all the enzymes have been detected with approximately the same activity in promastigotes of four species of Leishmania (Martin et al. 1976, Mukkada 1977). The activities of citrate synthase and α-ketoglutarate dehydrogenase were so low in all four of the species investigated, however, that doubt was expressed as to whether the pathway was significant in the metabolism of carbohydrates in vivo (Mukkada 1977, Meade et al. 1984).

The TCA cycle is also the entry point for the products of both protein and fatty acid degradation. When amino acids are used as fuel in mammalian cells they undergo the loss of their amino group and the carbon skeletons can either be converted into glucose via gluconeogenesis or oxidized to CO_2 via the TCA cycle. As Leishmania does not appear to contain carbohydrate stores (Marr 1980), it is unlikely that gluconeogenesis is important in this parasite. Enzymes involved in the two major mechanisms of amino acid utilization, transamination and oxidative deamination, however, have been detected in Leishmania. Three aminotransferases have been detected in L. donovani (Von Brand 1979, Le Blancq and Lanham 1984) and glutamate dehydrogenase was present in four species of Leishmania promastigotes (Martin et al. 1976) and L. donovani amastigotes (Meade et al. 1984). Amino acids may be provided, at least in part, by intracellular protein degradation of abnormal proteins. This protein degradation has been investigated in L. tropica promastigotes (Simon and Mukkada 1983) where a labile class of proteins (molecular weight 30,000-60,000) has been demonstrated to have a rapid turnover. L. m. mexicana has also been shown to have high proteinase activity

especially in the amastigote (Pupkis and Coombs 1984). Enzymes involved in β-oxidation of fatty acids have been reported in L. m. mexicana promastigotes and amastigotes and the product, acetyl CoA, can also enter the TCA cycle and be subsequently oxidized to CO_2 and H_2O (Coombs et al. 1982).

Leishmania is similar to many other trypanosomatids in possessing a classical respiratory chain containing a terminal cytochrome aa_3 oxidase (reviewed Bowman and Flynn 1976, Von Brand 1979). The use of respiratory inhibitors with known mechanisms of action have greatly helped the elucidation of the components of trypanosomatid respiratory chains. The respiration of both amastigotes and promastigotes of L. m. mexicana was sensitive to cyanide, azide, antimycin A and high concentrations of amytal indicating similarities in the respiratory chain between both stages of the parasite and also mammalian systems (Hart et al. 1981c). There is no evidence for any branched respiratory chain, with multiple terminal oxidases, in Leishmania promastigotes (Martin and Mukkada 1979, Hart et al. 1981c), whereas cytochrome O has been detected in various members of the Kinetoplastida including C. fasciculata and T. brucei (Hill and Cross 1973, Kronick and Hill 1974).

The two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, have been reported in promastigotes of five species of Leishmania (Mukkada 1977, Simon et al. 1978). These findings, together with the detection of the intermediate glyoxylate, suggested that the cycle is operative in vivo. The glyoxylate cycle is found in many prokaryotes, fungi and plants (Hogg and Kornberg 1963, Spector 1972). The role of the glyoxylate cycle in Leishmania is not established, but it could be important in utilizing substrates such as fatty acids. It is not known if the cycle is present in amastigotes, however, the high rate of utilization of fatty acids by

transforming amastigotes of L. m. mexicana (Hart et al. 1981a,c, Hart and Coombs 1982) suggests that this form of the parasite may benefit from such a cycle.

1.4.2.3.3. Carbon dioxide fixation

Succinate is an important end product of many parasites and can be formed by TCA cycle enzymes operating in either the forward or reverse directions (Von Brand 1979). Excretion of succinate means that oxaloacetic acid (OAA) must be regenerated in order for the TCA cycle to continue functioning and this may occur through a number of CO_2-fixing reactions. PEP carboxykinase is the major CO_2-fixing enzyme in many succinate-excreting parasitic worms, including Fasciola hepatica (Prichard and Schofield 1968), Hymenolepis diminuta (Bueding and Saz 1968) and Trichinella spiralis (Ward et al. 1969). This enzyme, and other CO_2-fixing enzymes, pyruvate carboxylase and malic enzyme (Table 4), have been discovered in various species of trypanosomatids (Bacchi et al. 1970, Klein et al. 1975, Martin et al. 1976, Cataldi de Flombaum et al. 1977, Cazzulo et al. 1980).

Table 4. Enzymes of CO_2-fixation in Leishmania

1. Pyruvate carboxylase (EC 4.6.1.1.)

   Pyruvate + HCO_3^- + ATP $\rightleftharpoons$ Oxaloacetic acid + ADP + Pi

2. Malic enzyme (EC 1.1.1.40.)

   Pyruvate + HCO_3^- + NADPH $\rightleftharpoons$ Malate + NADP

3. PEP carboxykinase (EC 4.1.1.49.)

   PEP + ADP + HCO_3^- $\rightleftharpoons$ Oxaloacetic acid + ATP

L. donovani promastigotes incorporated ^{14}C-labelled CO_2 into the end product succinate in the presence of glucose (Chatterjee and Datta 1973). Pyruvate was suggested as the major CO_2 acceptor (possibly via pyruvate carboxylase), the malate produced being subsequently converted to succinate. Radioactive assays, however, showed tha presence in L. donovani promastigotes of three CO_2-fixing enzymes, pyruvate carboxylase, malic enzyme and PEP carboxykinase (Chatterjee and Datta 1974). Pyruvate carboxylase was subsequently found with low activity in promastigotes of three other species of Leishmania (Martin et al. 1976, Berens and Marr 1977a) and more recently in amastigotes of L. donovani (Meade et al. 1984). Neither Martin et al. (1976) or Meade et al. (1984) assayed for PEP carboxykinase, although Berens and Marr (1977a) could not detect the enzyme using a spectrophotometric assay in either L. braziliensis or L. donovani. PEP carboxykinase, however, has been reported to be the major CO_2-fixing enzyme in C. fasciculata (Bacchi et al. 1970, Klein et al. 1975), T. cruzi epimastigotes (Cataldi de Flombaum et al. 1977, Cazzulo et al. 1980) and T. brucei procyclic trypomastigotes (Klein et al. 1975), all of which produce substantial quantities of succinate as an end product (Bowman 1974).

1.4.2.4. Variations in metabolism during the life-cycles of trypanosomatids

Of special interest in the study of intermediary metabolism, including energy metabolism, in digenetic trypanosomatids is the elucidation of the ways in which different parasite forms are adapted to the very different environments that they encounter during their life-cycle. The changes that occur in ultrastructural morphology and intermediary metabolism can be extreme and the most studied transformation is that between the long slender bloodstream and the

culture procyclic forms of T. brucei. The bloodstream form is totally dependant on glycolysis for energy production with glucose, entirely to pyruvate under aerobic conditions (Flynn and Bowman 1973). TCA cycle and respiratory chain enzymes and cytochromes are absent, and the NADH produced by glycolysis is reoxidized by a coupled glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate oxidase system that involves oxygen as the terminal electron acceptor but is cyanide insensitive (See Bowman and Flynn 1976, Bowman and Fairlamb 1976). On transformation to the procyclic stage, other energy substrates, such as amino acids, are utilized together with glucose and the mitochondrion is developed to contain an active TCA cycle and a respiratory chain involving cytochrome aa_3 as a terminal electron acceptor (See Bowman 1974, Bowman and Flynn 1976). These changes in morphology and substrate utilization are also paralleled by changes in the enzyme content of the glycosome (See section 1.4.3.1.). Trypanosoma cruzi, in comparison, has little change in mitochondrial structure and energy metabolism during its life-cycle with respiration being cyanide sensitive in both the cultured epimastigotes and the mammalian developmental forms of the parasite (Rogerson and Gutteridge 1979). Leishmania experiences changes in its environment probably as extreme as those to which T. brucei is exposed, however, from the information presently available, there appears to be only quantitative and not qualitative differences in energy metabolism between amastigotes and promastigotes (Hart et al. 1981c, Hart and Coombs 1982, Coombs et al. 1982, Meade et al. 1984).

1.4.3. The relationship between energy metabolism and subcellular structure of trypanosomatids

The subcellular organisation of trypanosomatids has been investigated biochemically in the last few years as an extension to

previous ultrastructural studies using electron microscopy and cytochemical staining. The principle techniques involved in these studies have been differential centrifugation and isopycnic centrifugation on linear sucrose gradients with the subsequent detection of various enzymes that have been assigned to different subcellular compartments of the cell. The majority of the work has been performed on T. brucei (Opperdoes and Borst 1977, See Opperdoes 1984b), although a number of other trypanosomatids are now being studied in detail (Cannata et al. 1982, Coombs et al. 1982), with emphasis being given to the characterisation of glycosomes. The part played by these organelles, however, must be considered together with the contributions of other cell components if the whole organism is to be understood. The mitochondrion and cytosol have been studied to an extent with some trypanosomatids, but there is comparatively little known about other organelles.

1.4.3.1. Glycosomes

Glycosomes are microbody-like organelles that were first discovered in T. brucei (Opperdoes and Borst 1977, Oduro et al. 1980a) and have subsequently been reported to be present in Crithidia spp. (Opperdoes et al. 1977a, Taylor et al. 1980), Leishmania spp. (Coombs et al. 1982, Hammond and Gutteridge 1982) and T. cruzi (Taylor et al. 1980, Cannata et al. 1982). The presence of glycosomes in these three major representatives of the Trypanosomatidae suggest that the organelles may be a general feature of this family of protozoa (Opperdoes 1982). Glycosomes have been separated from other subcellular components by isopycnic centrifugation (Opperdoes and Borst 1977) and have been purified to near homogeneity from bloodstream form T. brucei (Opperdoes et al. 1984) (See section

1.2. for analysis of purified glycosomes). Initial enzyme analysis of bloodstream form T. brucei fractionated on sucrose gradients showed that a number of glycolytic enzymes (hexokinase, glucose phosphate isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase) together with glycerol kinase and glycerol-3-phosphate dehydrogenase were associated with glycosomes (Opperdoes and Borst 1977). In addition, it was shown that the activity of these enzymes exhibited latency in the presence of the detergent Triton X-100 which suggested that the enzymes were present in an organelle and not absorbed to particles (Opperdoes and Borst 1977). At the same time, glycerol-3-phosphate oxidase was found to band with the mitochondrial marker oligomycin-sensitive ATPase suggesting it was located in the highly repressed mitochondrion of bloodstream T. brucei (Opperdoes and Borst 1977, Opperdoes et al. 1977a).

Trypanosoma brucei was also screened for enzymes which have been used as classical markers for other microbodies in other organisms (mammalian peroxisomes or plant glyoxysomes). Catalase, D-amino acid oxidase, malate synthase and isocitrate lyase were not detectable in the bloodstream form of T. brucei and this suggested that glycosomes are distinctly different from all other organelles previously isolated (Opperdoes et al. 1977b). Interestingly, catalase was detected in glycosomes of Crithidia spp. (Opperdoes et al. 1977a), and peroxidase in those of T. cruzi epimastigotes (Docampo et al. 1976). A number of recent advances, however, have suggested that glycosomes and mammalian peroxisomes may in fact be related (Opperdoes 1984a). Enzymes involved in alkoxy-phospholipid biosynthesis have been detected in glycosomes of bloodstream T. brucei (Opperdoes 1984a) and some enzymes of β-oxidation of fatty acids have been implicated with glycosomes of Leishmania

promastigotes (Hart and Opperdoes 1983); both pathways are present in peroxisomes of mammalian cells (Lazarow and De Duve 1976, Lazarow 1978).

A number of other enzymes have been found associated with glycosomes of trypanosomatids. Adenylate kinase, which in mammalian cells is found between the inner and outer mitochondrial membrane (Janski and Cornell 1980), has a dual mitochondrial and glycosomal distribution in both bloodstream and procyclic T. brucei (Opperdoes et al. 1981) and was also found in glycosomes of bloodstream T. rhodesiense (Mclaughlin 1981) and Leishmania promastigotes (Hart and Opperdoes 1983). The last two enzymes of pyrimidine biosynthesis, orotate phosphoribosyltransferase and orotidine carboxylase, were also found to be associated with glycosomes of bloodstream form T. brucei, T. cruzi culture epimastigotes, blood trypomastigotes and intracellular amastigotes, L. m. amazonensis promastigotes and also C. luciliae (Hammond et al. 1981, Hammond and Gutteridge 1982, Pragobpol et al. 1984). Hammond and Gutteridge (1982), however, were unable to detect latency of orotate phosphoribosyltransferase and they found that various inhibitors were as effective against the enzyme activity of intact glycosomes as that in broken glycosomal preparations. They suggested therefore that both enzymes are located on the outside of the glycosome and are freely accessible to the cytoplasm. As well as these pyrimidine biosynthetic enzymes, the purine phosphoribosyltransferases, hypoxanthine-guanine and xanthine, were detected in association with the glycosomes of L. m. mexicana promastigotes (Hassan and Coombs 1984) and T. cruzi epimastigotes (Gutteridge and Davies 1982), whereas adenine phosphoribosyltransferase was cytosolic (Gutteridge

and Davies 1982, Hassan and Coombs 1984). The CO_2-fixing enzyme PEP carboxykinase has been found in the glycosomes of T. brucei procyclics (Opperdoes and Cottem 1982, Broman et al. 1983), T. cruzi epimastigotes and C. fasciculata (Cannata et al. 1982). Malate dehydrogenase has also been detected in glycosomes of procyclic T. brucei (Opperdoes et al. 1981) and also epimastigotes of T. cruzi, where it has a dual mitochondrial and glycosomal distribution (Cannata and Cazzulo 1984).

In T. brucei, the large loss of mitochondrial activity that occurs upon transformation of the insect procyclic stage to the bloodstream trypomastigote is apparently compensated for by changes in the metabolic activity of the glycosome (Hart et al. 1984). Glycosomes of culture procyclic forms differ from those of bloodstream forms in polypeptide and phospholipid content (Hart et al. 1984) and analysis of the enzyme content also show a number of differences (Outlined in Fig. 3). Firstly, the activities of the glycolytic enzymes are considerably lower in the procyclic stage of T. brucei (Opperdoes et al. 1981), which may reflect the lesser importance of glycolysis to this form of the parasite (Hart et al. 1984). Secondly, phosphoglycerate kinase is found in the cytosol of procyclic forms but in the glycosome of bloodstream forms. Thirdly, the decrease in activity of the dihydroxyacetone phosphate, glycerol-3-phosphate shuttle in procyclics is associated with the production of glycosomal PEP carboxykinase and malate dehydrogenase (Opperdoes et al. 1981, Opperdoes and Cottem 1982). It has been suggested that this malate dehydrogenase acts to reoxidize glycolytically-produced NADH, reoxidized by glyceraldehyde-3-phosphate dehydrogenase in bloodstream forms, and PEP carboxykinase provides glycosomal ATP, provided by phosphoglycerate kinase in bloodstream forms (Opperdoes and Cottem 1982). Thus redox balance appears to be maintained in the

Fig. 3a.

Diagramatic representation of the compartmentation of glycolysis in Trypanosoma brucei bloodstream trypomastigotes. (from Opperdoes 1982)

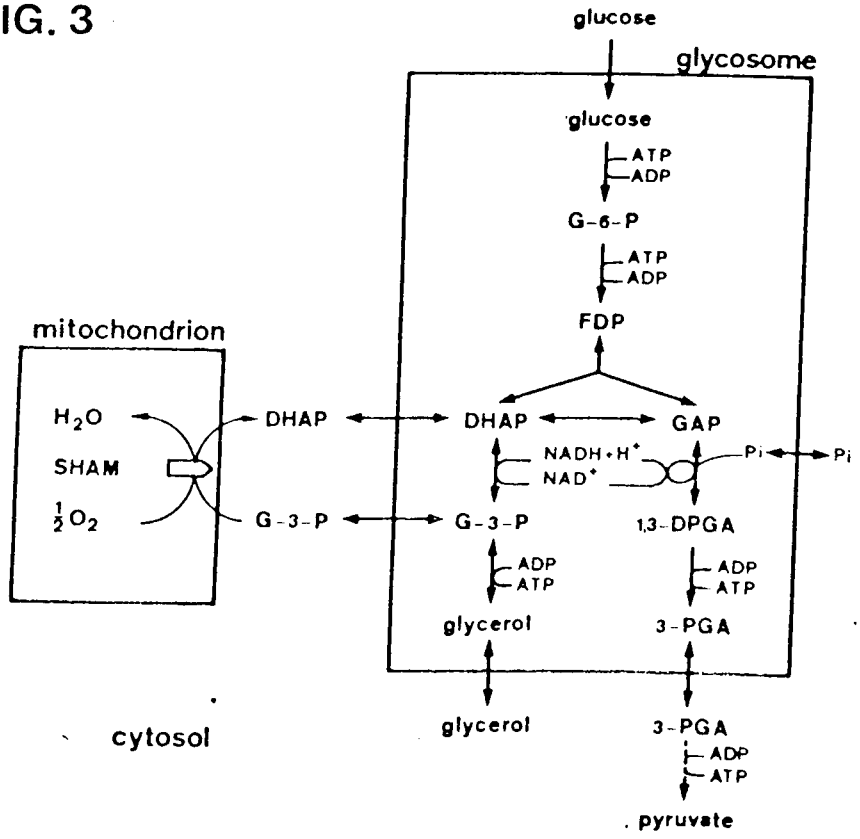
G-6-P, glucose-6-phosphate; FDP, fructose-1,6-diphosphate; DHAP, dihydroxyacetone phosphate; G-3-P, glycerol-3-phosphate; GAP, glyceraldehyde-3-phosphate; 1,3-DPGA, 1,3-diphosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; SHAM, salicylhydroxamic acid

Fig. 3b.

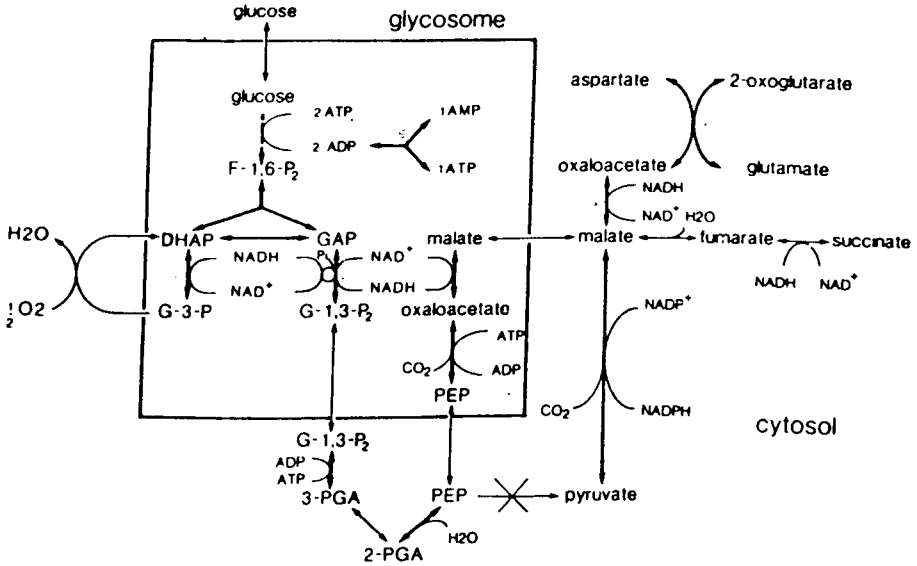
Diagramatic representation of the compartmentation of pathways within T. brucei procyclic trypomastigotes. (from Opperdoes and Cottem 1982)

FIG. 3

a)


glucose
glycosome
glucose
ATP
ADP
G-6-P
ATP
ADP
FDP
mitochondrion
H_2O
SHAM
$\frac{1}{2}O_2$
DHAP
G-3-P
DHAP
GAP
NADH+H+
NAD+
Pi
Pi
G-3-P
1,3-DPGA
ADP
ATP
ADP
ATP
glycerol
3-PGA
cytosol
glycerol
3-PGA
ADP
ATP
pyruvate

b)


glucose
glycosome
glucose
2 ATP
2 ADP
1 AMP
1 ATP
F-1,6-P_2
aspartate
2-oxoglutarate
oxaloacetate
glutamate
NADH
NAD+ H2O
H2O
DHAP
GAP
malate
malate
fumarate
succinate
NADH
NAD+
NADH
NAD+
NADH
NAD+
$\frac{1}{2}O_2$
G-3-P
G-1,3-P_2
oxaloacetate
ATP
CO_2
ADP
PEP
NADP+
CO_2
NADPH
cytosol
G-1,3-P_2
ADP
ATP
3-PGA
PEP
pyruvate
H2O
2-PGA

glycosomes of both forms of T. brucei (Fig. 3).

The changes that occur in glycosomal contents of other trypanosomatids during their life-cycles has not been well studied, but they are likely to be less extreme than with T. brucei which appears to exhibit the largest metabolic switch of any trypanosomatid studied to date. Information presently available, however, suggests that there is some variation in glycosomal structure between species as well as stages of the life-cycle (e.g. glycosome densities on sucrose gradients; see section 4.3. of the discussion), but the full extent is still to be elucidated.

All the glycolytic enzymes except glucose phosphate isomerase found in glycosomes of T. brucei are particulate and appear to be associated with each other (Opperdoes et al. 1977b, Oduro et al. 1980a,b, Opperdoes 1982). These enzymes apparently behave as a multienzyme complex, which is eluted in the void volume during Biogel A-5M column chromatography of cell homogenates (Oduro et al. 1980a). The complex can be dissociated into its individual components by 0.15M sodium chloride and this process can be reversed by removal of the salt by dialysis (Oduro et al. 1980a, Mclaughlin 1981).

It has been shown that glycosomal enzymes exhibit latency which was abolished by freezing and thawing, sonication or the addition of detergents such as Triton X-100 (Visser and Opperdoes 1980). This suggests that the glycosomal membrane is relatively impermeable to the substrates required for enzyme activity and therefore impermeable to certain cofactors and glycolytic intermediates. Further experiments with intact bloodstream form T. brucei pulse-labelled with ^{14}C-glucose showed the presence of two separate pools of glycolytic intermediates (Visser et al. 1981). One pool was rapidly labelled (in 15 seconds), represented 20-30% of the total cellular

metabolites, and was thought to be within the glycosome, whereas the other pool equilibrated only slowly (approximately 14 mins for labelling 90% of the cellular contents) and probably represented the cytosol (Visser et al. 1981). As the glycosomal volume only represents 9% of the cytoplasmic volume of T. brucei, but there is a glycosomal metabolite pool of 20-30% of the total pool size, this gives further evidence against a complete equilibration of metabolites between glycosome and cell sap (Visser et al. 1981). If the compartmentation of glycolysis outline in fig. 3. is to function then a number of metabolites including glycerate-3-phosphate, glycerol-3-phosphate, dihydroxyacetone phosphate and glucose must be able to cross the glycosomal membrane. It has been demonstrated that the glycosomal membrane is totally impermeable to hexose monophosphate (Opperdoes and Nwagwu 1980), suggesting a specific uptake mechanism for glucose. Glycerol-3-phosphate, however, unlike most other glycosomal intermediates, is rapidly equilibrated with the cytosolic pool, indicating that its crossing of the glycosomal membrane is facilitated by a specific translocator (Visser et al. 1981). Dihydroxyacetone phosphate must return to the glycosome to serve as an acceptor for reducing equivalents generated by glyceraldehyde-3-phosphate dehydrogenase, therefore the functioning of the shuttle would result in the rapid mixing of the dihydroxyacetone phosphate and glycerol-3-phosphate pools as indeed was observed (Visser et al. 1981). The presence of a multienzyme complex, together with this apparent compartmentation of intermediary metabolites and enzymes, may account for the extremely high rate of glycolysis that is found in bloodstream T. brucei. There is no comparable data concerning metabolite compartmentation in Leishmania.

1.4.3.2. Other organelles

With methods currently in use, the unitary mitochondrion of trypanosomatid flagellates is ruptured during cell lysis into vesicles that retain some of the soluble matrix enzymes. These mitochondrial vesicles have been found to have a lower density than glycosomes and the two organelles can therefore be separated by isopycnic centrifugation (Opperdoes and Borst 1977, Opperdoes et al. 1977a, Cannata et al. 1982). The inactive mitochondrion of bloodstream T. brucei was shown to contain oligomycin-sensitive ATPase, subsequently used as a marker for this organelle, and also glycerol-3-phosphate oxidase, isocitrate dehydrogenase, adenylate kinase and a particulate malate dehydrogenase. This latter enzyme represents about 10% of the total cellular malate dehydrogenase, the remainder of which was found in the cytosol (Opperdoes et al. 1977a, Opperdoes et al. 1981). Transformation to the procyclic stage is accompanied by the development of an extensively-branched mitochondrion with numerous plate-like cristae and an active respiratory chain. All the enzymes found in the bloodstream T. brucei mitochondrion were also detected in the procyclic organelle but in addition the TCA cycle enzymes, including succinate dehydrogenase, sn-glycerol-3-phosphate dehydrogenase and two enzymes involved in threonine metabolism, NAD-linked threonine dehydrogenase and carnitine acetyltransferase were also found (Opperdoes et al. 1981).

Trypanosoma brucei bloodstream and procyclic forms have also been screened for enzymes to serve as markers for the plasma membrane, flagellar pocket, lysosomes, endoplasmic reticulum and Golgi apparatus. α-glucosidase was found to be a good marker for the plasma membrane of both bloodstream and procyclic T. brucei. This

structure equibrated on sucrose gradients as a plasma membrane-microtubule complex at a density of 1.20-1.22 g/ml (Steiger et al. 1980, Opperdoes and Steiger 1981). Ouabain-sensitive Na^+/K^+-stimulated ATPase, adenylate cyclase, and 5'-nucleosidase have also been suggested as plasma membrane markers. The two former enzymes were considered good markers in bloodstream T. brucei by Rovis and Baekkeskov (1980), although Steiger et al. (1980) were unable to detect ouabain-sensitive ATPase. Voorheis et al. (1979) reported the presence of 5'-nucleosidase in T. brucei membranes, and this enzyme also has a surface membrane localization in L. donovani promastigotes (Dwyer and Gottlieb 1984). In addition to a 5'-nucleosidase, a 3'-nucleosidase, an enzyme that has not been demonstrated in mammalian cells, was also detected in L. donovani surface membranes (Gottlieb and Dwyer 1983, Dwyer and Gottlieb 1984). Membrane bound adenylate cyclase, which was thought to co-purify with plasma membranes in bloodstream T. brucei (Voorheis et al. 1979), was shown to be associated with some other membrane system, possibly the flagellar pocket (Walter and Opperdoes 1982).

Acid phosphatase was found in association with both the flagellar pocket and the endoplasmic reticulum in T. brucei bloodstream forms (Steiger et al. 1980), whereas in the procyclic form it was only found associated with microsomal membranes derived from the endoplasmic reticulum (Opperdoes and Steiger 1981). Using subcellular fractionation and ultrastructure cytochemistry, acid phosphatase, which is usually a lysosomal marker, was shown to have 50% of its activity on the plasma membrane of L. donovani promastigotes, where it was distributed over the entire surface of the parasite (Gottlieb and Dwyer 1981).

Galactosyl transferase, which is a marker for the Golgi

apparatus of higher eukaryotes, has also been found in this organelle of bloodstream T. brucei (Steiger et al. 1980) and also T. congolense and T. vivax (Grab et al. 1984), suggesting that the site of the major glycosylations of variable surface antigens is the Golgi apparatus in these three species of trypanosomes. There is no information at present on the enzymes of the Golgi apparatus in Leishmania.

Lysosomes have also been investigated in T. brucei bloodstream and procyclic forms with an acid proteinase and ∝-mannosidase being found associated with the organelles (Steiger et al. 1980, Opperdoes and Steiger 1981). A high cysteine proteinase activity found in L. m. mexicana amastigotes (Coombs 1982) has recently been shown to be located in organelles, possibly the lysosome-like "Megasomes" (Coombs and Pupkis 1984).

The study of these hydrolytic enzymes in trypanosomatids and their assignments to specific organelles as markers has helped to characterise further the subcellular organisation of the digestive systems of these parasites.

1.5. THE AIMS OF THE PROJECT

The overall aim of this project was to investigate aspects of the intermediary metabolism of Leishmania mexicana mexicana in order to correlate the differences in metabolism between the insect promastigote and mammalian amastigote stages to their respective environments and also to discover biochemical differences between the parasite and mammalian host. In this way it was hoped to find systems essential to the parasite which could be suitable for chemotherapeutic attack. The initial part of the project was to investigate further the carbohydrate metabolism of L. m. mexicana and

in particular the part played by CO_2 in leishmanial amastigote metabolism. A second aim was to elucidate the subcellular organisaton of the parasites metabolism and the enzyme distribution between glycosomal, mitochondrial and cytoplasmic compartments. A third object was to purify and characterise two of the enzymes found to be especially important to the amastigote; PEP carboxykinase and malate dehydrogenase. An additional aim was to gain direct information on the mechanism of antimonial action against Leishmania and the reasons for the specificity of pentavalent antimonials for the amastigote form

2.0 MATERIALS AND METHODS

2.1 PARASITES

2.1.1 Parasites and their cultivation.

The WHO-recommended strain of L. m. mexicana (code number M379) used in this study was first isolated in 1962 in Belize and was obtained from Dr. R. A. Neal (The Wellcome Research Laboratories, Beckenham, Kent) in 1972. The parasites, which were uncloned, were routinely passaged by subcutaneous injection of 3×10^7 purified amastigotes into the shaven rump of female NIH (Hacking and Churchill, Huntington, Cambridge, England) or CBA (Zoology Department, University of Glasgow) mice. The area surrounding the point of inoculation was shaved monthly and large cutaneous lesions developed in the mice 3 - 9 months after infection. The lesions that developed in CBA mice were generally large and unruptured and they grew more quickly than those in NIH mice, therefore CBA mice were used routinely after the first 3 months of this study.

Leishmania m. mexicana promastigotes were derived by transformation in vitro from amastigotes. A small piece of lesion was aseptically removed from a CBA mouse, inoculated into 5ml HOMEM medium (Berens et al. 1976) supplemented with 20% (v/v) heat inactivated foetal calf serum (HIFCS) and 25µg/ml gentamycin sulphate to prevent bacterial growth, and incubated at 25°C for 72h. The promastigotes produced were then routinely subpassaged into HOMEM medium when at mid-log phase of growth (approximately every 3 days) and incubated at 25°C in sealed universals using air as the gas phase throughout. Only promastigotes that had been subpassaged less than 11 times were used in this study.

Promastigotes of L. m. mexicana clone (2B(sub1)), isolated from M379 promastigotes by Mrs M.Y. Ghafoor, were stored as a stabilate in liquid nitrogen until use, when they were grown in HOMEM medium as above. Amastigotes of the same clone were grown in female CBA mice

by inoculation of 4 x 10^7 promastigotes. Amastigote lesions, which took between 5 - 7 months to grow, were harvested as described in section 2.1.2.

Two species of Leishmania were kindly supplied by Dr. R. Stokes (Liverpool School of Tropical Medicine). Amastigotes isolated from mice and culture promastigotes of L. m. amazonensis (LV 78) and culture promastigotes of L. donovani (LV 9), were obtained as washed pellets frozen at -70°C and ready for use. Leishmania donovani amastigotes were supplied in infected Cotton rats.

Procyclic T. b. brucei Antat 1.8 and T. b. rhodesiense Eatro 2340 were grown in Hills medium at 25°C (Cunningham 1977) with 10% (v/v) HIFCS and 25µg/ml gentamycin sulphate and harvested in late log phase of growth (approximately 10^7 cells/ml). Trypanosoma b. brucei and T. b. rhodesiense pleomorphic bloodstream trypomastigotes, derived from the same stock as the procyclics, were grown in NIH mice after inoculation of 10^6 bloodstream forms and harvested for use 5 days later.

2.1.2 Harvesting parasites

Leishmania m. mexicana promastigotes, grown in bulk in 500ml sealed bottles containing 200ml of medium, were harvested when at mid-log phase of growth (1-2 x 10^7 cells/ml) by centrifugation at 1600g for 10 min at 4°C on an MSE Chilspin centrifuge. The parasites were washed three times in 0.25M sucrose and then either used immediately or stored as pellets at -70°C.

Amastigotes of L. m. mexicana were purified essentially as described by Hart et al. (1981b) with the following procedure: The lesion was aseptically excised from the mouse, placed in 20ml of PSGEMKA buffer and cut into small pieces. The chopped lesion was then homogenised between two gauze meshes to release the parasites

and the suspension filtered through No 1 Whatman filter paper to remove large pieces of debris. Saponin (0.05% w/v) was added to lyse red blood cells, the parasites washed three times in PSGEMKA and then gently dispersed by two strokes of a Potter homogeniser working at low speed. The cells were then diluted with buffer to give a parasite density of approximately 10^8 /ml and so minimise parasite clumping. The cells were passed down an ion-exchange column containing 2g of CM-Sephadex C25 to remove the contaminating host white cells, washed once in PSGEMKA and then once in 0.25M sucrose. They were either used immediately or stored as pellets at -70°C. Amastigote numbers and the percentage contamination with other cells were determined using an improved Neubauer haemocytometer. This procedure routinely gave preparations with less than 0.1% (cell/cell) contamination.

Leishmania donovani amastigotes were isolated from the spleens of Cotton rats and purified using the same method as for L. m. mexicana.

Bloodstream forms of T. b. brucei and T. b. rhodesiense were purified by ion-exchange chromatography using the method of Lanham (1968). Blood was removed by cardiac puncture, collected in heparin to prevent clotting, and the trypanosomes separated from blood cells on a DEAE cellulose column using phosphate saline glucose buffer pH 8.05. All harvested trypanosomes were washed twice in 0.25M sucrose before use.

Adult Schistosoma mansoni, kindly supplied by Dr. A. McGregor (Biochemistry Department, University of Glasgow), were isolated from Balb/c mice 60 days after infection, washed and suspended in Hanks Saline and stored for 7-14 days at -20°C.

2.1.3 Preparation of parasite homogenates and mouse liver extracts

To prepare parasite homogenates for use in many experiments, Leishmania amastigotes and promastigotes and trypanosomes were suspended in 0.25M sucrose and lysed by either the addition of 0.1% (v/v) Triton X-100 or by 4 cycles of freezing at -70°C and thawing at 25°C. In some experiments, as specified in the text, enzyme activities were determined for parasite lysates produced in the presence of 100µg/ml of the proteinase inhibitor leupeptin. Parasites were lysed by mixing to a thick paste with acid-washed defined alumina (Sigma type 305) for differential and isopycnic centrifugation fractionation experiments.

Homogenates of S. mansoni were prepared by twenty strokes of a Dounce homogeniser and used immediately. Mouse liver extracts were prepared using female CBA mice. The livers were excised, washed twice in 0.1M Tris-HCl buffer pH 7.3, and homogenised with twenty passes of a Potter homogeniser. The homogenate produced was centrifuged at 4°C for 1h at 240,000g and the supernatant stored at -70°C until use.

2.2 ASSAYS

2.2.1 Enzyme assays.

All enzyme assays were performed on either a Pye Unicam SP 8000 or a Perkin-Elmer Lambda 5 UV/VIS spectrophotometer, both incorporating a temperature controlled cell holder. All assays were in 1.5ml total volume unless otherwise specified. For the determination of specific activities, all assays were carried out at 25°C. For inhibitor studies, promastigote extracts were assayed at 25°C, amastigote extracts at 34°C and both mouse liver and S. mansoni extracts at 37°C. The following assays were used as standard except where specified.

Hexokinase (EC 2.7.1.2) was assayed at 340nm (Martin et al. 1976) in a reaction mixture containing 100mM Tris-HCl pH 7.3, 0.66mM EDTA, 6.6mM $MgCl_2$, 15mM glucose, 0.33mM NADP, 1.4 units yeast glucose-6-phosphate dehydrogenase and 30-50µg sample protein. The reaction was initiated by 1mM ATP.

Glucose phosphate isomerase (EC 5.3.1.9) was assayed at 340nM (Martin et al. 1976) in a reaction mixture containing 100mM Tris-HCl pH 8.0, 0.33mM NADP, 0.7 units yeast glucose-6-phosphate dehydrogenase and 3.3mM fructose-6-phosphate. The reaction was initiated by 30-60µg sample protein.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed at 340nm (Bergmeyer 1974) in a reaction mixture containing 100mM triethanolamine buffer pH 7.5, 0.33mM NADP, 13.3mM EDTA and 10-20µg sample protein. The reaction was initiated with 2mM glucose-6-phosphate.

Phosphofructokinase (EC 2.7.1.11) was assayed at 340nm (Mukkada et al. 1974) in a reaction mixture containing 100mM Tris-HCl pH 7.3, 0.1mM NADH, 6.6mM $MgCl_2$, 3.3mM fructose-6-phosphate, 0.2 units rabbit muscle aldolase, 2 units rabbit muscle triosphosphate isomerase/α-glycerophosphate dehydrogenase and 100µg sample protein. The reaction was started with 0.4mM ATP.

Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) was assayed at 340nm (Bergmeyer 1974) in a reaction mixture containing 100mM triethanolamine buffer pH 7.5, 1mM ATP, 3.3mM glycerate-3-phosphate, 0.1mM NADH, 1mM EDTA, 2mM $MgSO_4$ and 3.3 units of rabbit muscle phosphoglycerate kinase. The reaction was started by the addition of 50-100µg of sample protein.

Phosphoglycerate kinase (EC 2.7.2.3) was assayed at 340nm (Bergmeyer 1974) in a reaction mixture containing 100mM triethanolamine buffer

pH 7.5, 1.66mM $MgSO_4$, 0.6mM ATP, 2.66mM glycerate-3-phosphate, 0.1mM NADH and 2.5 units of yeast glyceraldehyde-3-phosphate dehydrogenase. The reaction was started by the addition of 20-50µg sample protein.

Pyruvate kinase (EC 2.7.1.40) was assayed at 340nm (Bucher and Pfleiderer, 1962) in a reaction mixture containing 80mM Tris HCl 7.3, 8mM $MgSO_4$, 75mM KCl, 0.1mM NADH, 4mM PEP, 15 units of pig heart lactate dehydrogenase and 30-150µg sample protein. The reaction was started with 1mM ADP.

Isocitrate dehydrogenase (EC 1.1.1.42) was assayed at 340nm (Bergmeyer 1974) in a reaction mixture containing 100mM triethanolamine HCl buffer pH 7.5, 4mM $MnSO_4$, 0.33mM NADP, and 80-100µg sample protein. The reaction was started by the addition of 3.3mM isocitrate.

∝-Ketoglutarate dehydrogenase (EC 1.2.4.2) was assayed at 340nm (Martin et al. 1976) in a reaction mixture containing 100mM Tris HCl pH 7.0, 0.33mM thiamine pyrophosphate, 0.1mM coenzyme A, 3.3mM L-cysteine, 0.33mM NAD and 150µg sample protein. The reaction was initiated with the addition of 3.3mM ∝-ketoglutarate.

Malate dehydrogenase (EC 1.1.1.37) was assayed at 340nm in both the forward and reverse directions. For oxaloacetic acid reduction (Martin et al. 1976) the reaction mixture contained 100mM triethanolamine HCl buffer pH 7.5, 0.2mM NADH and 5-20µg sample protein. The reaction was started with 0.83mM oxaloacetic acid. For malate oxidation (Bergmeyer 1974) the reaction mixture contained 1M glycine, 0.4M hydrazine sulphate, NaOH buffer pH 9.5, 2mM NAD, and 5-20µg sample protein. The reaction was initiated with 133mM sodium malate.

Phosphoenolpyruvate carboxykinase (PEP carboxykinase) (EC 4.1.1.49) was assayed at 340nm by following the oxidation of NADH after coupling the production of oxaloacetic acid to bovine malate dehydrogenase (Bentle and Lardy 1976). The reaction mixture

contained 100mM imidazole buffer pH 6.6, 4mM $MnSO_4$, 20mM $NaHCO_3$, 0.1mM NADH, 4mM PEP, 15 units of bovine malate dehydrogenase (in glycerol) and approximately 40-150µg sample protein. The reaction was started by the addition of 1mM ADP. In order to avoid interference from the high promastigote NADH oxidase activity that is stimulated by Mn^{2+} and HCO_3^-, promastigote PEP carboxykinase was assayed anaerobically with the same incubation mixture as above but with 0.7ml total volume. Amastigote Mn^{2+} stimulated NADH oxidase was low in comparison to the PEP carboxykinase activity and was simply subtracted to obtain the PEP carboxykinase activity.

Pyruvate carboxylase (EC 6.4.1.1) was assayed at 340nm (Martin et al. 1976) in a reaction mixture containing 100 mM HEPES buffer pH 7.8, 6.6mM $MgCl_2$, 20mM $NaHCO_3$, 0.1mM NADH, 0.4mM acetyl CoA, 1mM ATP, 11 units bovine malate dehydrogenase and 100-150µg sample protein. The reaction was initiated with 10mM pyruvate.

Malic enzyme (carboxylating) (EC 1.1.1.40) was assayed at 340nm (Klein et al. 1975) in a reaction mixture containing 100mM imidazole buffer pH 6.6, 4mM $MnSO_4$, 0.1mM NADPH, 20mM $NaHCO_3$ and 100-150µg sample protein. The reaction was initiated with 10mM pyruvate.

Malic enzyme (decarboxylating) (EC 1.1.1.40) was assayed at 340nm (Martin et al. 1976) in a reaction mixture containing 100mM triethanolamine buffer pH 7.5, 4mM $MnSO_4$, 0.33mM NADP and 60-150µg sample protein. The reaction was initiated with 3.3mM malate.

Glutamate dehydrogenase (EC 1.4.1.3) was assayed at 340nm (Martin et al.. 1976) in a reaction mixture containing 100mM Tris HC1 pH 8.5, 320mM $(NH_4)_2SO_4$, 0.2mM NADH or NADPH and 6-60µg sample protein. The reaction was started with 6.6mM ∝-ketoglutarate.

Glutathione reductase (EC 1.6.4.2) was assayed at 340nm (Bergmeyer 1974) in a reaction mixture containing 80mM sodium phosphate buffer

pH 6.6, 0.1mM NAD(P)H, and 100µg sample protein. The reaction was started with 0.3mM oxidized gluthatione.

Fumarate reductase (EC 1.3.99.1) (NADH-dependant reversal of succinate dehydrogenase) was assayed at 340nm (Klein et al. 1975) in a reaction mixture containing 80mM Tris HCl pH 8.0, 0.1mM NADH and 150µg sample protein. The reaction was initiated with 3.3mM sodium fumarate.

Isocitrate lyase (EC 4.1.3.1) was assayed according to Dixon and Kornberg (1959) with 100mM imidazole buffer pH 6.6, 6.6mM $MgSO_4$ or $MnSO_4$, 0.66mM EDTA, 3.3mM isocitrate and 3.3mM phenylhydrazine hydrochloride. The production of glyoxylate phenylhydrazone was followed at 324nm.

Alanine aminotransferase (EC 2.6.1.2) was assayed at 340nm (Bergmeyer 1974) in a reaction mixture containing 85mM sodium phosphate buffer pH 7.4, 13.2mM L-alanine, 0.1mM NADH, 1.5 units of rabbit muscle lactate dehydrogenase and 10-80µg sample protein. The reaction was started with 6.6mM α-ketoglutarate dehydrogenase.

ATPase (EC 3.6.1.4) was determined according to Frasch et al. (1978). In the assays, which were performed in duplicate, approximately 50ug of sample protein was incubated in a total volume of 1.0ml containing 100mM Tris-HCl pH 7.3, 4 µmoles $MgCl_2$ and 3µmoles ATP. After 30 minutes incubation, the reaction was stopped by the addition of 0.1ml 50% (w/v) trichloracetic acid, centrifuged at 10,000g for 5 mins at 18°C and the supernatant estimated for phosphate by the method of Rathburn and Betlach (1969). 0.8ml of supernatant was added to 0.55ml of buffer (3M acetate buffer, 37% formaldehyde in a ratio of 1 : 0.1) and mixed immediately. 50µl ammonium molybdate (2% w/v) and 100µl 6.75mM stannous chloride were added, the mixture left for 5 min and the absorbance read at 735nm. Sample phosphate

concentrations were determined by comparison with a standard curve prepared with 0-200μmoles (in 25μg steps) of K_2HPO_4. To assay oligomycin-sensitive ATPase, the enzyme sample was pre-incubated with 20μg/ml of oligomycin for 10 minutes at 25°C before the addition of ATP. Controls in which trichloracetic acid was added at time zero were included for all the ATPase assays.

2.2.2 Protein determinations

Protein concentrations were determined by one of two methods. In the enzyme activity and fractionation studies the method of Lowry et al. (1951) was used. 1.0ml of reagent 1 (2% (w/v) Na_2CO_3 in 0.1M NaOH, 1% (w/v) $CuSO_4$, and 2% (w/v) NaK tartrate mixed in the ratio of 98:1:1) was added to 0.2ml of sample and left to stand for at least 10 min at room temperature. 0.1ml of 1N Folin and Ciocalteu's phenol reagent was then added, mixed well and the mixture left for 1 h at room temperature and the absorbance read at 700nm. Sample protein concentrations were determined by comparison with a standard curve prepared with 0-50μg (in 5μg steps) of bovine serum albumin (BSA).

The Coomassie blue method of Bradford (1976) as modified by Read and Northcote (1981) is 5-10 times as sensitive as the method of Lowry et al. (1951) and was therefore used for determining protein concentrations during the enzyme purifications and the digitonin experiments. 950μl of dye (100mg Coomassie blue G-250 dissolved in 8.8% (v/v) orthophosphoric acid and 4.67% (v/v) ethanol and filtered through Watman No 1 filter paper) was added to 50μl of sample protein, left for 5 min at room temperature and the absorbance read at 595nm. Sample protein concentrations were determined by comparison with a standard curve prepared with 0-10 μg (in 0.5μg steps) of BSA.

2.3. INHIBITOR STUDIES WITH ANTIMONIALS AND ARSENICALS.

Melarsen and 1714 were dissolved in distilled, deionised water, melarsen oxide in 0.1M NaOH and Mel B in propylene glycol, all to 3mM. Before use melarsen oxide was diluted 1:5 in 0.1M Tris-HCl pH 7.3. 5822 was prepared as a cloudy suspension in absolute ethanol at a concentration of 3mM. Pentostam was dissolved at 1.0mg/ml (1.1mM) in 0.1M gluconic acid and Triostam at 2.0mg/ml (6.0mM) in 0.01M gluconic acid. The effect of inhibitors upon enzymes was tested by pre-incubation with the enzyme at the appropriate temperature for one hour before the enzyme activity was measured. Only concentrations of inhibitors that did not significantly effect the activities of the linkage enzymes were used. In all cases, a cuvette containing an equal concentration of the solvent used to dissolve the inhibitor was assayed in parallel to measure the control enzyme activity. The activity of the inhibitors against enzymes was expressed as the I_{50}, the concentration that inhibited the activity by 50%, and was determined from a curve drawn by eye to fit a series of at least 5 inhibitor concentrations.

The effect of the inhibitors on L. m. mexicana promastigote growth was assessed using a range of doubling concentrations with a three day incubation period. The number of motile cells after 72 hours were counted and the LD_{50} (the concentration of inhibitor that reduced the number of parasites to 50% of the control) and the MLC (minimum concentration that killed all the parasites) determined. The time taken for Triostam (400µg/ml), and melarsen oxide (100µg/ml) to exert an effect on promastigotes in vitro was monitored by observations of cell morphology and mobility using phase contrast microscopy. The effects of cysteine on the growth of promastigotes and the inhibitory action of Triostam was assessed using a range of cysteine concentrations in the incubations.

2.4 STUDIES ON THE SUBCELLULAR LOCATION OF LEISHMANIAL ENZYMES

2.4.1 Particulate and soluble activities.

The distribution of enzyme activities between the particulate and soluble parts of the parasite was determined for amastigotes and promastigotes of L. m. mexicana. Parasites were lysed by the addition of 0.1% (v/v) Triton X-100 as described in section 2.1.3. The homogenates were centrifuged for 2 min at 10,000g at 18°C in an Eppendorf centrifuge and the supernatant removed. After suspending the pellet in 0.25M sucrose, to the same volume as the supernatant, the enzyme activity in each fraction was determined and the values expressed as the percentage of the total activity recovered in both fractions.

2.4.2 Differential centrifugation

All procedures were performed at 4°C. Washed L. m. mexicana promastigotes and amastigotes were lysed with alumina as described in section 2.1.3. The degree of cell breakage was assessed using phase contrast microscopy and when >95% of the cells were lysed the paste was resuspended in 2-3 volumes of breakage buffer (0.25M sucrose, 25mM Tris-HCl pH 7.3, 1mM EDTA). The alumina was removed by centrifugation at 1,000 rpm (180g) for 5 min on an MSE Chilspin centrifuge and the supernatant taken as the homogenate (H). This was then fractionated in 3 steps: 2,100g for 10 mins to give pellet 1 (P1); 15,800g for 10 mins to give pellet 2 (P2); 240,000g for 1 hour to give pellet 3 (P3) and the supernatant (S). Each pellet was resuspended in 1.0ml of breakage buffer. In most cases the fractions were assayed immediately but on occasions they were frozen at -70°C until use. All fractions were assayed for enzyme activity in the presence of 0.1% Triton X-100 to ensure organelle lysis.

2.4.3 Sucrose density gradient centrifugation

All procedures were performed at 4°C. Promastigotes of L. m. mexicana were lysed and the alumina removed in the same manner as described above for differential centrifugation. The post-alumina homogenate (H) was centrifuged at 2,100g for 3 mins and the resultant supernatant (H2) removed. 1.15-1.24g/ml linear sucrose gradients containing 25mM Tris-HCl pH 7.3 and 1mM EDTA were prepared using a Pharmacia P-3 peristaltic pump and an LKB gradient mixer. 33ml of gradient were layered onto 1.0ml of 2.5M sucrose and cooled to 4°C. Subsequently, 2-3ml of H2 was layered onto the pre-cooled gradient which was then centrifuged in an MSE Prepspin 50 ultracentrifuge using a MSE titanium vertical tube rotor. The rotor was accelerated at minimum setting to 1,000 r.p.m. and then at maximum acceleration to 50,000 r.p.m. (200,000g). After 105 mins, the rotor was deaccelerated with the brake on to 1,000 r.p.m. and then without the brake to rest. The gradient was collected in 2.0ml aliquots by suction after piercing the bottom of the tube with a needle. After mixing each fraction, 1.0ml was taken and weighed to determine the density and then assayed immediately or frozen at -70°C. Enzyme assays were performed in the presence of 0.1% (v/v) Triton X-100, or by 4 cycles of freezing at -70°C and thawing at 25°C in the case of ATPase, to ensure organelle lysis. The enzyme data was calculated and presented essentially as described by Beaufay and Amar-Costesec (1976) using the formula:

$$\text{Frequency} = \frac{\%\ \text{of total activity}}{\text{computed difference in density}} \times \frac{1}{100}$$

In order to combine the results of different experiments, the density distribution histograms were normalised according to the method of Bowers and De Duve (1967). The distribution histograms

were divided into 18 sections with equal density increments of 0.05g/ml. Taking a starting density of 1.24 g/ml, the fractional area included in each of the 18 sections was calculated and normalised according to the density range for the individual fractions from each experiment. As the linear gradient had a final density of 1.15 g/ml, all the activity recovered at a lower density than this was combined and is presented as 1 fraction constituting the soluble activity (ρ = 1.15 - 1.07 g/ml). In the figures presented (Fig. 7 and 8), the left hand axis represents the frequency of the enzymes on the gradient (ρ = 1.24 - 1.15 g/ml), and the right hand axis represents the frequency for the single soluble fraction(ρ = 1.15 - 1.07 g/ml). All results were calculated and plotted using a BASIC program written for a Commodore Pet 2 series 2001 computer and linked to a Watanabe WX 4671 Digiplot.

2.4.4 The release of Leishmania m. mexicana enzymes using digitonin.

The release of soluble enzymes from L. m. mexicana amastigotes and promastigotes was studied by suspending purified amastigotes (10^9 parasites/sample) or washed promastigotes (2×10^8 parasites/sample) in breakage buffer and exposing them at 25°C to digitonin at a range of concentrations and for different times. The digitonin was dissolved in dimethylformamide and it was confirmed that controls with the solvent did not cause enzyme release. After the appropriate incubation period, the parasites were centrifuged at 10,000g for 5 mins at 18°C, the supernatant removed, and the pellet resuspended in breakage buffer containing 0.1% (v/v) Triton X-100. Enzyme assays were performed immediately for each fraction and the activity in each expressed as a percentage of the total activity recovered in the two fractions.

2.4.5 Isoelectric focusing

Flat bed agarose isoelectric focusing (IEF) was performed using pH range 3.5-10 as outlined in the Pharmacia guide to IEF.

2.4.5.1 Preparation of agarose gels

Agarose IEF was performed by casting 27ml of gel onto a hydrophilic polyester sheet (Gelbond). The gel contained the following components:

0.275g Agarose IEF;

3.3g sorbitol;

24.75ml distilled deionized water;

1.75ml LKB Ampholine pH 3.5-10.

The agarose and sorbitol were dissolved in the water by heating in a boiling water bath. When the solids were dissolved the gel was cooled to 75^{o}C, the Ampholine added and mixed, and the solution poured into a 114 x 185 mm mould. Once the gel was set (about 10-15 min) it was placed at 4^{o}C for at least 1hr before use.

2.4.5.2 Preparation of sample protein

2 x 10^{9} amastigotes and 1 x 10^{9} promastigotes of L. m. mexicana clone 2B (sub1) were each lysed in 400µl 0.25M sucrose containing 100µg/ml leupeptin and 0.2% (v/v) Triton X-100. Both the amastigote and promastigote samples were then split into two equal fractions. To the first fraction was added 50µl 1M NaCl containing 10% (v/v) ethylene glycol. After 5 min incubation at 4^{o}C, the sample was centrifuged at 10,000g for 5 min at 18^{o}C and the resultant supernatant taken as the homogenate (H). The second sample was centrifuged at 10,000g for 5 min and the resultant supernatant taken as S. The pellet was washed once in 0.25M sucrose and then resuspended in 100µl 200mM NaCl containing 10% (v/v) ethylene glycol

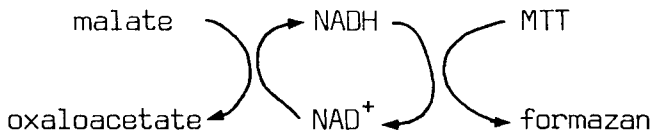
and 100µg/ml leupeptin. After 5 min incubation at 4°C, the sample was recentrifuged and the resultant supernatant taken as P.

2.4.5.3 Running the agarose gel

The gel was placed on the cooling plate of a flat bed electrophoresis apparatus (Pharmacia FBE 3000). Electrode strips were soaked in the appropriate electrode solutions (Anode: 0.05M H_2SO_4 : Cathode: 1M NaOH), blotted well, and placed on the gel. 15µl of the sample proteins were applied to filter paper applicators which were then placed on the gel at intervals of approximately 1cm. The constant power supply (Pharmacia ECPS 3000/150) was set to deliver a maximum of 5W and 500 volts with the current unlimited. The sample applicators were removed after 40 min and the electrophoresis continued for a total of 2.5 hr by which time the haemoglobin marker had focused.

2.4.5.4 Staining for malate dehydrogenase

Malate dehydrogenase activity in the focused gel was detected using a zymogram technique of overlaying the agarose gel with a reaction mixture, dissolved in agar, that forms a visible insoluble product (formazan). The reactions involved were:



5mg NAD^+, 5mg MTT (thiazolyl blue) and 2ml of 1M sodium malate (neutralized to pH 7.0 with NaOH) were dissolved in 20ml of 0.1M Tris-HCl pH 8.0, mixed with 20ml of 2% (w/v) agar and 0.5mg phenazine methosulphate (PMS) and poured over the gel. After the overlay had set, the gel was placed in a 37°C incubator until the blue formazan product was visible.

2.5 SOUBILIZATION OF LEISHMANIA M. MEXICANA PARTICULATE ENZYMES

10^9 amastigotes or 2×10^8 promastigotes were lysed at 4°C in 0.25M sucrose containing 0.1% (v/v) Triton X-100 and centrifuged for 5 min at 18°C at 10,000g. The pellet was resuspended in 0.25M sucrose and aliquots exposed to different concentrations of NaCl. After 5 min incubation at 4°C, the samples were centrifuged for 5 min at 18°C at 10,000g, the supernatant removed, the pellet resuspended in 0.25M sucrose, and both fractions assayed for enzyme activity.

2.6 ENZYME PURIFICATION AND CHARACTERISATION

All procedures were performed at 4°C. 5×10^{10} purified L. m. mexicana amastigotes were lysed by 10 min incubation in 0.25M sucrose containing 0.2% (v/v) Triton X-100 and 100µg/ml leupeptin, the latter to protect the enzymes from proteolytic attack. The resultant homogenate (H) was centrifuged at 10,000g for 5 min and the pellet (P1), after one wash in 0.25M sucrose, was resuspended in 4ml of 0.12M triethanolamine buffer, pH 7.5, containing 0.5M NaCl. After 5 min incubation, the sample was centrifuged for 30 min at 240,000g on an MSE Prepspin 50 ultracentrifuge and the resultant supernatant (S2) used as the source of solubilized enzyme.

2.6.1 Hydrophobic interaction chromatography.

The supernatant S2 was loaded onto a Phenyl-Sepharose Cl-4B column (1.6 x 15.5cm, bed volume 31ml) which had been pre-equilibrated with buffer A (0.12M triethanolamine buffer, pH 7.5, with 1.0M NaCl). Unbound protein was eluted with 80ml buffer A (flow rate 60ml/h, eluant collected in 8ml fractions) and the bound enzymes were eluted with 150ml of a concomitantly increasing gradient of ethylene glycol (30-70% v/v) and decreasing gradient of NaCl (700-300mM) with a flow rate of 30ml/h. The eluant was collected in 3ml

fractions, and those containing malate dehydrogenase or PEP carboxykinase and hexokinase activities were combined and concentrated on an Amicon filtration unit (model 8010) using a YM5 Diaflow ultrafiltration membrane with an exclusion range of 5000 daltons. The Phenyl-Sepharose Cl-4B gel was regenerated with the following procedure:

1) 20ml 70% (v/v) ethylene glycol;
2) 2 volumes distilled water;
3) 2 volumes absolute ethanol;
4) 4 volumes distilled water.

The column was stored in 0.02% (w/v) sodium azide to prevent bacterial growth.

2.6.2 Metal chelate affinity chromatography.

Metal chelate affinity chromatography was attempted in an effort to separate malate dehydrogenase and PEP carboxykinase activities that coeluted from the Phenyl-Sepharose column. In order not to waste any semi-purified sample, the technique was tested with solubilized pellet (S2). The Chelating Sepharose 6B (1.6 x 4cm, bed volume 10ml) was charged with metal ions by washing with 20ml of 5mg/ml $MnSO_4$ or $ZnCl_2$ dissolved in buffer A (flow rate 30ml/h). Excess ions were removed with 20ml buffer A and then 0.8ml S2 was loaded onto the column. Unbound proteins were then eluted with 20ml of buffer A containing 1M NaCl (buffer X) and bound proteins with either 0.05M sodium acetate buffer pH 4.0 or 0.12M triethanolamine buffer pH 7.5 containing 1M NaCl and 50mM EDTA (buffer Y). Eluant was collected in 2ml fractions. The final elution conditions also served to regenerate the column which was stored in 0.02% (w/v) sodium azide.

2.6.3 Affinity chromatography.

As the metal chelate column was unsuccessful in separating malate dehydrogenase and PEP carboxykinase (see section 3.6), further purification was achieved using affinity chromatography. 3ml of concentrated enzyme preparation from the Phenyl-Sepharose column was loaded onto a 5' AMP-Sepharose 4B column (1.6 x 3.5cm; bed volume 7ml) which had been pre-equilibrated with buffer B (0.12M triethanolamine buffer, pH 7.5, 200mM NaCl, 1.0mM DTT and 10% (v/v) ethylene glycol). Unbound protein, which included PEP carboxykinase and hexokinase, was eluted with 40ml of buffer B (flow rate 15ml/h) and protein bound to the column was eluted with 40 ml buffer C (0.12M triethanolamine buffer pH 7.5, 1M NaCl, 1mM DTT, 10% (v/v) ethylene glycol). The eluants were collected in 2ml fractions and those containing malate dehydrogenase or PEP carboxykinase and hexokinase activities were pooled and concentrated on an Amicon filtration unit (YM5 membrane).

The column was regenerated in three steps.

1) 10 volumes of 0.1M Tris-HCl pH 8.5 + 0.5M NaCl;
2) 10 volumes of 0.1M sodium acetate buffer pH 4.5 + 0.5M NaCl;
3) 10 volumes of distilled water.

The column was stored in 0.02% (w/v) sodium azide.

2.6.4 Gel filtration chromatography.

PEP carboxykinase and hexokinase were further purified by gel filtration. 1.5ml of concentrated eluate containing PEP carboxykinase and hexokinase activities from the 5' AMP-Sepharose 4B column was loaded onto a Sephadex G-100 superfine column (2.6 x 50cm; bed volume 265ml) which had been pre-equilibrated with buffer C. The column was eluted with the same buffer in an upwards direction (20ml/h) and 2.5ml fractions were collected. The column

was calibrated using the low molecular weight calibration kit from Pharmacia Ltd. The standard proteins and molecular weights were:

Blue dextran 2000,	2,000,000;
Albumin,	67,000;
Ovalbumin,	43,000;
Chymotrypsinogen A,	25,000;
Ribonuclease A,	13,700.

3mg of each standard protein was dissolved in buffer C, which was also used to elute the proteins. The molecular weights of purified enzymes were calculated from a plot of $\log_{10}$ molecular weight of calibration proteins against fraction number.

The Sephadex G-100 was also used to determine the molecular weight of malate dehydrogenase isoenzymes from cell free homogenates of L. m. mexicana amastigotes. 3×10^9 amastigotes were lysed by four cycles of freeze and thawing in 1ml buffer C. The homogenate was centrifuged for 5 min at 10,000g at $18^{o}C$ and the supernatant loaded and run on the Sephadex column as described above.

The column was stored in buffer C containing 0.02% (w/v) sodium azide.

2.6.5 pH and temperature profiles of purified malate dehydrogenase

The activity of purified malate dehydrogenase in both forward and reverse directions was determined in the pH range 4.5 to 11.0. The following buffers were used for oxaloacetic acid reduction: pH 4.5 - 6.0, 0.1M sodium succinate; pH 6.0 - 7.5, 0.1M imidazole-HCl; pH 7.5 - 9.0, 0.1M Tris HCl; pH 9.0 - 11.0, 0.1M glycine-NaOH. Assays for malate oxidation incorporated the following buffers; pH 4.0 - 7.0, 0.5M citric acid/0.4M hydrazine sulphate; pH 7.0 - 10.5, 1.0M glycine/0.4M hydrazine sulphate. The temperature profile was

determined for the range 15°C - 50°C using the standard assay conditions for oxaloacetic acid reduction. The enzyme was pre-equilibrated for 5 min at the required temperature before addition of substrate.

2.6.6 Enzyme kinetics.

Purified malate dehydrogenase was assayed in the direction of oxaloacetic acid reduction in 1.5ml total volume with extracts diluted 50-fold. For malate oxidation, malate dehydrogenase was assayed in 1.5ml total volume with extract diluted 6-fold. The K_m and V_{max} of purified malate dehydrogenase were determined in both directions, with oxaloacetic acid and malate as substrates, using the method of Lineweaver and Burk (1934). Double-reciprocal plots were constructed using data for 5 NADH (0.20 - 0.03mM) or NAD (2.0 - 0.05mM) and six oxaloacetic acid (0.17 - 0.017mM) or malate (133 - 3.3mM) concentrations. The V_{max} and apparent K_m were determined for each line by least squares regression analysis (Wilkinson 1961). Plots of the intercepts of the ordinate from the double-reciprocal plots against the reciprocals of the concentration of NADH or NAD were used to obtain the K_m NADH or K_m NAD and respective V_{max}. A plot of the slope from the double reciprocal plots against the reciprocals of the concentration of NADH or NAD was also used to determine the K_m oxaloacetic acid and K_m malate (Fromm 1975).

2.7 POLYACRYLAMIDE GEL ELECTROPHORESIS

Discontinuous SDS polyacrylamide gel electrophoresis (Disc-PAGE) was performed according to the method of Laemmli (1970) using an apparatus designed for microelectrophoresis (Amos 1976).

2.7.1 Gel preparation

Disc-PAGE relies on the casting of two gels with different pore sizes, a large pore stacking gel and a small pore running or separating gel. The separating gel was cast first and contained as a final concentration in 25% separating gel buffer (see Table 5):

10% (w/v) total acrylamide (see Table 5);

0.1% (v/v) N,N,N',N'-tetramethyl-ethylenediamine (TEMED);

0.1% (w/v) ammonium persulphate;

0.1% (w/v) lauryl sulphate (SDS).

Oxygen inhibits polyacrylamide polymerisation, therefore the mixture was degassed under partial vacuum before the addition of the ammonium persulphate catalyst. After pouring the gel into 8 x 8 cm glass holders, separating buffer was layered on top of the gel mixture to ensure that it set to give a smooth interface for the stacking gel. The stacking gel, which was prepared as above except with 4% (w/v) total acrylamide and stacking gel buffer (See table 5), was subsequently layered over the separating gel. A nine teeth well-former was placed in the gel, which was left for at least one hour at room temperature to set before use.

2.7.2 Sample preparation

45ul of sample protein was incubated for 5 min at 100^{o}C with 15µl of sample buffer (4x) (see table 5). The low molecular weight (LMW) gel electrophoresis calibration protein mixture was dissolved in 300µl of sample buffer (x1), in which the SDS concentration had been increased to 2.5%, and heated for 5 min at 100^{o}C to completely reduce and denature the proteins.

Table 5.

Solutions for discontinuous polyacrylamide gel electrophoresis.

---

(1) Concentrated Acrylamide: 30.8% (w/v) total with 2.7% cross-linker (N,N'-Methylene-bis-acrylamide)

(2) Separating gel buffer: 1.5M Tris-HCl, pH 8.8

(3) Stacking gel buffer: 0.5M Tris-HCl, pH 6.8

(4) Sample buffer (4X): 60% sucrose, 40mM Tris-HCl pH 8.8, 4mM EDTA, 160mM DTT, 4mg/ml bromophenol blue, 4% SDS.

(5) Electrophoresis buffer: 0.05M Tris-HCl, 0.385M glycine, pH 8.3, 0.1% SDS.

Table 6.

Gel destaining procedure for discontinuous polyacrylamide gel electrophoresis

---

Step 1:	40% ethanol, 3% acetic acid	1-2 hours
Step 2:	30% ethanol, 3.5% acetic acid	1-2 hours
Step 3:	Repeat step 2	
Step 4:	20% ethanol, 4% acetic acid	1-2 hours
Step 5:	5% acetic acid	Store

2.7.3 Electrophoresis and staining

Either 20-30µl of denatured sample protein or 10µl LMW calibration protein solution were gently settled into each well using a micropipette. Anode and cathode reservoirs were filled with electrophoresis buffer (Table 5) and the gel run at 18mA constant current using a Volkam SAE 2761 powerpack until the tracking dye, bromophenol blue, was close to the bottom edge of the gel (approximately 3.5 hours). The gels were stained overnight in a solution of 0.2% (w/v) coomassie blue R-250 in 95% (v/v) ethanol which was diluted 1:1 before use with 10% (v/v) acetic acid. The gel was subsequently destained using the procedure outlined in table 6.

2.7.4 Molecular weight determinations

The molecular weights of sample enzymes were determined from a standard curve prepared using standard proteins. The relative migration rates (Rf) for each of the calibration proteins was measured using the dye front as the reference point. The $\log_{10}$ molecular weight of marker proteins (phosphorylase b (94,000); albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); trypsin inhibitor (20,100); lactalbumin (14,400)) was plotted against Rf values and a line drawn by regression analysis. The molecular weights of sample enzymes were then determined from their corresponding Rf values.

2.8 MATERIALS

Pentostam (sodium stibogluconate) and Triostam (sodium antimonyl gluconate) were gifts from The Wellcome Research Laboratories, Beckenham, Kent, UK. Sodium melarsen; melarsen oxide; Melarsoprol (Mel B); monosodium p(2,4 diamino-1,3,5 triazin-6-ylamino)phenyl-stibonate octahydrate (1714, a pentavalent antimonial analogue of

sodium melarsen); 2-hydroxy-4-methylol-4,5-dihydro-1,3,2-dithiastibole (5822, a trivalent antimonial analogue of Mel B) were gifts from May & Baker Ltd, Dagenham, Essex, UK.

Growth culture medium including foetal calf serum, MEM Eagle suspension powder, MEM (50x) amino acids solution and MEM (100x) non-essential amino acids solution were obtained from Gibco-Biocult, Paisley, Scotland.

Phenyl-Sepharose Cl-4B (Cat. No. 17-0810-01), Chelating Sepharose 6B (Cat.No. 17-0527-01), 5' AMP-Sepharose 4B (Cat.No. 17-0620-01), low molecular weight gel filtration calibration kit (Cat.No. 17-0442-01), low molecular weight PAGE calibtration kit (Cat.No. 17-0446-01), agarose IEF (Cat.No. 17-0468-01), CM-Sephadex C25 and Gelbond were obtained from Pharmacia Fine Chemicals Ltd, Milton Keynes, England.

Digitonin, dimethylformamide, sorbitol, alumina (Type 305) and Sephadex G-100 superfine (Cat.No. G-100-50) were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, England.

Leupeptin was obtained from The Protein Research Foundation, Osaka, Japan.

All other chemicals and linkage enzymes were purchased from Sigma Chemical Co. Ltd., The Boehringer Corporation (London) Ltd., Lewes, East Sussex, England, or BDH Chemicals Ltd., Poole, Dorset, England.

3.0 RESULTS

3.1 THE ACTIVITIES OF GLYCOLYTIC , TCA CYCLE AND ASSOCIATED ENZYMES IN LEISHMANIA AND TRYPANOSOMES.

The activities detected in L. m. mexicana promastigotes and amastigotes of a number of enzymes involved in glycolysis, the TCA cycle and related metabolic pathways are given in Table 7. The glycolytic enzymes hexokinase, glucose phosphate isomerase, phosphofructokinase and phosphoglycerate kinase were at high activities in both forms of the parasite and this was also found for glucose-6-phosphate dehydrogenase, the first enzyme of the pentose phosphate pathway. The presence of TCA cycle enzymes was confirmed with isocitrate dehydrogenase and malate dehydrogenase activities being present, although α-ketoglutarate dehydrogenase could not be detected in either form. Isocitrate dehydrogenase was found to have NADP-linked activity but no activity was detected with NAD^+ as substrate.

One of the major differences found between the two stages of the parasite was found in the area of phosphoenolpyruvate metabolism and CO_2 fixation. PEP carboxykinase and malate dehydrogenase were detected at much higher activity in amastigotes than promastigotes of L. m. mexicana (Table 7). Neither of the other CO_2-fixing enzymes investigated, pyruvate carboxylase and malic enzyme (carboxylating), could be detected in amastigotes although the latter activity was found at low levels in promastigotes. Pyruvate kinase was confirmed as having significantly higher activity in promastigotes than amastigotes (Table 7), whilst fumarate reductase, which functions as a reversal of succinate dehydrogenase and can be important as a metabolic route for reverse flow of the TCA cycle to succinate, could only be detected in amastigotes and then at low activity.

Table 7.

Enzyme activities of Leishmania m. mexicana promastigotes and amastigotes.

ENZYME	SPECIFIC ACTIVITY[a] PROMASTIGOTE		AMASTIGOTE	
Hexokinase	138 ± 38	(4)	89 ± 15	(3)
Glucose phosphate isomerase	159 ± 10	(2)	430 ± 112	(3)
Phosphofructokinase	106 ± 25	(3)	55 ± 9	(2)
Phosphoglycerate kinase	235	(1)	102 ± 7	(2)
Pyruvate kinase	100 ± 10	(5)	12 ± 8	(4)
Glucose-6-phosphate dehydrogenase	105 ± 20	(2)	92 ± 24	(3)
PEP carboxykinase	26 ± 9	(4)	172 ± 58	(7)
Malate dehydrogenase	470 ± 160	(4)	3760 ± 690[b]	(5)
Isocitrate dehydrogenase	100 ± 31	(6)	71 ± 22	(7)
∝-ketoglutarate dehydrogenase	ND[c] < 1.0	(2)	ND < 0.8	(2)
Glutamate dehydrogenase (NAD)	980 ± 235	(5)	112 ± 13	(5)
Glutamate dehydrogenase (NADP)	ND < 1.0	(3)	33 ± 6	(3)
Alanine aminotransferase	137 ± 7	(2)	35	(1)
Malic enzyme (decarboxylating)	49 ± 20	(5)	3 ± 2	(3)
Malic enzyme (carboxylating)	3 ± 2	(3)	ND < 0.4	(4)
Pyruvate carboxylase	ND < 1.1	(2)	ND < 0.8	(3)
Isocitrate lyase	ND < 0.7	(4)	ND < 0.9	(7)
Glutathione reductase	ND < 0.8	(2)	ND < 0.8	(2)
Fumarate reductase	ND < 1.0	(3)	1.6 ± 0.2	(2)

[a] The activities are expressed in nmoles/min/mg protein and are the means (± SD or range) from the number of experiments given in parentheses. Parasites were lysed by incubation in 0.25M sucrose with 0.1% (v/v) Triton X-100 for 10 mins at 4°C.

[b] Assayed in the presence of 100µg/ml leupeptin

[c] Not detected with limit of detection given.

In order to see if these large difference between the two stages of the parasite were found in other species of Leishmania and also in trypanosomes, the activities of PEP carboxykinase, malate dehydrogenase and pyruvate kinase were determined for both L. m. amazonensis and L. donovani (Table 8) and T. b. brucei and T. b. rhodesiense (Table 9). Both malate dehydrogenase and PEP carboxykinase ofL. donovani were at significantly higher activity in amastigotes than promastigotes which was similar to the situation found in L. m. mexicana. Pyruvate kinase from L. donovani amastigotes, however, was higher than detected for L. m. mexicana but still lower than the promastigote activity. Although the promastigote activities of L. m. amazonensis agreed well with the other two Leishmania species, there was only sufficient amastigote material for one determination. The activities detected were similar to those of the promastigote. The procyclic stage of T. b. brucei and T. b. rhodesiense both showed substantially higher malate dehydrogenase and PEP carboxykinase activities when assayed at both 25°C and 37°C than the mammalian bloodstream form of the parasite (Table 9), whereas the reverse was true of pyruvate kinase.

Malate dehydrogenase activity in L. m. mexicana amastigote homogenates maintained at 4°C was found to be very unstable and the addition of leupeptin had a marked effect on the enzyme's activity. In the absence of leupeptin, only 20% of the amastigote activity remained 30 mins after amastigote lysis, whereas 64% remained when amastigotes were lysed in the presence of 100μg/ml leupeptin (Table 10). In contrast, homogenate activities of PEP carboxykinase, pyruvate kinase and hexokinase were stable, even in the absence of leupeptin, for several hours at 4°C.

Two enzymes involved in the utilization and metabolism of amino

Table 8.

Malate dehydrogenase, PEP carboxykinase, and pyruvate kinase activities in different species of *Leishmania*

ISOLATE	ENZYME	Specific activity[a] PROMASTIGOTE	AMASTIGOTE
L. m. mexicana	Malate Dehydrogenase	470 ± 160 (4)	3760 ± 690[b] (2)
	PEP Carboxykinase	26 ± 9 (4)	172 ± 58 (2)
	Pyruvate Kinase	100 ± 10 (5)	12 ± 8 (2)
L. m. amazonensis	Malate Dehydrogenase	222 ± 100 (3)	241 (1)
	PEP Carboxykinase	39 ± 18 (3)	42 (1)
	Pyruvate kinase	60 ± 35 (3)	46 (1)
L. donovani	Malate Dehydrogenase	219 ± 73 (2)	1440 ± 330 (4)
	PEP Carboxykinase	45 ± 6 (2)	115 ± 12 (4)
	Pyruvate kinase	77 ± 14 (2)	46 ± 9 (4)

[a] The activities are expressed in nmoles/min/mg protein and are the means (± SD or range) for the number of experiments given in parentheses.

[b] Assayed in the presence of 100µg/ml leupeptin.

Table 9.

Malate dehydrogenase, PEP carboxykinase, and pyruvate kinase enzyme activities in Trypanosoma b. brucei and Trypanosoma b. rhodesiense[a]

	T. b. brucei		T. b. rhodesiense	
25°C	Procyclic	Bloodstream	Procyclic	Bloodstream
Malate dehydrogenase	1260 ± 35 (2)	150 ± 5 (2)	1390 ± 30 (2)	145 ± 1 (2)
PEP carboxykinase	40 ± 0 (2)	15 ± 3 (2)	41 ± 10 (2)	6 ± 1 (2)
Pyruvate kinase	49 ± 2 (2)	165 ± 5 (2)	2 (1)	Not Done
37°C				
Malate dehydrogenase	1730 ± 120 (2)	280 ± 10 (2)	1380 ± 120 (2)	250 ± 14 (2)
PEP carboxykinase	90 ± 10 (2)	25 ± 2 (2)	188 ± 4 (2)	3.9 ± 0.1 (2)
Pyruvate kinase	Not Done	250 ± 0 (2)	Not Done	425 ± 10 (2)

[a] The activities are expressed in nmoles/min/mg protein and are the means (± range) from the number of experiments given in parentheses. Parasites were lysed in 0.1% (v/v) Triton X-100.

Table 10.

Loss of Leishmania m. mexicana amastigote homogenate malate dehydrogenase and PEP carboxykinase activities during incubations in the presence and absence of 100µg/ml leupeptin.[a]

		TIME				
	Leupeptin	10min	30min	1hr	2hr	7hr
Malate dehydrogenase	-	87	20	9	6	2
Malate dehydrogenase	+	100	64	45	40	ND[b]
PEP carboxykinase	-	100	108	105	130	70

[a] Values are expressed as a percentage of activity measured at time zero. Homogenates were prepared by lysis with 0.2% (v/v)Triton X-100 in 0.25M sucrose.

[b] Not determined

acids were also investigated, with both glutamate dehydrogenase and alanine aminotransferase being detected in both forms of L. m. mexicana (Table 7). The promastigote NADH-linked glutamate dehydrogenase was 10-fold higher in activity than the equivalent amastigote enzyme; in contrast no NADPH-linked activity could be detected in the promastigote, although significant activity was detected in the amastigote. Neither isocitrate lyase nor glutathione reductase could be detected in amastigotes or promastigotes, either in the presence or absence of leupeptin.

The distribution of the enzyme activities between the particulate and soluble fractions of the parasite are given in Table 11. Hexokinase, malate dehydrogenase, PEP carboxykinase, glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase were all found to be particulate to a large extent. Pyruvate kinase, glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase, glutamate dehydrogenase, malic enzyme and alanine aminotransferase were all soluble. For most of the enzymes there was little difference in distribution between the two parasite forms, however malate dehydrogenase had a higher proportion of particulate activity in the amastigote. When the cells were lysed with Triton X-100 instead of freezing and thawing, the fractionation gave similar results.

Following the discovery that the amastigote contained much higher activities of PEP carboxykinase and malate dehydrogenase than the culture promastigotes and because of the suggested involvement of glycolytic kinases as targets for the antileishmanial antimonials, these enzymes were studied in more detail. Preliminary studies on PEP carboxykinase in parasite homogenates showed that it is ADP-specific and has an absolute requirement for manganese that could not

Table 11.

Particulate nature of Leishmania m. mexicana amastigote and promastigote enzymes.[a]

ENZYME	PROMASTIGOTE P	S	SD		AMASTIGOTE P	S	SD	
Protein	34	66	± 6	(7)	42	58	± 4	(4)
Hexokinase	73	27	± 13	(3)	83	17	± 5	(3)
Glucose phosphate isomerase	17	83	± 3	(2)	4	96		(1)
Glucose-6-phosphate dehydrogenase	15	85	± 3	(2)	Not Done			
Phosphofructokinase	62	38	± 5	(2)	61	39	± 2	(2)
Phosphoglycerate kinase	20	80		(1)	17	83	± 3	(2)
Glyceraldehyde-3-phosphate dehydrogenase	49	51		(1)	57	43	± 4	(2)
Pyruvate kinase	5	95	± 4	(3)	5	95	± 5	(3)
Malate dehydrogenase[b]	44	56	± 11	(3)	80	20	± 15	(4)
Isocitrate dehydrogenase	11	89	± 3	(3)	8	92	± 4	(3)
Glutamate dehydrogenase (NAD)	2	98	± 2	(4)	2	98	± 5	(5)
Malic enzyme	9	91	± 2	(3)	Not Done			
Alanine aminotransferase	7	93	± 2	(2)	14	86		(1)
PEP carboxykinase	40	60	± 5	(2)	91	9	± 8	(4)

[a] Values are expressed as the percentage of the total activity recovered in the pellet (P) and supernatant (S) fractions after 2 min centrifugation at 10,000g. The results are the mean (± SD or range) from the number of experiments shown in parentheses.

[b] Amastigote malate dehydrogenase assayed in the presence of 100µg/ml leupeptin.

be replaced by magnesium. It was also unaffected by nucleoside triphosphates (ATP, ITP, GTP), nucleoside diphosphates (IDP, GDP) and the end products malate and succinate. In addition the activity of PEP carboxykinase was considerably higher when assayed in imidazole buffer pH 6.6 than Tris-HCl pH 7.5.

3.2 THE EFFECT OF ANTIMONIALS AND ARSENICALS ON GLYCOLYTIC KINASES

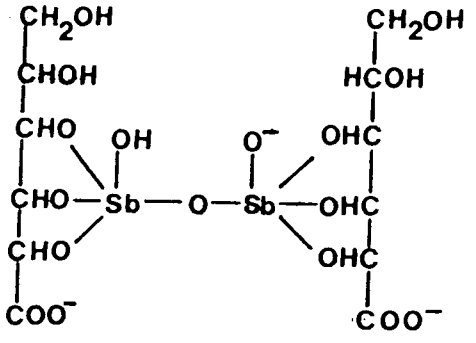
A number of organic antimonials and arsenicals were tested for inhibitory activity against glycolytic kinases thought to be possible targets for these antileishmanial chemotherapeutic agents. The structures of the drugs used are shown in Fig. 4. The effects of these antimonials and arsenicals on phosphofructokinase from several sources are given in Table 12. Triostam was active only against the schistosome enzyme, with an I_{50} of 200 $\pm$ 25 µm and 74% inhibition at 500µm (Fig. 5), whereas the pentavalent antimonial 1714 was equally active against the enzyme from schistosome and mouse liver (Table 12). None of the antimonials, pentavalent or trivalent, had an inhibitory effect against the leishmanial enzymes. Melarsen oxide was an inhibitor of all the phosphofructokinases tested whereas Mel B inhibited all except the promastigote enzyme; schistosomal phosphofructokinase was especially sensitive to both trivalent arsenicals.

Melarsen oxide had a broad spectrum of inhibitory activity (Table 13). It inhibited pyruvate kinase and malate dehydrogenase from L. m. mexicana at low µM concentrations, with the mouse liver pyruvate kinase being less sensitive. Leishmanial hexokinase was also inhibited. The other compounds were found to be much poorer inhibitors than melarsen oxide (Table 13). Mel B inhibited pyruvate kinase, but only at relatively high concentrations, and

Fig. 4 The structures of the organic antimonial and arsenical drugs used in the inhibitor studies.

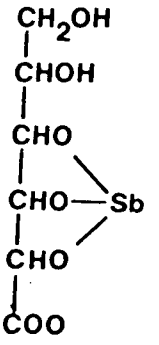
ORGANIC ANTIMONIALS

(1)



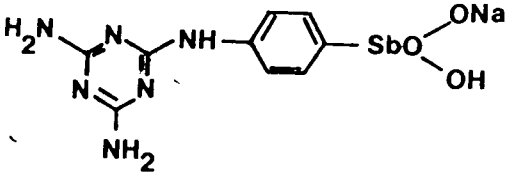
PENTOSTAM

(2)



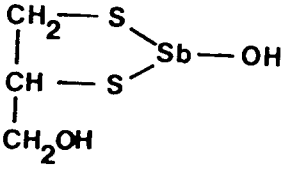
TRIOSTAM

(3)



M&B1714

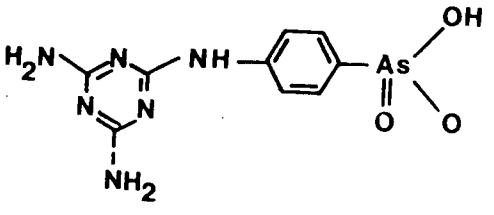
(4)



M&B 5822

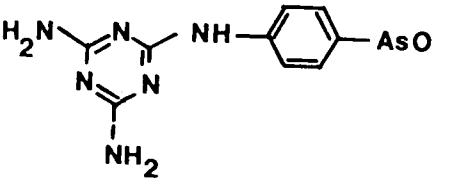
ORGANIC ARSENICALS

(5)



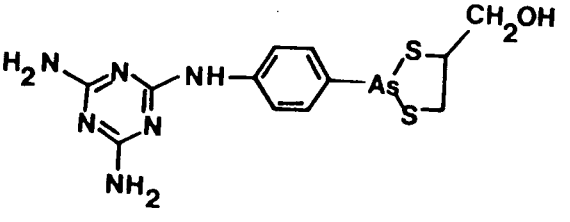
MELARSEN

(6)



MELARSEN OXIDE

(7)



MEL B

Fig. 5. Inhibition of Schistosoma mansoni phosphofructokinase by Triostam.

Fig 6. The effect of Triostam and melarsen oxide on the growth of Leishmania m. mexicana promastigotes.

Promastigotes were grown in HOMEM medium for 72hr incubations with 0-200µg/ml Triostam (■) or 0-100µg/ml melarsen oxide (▲). % growth represents the parasite numbers as a percentage of the control with no drug.

Fig. 5

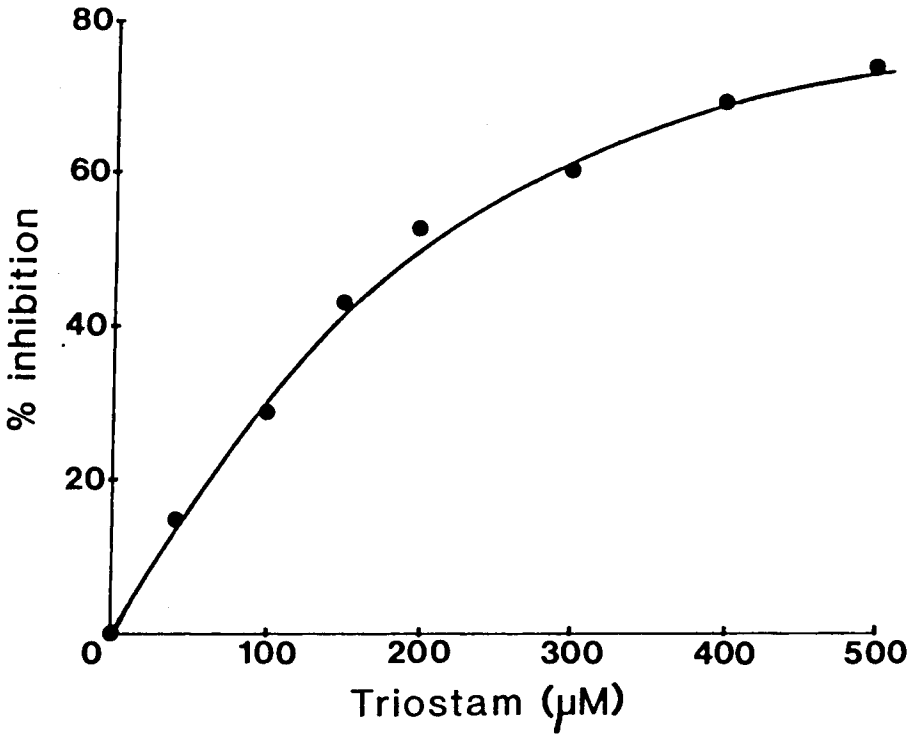

80
60
40
20
0
% inhibition
100
200
300
400
500
Triostam (μM)

Fig. 6

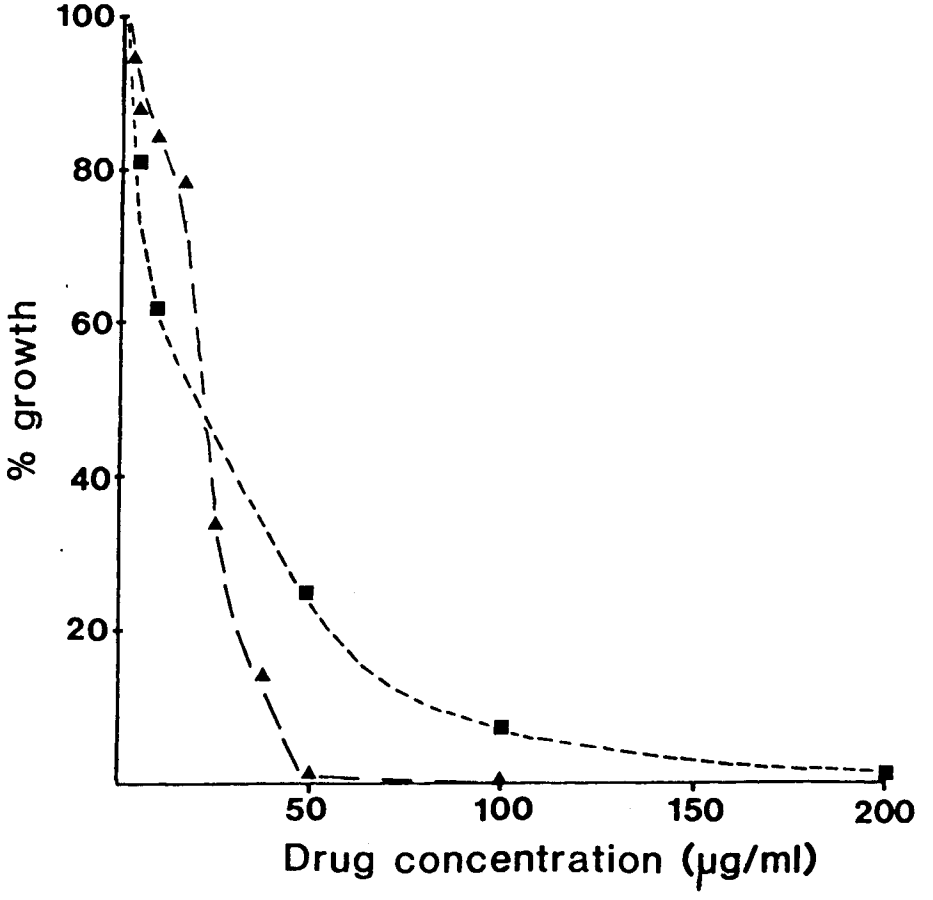

100
80
60
40
20
% growth
50
100
150
200
Drug concentration (μg/ml)

Table 12.

Effects of antimonials and arsenicals on phosphofructokinase activities[a].

Compound	*Leishmania m. mexicana*		Mouse liver	Rabbit muscle	*Schistosoma mansoni*
	Amastigote	Promastigote			
Triostam	NI[b]	NI	NI	NI	200 ± 25 (3)
1714	NI	NI	275 ± 35 (3)	NI	275 (1)
Melarsen oxide	8 ± 1 (2)	7 ± 1 (2)	9 ± 2 (2)	160 ± 15 (2)	0.6 ± 0.2 (2)
Mel B	300 ± 25 (2)	NI	275 ± 25 (2)	400 (1)	13 ± 3 (2)

[a] The figures given are the I_{50} in μM and are the means (± S.D. or range) from the number of experiments given in parentheses. The following drugs gave no inhibition at the concentrations tested (given in parentheses): Melarsen (400μM); pentostam (110μM).

[b] NI. No inhibition at the maximum concentration tested: Triostam (600μM); 1714 (400μM); Mel B (200μM).

Table 13.

Effects of antimonials and arsenicals on hexokinase, pyruvate kinase, malate dehydrogenase and PEP carboxykinase activities.[a]

Compound	HEXOKINASE			PYRUVATE KINASE			MALATE DEHYDROGENASE	
	Leishmania m. mexicana		Mouse Liver	Leishmania m. mexicana		Mouse Liver	Leishmania m. mexicana	
	Amastigote	Promastigote		Amastigote	Promastigote		Amastigote	Promastigote
1714	820 ± 30 (2)	550 (1)	NI[b]	ND[c]	ND	NI	NI	NI
Melarsen oxide	290 ± 140 (3)	110 ± 38 (2)	>400 (38%)	8 ± 2 (2)	7 ± 4 (3)	165 ± 15 (2)	4 ± 2 (2)	8 (1)
Mel B	NI	>400 (28%)	NI	400 (1)	380 ± 5 (2)	>200 (10%)	100 (1)	NI

[a] The figures given (means ± S.D. or range from the number of experiments in parentheses) are the I_{50} in μM except where indicated >, when the percentage inhibition at the highest concentration tested is given. The following drugs gave no inhibition at the concentrations tested (given in parentheses): Melarsen (400μM); 5822 (200μM); Pentostam (110μM); Triostam (600μM). L. m. mexicana amastigote PEP carboxykinase was not inhibited by any of the drugs tested except Pentostam which gave 34% inhibition at 366μM.

[b] No inhibition at the maximum concentration tested: 1714 (400μM); Mel B (200μM).

[c] Not determined.

amastigote, but not promastigote, malate dehydrogenase. The pentavalent antimonial 1714 inhibited leishmanial hexokinase, whereas Triostam, 5822 and melarsen had no effect on hexokinase, pyruvate kinase, malate dehydrogenase or PEP carboxykinase from any source. The only effect of Pentostam was a slight inhibition of amastigote PEP carboxykinase, with 34% inhibition at the maximum concentration tested which was 366μM. The peak serum level of Pentostam obtained in humans being treated for cutaneous leishmaniasis was reported to be approximately 15μg/ml (16.5μM) (Berman and Wyler 1980). At this concentration, Pentostam had no inhibitory action on PEP carboxykinase.

3.3 THE EFFECT OF ANTIMONIALS AND ARSENICALS ON THE GROWTH OF *LEISHMANIA M. MEXICANA* PROMASTIGOTES

The lack of a marked effect of Pentostam on *Leishmania* promastigote growth reported by previous workers (Mattock and Peters 1975, Berman and Wyler 1980 and Coombs *et al.* 1983) was confirmed, with promastigote numbers being reduced by less than 10% by drug at 100μg/ml in comparison with the control after a 72 hour incubation. Neither the pentavalent arsenical melarsen nor its antimonial analogue 1714, at concentrations of 200μg/ml, had any appreciable effect on growth. In contrast, the trivalent analogue of Pentostam, Triostam, had potent antileishmanial activity (Fig. 6, pg. 91); the LD_{50} was 20μg/ml and the MLC was 400μg/ml. It was found to be almost as effective, on a weight/weight basis, as melarsen oxide (LD_{50}, 20μg/ml; MLC, 100μg/ml). Triostam at 400μg/ml caused promastigotes to round up by 4 hours and immotile parasites were observed from 8 hours onwards: over 90% of the cells appeared dead within 24 hours. Melarsen oxide acted less rapidly, with dead cells

Table 14.

The effect of cysteine on the inhibitory action of Triostam against promastigotes of Leishmania m. mexicana.

Cysteine (mM)	without Triostam[a]	with Triostam[b]		
		400µg/ml	200µg/ml	100µg/ml
10	11 ± 5	41 ± 2	111 ± 75	105 ± 37
5	18 ± 2	18 ± 9	41 ± 18	79 ± 13
3	21 ± 5	1 ± 1	18 ± 10	76 ± 18
1	78 ± 23	0	1 ± 1	20 ± 9
0	100	0	0.4 ± 0.2	5 ± 2

[a] Parasite numbers as a percentage of the control minus cysteine and Triostam after 72 hrs incubation at 25°C.

[b] Parasite numbers as a percentage of that in cultures containing the same concentration of cysteine but no drug. The results are the mean (± S.D.) from at least three experiments.

appearing by 12 hours but only 60% of the promastigotes were killed by 24 hours. The activity of Triostam was found to be antagonised by cysteine (Table 14). The addition of 10mM cysteine to promastigote cultures with Triostam at 400µg/ml reduced the inhibitory action of the drug on parasite growth by 41% and abolished the effect of the drug at 200µg/ml. With cysteine at this concentration, however, there was only 11% growth compared to the control with no cysteine. 1mM cysteine, which by itself reduced the growth of L. m. mexicana by 22%, had no significant effect on the action of Triostam at 400 or 200µg/ml, even though the molar ratio of cysteine to drug was 1:1.2 and 1:0.6 respectively, and only antagonised the drug at 100µg/ml by 15%.

3.4 STUDIES ON ENZYME LOCATIONS IN LEISHMANIA M. MEXICANA

3.4.1 Differential centrifugation

The distributions of enzymes and protein in the fractions of L. m. mexicana promastigotes produced by differential centrifugation are given in Table 15. Hexokinase, glucose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase and PEP carboxykinase were recovered in large amounts in the small organelle fraction P2 and all exhibited latency. Isocitrate dehydrogenase, malic enzyme, glutamate dehydrogenase (NAD-linked) and alanine aminotransferase were present at high activity in both P1 and the soluble fraction, whereas almost 70% of the malate dehydrogenase activity was recovered in P1. A small but significant proportion of phosphoglycerate kinase activity was found in the pellets with the majority, 69%, in S. Glucose-6-phosphate dehydrogenase and pyruvate kinase were found almost exclusively in S.

It was found impossible to fractionate L. m. mexicana amastigotes successfully by applying the methods used with promas-

Table 15. Distribution of protein and enzymes in the subcellular fractions of *Leishmania m. mexicana* promastigotes prepared by differential centrifugation.

Component	% of total activity[a]					No.of Expts.
	P1	P2	P3	S	% Recovery	
Protein	23±6	18±7	14±4	45±9	103±7	(7)
Hexokinase	15±6	58±7	11±4	16±8	87±7	(7)
Glucose phosphate isomerase	12±5	32±4	4±0	52±3	94±4	(3)
Glucose-6-phosphate dehydrogenase	10±1	7±3	2±1	81±5	99±5	(2)
Glyceraldehyde-3-phosphate dehydrogenase	20±8	42±5	2±1	36±9	109±10	(3)
Phosphoglycerate kinase	12±7	13±6	6±1	69±12	86±13	(4)
Pyruvate kinase	6±0	2±1	3±1	89±2	106±14	(4)
PEP carboxykinase	7±3	59±13	8±3	26±9	63±19	(3)
Malate dehydrogenase	68±5	22±6	1±1	9±3	90±22	(6)
Isocitrate dehydrogenase	52±7	15±8	1±1	32±4	99±19	(4)
Malic enzyme	39±4	15±2	2±0	44±6	104±26	(2)
Glutamate dehydrogenase (NAD)	50±4	17±5	5±2	28±3	99±20	(3)
Alanine aminotransferase	46±6	15±6	3±2	35±7	93±15	(3)

a. The values given (mean ± SD or range) are the activities found in each fraction as a percentage of the total activity recovered.

tigotes. Attempts at fractionation resulted in fractions in which there was no latency detected for either of the glycosomal markers hexokinase or glucose phosphate isomerase, presumably due to lysis of the organelle and consequent release and exposure of their contents. The distributions of a number of amastigote enzymes produced by differential centrifugation are shown in Table 16. Hexokinase, which has particulate activity (Table 11), showed an even distribution between P1 and P2 whereas glucose phosphate isomerase, a putative soluble glycosomal enzyme, was mainly recovered in the soluble fraction S. Glutamate dehydrogenase, which is a soluble mitochondrial matrix enzyme and showed 50% of its activity in P1 of promastigotes (Table 15), was recovered to the extent of only 15% of its activity in the P1 fraction of amastigotes (Table 16). In an attempt to fractionate amastigotes successfully, various modifications of the methods were tested, including lysis using glass beads, the detergent digitonin, ground glass homogenisers and hypotonic conditions, but none proved better than the method using alumina. Breakage buffers of different compositions (eg. replacing sucrose with mannitol) and containing combinations of inhibitors of proteinases (leupeptin and antipain) and phospholipases (mepacrine, chelating agents), enzymes which were thought to be responsible at least in part for the breakdown of the organelles, were also tested but without success. Unfortunately, none of these methods produced cell lysates containing intact amastigote organelles as determined by latency of glucose phosphate isomerase and hexokinase.

3.4.2 Sucrose density centrifugation

The enzyme distribution profiles of some of the glycolytic enzymes on 1.24 - 1.15 g/ml linear sucrose gradients are shown in Figures 7 and 8. Hexokinase, glucose phosphate isomerase, phospho-

Table 16.

Distribution of protein and enzymes in the subcellular fractions of *Leishmania m. mexicana* amastigotes prepared by differential centrifugation.

Component	% of total activity[a]					
	P1	P2	P3	S	% Recovery	No. of Expts
Protein	34±4	18±7	5±2	43±4	101±11	(5)
Hexokinase	35±8	38±12	17±6	10±11	78±5	(5)
Glucose phosphate isomerase	8	11	4	77	66	(1)
Glucose-6-phoshate dehydrogenase	9	3	2	86	94	(1)
PEP carboxykinase	38±13	22±15	8±4	32±23	81±21	(4)
Malate dehydrogenase	74±11	12±10	2±3	12±2	70±33	(4)
Glutamate dehydrogenase	15±5	7±4	20±8	58±9	71±5	(2)

[a] The values given (mean ± SD or range) are the activities found in each fraction as a percentage of the total activity recovered.

Figs. 7 and 8.

Distribution profiles of Leishmania m. mexicana promastigote enzymes after isopycnic centrifugation of lysates on 1.24-1.15g/ml linear sucrose gradients. The left hand axis represents the frequency of the gradient (ρ= 1.24-1.15g/ml) and the right hand axis represents the frequency of the soluble fractions combined as one histogram bar (ρ = 1.15 -1.07g/ml). Histograms show the means (± S.E. or range) from the number of experiments in parentheses. Mean percentage recoveries ranged from 87% (glucose phosphate isomerase) to 138% (malate dehydrogenase).

FIG. 7

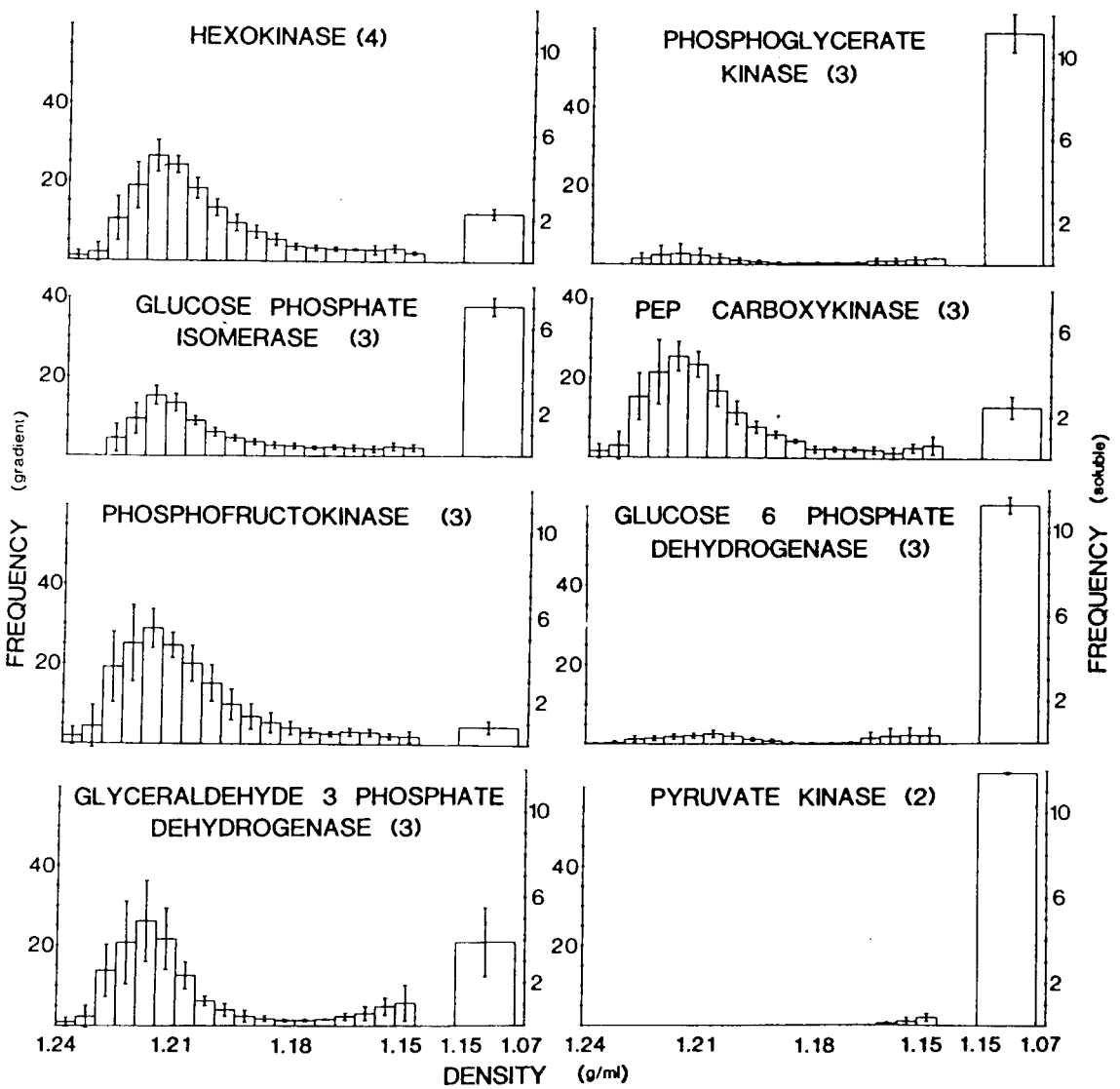
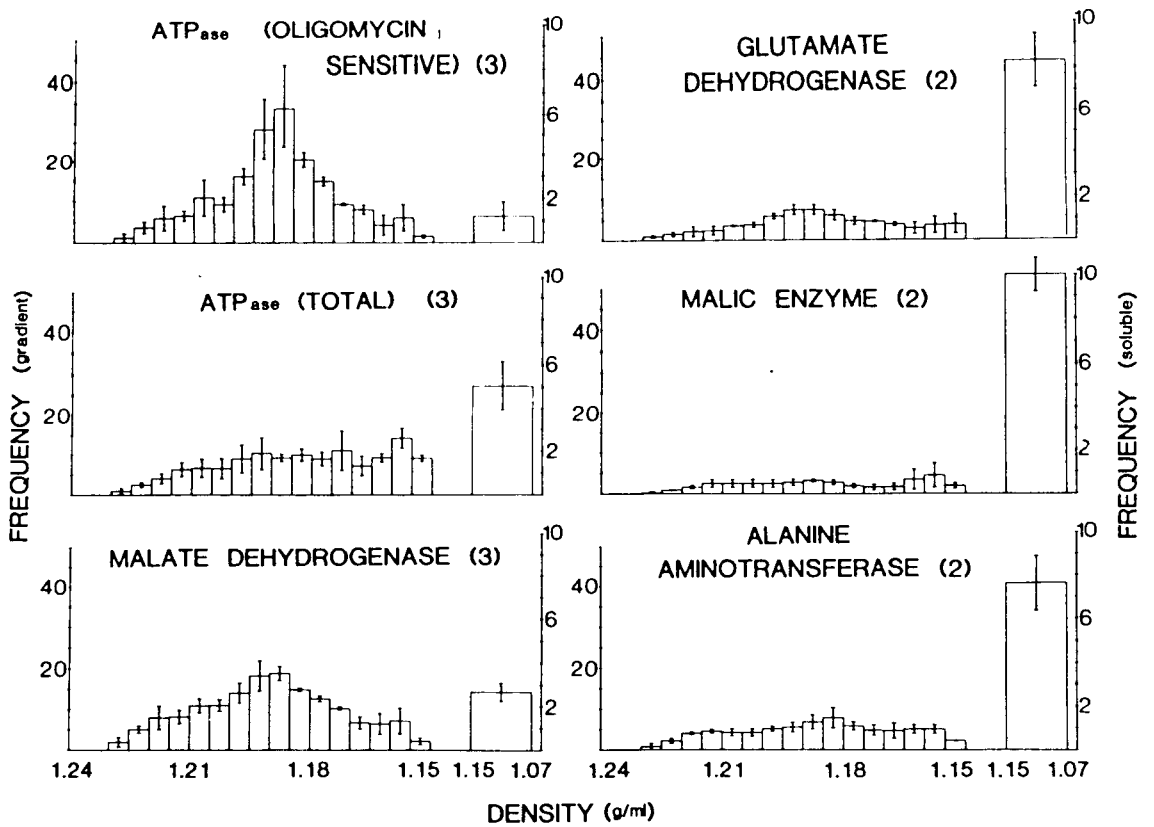

HEXOKINASE (4)
PHOSPHOGLYCERATE KINASE (3)
GLUCOSE PHOSPHATE ISOMERASE (3)
PEP CARBOXYKINASE (3)
PHOSPHOFRUCTOKINASE (3)
GLUCOSE 6 PHOSPHATE DEHYDROGENASE (3)
GLYCERALDEHYDE 3 PHOSPHATE DEHYDROGENASE (3)
PYRUVATE KINASE (2)
FREQUENCY (gradient)
FREQUENCY (soluble)
40
20
10
6
2
1.24
1.21
1.18
1.15
1.15
1.07
DENSITY (g/ml)

FIG. 8


ATPase (OLIGOMYCIN SENSITIVE) (3)
GLUTAMATE DEHYDROGENASE (2)
ATPase (TOTAL) (3)
MALIC ENZYME (2)
MALATE DEHYDROGENASE (3)
ALANINE AMINOTRANSFERASE (2)
FREQUENCY (gradient)
FREQUENCY (soluble)
40
20
10
6
2
1.24
1.21
1.18
1.15
1.15
1.07
DENSITY (g/ml)

fructokinase, glyceraldehyde-3-phosphate dehydrogenase, and PEP carboxykinase showed large distinct peaks with a density of 1.215 - 1.220 g/ml (Fig. 7). For each enzyme some of the activity was found remaining on top of the gradient and in the case of glucose phosphate isomerase this amounted to about 50% of the total activity. Small amounts of phosphoglycerate kinase and glucose-6-phosphate dehydrogenase activities were also detected on the gradient at a similar density to hexokinase. Pyruvate kinase was recovered exclusively in the soluble fraction. The distribution profile for oligomycin-sensitive ATPase, a mitochondrial marker enzyme, shows a sharp peak of activity at a density of 1.190 - 1.195 g/ml (Figure 8). Total ATPase activity, however, was spread through most of the gradient. Malate dehydrogenase also showed a distinct but smaller peak at 1.190 - 1.195 g/ml. Glutamate dehydrogenase, alanine aminotransferase and malic enzyme, probably all mitochondrial enzymes, showed very small peaks at this density, with most of the activity in the soluble fractions and spread out on the gradient.

3.4.3 Release of soluble enzymes of Leishmania m. mexicana by digitonin.

As an alternative approach to the study of enzyme locations in L. m. mexicana, the detergent digitonin was used: this lysed the various parasite membranes at different rates and so caused the sequential release of soluble enzymes from within the organelles. The results of these studies with both amastigotes and promastigotes are given in Figures 9, 10 and 11. In the amastigote, three subcellular compartments were distinguished. After 10 min incubation with digitonin at 150µg per 10^9 amastigotes, more (95%) of glucose-6-phosphate dehydrogenase activity was released than the putative

Fig. 9.

Release of soluble enzymes and protein from Leishmania m. mexicana amastigotes treated for 10 mins with digitonin at different concentrations. The experiment was performed twice with one representative set of experimental data shown. 10^9 amastigotes contained approximately 1.8mg protein. Key to enzymes: Glucose-6-phosphate dehydrogenase (▲); glucose phosphate isomerase (O); phosphoglycerate kinase (●); glutamate dehydrogenase (△); alanine aminotransferase (■); protein (□).

Fig. 10.

Release of soluble enzymes and protein from Leishmania m. mexicana amastigotes treated for different lengths of time with 150µg digitonin/10^9 cells. The experiment was performed 3 times, one representative set of data are shown. Legends for enzymes as for Fig. 9.

Fig. 11.

Release of soluble enzymes and protein from Leishmania m. mexicana promastigotes treated for 10 mins with digitonin at different concentrations. 2×10^8 promastigotes contained approximately 1.6mg protein. Pyruvate kinase (×). Legends for other enzymes as for Fig. 9.

FIG. 9

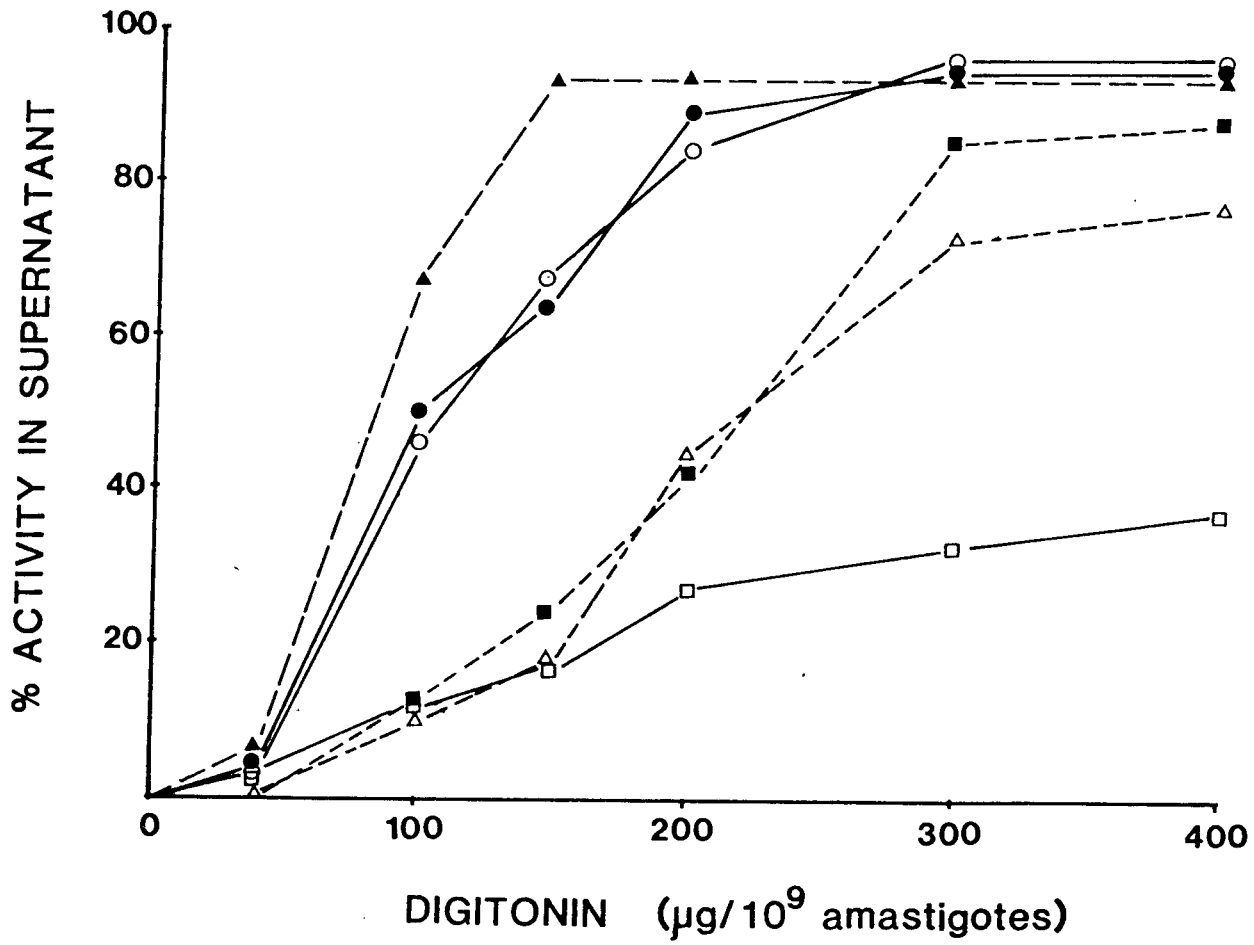

100
80
60
40
20
0
100
200
300
400
% ACTIVITY IN SUPERNATANT
DIGITONIN (µg/10^9 amastigotes)

FIG. 10

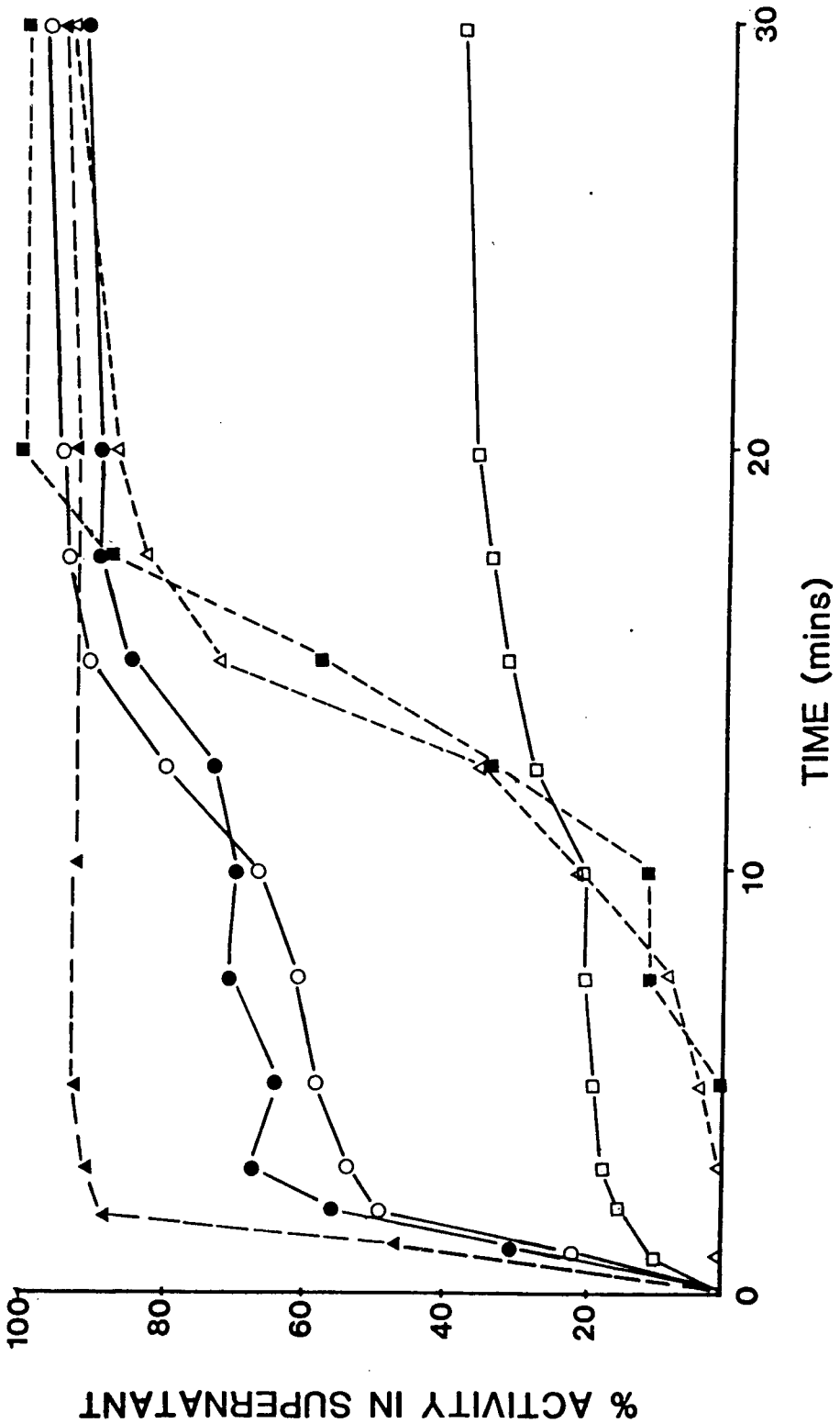
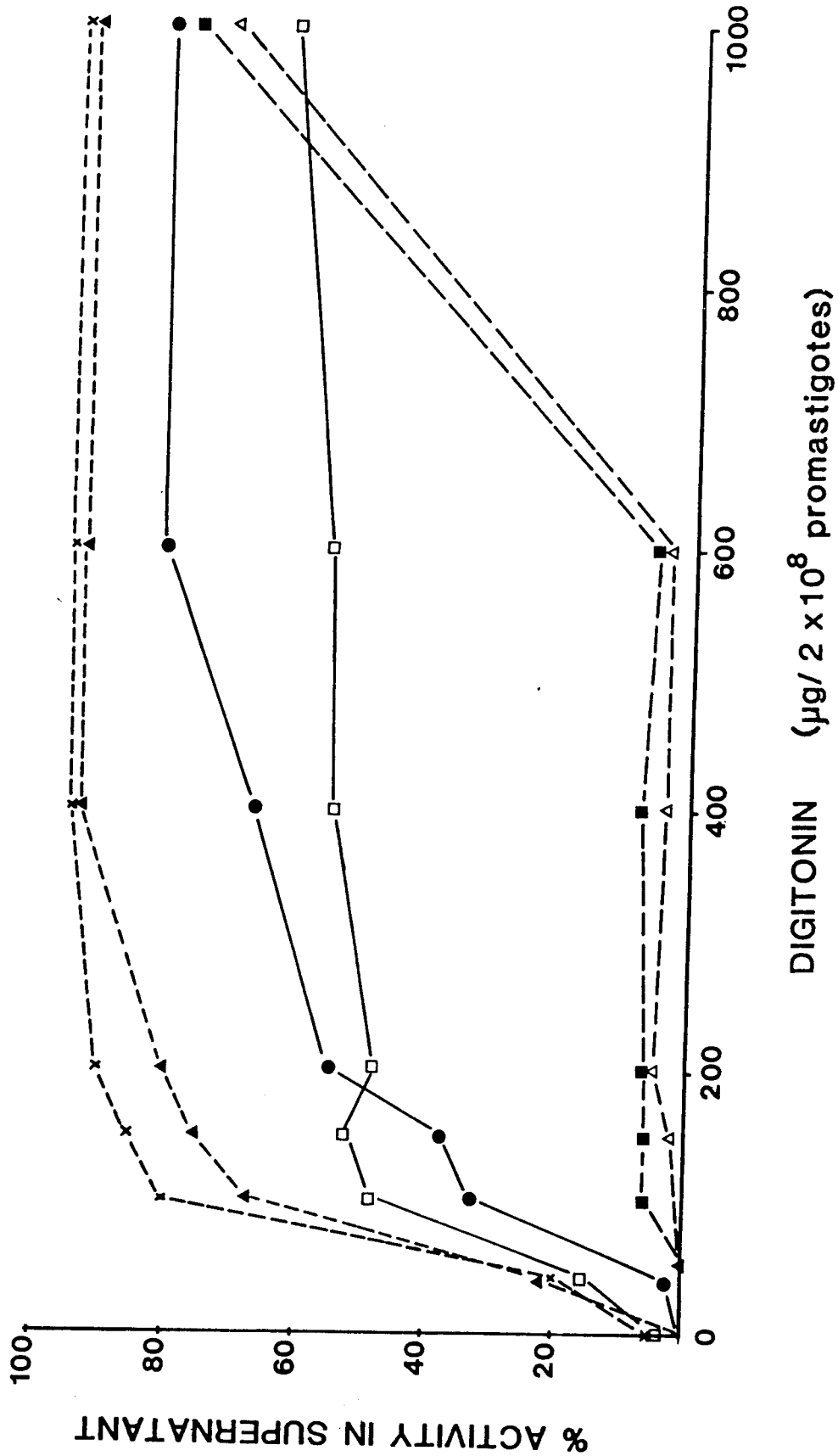


FIG. 11


% ACTIVITY IN SUPERNATANT
100
80
60
40
20
0
200
400
600
800
1000
DIGITONIN (µg/ 2 x10^8 promastigotes)

glycosomal enzymes glucose phosphate isomerase and phosphoglycerate kinase (each approximately 70%), which in turn were released to a greater extent than the mitochondrial enzymes glutamate dehydrogenase and alanine aminotransferase (only about 20% release) (Fig. 9). Following the release of enzymes during 30 min incubations of 10^9 amastigotes with 150μg digitonin revealed even greater differences between enzymes from different organelles (Fig. 10). 90% of glucose-6-phosphate dehydrogenase activity was released within 2 min, whereas only about 50% of both glucose phosphate isomerase and phosphoglycerate kinase activities were released within 3 min with the remainder of the activities (up to 90% maximum) being released over the next 20 minutes. Less than 20% of glutamate dehydrogenase and alanine aminotransferase activities were released during the first 10 mins incubation, but between 10 and 20 mins there was a rapid release such that greater than 90% of each activity was recovered in the supernatant by 30 minutes. Comparative studies carried out with L. m mexicana promastigotes produced a similar enzyme release profile to amastigotes but there were some differences in concentrations of digitonin involved (Fig. 11). Glucose-6-phosphate dehydrogenase was released from both parasite forms at approximately the same digitonin concentration (per mg protein), but the mitochondrial glutamate dehydrogenase and alanine aminotransferase and the glycosomal glucose phosphate isomerase were released from promastigotes at higher digitonin concentrations in comparison to the amastigote enzymes. In digitonin-treated promastigotes, pyruvate kinase was released in parallel with glucose-6-phosphate dehydrogenase (Fig. 11).

3.4.4 Isoelectric focusing of malate dehydrogenase isoenzymes

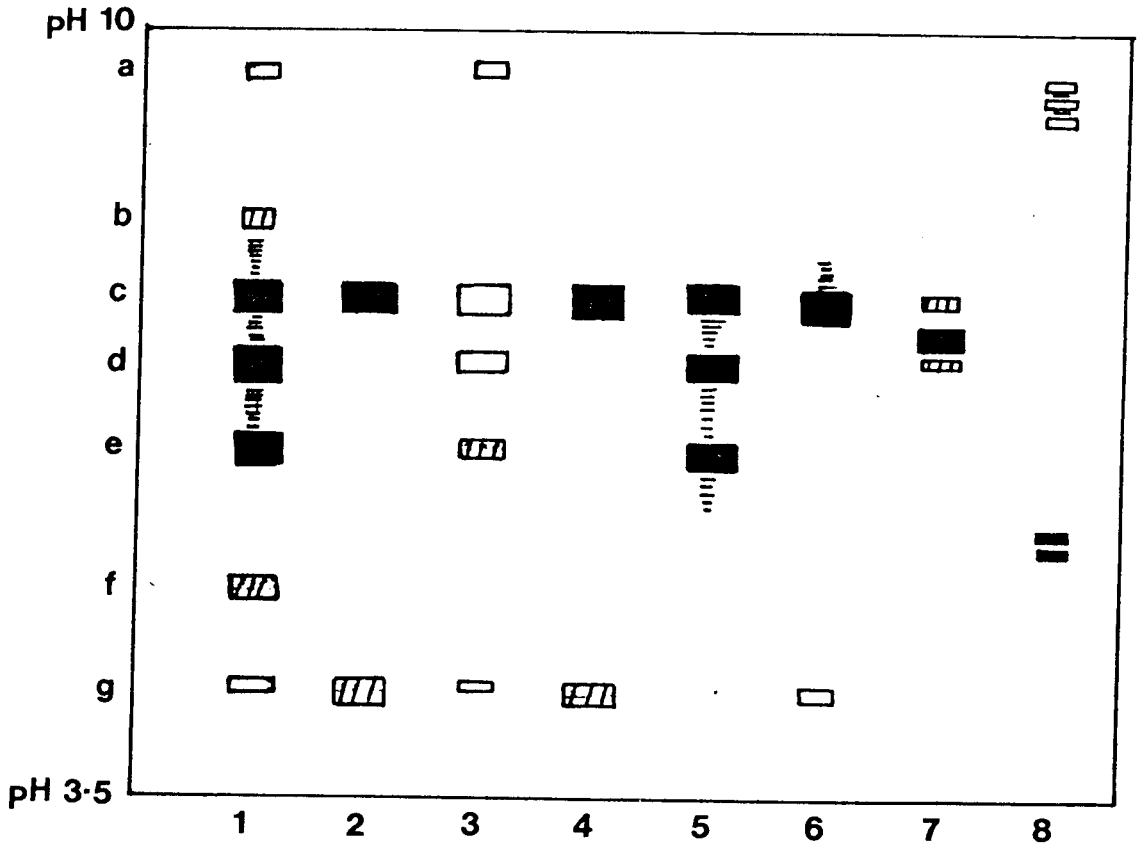
A diagramatic representation of the zymogram of malate dehydrogenase for various fractions of L. m. mexicana amastigote and promastigote clones is shown in Fig. 12. The major bands were not distinctly focused but ran together to a certain extent. This may have been due to the 200mM NaCl which was required to solubilize all the particulate isoenzymes. If this was removed by dialysis before applying the samples to the agarose gel, the enzymes precipitated and lost activity. Despite these limitations in the use of the technique there were clearly large differences observed between amastigote and promastigote malate dehydrogenase isoenzymes. Amastigote homogenate (Track 1) and solubilised pellet (Track 5) showed 3 major bands (c,d,e,) which stained within 20 min. These were possibly due to particulate enzymes although they also appeared in amastigote supernatants but at much lower activity. Only one of these bands (c) was detected in promastigote homogenates (Track 2) and pellets (Track 6). Amastigote homogenates showed a further 2 bands which appeared after 1 hr (b and f), which were not detected in any other sample. Amastigote homogenate and supernatant both showed a weak band (a) very close to the cathode which only appeared after 2 h incubation and which was not found in the amastigote pellet or promastigote fractions. The band g isoenzyme, which may also represent a soluble isoenzyme, had higher activity in promastigote homogenate and supernatant than those from amastigotes and was not found in amastigote pellet. Purified malate dehydrogenase showed one major band (mid-way between band c and d) with 2 smaller bands on either side, however the three bands appeared to overlap.

Fig. 12.

Diagramatic representation of isoelectric focusing of malate dehydrogenase isoenzymes. Samples were run on 1mm thick IEF agarose gels (114 x 185mm) for 2.5 hr at a maximum of 5W and 500V with current unlimited. Gels were stained for malate dehydrogenase activity with a zymogram overlay in 0.2% agar. Key to the time in which the malate dehydrogenase stain appeared: Within 20 mins (■); 1hr (▨); 2hr (□). The shading represents the bands that ran together to a certain extent.

Key to tracks:

1) Amastigote homogenate
2) Promastigote homogenate
3) Amastigote supernatant
4) Promastigote supernatant
5) Amastigote pellet
6) Promastigote pellet
7) Purified malate dehydrogenase
8) Lysed human blood cells


pH 10
a
b
c
d
e
f
g
pH 3·5
1
2
3
4
5
6
7
8

3.5 SOLUBILIZATION OF PARTICULATE ENZYMES

The effect of ionic strength on particulate enzymes from amastigotes and promastigotes of L. m. mexicana was investigated using 0-1M sodium chloride. Malate dehydrogenase, PEP carboxykinase and hexokinase activities sedimented from amastigote homogenates by centrifugation at 10,000g for 2 min were found to require different concentrations of NaCl for solubilization (Fig. 13a). Malate dehydrogenase was solubilized by lower concentrations of NaCl than PEP carboxykinase or hexokinase but all three enzymes were solubilized to greater than 80% by 0.5M NaCl; consequently this concentration of salt was used in the subsequent purification procedure for amastigote enzymes. In a comparative experiment promastigote particulate enzymes were found to behave in a manner similar to those from amastigotes (Fig 13b) although 1M NaCl solubilized more protein from amastigotes (65%) than promastgotes (40%).

3.6 PURIFICATION AND CHARACTERISATION OF PARTICULATE MALATE DEHYDROGENASE AND PEP CARBOXYKINASE FROM LEISHMANIA M. MEXICANA AMASTIGOTES

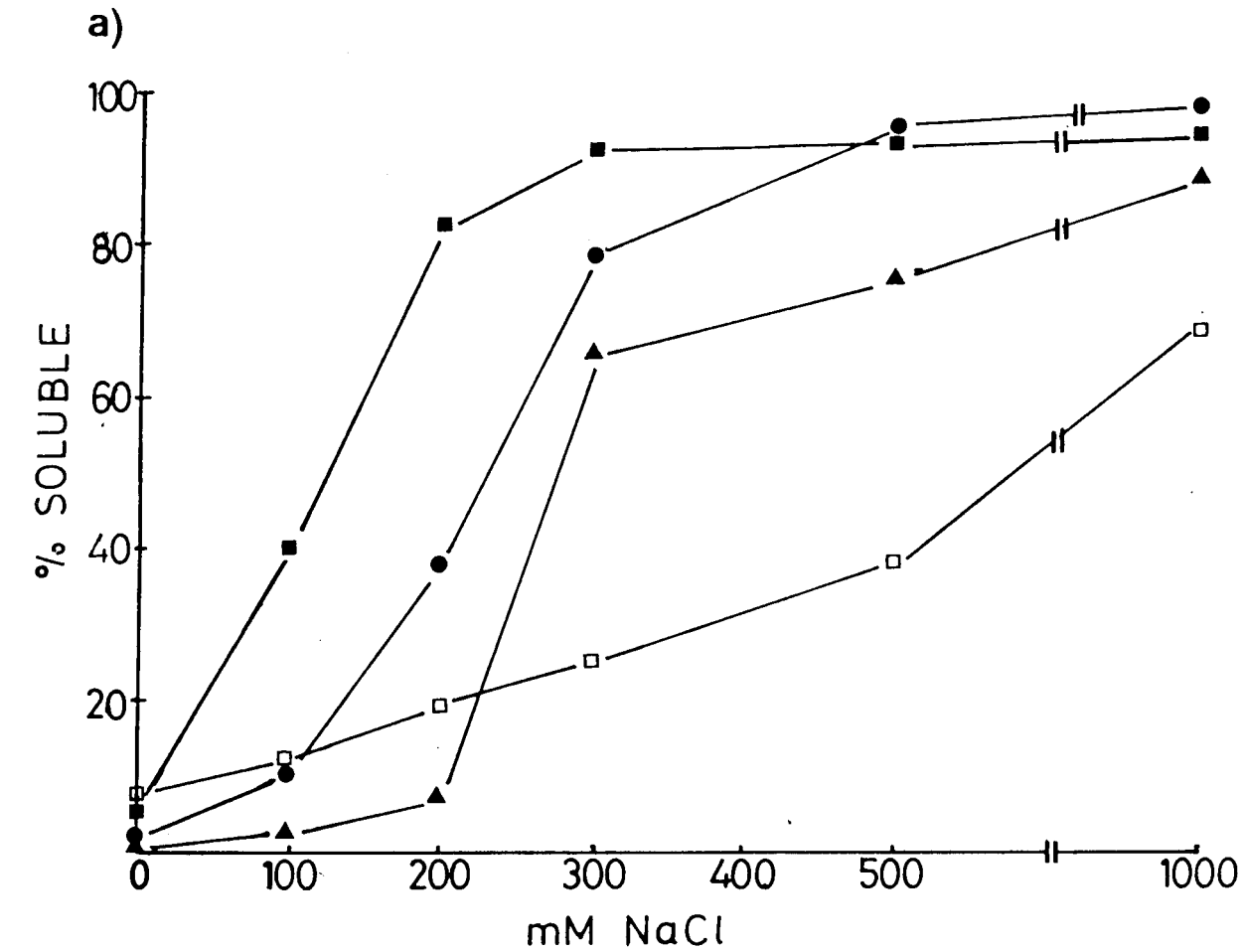
Particulate malate dehydrogenase and PEP carboxykinase were purified to electrophoretic homogeneity as summarised in Tables 17 and 18. Malate dehydrogenase was purified 150-fold overall with a final specific activity of 1230 units/mg protein and a recovery of 63% (Table 17). Most of the loss of activity occurred during the concentration step using the Amicon filtration unit. This may have been due in part to the process taking several hours because of the high viscocity of the mixture containing 50% ethylene glycol, and also possibly to non-specific adsorption to the membrane filter. Less activity was lost when the eluates were reduced less in volume, however the protein concentrations in these samples were too low for SDS-gel electrophoresis. For routine purifications, the samples

Fig. 13a.

Solubilization of particulate enzymes of Leishmania m. mexicana amastigotes. Parasites were lysed in 0.25M sucrose containing 0.1% (v/v) Triton X-100, the lysate centrifuged at 18°C for 5 min at 10,000g and the pellet exposed to a range of NaCl concentrations. The supernatant and resuspended pellet from a subsequent 5 min centrifugation at 10,000g were assayed for malate dehydrogenase (■), PEP carboxykinase (●), hexokinase (▲) and protein (□) and the soluble activity expressed as a percentage of the total activity recovered in both fractions.

Fig 13b.

Solubilization of particulate enzymes of Leishmania m. mexicana promastigotes. The experimental procedures and representation of data is as shown in Fig. 13a.


a)
100
80
60
40
20
% SOLUBLE
0
100
200
300
400
500
1000
mM NaCl

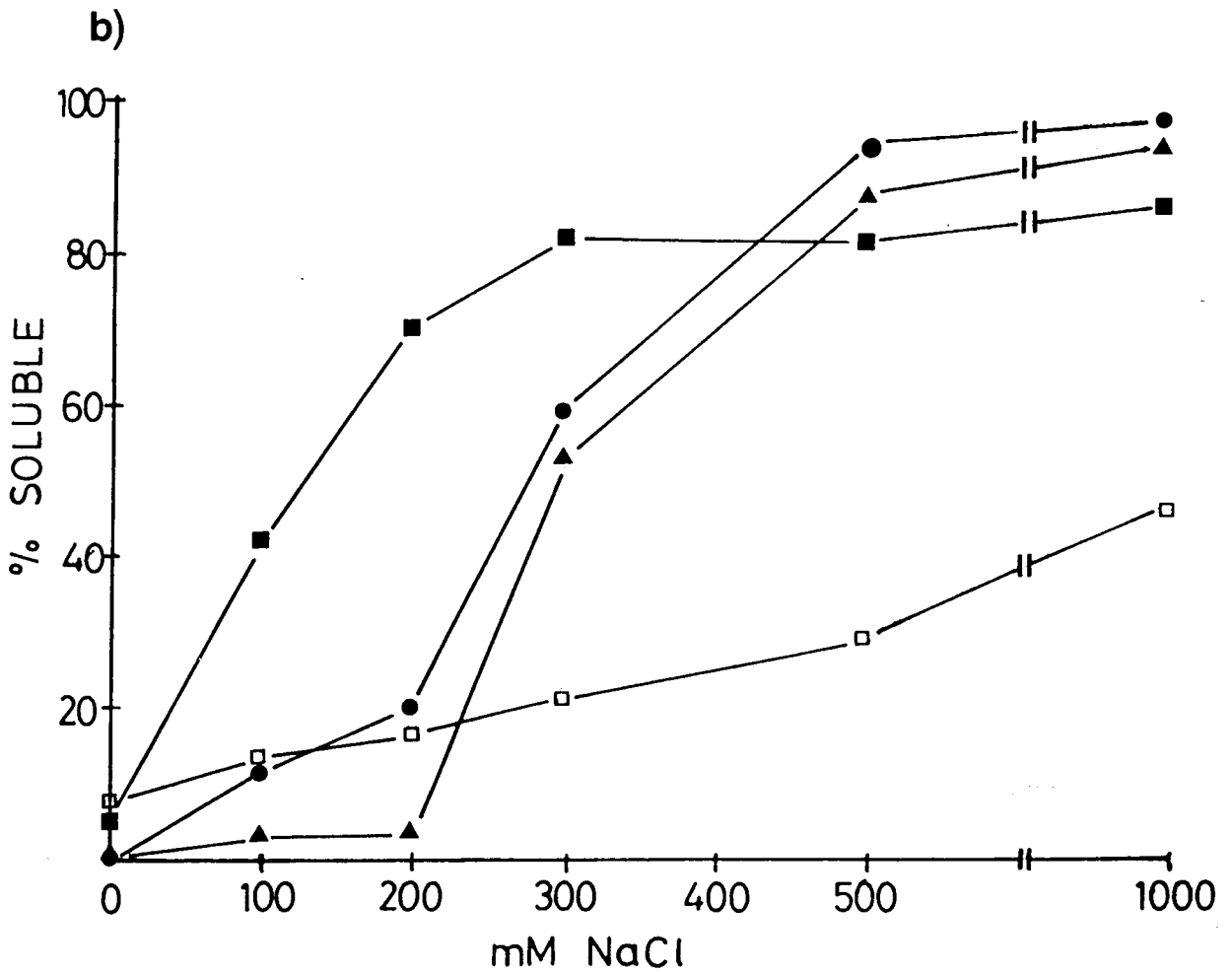

b)
100
80
60
40
20
% SOLUBLE
0
100
200
300
400
500
1000
mM NaCl

Table 17. Summary of the purification of Leishmania m. mexicana amastigote particulate malate dehydrogenase.

Purification step	Volume (ml)	Total protein (mg)	Total Activity (units)	Specific[a] Activity	Yield (%)	Purification Factor
Homogenate	6.0	60	499	8.3		
Pellet 1	4.4	37	424	11.5	85	1.4
Supernatant S2	3.9	24	458	19.3	92	2.3
Phenyl-Sepharose Cl-4B	44	2.1	455	219	92	35
Phenyl-Sepharose Cl-4B[b]	19	0.9	195	218		
Amicon filtration 1	5.3	0.9	120	133	(56)[c]	16
5' AMP Sepharose 4B	18	0.11	135	1230	(63)[c]	150

[a] The specific activity is given in units/mg protein.

[b] Only 43% of the eluate containing malate dehydrogenase activity from the Phenyl-Sepharose column was further purified.

[c] Figures for yield shown in parentheses take into account that only 43% of the initial activity was fully purified (see b).

Table 18. Summary of the purification of *Leishmania m. mexicana* amastigote PEP carboxykinase

Purification step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific[a] Activity	Yield (%)	Purification Factor
Homogenate	6.0	60	13.7	0.23		
Pellet 1	4.4	37	13.7	0.37	99	1.6
Supernatant S2	3.9	24	13.9	0.58	101	2.6
Phenyl-Sepharose Cl-4B	44	2.1	7.8	3.7	57	21.5
Phenyl-Sepharose Cl-4B[b]	19	0.9	3.4	3.7		
5' AMP Sepharose 4B	18	0.44	3.8	8.7	(65)[c]	38
Amicon Filtrate	1.7	0.44	1.3	2.8	(21)[c]	12
Sephadex G-100	12.5	0.04	1.2	30.3	(20)[c]	132

[a] The specific activity is given in units/mg protein.

[b] Only 43% of the eluate containing PEP carboxykinase activity from the Phenyl-Sepharose column was further purified.

[c] Figures for yield shown in parentheses take into account that only 43% of the initial activity was fully purified (see b).

would require less concentrating and the percentage recoveries would be accordingly better. Partially purified and pure malate dehydrogenase when washed free of ethylene glycol and suspended in 0.12M triethanolamine buffer, pH 7.5, containing 200 mM NaCl and 1mM DTT were found to be very unstable at both 4°C and -70°C, all activity being lost within 24 hr. Solubilised particulate malate dehydrogenase (S2) lost 78% activity when stored at 4° for 5.5 hr. When the enzyme was kept in 1% (v/v) ethylene glycol, there was still a loss of 70% of the enzyme activity in 5.5hr at 4°C, whereas in 5% (v/v) ethylene glycol 35% of the activity was lost under the same conditions (Table 19). It was found, however, that full activity was maintained when the enzyme was stored in 10% (v/v) ethylene glycol at -70°C for up to 3 months or overnight at 4°C; consequently 10% ethylene glycol was included in all buffers used for further purification procedures.

PEP carboxykinase was purified 132-fold overall with a final specific activity of 30.3 units/mg protein and a recovery of 20% (Table 18). The percentage recovery from the Phenyl-Sepharose column was lower in the experimental example presented (57%) than in other similar experiments (80-90%). In all cases, however, the majority of the enzyme loss occurred during Amicon filtration.

Malate dehydrogenase, hexokinase and PEP carboxykinase co-eluted from the Phenyl-Sepharose Cl-4B column when the gradient had reached 50% ethylene glycol and 500mM NaCl. Glyceraldehyde-3-phosphate dehydrogenase was eluted earlier (Fig. 14).

Metal chelate affinity chromatography was attempted in an effort to separate PEP carboxykinase from the enzymes that co-eluted with it from the Phenyl-Sepharose Cl-4B column. The separation method is based on the differing ability of sample proteins to bind heavy metal

Table 19.

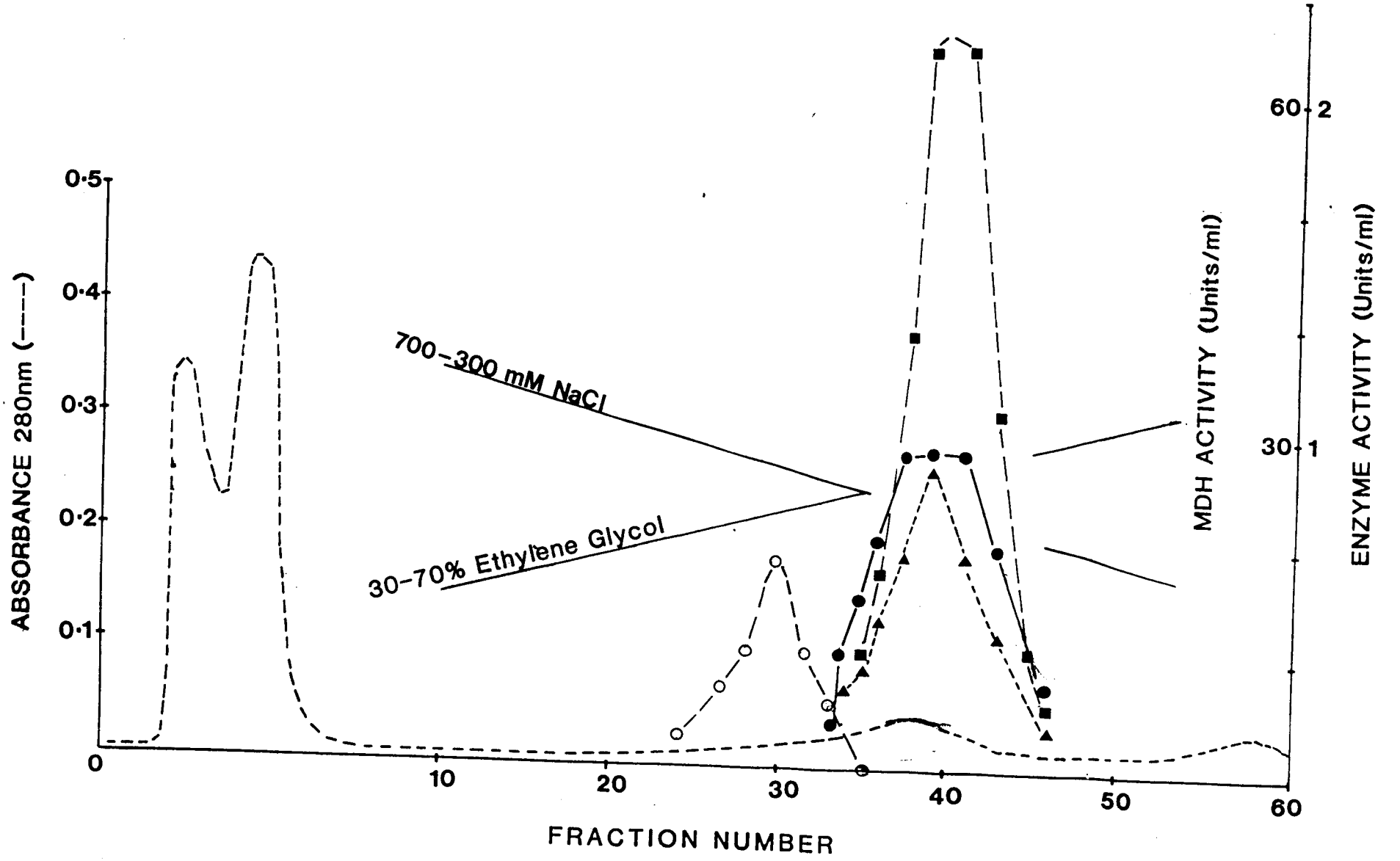
The effect of ethylene glycol on the loss of Leishmania m. mexicana amastigote solubilised particulate malate dehydrogenase activity.[a]

	TIME (Hours)				
% Ethylene glycol	0	0.5	1	2	5.5
0	100	69	76	64	22
1	100	74	60	64	30
5	100	83	93	91	65
10	100	91	83	85	100
20	100	104	112	104	131

[a] The values represent the percentage of the activity present at time zero remaining after incubation at 4°C. The solubilised enzyme (S2) was prepared as described in section 2.6 of the methods.

Fig. 14.

Purification of Leishmania m. mexicana amastigote enzymes using hydrophobic interaction chromatography. Solubilized particulate enzyme (S2) was applied to a column (1.6 x 15.5cm) of Phenyl-Sepharose CL-4B pre- equilbrated with 0.12M triethanolamine buffer pH 7.5 and 1M NaCl (Buffer A). The column was then washed with 80ml of buffer A (flow rate 60ml/hr, 8ml fractions collected) followed by 150ml of an increasing gradient of ethylene glycol (30-70% v/v) and a decreasing gradient of NaCl (700-300mM) in 0.12M triethanolamine buffer pH 7.5 (flow rate 30ml/hr, 3ml fractions collected). Fractions were assayed for malate dehydrogenase (■), PEP carboxykinase (●), hexokinase (▲) and glyceraldehyde-3-phosphate dehydrogenase (O). Protein (----) was measured at 280nm. The malate dehydrogenase activity was approximately 60-fold higher than the other enzymes assayed and is represented on a separate scale.


ABSORBANCE 280nm (——)
0·5
0·4
0·3
0·2
0·1
0
700–300 mM NaCl
30–70% Ethylene Glycol
10
20
30
40
50
60
FRACTION NUMBER
MDH ACTIVITY (Units/ml)
60
30
2
1
ENZYME ACTIVITY (Units/ml)

ions. The binding to the column is pH dependent and can be reversed by reducing the pH, increasing the ionic strength or using a chelating agent such as EDTA. A chelating Sepharose 6B column loaded with Zn^{2+} showed separation of the test sample (solubilised pellet S2) into three protein peaks under different elution conditions (Fig. 15a), however the column was not suitable for purification of the enzymes under study as both PEP carboxykinase and malate dehydrogenase, which are inhibited by 0.5mM $ZnCl_2$ by 93% and 95% respectively, were inhibited by $ZnCl_2$ from the column. Leishmania m. mexicana PEP carboxykinase is manganese dependent and a Chelating Sepharose column loaded with Mn^{2+} might therefore be expected to give a good purification of PEP carboxykinase. However Mn^{2+} only binds poorly to the Chelating Sepharose (Porath et al. 1975) and it was found that only a small concentration of protein bound to the column, the majority, including all malate dehydrogenase and PEP carboxykinase activities, was washed straight through (Fig. 15b).

7% of the malate dehydrogenase activity was eluted immediately from the 5' AMP-Sepharose column, together with all detectable PEP carboxykinase and hexokinase activities (Fig. 16). The 93% of malate dehydrogenase activity that remained bound to the column was eluted with 1M NaCl in 0.12M triethanolamine buffer pH 7.5 and was shown to be electrophoretically homogeneous on SDS polyacrylamide gels (Fig. 17). This purified enzyme preparation was used in the characterisation studies.

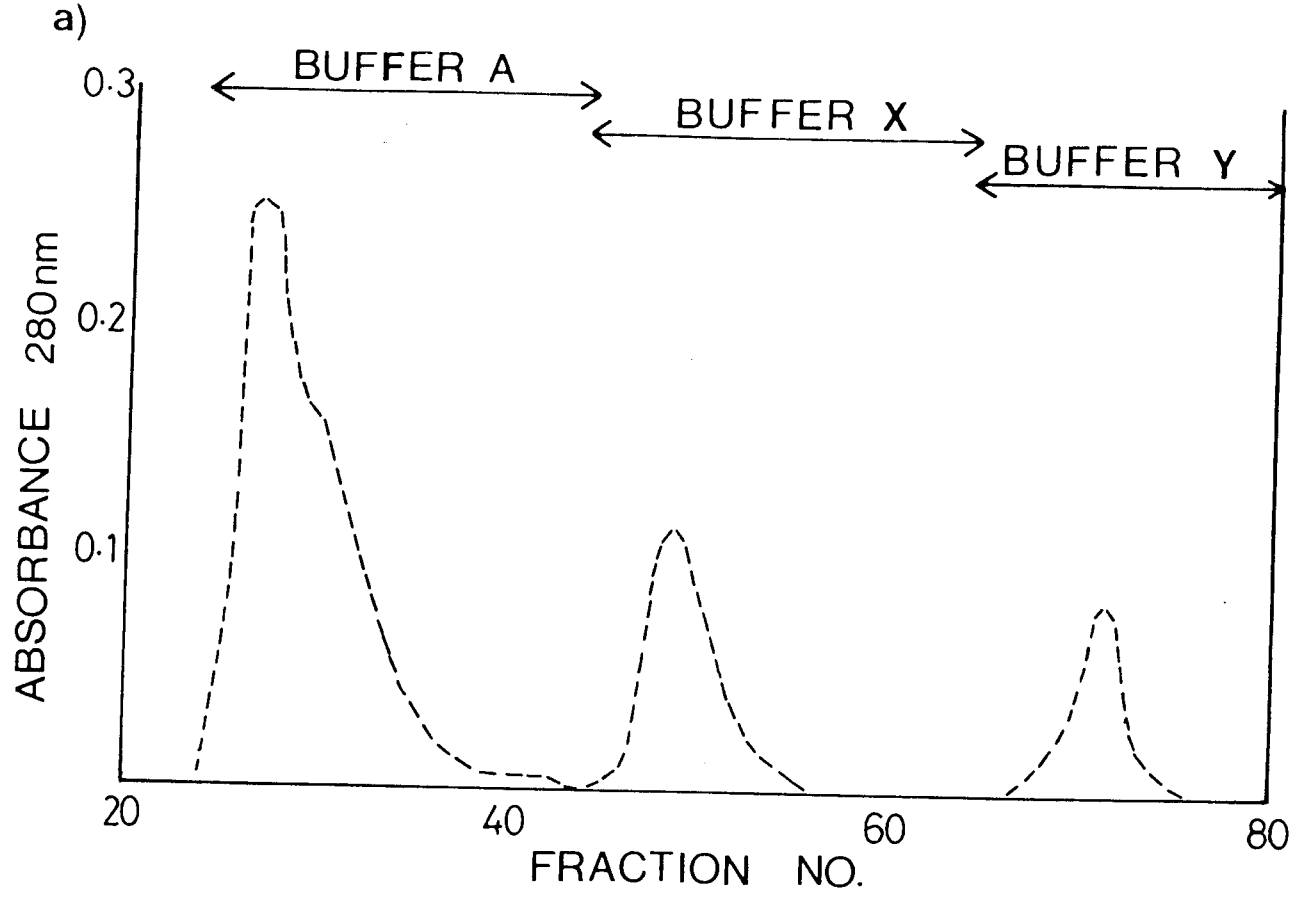
PEP carboxykinase, hexokinase and the 7% of malate dehydrogenase activity which did not bind to 5' AMP-Sepharose were subsequently separated by gel filtration on Sephedex G-100 (Fig. 18). The eluted PEP carboxykinase and hexokinase both appeared to be homogeneous on SDS polyacrylamide gels; however due to the very low protein concentration loaded onto the gels the bands were too weak to be reproduced

Fig. 15a.

Attempted purification of Leishmania m. mexicana PEP carboxykinase using metal chelate affinity chromatography (MCAC) with Zn^{2+}. 0.8ml of solubilized pellet (S2) was applied to a column (1.6 x 4cm) of Chelating-Sepharose 6B charged with 5mg/ml $ZnCl_2$ and pre-equilibrated with buffer A. The column was washed with 20ml of the same buffer (flow rate 60ml/hr; 2ml fractions collected). Bound enzymes were subsequently eluted with 20ml buffer X (0.05M sodium acetate buffer pH 4.0, 1M NaCl) and 20ml buffer Y (0.12M triethanolamine buffer pH 7.5, 1M NaCl, 50mM EDTA). Protein was determined at 280nm (------).

Fig. 15b.

Attempted purification of Leishmania m. mexicana PEP carboxykinase using MCAC with Mn^{2+}. Procedure was as outlined above except that the column was charged with 5mg/ml $MnSO_4$. Malate dehydrogenase (•••□••), PEP carboxykinase (O), protein (------).


a)
BUFFER A
BUFFER X
BUFFER Y
0·3
0·2
0·1
ABSORBANCE 280nm
20
40
60
80
FRACTION NO.

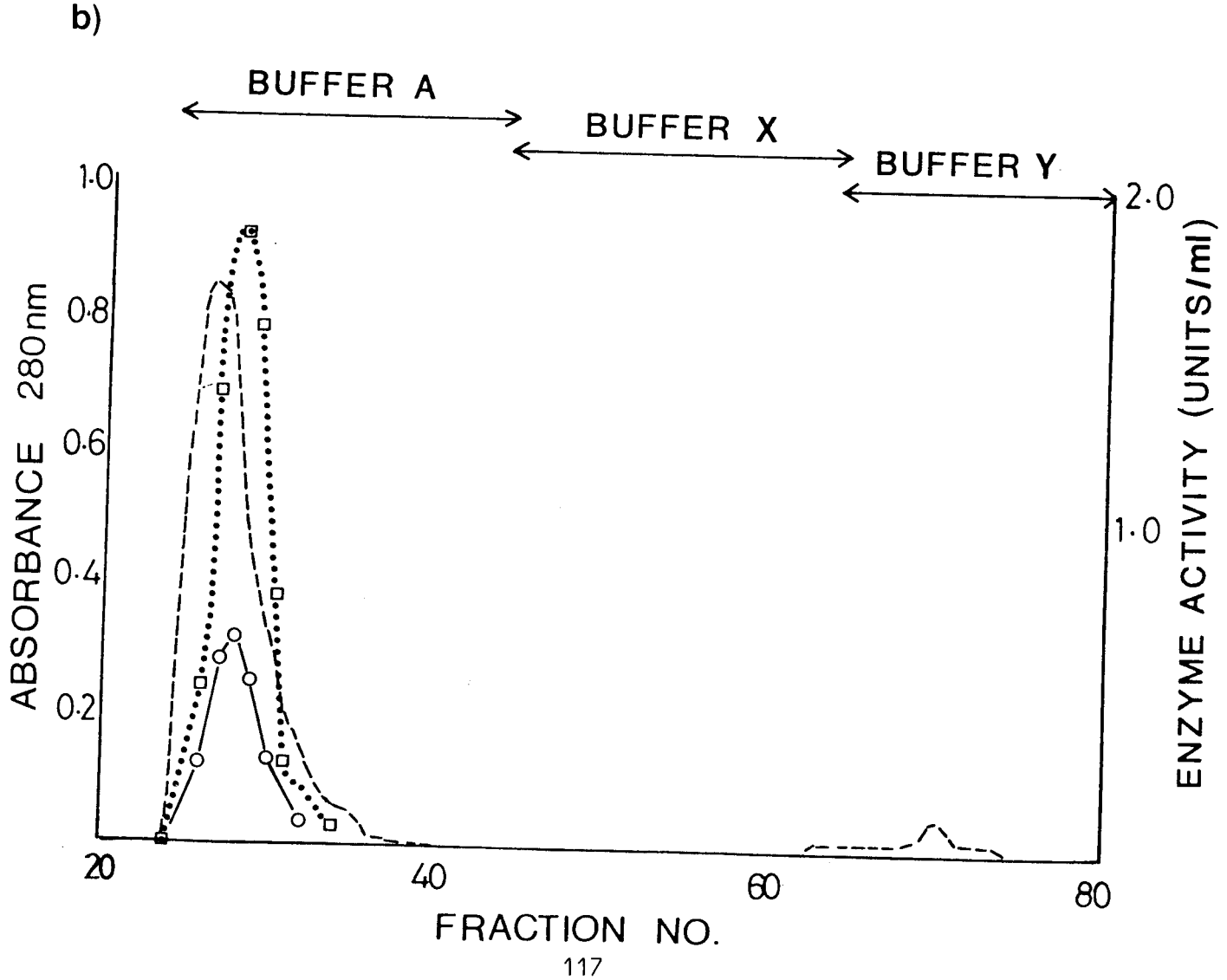

b)
BUFFER A
BUFFER X
BUFFER Y
1·0
0·8
0·6
0·4
0·2
ABSORBANCE 280nm
2·0
1·0
ENZYME ACTIVITY (UNITS/ml)
20
40
60
80
FRACTION NO.

Fig. 16.

Purification of Leishmania m. mexicana malate dehydrogenase using affinity chromatography. The concentrated sample from the Phenyl-Sepharose Cl-4B column was applied to a column (1.6 x 3.5cm) of 5' AMP-Sepharose 4B pre-equilibrated with 0.12M triethanolamine buffer pH 7.5, 200mM NaCl, 1mM DTT and 10% (v/v) ethylene glycol. The column was washed with 20ml of the same buffer, 2.5ml fractions being collected. Bound enzyme was subsequently (arrowed) eluted with 20ml of buffer containing 1M NaCl and the fractions collected assayed for malate dehydrogenase (■), PEP carboxykinase (●) and hexokinase (▲). Protein (----) was measured at 280nm.

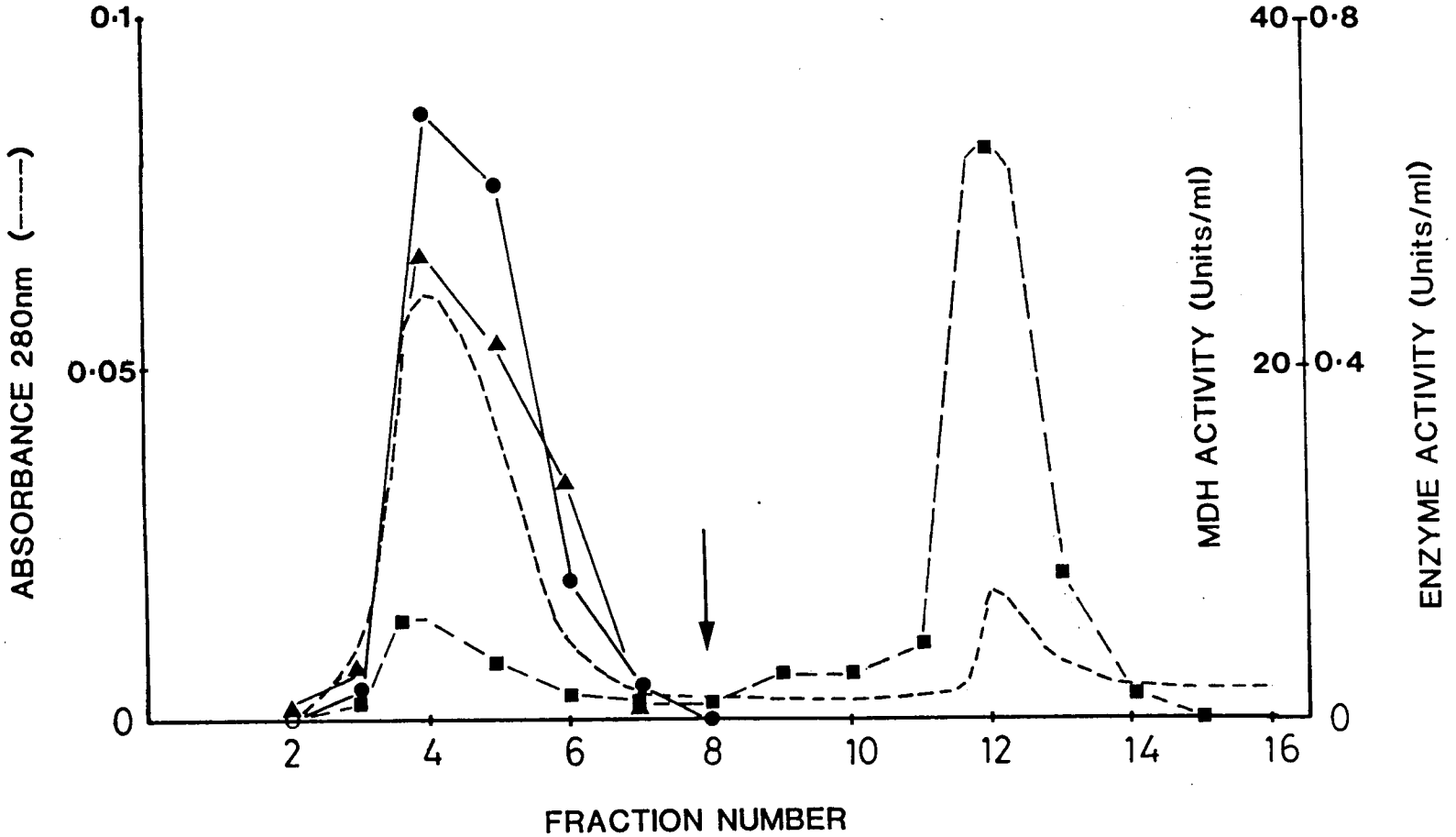
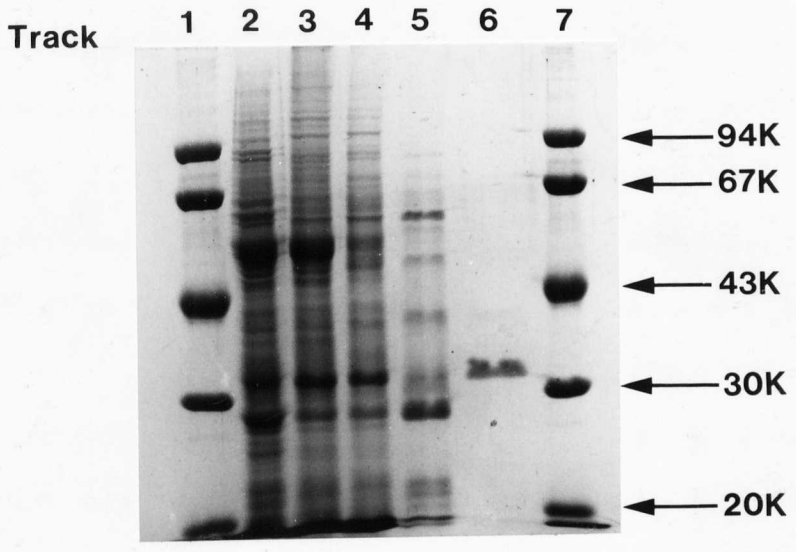

0·1
0·05
0
ABSORBANCE 280nm (——)
40
20
0
MDH ACTIVITY (Units/ml)
0·8
0·4
0
ENZYME ACTIVITY (Units/ml)
2
4
6
8
10
12
14
16
FRACTION NUMBER

Fig. 17.

a) SDS gel electrophoresis of samples from different stages during the purification of malate dehydrogenase. Key to tracks (protein applied): 1, low molecular weight (LMW) marker proteins (20µg); 2, homogenate H (6µg); 3, pellet P1 (8.5µg); 4, supernatant S2, (6.1µg); 5, unbound protein eluted from 5' AMP-Sepharose column (1.3µg); 6, purified malate dehydrogenase (0.32µg); 7, LMW marker proteins (20µg).

b) Molecular weight determinations using a calibration curve of low molecular weight protein markers. 1, phosphorylase b. 2, albumin. 3, ovalbumin. 4, carbonic anhydrase. 5, trypsin inhibitor.

a)


Track
1
2
3
4
5
6
7
94K
67K
43K
30K
20K

b)

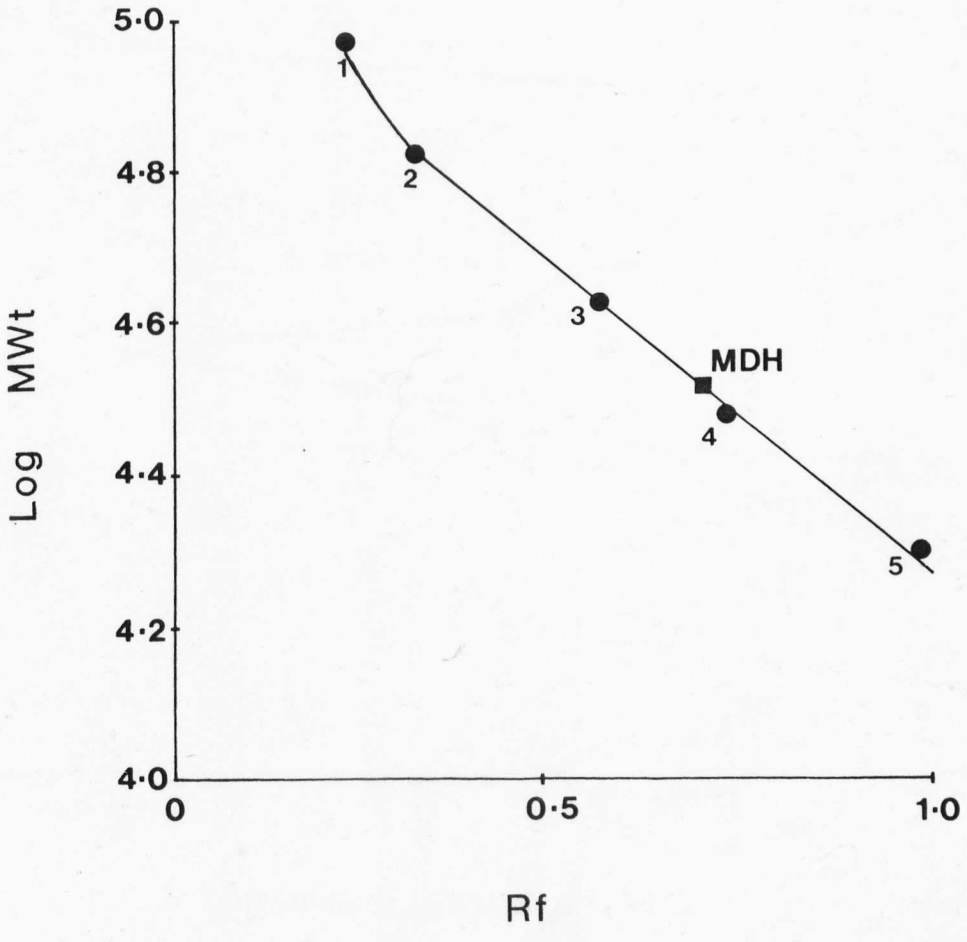
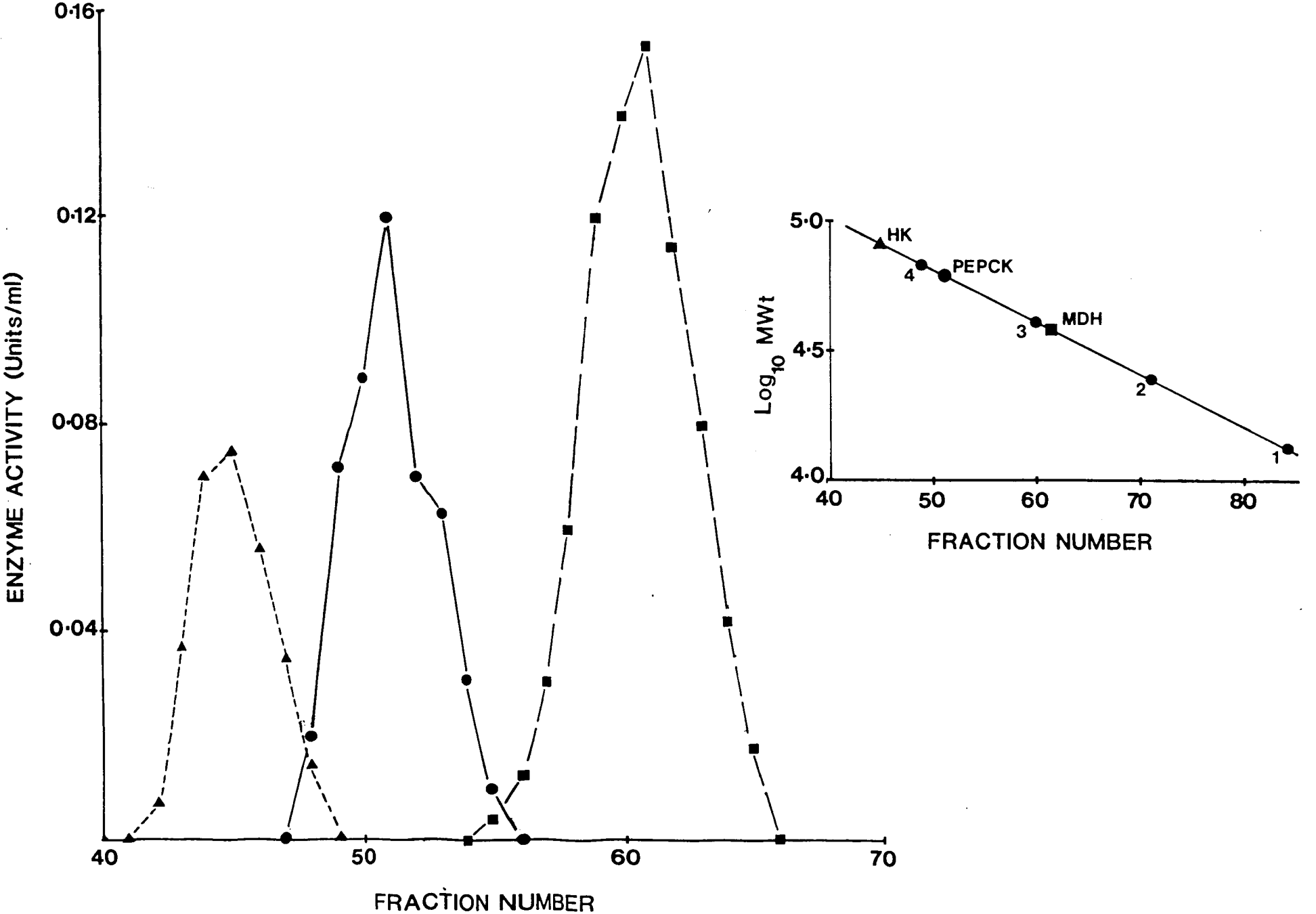

5·0
4·8
4·6
4·4
4·2
4·0
Log MWt
0
0·5
1·0
Rf
1
2
3
MDH
4
5

Fig. 18.

Purification of Leishmania m. mexicana enzymes using gel filtration. 1.7ml of unbound protein from the 5' AMP-Sepharose 4B column was concentrated and applied to a column (2.6 x 50cm) of Sephadex G100 equilibrated with 0.12M triethanolamine buffer pH 7.5 with 10% (v/v) ethylene glycol, 1M NaCl and 1mM DTT. The column was eluted in an upwards direction (20ml/hr) and 2.5ml fractions were collected and assayed for malate dehydrogenase (■), PEP carboxykinase (●) and hexokinase (▲).

Insert: Molecular weight determination using a calibration curve of low molecular weight (MWt) protein markers. 1, ribonuclease A - 13,700; 2, chymotrypsinogen A - 25,000; 3, ovalbumin - 43,000; 4, albumin - 67,000.


0·16
0·12
0·08
0·04
ENZYME ACTIVITY (Units/ml)
40
50
60
70
FRACTION NUMBER
5·0
4·5
4·0
Log10 MWt
HK
PEPCK
MDH
4
3
2
1
40
50
60
70
80
FRACTION NUMBER

by photography.

3.6.1 Molecular weight determinations

The molecular weights of the purified enzymes were estimated by two methods: Chromatographic mobility on Sephadex G-100 (Fig. 18), which gave molecular weights of 39,800 for malate dehydrogenase, 63,100 for PEP carboxykinase and 83,000 for hexokinase, and SDS polyacrylamide gel electrophoresis, which gave molecular weights of 33,300 and 65,100 for malate dehydrogenase (Fig. 17) and PEP carboxykinase, respectively.

The molecular weights of all malate dehydrogenase isoenzymes were investigated using Sephadex G-100. A homogenate of L. m. mexicana amastigotes containing all the isoenzymes of malate dehydrogenase, including solubilized particulate enzyme, eluted as a single peak during gel filtration (Fig. 19) with a molecular weight of 39,800.

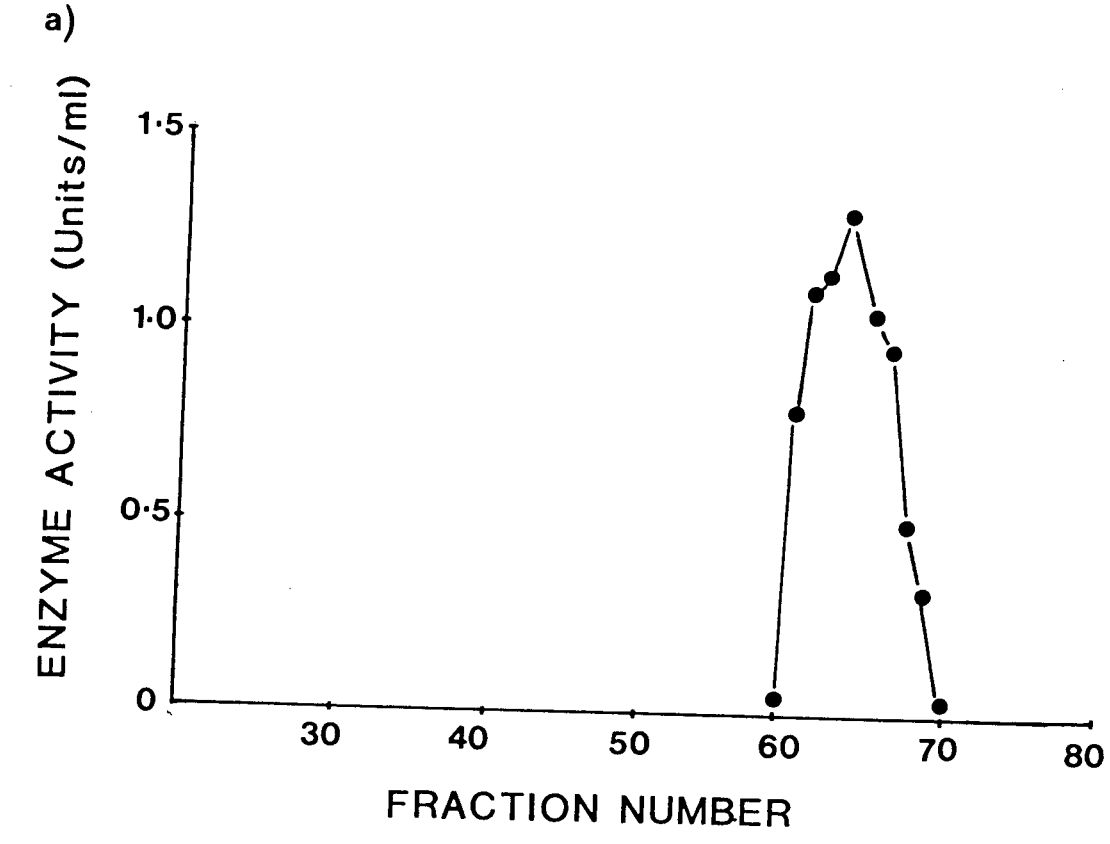
3.6.2 pH and temperature profiles

The pH profiles for malate dehydrogenase assayed in both directions (oxaloacetic acid (OAA) reduction and malate oxidation) are shown in Fig. 20(a). The former reaction was active over a broad pH range (6.0 - 9.5) with a maximum at pH 7.5. For malate oxidation, the optimum pH was 8.5. The enzyme, assayed with OAA as substrate, increased in activity as the temperature was increased from 15^{o}C up to 40^{o}C (Fig. 20b) but was inactivated by higher temperatures.

Fig 19.

a) Molecular weight determinations using Sephadex G-100 of malate dehydrogenase in Leishmania m. mexicana amastigote total homogenate solubilized with 0.12M triethanolamine buffer pH 7.5 with 10% (v/v) ethylene glycol, 1M NaCl and 1mM DTT. The column was run as described in Fig. 18.

b) Calibration curve of low molecular weight markers (see fig. 18)


a)
ENZYME ACTIVITY (Units/ml)
1·5
1·0
0·5
0
30
40
50
60
70
80
FRACTION NUMBER

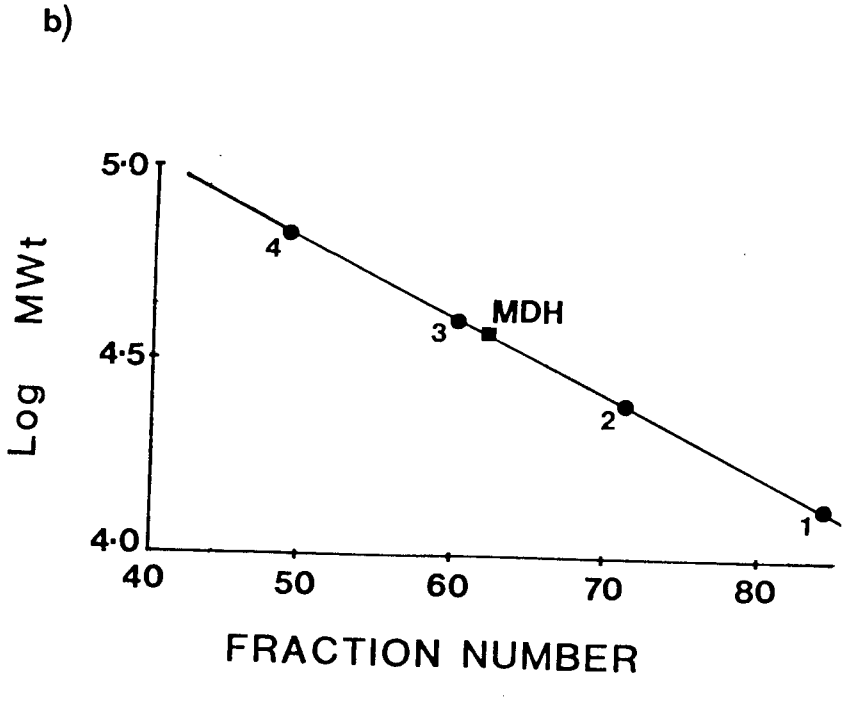
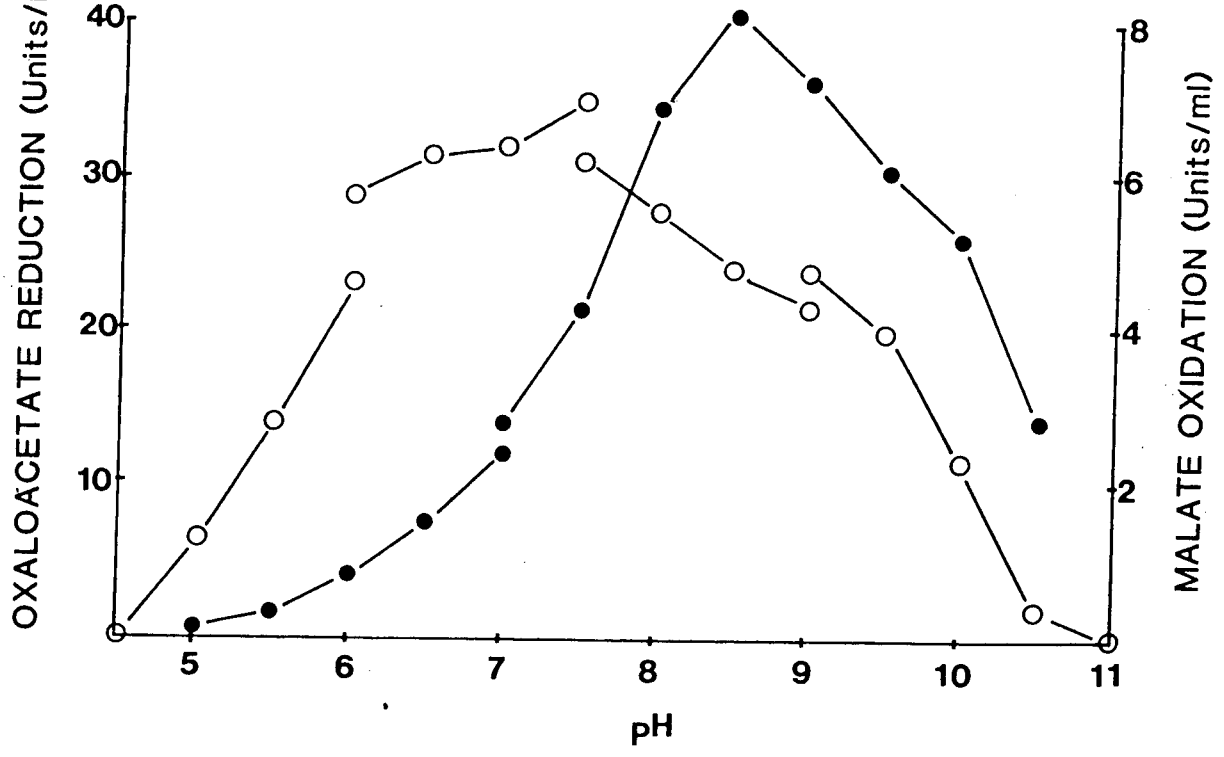

b)
Log MWt
5·0
4·5
4·0
4
3
MDH
2
1
40
50
60
70
80
FRACTION NUMBER

Fig. 20.

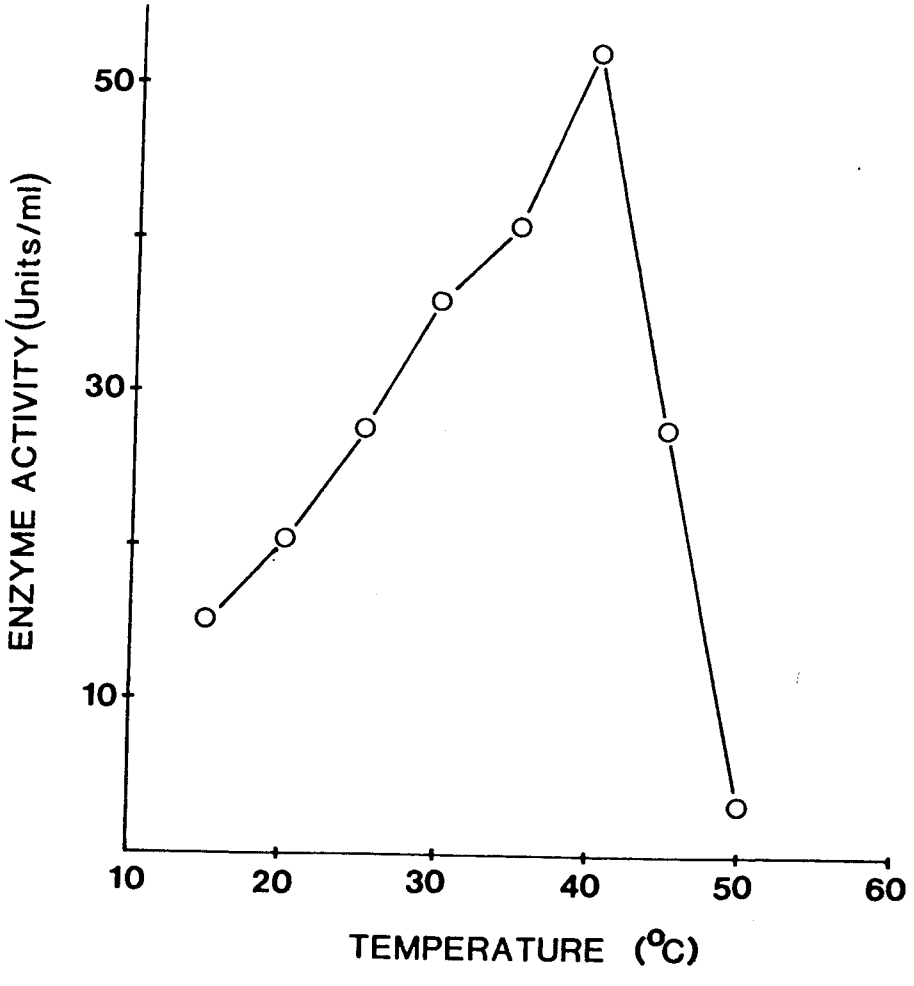
a) pH profile for purified malate dehydrogenase assayed in both the malate oxidation (●) and oxaloacetic acid reduction (O) directions.

b) Temperature profile for purified malate dehydrogenase assayed in the direction of oxaloacetic acid reduction.

a)


OXALOACETATE REDUCTION (Units/ml)
MALATE OXIDATION (Units/ml)
40
30
20
10
8
6
4
2
5
6
7
8
9
10
11
pH

b)


ENZYME ACTIVITY (Units/ml)
50
30
10
10
20
30
40
50
60
TEMPERATURE (°C)

3.6.3 Enzyme Kinetics

The V_{max} (OAA reduction) and apparent K_m OAA values for malate dehydrogenase for a range of NADH concentrations are given in Table 20. The results are shown graphically as Lineweaver-Burk plots, which intercepted to the left of the x-axis suggesting a single displacement reaction (Fig. 21). A plot of the intercepts with the ordinate against the reciprocal of NADH concentration gave an apparent K_m NADH of 41μM, and a V_{max} (oxaloacetic acid reduction) of 576 units/mg protein (Fig. 22a) and a plot of the slope against the NADH concentration gave a K_m OAA of 38μM (Fig. 22b). The V_{max} (malate oxidation) and apparent K_m malate values for a range of NAD^+ concentrations are given in Table 21. The results are shown graphically as Lineweaver-Burk plots in Fig. 23. From plots of the reciprocal of NAD^+ concentration against the intercepts (Fig. 24a) and the slope (Fig. 24b) taken from Fig. 23, the apparent K_m NAD^+ was 0.79mM, the K_m malate 3.6mM and the V_{max} (malate oxidation) 165 units/mg protein.

The activity for malate oxidation with both the pure or crude preparations of malate dehydrogenase was reduced 3-fold by the omission from the assay mixture of the high concentrations of hydrazine sulphate, which reacts with the end product oxaloacetic acid. Oxaloacetic acid exhibited substrate inhibition at concentrations greater than 0.83mM (Fig. 25) and malate was found to act as a product inhibitor at high concentrations with 82% inhibition at 399mM and 27% inhibition at 33mM (Table 22).

Metabolic intermediates, cofactors and ions were tested as possible modifiers of enzyme activity for malate dehydrogenase assayed in the oxaloacetic acid reduction direction. PEP, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6,-diphosphate,

Table 20.

The V_{max} and apparent K_m oxaloacetic acid (OAA) for purified amastigote malate dehydrogenase with different NADH concentrations.[a]

NADH (mM)	V_{max}[b]	Apparent K_m OAA (μM)
0.2	0.89	50.9
0.1	0.84	53.2
0.075	0.81	60.9
0.05	0.69	64.6
0.04	0.51	63.0
0.03	0.50	74.0

[a] Taken from fig. 21.

[b] Units= mmoles/min.

Table 21.

The V_{max} and apparent $K_m$malate for purified amastigote malate dehydrogenase with different NAD^+ concentrations.[a]

NAD (mM)	V_{max}[b]	Apparent K_m malate (mM)
2	1.15	4.9
1	0.90	7.3
0.5	0.60	9.0
0.2	0.40	12.7
0.1	0.24	31.1
0.05	0.11	22.4

[a] Taken from fig. 23.

[b] Units= mmoles/min.

Fig. 21.

Lineweaver-Burk plots for the activity of purified malate dehydrogenase at a range of oxaloacetic acid and NADH concentrations.

Fig. 22.

a) Secondary plot of the reciprocals of NADH concentrations against the intercept of the ordinate from Fig. 21.

b) Secondary plot of the reciprocals of NADH concentrations against the slope of the lines from Fig. 21.

Fig. 23.

Lineweaver-Burk plots for the activity of malate dehydrogenase at a range of malate and NAD^+ concentrations.

Fig. 24.

a) Secondary plot of the reciprocals of NAD^+ concentrations against the intercept of the ordinate from Fig. 23.

b) Secondary plot of the reciprocals of NAD^+ concentrations against the slopes of the lines from Fig. 23. Key: mal., malate.

FIG. 21

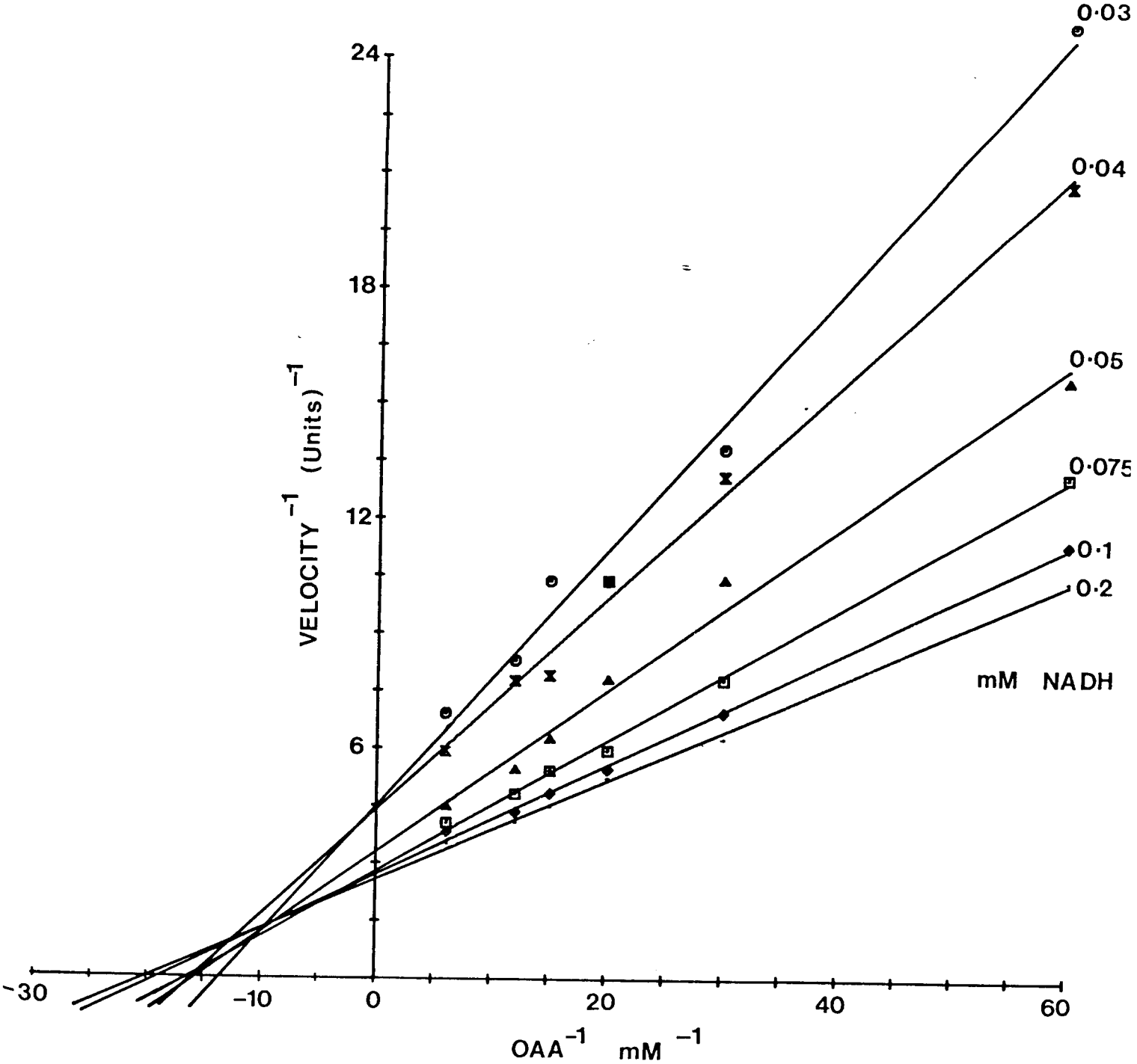
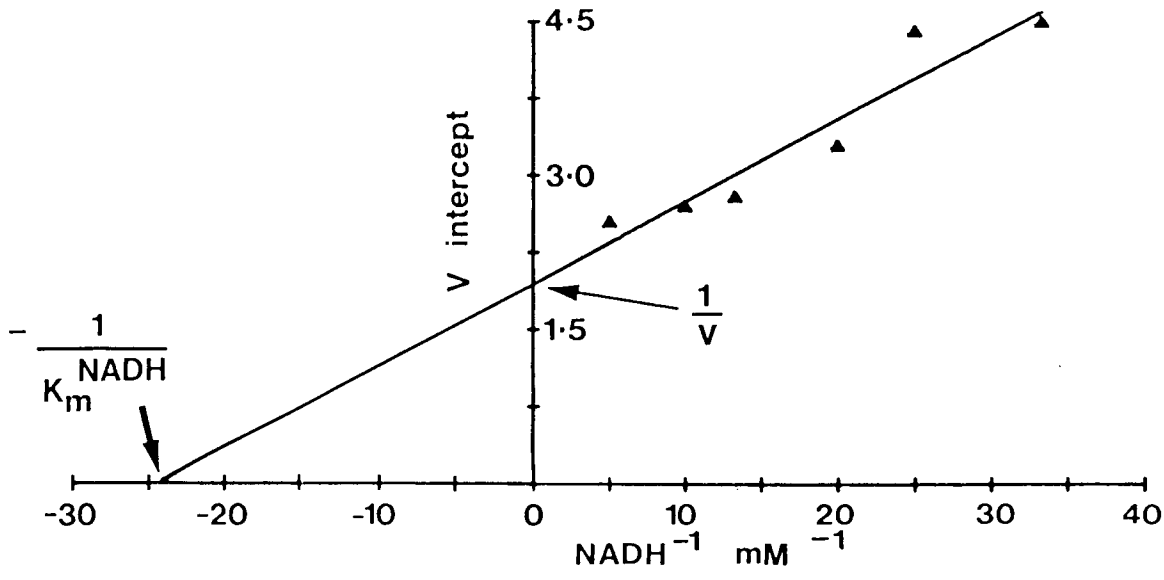

0·03
0·04
0·05
0·075
0·1
0·2
mM NADH
24
18
12
6
VELOCITY^{-1} (Units)$^{-1}$
-30
-10
0
20
40
60
OAA^{-1} mM^{-1}

FIG. 22

a)


4·5
3·0
1·5
V intercept
1/V
-1/Km NADH
-30
-20
-10
0
10
20
30
40
NADH-1 mM-1

b)

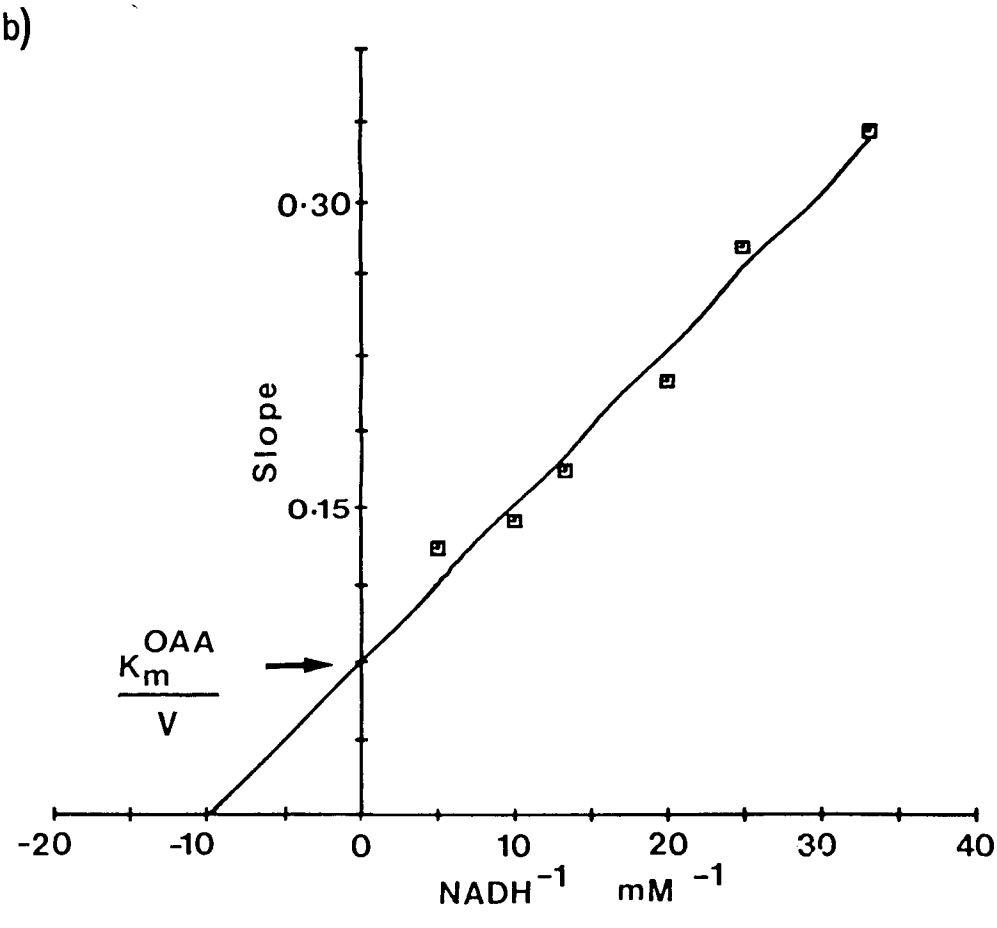

0·30
0·15
Slope
Km OAA/V
-20
-10
0
10
20
30
40
NADH-1 mM-1

Fig. 23

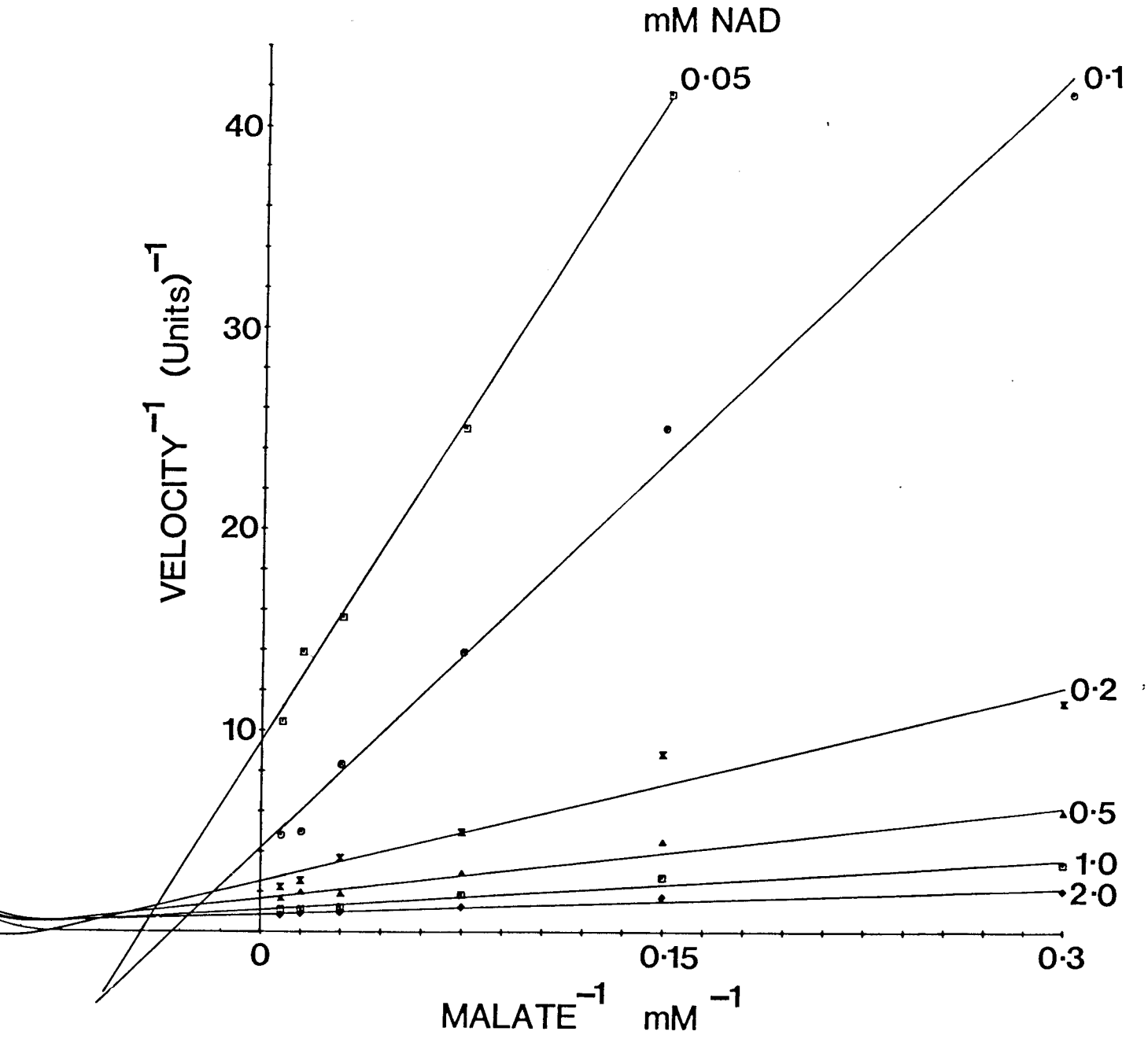
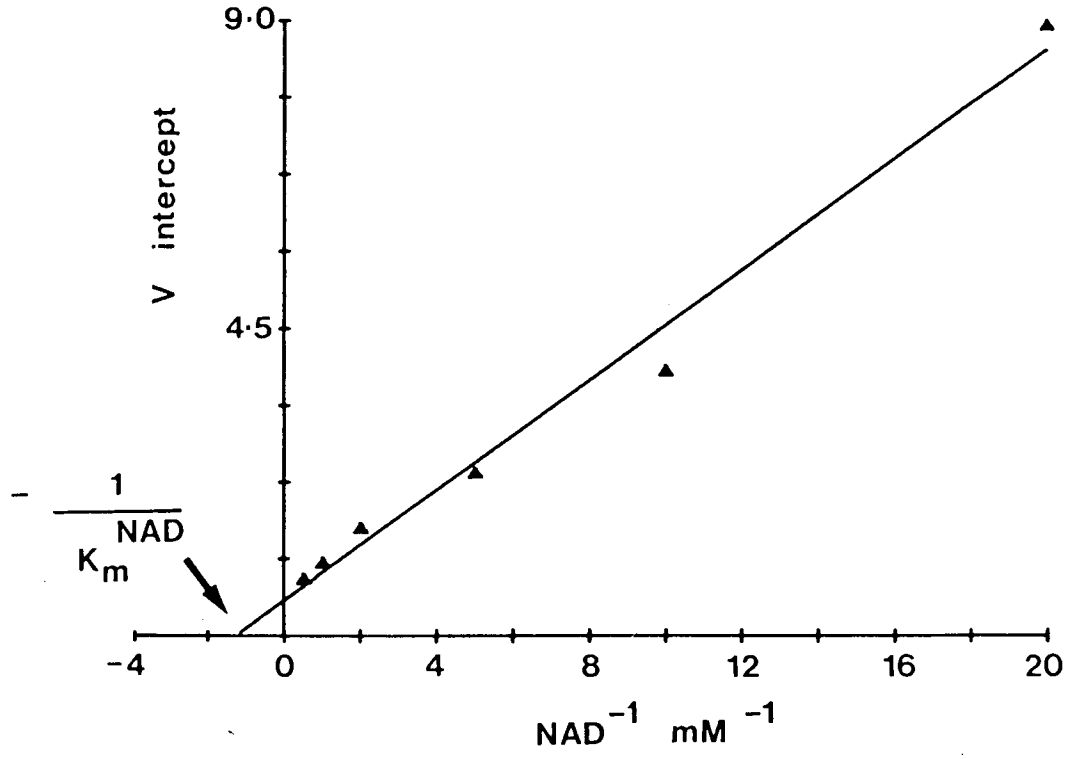

mM NAD
0·05
0·1
0·2
0·5
1·0
2·0
40
30
20
10
0
0·15
0·3
VELOCITY^{-1} (Units)$^{-1}$
MALATE^{-1} mM^{-1}

FIG. 24

a)


9·0
4·5
V intercept
$-\frac{1}{K_m^{NAD}}$
-4
0
4
8
12
16
20
NAD^{-1} mM^{-1}

b)

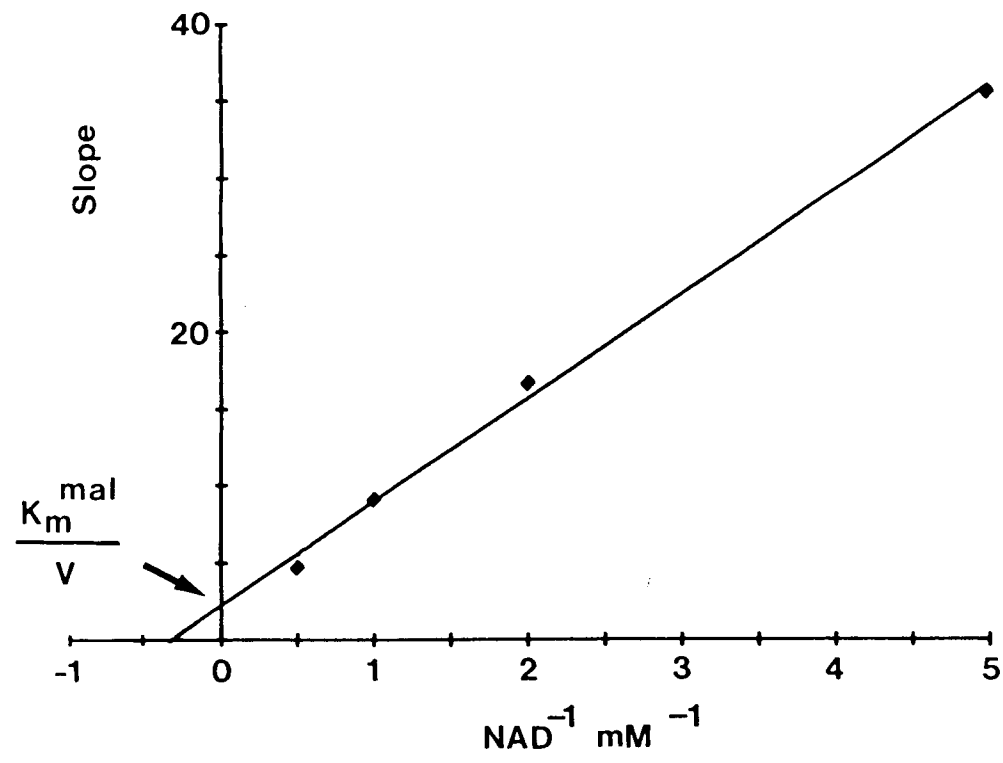

40
20
Slope
$\frac{K_m^{mal}}{V}$
-1
0
1
2
3
4
5
NAD^{-1} mM^{-1}

Fig. 25.

The effect of the substrate oxaloacetic acid on malate dehydrogenase activity.

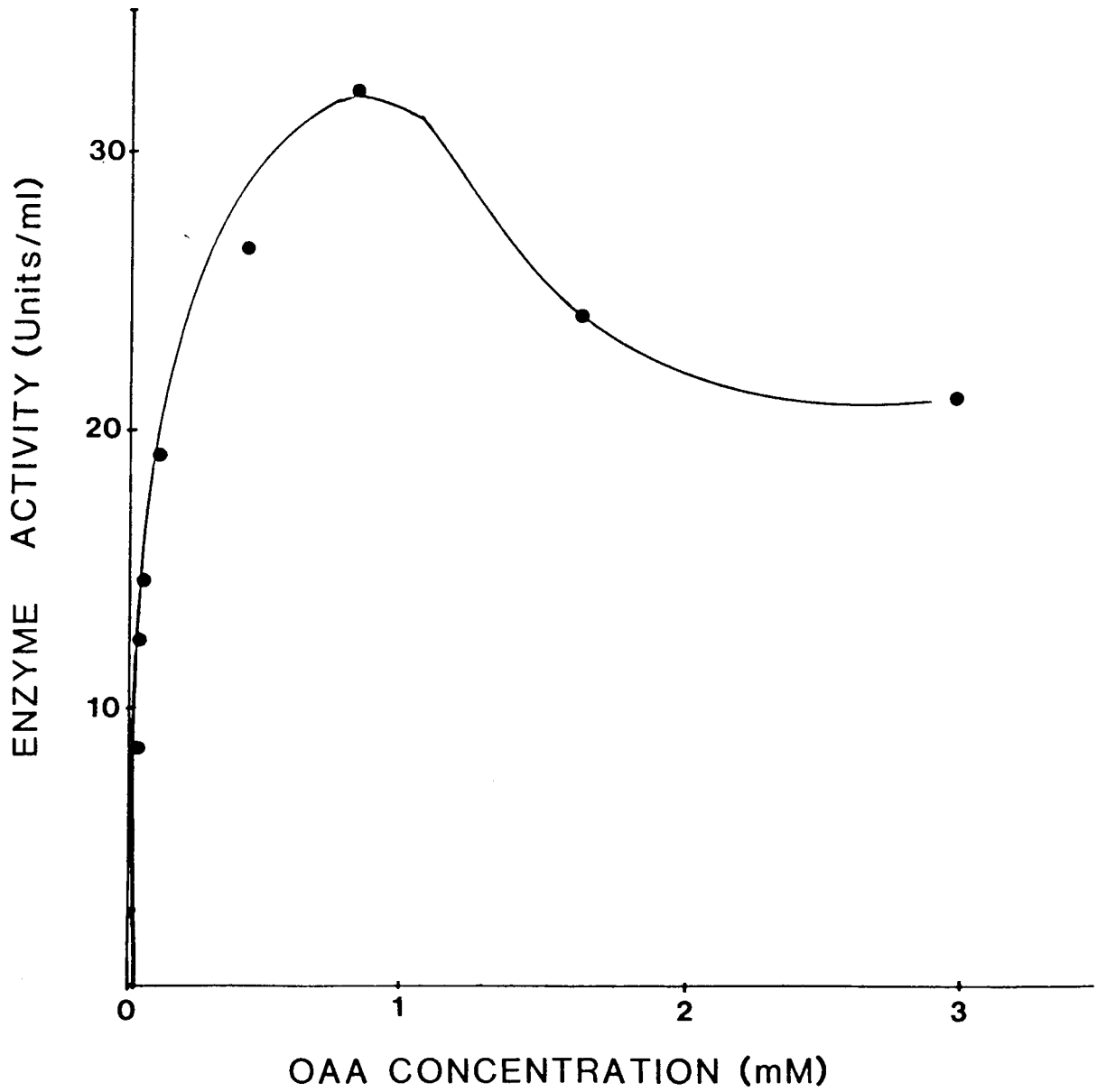


Table 22.

Inhibition of L. m. mexicana amastigote malate dehydrogenase by malate.[a]

Concentration (mM)	Activity (Units/ml)	% inhibition
0	19.0	0
33	13.9	27
66	10.5	45
133	7.5	61
266	4.8	75
399	3.4	82

[a] Assayed in the direction of oxaloacetate reduction.

fumarate, succinate, alanine, citrate, NAD^+, 5'AMP, ATP, ADP, GTP and GDP (all at 1mM) showed no effect, whereas 1mM $CuSO_4$ and $ZnSO_4$ inhibited the enzyme activity by 90% and 91% respectively.

Leishmania m. mexicana amastigotes contain high particulate malate dehydrogenase activity compared with PEP carboxykinase and hexokinase, consequently large amounts of malate dehydrogenase were recovered with this procedure which allowed the enzyme to be characterized in some detail. Only small quantities of PEP carboxykinase and hexokinase could be isolated, however, and so characterisation of these enzymes was not possible in the time available in this study.

4.0 DISCUSSION

4.1 STUDIES ON THE GLYCOLYTIC SEQUENCE, THE TRICARBOXYLIC ACID CYCLE AND ASSOCIATED ENZYMES IN *LEISHMANIA*

The vast majority of work on *Leishmania* carbohydrate metabolism, and indeed the general biochemistry of *Leishmania*, has been performed on the promastigote stage of the parasite as it can easily be cultured in large numbers axenically *in vitro* (see Gutteridge and Coombs 1977, Marr 1980). The recent development of techniques to isolate, free from host cell contamination, amastigotes which had been grown in mice has made possible comparative studies of the biochemistry of the amastigote, the cultured promastigote and the host cell, the macrophage (Hart *et al.* 1981a,b,c, Hart and Coombs 1982, Coombs *et al.* 1982, Meade *et al.* 1984).

Previous studies on some enzymes involved in the intermediary metabolism of *L. m. mexicana* showed that glycolytic enzymes were generally lower in activity in amastigotes than promastigotes whereas the reverse was true for some enzymes involved in the β-oxidation of fatty acids. (Coombs *et al.* 1982). The first part of my study, therefore, was to investigate further the metabolism of the mammalian form of the parasite by comparing the amastigote activities of enzymes involved in central metabolism with those of the promastigote.

The detection of the high PEP carboxykinase activity together with the confirmation of the low pyruvate kinase activity in amastigotes of *L. m. mexicana*, which is the reverse of the situation found in culture promastigotes (Table 7, pg. 82), suggests that CO_2 fixation in the conversion of PEP to oxaloacetic acid is a key reaction in the metabolism of the mammalian form of *L. m. mexicana*. This correlates well with the observation that high CO_2 levels stimulate the transformation of *L. m. mexicana* amastigotes to promastigotes but have no effect on the growth *in vitro* of

promastigotes (Hart and Coombs 1981).

The suggested importance of PEP carboxykinase in CO_2 fixation in the amastigote is reinforced by the apparent absence from this stage of two other CO_2-fixing enzymes, pyruvate carboxylase and malic enzyme (carboxylating) (Table 7). Pyruvate carboxylase has been reported to be present at low levels in culture promastigotes of four species of Leishmania (Chatterjee & Datta 1974, Martin et al. 1976) as well as amastigotes of L. donovani (Meade et al. 1984). It is likely that these apparently contradictory results are due to differences in methodology or possibly the parasite strains. Chatterjee and Datta (1974) detected PEP carboxykinase activity in L. donovani using a radioactive assay method, but they were unable to demonstrate spectrophotometrically oxaloacetate production from PEP. Berens and Marr (1977a) were also unable to detect PEP carboxykinase spectrophotometrically in L. donovani or L. braziliensis cell-free extracts using the method of Bacchi et al. (1970). Both groups, however, were using an assay system with a buffer at pH 7.4 which favours the reverse decarboxylation reaction (Klein et al. 1975). In this study L. m. mexicana, L. m. amazonensis and L. donovani had large PEP carboxykinase activities when assayed with imidazole buffer at pH 6.6. (Table 8, pg. 84). Limitations in the continuous spectrophotometric assay for PEP carboxykinase have been reported in crude extracts of muscle (Duff and Snell 1982) where NADH oxidation, attributed to the PEP carboxykinase assay, was found to be caused by a linked pyruvate kinase and lactate dehydrogenase. Lactate dehydrogenase, however, has not been detected in Leishmania (Martin et al. 1976), therefore this problem should not arise in PEP carboxykinase assays in Leishmania. Berens and Marr (1977a) attributed the small PEP carboxykinase activity detected in

promastigotes of L. donovani by Chatterjee and Datta (1974) to a linked pyruvate kinase and pyruvate carboxylase. Although pyruvate kinase has high activity in L. m. mexicana promastigotes, and may compete for substrates, it is considerably lower than the PEP carboxykinase activity detected in amastigotes. In addition, no pyruvate carboxylase activity was detected in either amastigotes or promastigotes of this strain of L. m. mexicana (Table 7), suggesting that the PEP carboxykinase activity detected is a true representation of the enzyme in this organism.

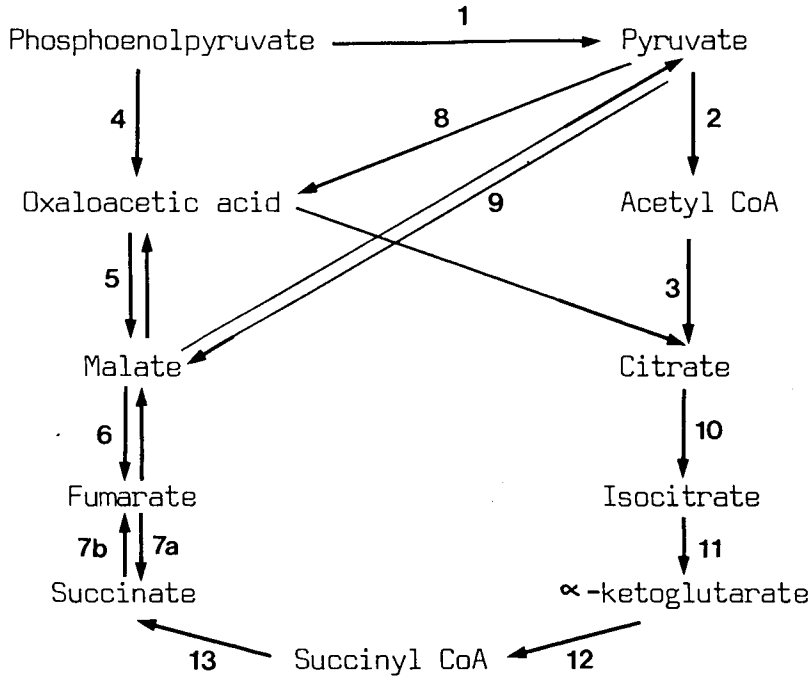
The results for L. donovani (Table 8) suggest that this metabolic switch involving PEP metabolism may be a general feature of the leishmanias, even though the data for L. m. amazonensis does not show marked differences between amastigotes and promastigotes. There was, however, only one set of experimental data for amastigotes of L. m. amazonensis due to the lack of material, therefore the amastigote result cannot be interpreted with confidence. The activity of PEP carboxykinase detected in L. donovani in this study was considerably higher than the activity of pyruvate carboxylase detected by Meade et al. (1984), who did not assay PEP carboxykinase, suggesting that it may be the most important CO_2-fixing enzyme in L. donovani as well as L. m. mexicana.

The high PEP carboxykinase activities detected in the culture procyclic forms of T. b. brucei and T. b. rhodesiense (Table 9, pg. 85) are in good agreement with previous reports on the importance of this CO_2-fixing enzyme in other trypanosomatids (Klein et al. 1975, Cataldi de Flombaum et al. 1977, Cazzulo et al. 1980, Opperdoes and Cottem 1982). It is interesting to note that CO_2 fixation appears to be more important in the mammalian amastigote than the cultured promastigote form of Leishmania, in contrast to the trypanosomes in which PEP carboxykinase is found at high activity only in the insect

procyclic stage of T. b. brucei and the epimastigote form of T. cruzi (Table 9, Cataldi de Flombaum et al. 1977, Opperdoes and Cottem 1982, Cannata et al. 1982). This difference is difficult to explain in terms of our present knowledge of environmental factors, as the Leishmania-infected gut of a sandfly could be expected to be similar to a trypanosome-infected gut of a tsetse fly or kissing bug. The trypanozoon trypanosomes such as T. brucei, however, undergo a much more dramatic metabolic switch between mammalian and insect stages in comparison to the changes that occur in Leishmania (see section 1.4.2.4). It seems likely, therefore, that the various changes in PEP metabolism that occur are related to the ability of each of the trypanosomatids to transform and adapt to their new environment. In addition, there is an assumption that the metabolism of culture forms is a true representation of the parasites which live in the gut of the insect and are not an adaptation to the medium in which they are grown. This is yet to be confirmed.

The possible routes of PEP metabolism in Leishmania are outlined in fig 26. The presence of very high malate dehydrogenase activities in amastigotes of L. m. mexicana (Table 7) suggests that this enzyme may act in conjunction with PEP carboxykinase, fumarase and fumarate reductase to bring about the conversion of phosphoenolpyruvate to succinate, a major end product of amastigote metabolism (Hart & Coombs 1982). In contrast, conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase appears to be the more important catabolic route in culture promastigotes of L. m. mexicana. The reasons for the change in metabolism between the two forms of Leishmania are not known, but it is possible that the amastigote metabolism is an adaptation to an environment containing high CO_2

Fig. 26 Phosphoenolpyruvate catabolism in Leishmania m. mexicana.



Key to enzymes: 1) pyruvate kinase, 2) pyruvate dehydrogenase, 3) citrate synthase, 4) PEP carboxykinase, 5) malate dehydrogenase, 6) fumarase, 7a) fumarate reductase, 7b) succinate dehydrogenase 8) pyruvate carboxylase, 9) malic enzyme, 10) aconitase, 11) isocitrate dehydrogenase, 12) ∝-ketoglutarate dehydrogenase, 13) succinyl CoA synthetase.

concentrations but low O_2 levels. In a number of parasitic helminths including Ascaris lumbricoides and Fasciola hepatica the NADH-linked reduction of fumarate to succinate is coupled to the electron transport chain resulting in the formation of ATP (see Barrett 1981). It is possible that a similar mechanism occurs in Leishmania and other trypanosomatids, although there is at present no evidence to support this theory (Klein et al. 1975). Fumarate reductase was only detected with low activity in amastigotes of L. m. mexicana (Table 7) and no activity was found with promastigotes. CO_2 fixation may play a part in maintaining the CO_2 concentration at an acceptably low level, as has been postulated for some intestinal helminths (See Barrett 1981). In addition, the switch in the metabolism of PEP may be related to the observed differences in substrate utilization by the two forms of L. m. mexicana (Hart and Coombs 1982). Transforming amastigotes utilize fatty acids at 10 times the rate of promastigotes growing in vitro (Hart and Coombs 1982). CO_2 fixation may then provide C4 intermediates, such as oxaloacetic acid, in order to replenish the C4 compounds removed in the production of the end-product succinate from the extra acetyl CoA produced from fatty acid metabolism.

The discovery of the rapid loss of malate dehydrogenase activity in extracts of L. m. mexicana amastigotes and the protective effect of leupeptin on the enzyme (Table 10, pg. 86) confirms that this stage of the parasite contains high proteolytic activity and emphasizes the importance of taking precautions to limit proteolysis when studying amastigote proteins (Pupkis & Coombs 1984). Although other enzymes have been shown to be susceptable to proteolytic degradation in homogenates of L. m. mexicana amastigotes (Hassan and Coombs 1984), malate dehydrogenase appears to be the only enzyme in

this study that was degraded. Malate dehydrogenase activity in L. m. mexicana amastigote homogenates was also found to be unstable even in the presence of 100μg/ml leupeptin, in that 55% of the activity was lost during a 1 hour incubation at 4°C (Table 19, pg. 114). This loss of activity was found to be greatly reduced by the addition of 10% (v/v) ethylene glycol, however, a procedure which was found to be essential for maintaining enzyme activity during the purification procedure (See section 4.4).

The detection of several TCA cycle enzymes including isocitrate dehydrogenase and malate dehydrogenase in both amastigotes and promastigotes of L. m. mexicana, together with previous data on the respiration of L. m. mexicana (Hart et al. 1981c), indicate that the TCA cycle is probably functional in both amastigotes and promastigotes of this species. It has been suggested previously that this is the case with culture promastigotes of several other species (Martin et al. 1976, Mukkada 1977), and also purified L. donovani amastigotes (Meade et al. 1984). Even though ∝-ketoglutarate dehydrogenase could not be detected in this study, it seems likely that this enzyme was present but only at levels below the limit of detection; Martin et al. (1976) found it at very low levels in culture promastigotes of L. m. mexicana and 3 other species of Leishmania.

Under conditions of low oxygen tension the TCA cycle would be inoperative due to a build up of reduced coenzymes that could not be reoxidized by the respiratory chain. In this case, a partial reversal of the TCA cycle could occur with NADH being reoxidised by malate dehydrogenase and fumarate reductase. The extremely low activities in L. m. mexicana of a number of TCA cycle enzymes suggests that the TCA cycle and respiratory chain may not cope even with the flux of reduced coenzyme produced by glycolysis under high

oxygen tensions. Thus, succinate may be produced by either the forward or reverse cycle depending on the efficiency of the TCA cycle and respiratory chain and the level of oxygen available to the organism. Succinate excretion potentially results in the depletion of TCA cycle intermediates and so the cycle being stopped, therefore oxaloacetic acid must be replenished if the TCA cycle is to continue function in the forward direction.

The production of pyruvate, either from pyruvate kinase, the decarboxylation of malate by malic enzyme or transamination of alanine, and the regeneration of oxaloacetic acid through one of the CO_2-fixing enzymes or aspartate-glutamate transaminase is clearly an important area of intermediary metabolism in Leishmania and one which is presumably under rigorous control.

The regulation of glycolysis has been extensively studied in mammalian cells where control is maintained by a complex system that involves substrate availability, levels of enzyme activity, and allosteric control of individual enzymes with activation and feedback inhibition by metabolic intermediates (see Newsholme and Start 1973). Initial studies on Leishmania promastigotes suggests that glycolysis is not regulated in the same way as mammalian systems as several key glycosomal glycolytic enzymes including hexokinase and phosphofructokinase are apparently not tightly regulated (see section 1.4.2.3.1). Subcellular compartmentation of both enzymes and metabolic intermediates, together with the organisation of these enzymes in a multienzyme complex within the glycosome, therefore are likely to be essential elements in the control of glycolysis in trypanosomatids (see section 4.4).

The fate of phosphoenolpyruvate is clearly a key branch point in leishmanial intermediary metabolism and it would be expected that

control of metabolic flux may be mediated by regulation of the first two enzymes past the branchpoint. PEP carboxykinase is thought to be the rate-limiting enzyme in glyconeogenesis in mammalian cells where it catalyses the first step of the pathway, the decarboxylation of oxaloacetic acid to PEP (Newsholme and Start 1973, Hers and Hue 1983). Its intracellular distribution varies between species; in rat and mouse liver it is exclusively cytosolic, in rabbit liver totally mitochondrial, and in man equally distributed between the cytosol and mitochondria (Utter and Kolenbrander 1972, Hers and Hue 1983). There is apparently no modulation of PEP carboxykinase activity in mammals by feedback inhibition, although a protein factor, that has been isolated from rat liver, is required for optimal activation of the enzyme by low concentrations of ferrous ions (Bentle et al. 1976). There is more long-term control of the enzyme, however, at the level of enzyme synthesis, where the level of PEP carboxykinase mRNA is regulated by hormonal and environmental stimuli including insulin and glucagon (Cimbala et al. 1981). In Leishmania no activators or inhibitors of PEP carboxykinase in crude homogenates of L. m. mexicana amastigotes were found, but it seems likely that CO_2 concentration is an important regulatory factor. The role of PEP carboxykinase in Leishmania clearly differs from that in mammalian cells; it is possible that the ways in which it is regulated are also distinct.

It has been reported that various species of Leishmania possess isocitrate lyase and malate synthase, two of the enzymes involved in the glyoxylate cycle, and also the intermediate glyoxylate (Mukkada 1977, Simon et al. 1978). The glyoxylate cycle has been found in the microbodies of higher plants (Beevers 1979), in bacteria and fungi (Kornberg 1966, Spector 1972) and also in the protozoan Tetrahymena (Hogg and Kornberg 1963), where it is important in providing

oxaloacetic acid for gluconeogenesis from 2 carbon units such as acetyl coenzyme A. Isocitrate lyase could not be detected in either the procyclic or bloodstream forms of T. brucei (Opperdoes et al. 1977b, Opperdoes and Cottem 1982). The functional significance of a glyoxylate cycle in Leishmania is not clearly understood, and although the enzymes fructose-1,6-bisphosphatase and glucose-6-phosphatase have been detected in both forms of L. m. mexicana at approximately the same activity (Coombs et al. 1982), it is not clear if gluconeogenesis occurs in vivo in Leishmania. The presence of a glyoxylate cycle, however, could correlate with the reported use of fatty acids as an energy substrate by the amastigote (Hart and Coombs 1982). My inability, however, to detect isocitrate lyase (Table 7), a key glyoxylate cycle enzyme, in L. m. mexicana suggests that, at least in this species of Leishmania, the cycle plays little part.

Other large differences between enzyme activities in amastigotes and promastigotes of L. m. mexicana were found with glutamate dehydrogenase and malic enzyme (decarboxylating). The latter enzyme is present in promastigotes and possibly functions in anapleurotic reactions, possibly as a source of NADPH and pyruvate, whereas it appears to have little role in amastigotes. The high promastigote, NAD-specific glutamate dehydrogenase activity suggests that the catabolism of amino acids may be of importance to this form of the parasite. Previous investigations with L. m. mexicana gave no evidence of the use of any amino-acids as a major energy substrate (Hart and Coombs 1982), however the enzyme may be responsible, at least in part, for the high rate of ammonia excretion from the parasite (Coombs and Sanderson, 1984). It has been postulated that this excretion may play a part in protecting the parasite against the antimicrobial activity of the host macrophages (see section 1.1.3.2).

The presence of both NAD- and NADP-specific glutamate dehydrogenase activities in the amastigote but the absence of a NADP-specific activity in promastigotes suggests that two distinct enzymes are present in the amastigote. This is similar to T. cruzi, which contains two enzymes with different specificities and kinetic properties (Juan et al. 1978), and in contrast to mammalian cells, where the enzyme is coenzyme unspecific (Frieden 1963).

The detection of high activities of an enzyme is not proof of an active metabolic pathway and confirmation that the pathways discussed operate in vivo in Leishmania await further studies on the fate of intermediate substrates in each developemental form.

4.2 STUDIES ON THE MODE OF ACTION OF ANTIMONIALS AND ARSENICALS.

The lack of activity of Pentostam against Leishmania growing in vitro is well established (Mattock and Peters 1975, Berman and Wyler 1980, Coombs et al. 1983) and for this reason Triostam, the trivalent analogue of Pentostam, and melarsen oxide, a trivalent arsenical were included in this study. Both of these drugs were found to be active against culture promastigotes (Fig. 6, pg. 91). The reason for the differences in susceptability of L. m. mexicana promastigotes to the pentavalent and trivalent antimonial drugs is not known although there may be some differences in uptake or metabolism (possibly to an active trivalent form) of Pentostam. The intracellular amastigote, however, is sensitive to Pentostam in vitro when incubated in monocyte-derived macrophages with the drug (Berman and Wyler 1980) as are amastigotes transforming in vitro (Coombs et al. 1983). In an attempt to explain both the antileishmanial activity of antimonials and arsenicals and the specificity of Pentostam to the amastigote, the relative sensitivities of L. m.

mexicana amastigote and promastigote enzymes and mammalian isofunctional enzymes to inhibition by these drugs were investigated. The antileishmanial activity of antimonials could not, however, be correlated with any enzyme inhibitory activity (Tables 12 and 13, pgs. 92,93). Neither Pentostam nor Triostam inhibited any of the five leishmanial enzymes tested (hexokinase, phosphofructokinase, pyruvate kinase, malate dehydrogenase and PEP carboxykinase) suggesting that these antileishmanial drugs may have a target other than glycolysis, a conclusion suggested previously because of the failure of Pentostam to interfere with the catabolism of glucose to CO_2 (Hart and Coombs 1982). This appears to contrast with the situation found with Schistosoma mansoni where Triostam, but not Pentostam, markedly inhibited schistosomal phosphofructokinase (Table 12). This confirms the results of Mansour and Bueding (1954) using the trivalent antimonial Stibophen (sodium antimony bis (pyrocatechol-2,4-disulphonate)) who suggested that phosphofructokinase was the primary site of action of antischistosomal antimonials. Thus antimonials may act in different ways against Leishmania and schistosomes; alternatively the drugs may have a similar, as yet undiscovered, target in both groups of parasites, a target unrelated to the glycolytic kinases. The relatively long time taken for Triostam to exert its leishmanicidal effects against promastigotes (immotile parasites were not observed until 8 hrs after the addition of the drug) suggests that anabolic reactions such as DNA replication cannot be excluded from being a primary target. The antagonistic effect of cysteine on the action of Triostam (Table 14, pg. 95) may have been due simply to a direct interaction with the drug, but possibly the effect was mediated through cysteine protecting thiol groups in the parasites that are primary targets for

the drug. Thus, it is feasible but still to be confirmed that thiol groups are involved as primary targets of antiparasitic antimonials.

The finding that Triostam had no effect on either mouse liver or rabbit muscle phosphofructokinase confirms that there are differences in susceptability of the mammalian and schistosomal enzymes to the drugs that may be the basis of the selective toxicity towards schistosomes. In addition the pentavalent antimonial 1714 was found to inhibit mouse liver, but not leishmanial, phosphofructokinase and leishmanial, but not mouse liver, hexokinase (Tables 12 and 13). This suggests that the structure of the organic moiety of the antimonial drugs is important in determining their effects against these glycolytic enzymes.

Stibophen also has been found to inhibit T. rhodesiense phosphofructokinase (Jaffe et al. 1971), although the drug had an I_{50} of 0.5mM, five times that against the schistosomal enzyme. In contrast to the trivalent arsenical oxophenarsine, however, this action was not nullified by the addition of mercaptoethanol, suggesting that its action on this enzyme was not dependant on binding thiol groups.

Arsenical drugs, such as melarsen, Mel B and tryparsamide, have been used in the treatment of sleeping sickness in man, even though they have a low chemotherapeutic index (the ratio of toxic to curative dose), as they are one of the few groups of drugs available which can cross the cerebrospinal membrane and so control the later stages of the disease. The pentavalent arsenicals are believed to be reduced to the trivalent forms in vivo before the drug is active (Crawford 1947), therefore both melarsen and melarsen oxide were included in this study. The antitrypanosomal action of these drugs has been studied to some extent. When whole cells of T. brucei were

exposed to concentrations of melarsen oxide that have little effect on glucose utilization or respiration, phosphoenolpyruvate was shown to accumulate within the cells (Flynn and Bowman 1974). It was suggested therefore that the mode of action of melarsen oxide against bloodstream T. brucei was through inhibition of pyruvate kinase. A number of other enzymes, including glycerol kinase, glycerophosphate oxidase, pyruvate and ∝-ketoglutarate decarboxylases , however, were shown to be more sensitive than pyruvate kinase to inhibition by the drug (Flynn and Bowman 1974, Bowman and Flynn 1976, Fairlamb 1982). It is presumed that the membranes of the glycosome and mitochondrion present a permeability barrier to the drug that leaves pyruvate kinase, a cytosolic enzyme, the most susceptable target.

In this study, melarsen was found to have no effect on L. m. mexicana promastigotes growing in culture. Melarsen oxide, however, was found to be more toxic to L. m. mexicana culture promastigotes than Triostam (MLCs: 100µg/ml and 400µg/ml, respectively), although at these concentrations the antimonial had a more rapid effect than the arsenical. Melarsen oxide was found to be an inhibitor of all the enzymes tested except mouse liver hexokinase and L. m. mexicana amastigote PEP carboxykinase. Phosphofructokinase, malate dehydrogenase and pyruvate kinase from Leishmania were about equally sensitive (I_{50}s, 4-8µM), although mouse liver pyruvate kinase (I_{50} 165µM) and rabbit muscle phosphofructokinase (I_{50} 160µM) were less sensitive to the drug. Mel B was also inhibitory, but higher concentrations were required than for melarsen oxide. The different sensitivities of the host and leishmanial enzymes, especially pyruvate kinase, to melarsen oxide suggests that, if pyruvate kinase was the primary target, the drug could have some specificity for the parasite in leishmaniasis. The results also agree with those found

previously with T. brucei (Flynn and Bowman 1974) and although phosphofructokinase, malate dehydrogenase and pyruvate kinase from L. m. mexicana appeared to be equally susceptable to inhibition by melarsen oxide, pyruvate kinase is probably the only leishmanial enzyme tested that is not contained within an organelle and so is likely to be more susceptable to inhibition by the drug. The high sensitivity of some mammalian enzymes to melarsen oxide and the fact that glycolytic enzymes are cytosolic in mammalian cells, probably accounts for the high toxicity of this drug to man.

One of the characteristics of arsenic is its ability to form metallo-thiol bonds with sulphydryl groups, thus accounting for the inhibition of various sulphydryl enzymes by arsenical drugs (Peters et al. 1946, Flynn and Bowman 1974). For this reason, glutathione metabolism in the rodent filaria Litosomoides carinii has been investigated as the possible target for arsenical drugs that affect this organism (Bhargava et al. 1983). Arsenicals were found to readily inhibit glutathione reductase from L. carinii but had no effect on the mammalian host enzyme, and administration of Mel B to filaria infected gerbils caused decreases in filarial, but not host, glutathione reductase and reduced glutathione levels. Glutathione reductase could not, however, be detected in amastigote or promastigote homogenates of Leishmania m. mexicana with either NADPH or NADH as the reduced cofactor (Table 7, pg. 82). Recently, a preliminary report showed that glutathione reductase in the trypanosomatids T. brucei and C. fasciculata required a soluble cofactor for activity that was heat stable with an apparent molecular weight of 400 (Fairlamb and Cerami 1983). This cofactor could not be replaced by FAD, FMN, riboflavin or various pterins. It is possible that this cofactor is a general feature of trypanosomatids and may therefore be present in Leishmania species as well. A possible explanation of why

this enzyme was not detected in this study is that the cofactor was either denatured under the lysis conditions used or that it was too dilute in the assay mixture to have its effect. A more detailed investigation is therefore required to elucidate if glutathione metabolism is a target for arsenical and possibly antimonial drugs in *Leishmania*.

4.3 INVESTIGATIONS INTO THE SUBCELLULAR ORGANISATION OF *LEISHMANIA*

The glycosomes of *T. brucei* and *T. cruzi* have been studied in some detail (see section 1.4.3.1) and the enzyme contents of the organelles investigated with respect to the changes in metabolism that occur during the life-cycle of these trypanosomatids (see Opperdoes 1982, 1984b, Cannata *et al.* 1982, Hart *et al.* 1984). Previously there has been little work on the glycosomes of *Leishmania*, although the presence of the organelles has been suggested from simple fractionation studies (Coombs *et al.* 1982), the recovery of hexokinase and two pyrimidine biosynthetic enzymes on sucrose gradients (Hammond *et al.* 1981) and electron microscope studies. In order to confirm the presence of glycosomes in *Leishmania* and to establish how they differ in the two main forms of the parasite and the parts they play in their metabolism, the subcellular organisation of the culture promastigote of *L. m. mexicana* was investigated and compared to that of the amastigote. The results obtained from differential centrifugation (Table 15, pg. 97) and isopycnic centrifugation on linear sucrose gradients (Figs. 7 and 8, pgs. 100,101) confirm the existence of glycosomes in *Leishmania* promastigotes.

Fractionation studies with amastigotes using the same techniques of cell breakage as used for promastigotes, that is mixing the

parasites to a thick paste with de-fined alumina, were unsuccessful, apparently due to lysis of the organelles. The presence of "Megasomes", organelles found only in the amastigote, together with the presence of high amastigote proteinase activity (Coombs 1982), suggested that lysosomal type enzymes may be degrading the organelles and releasing their contents before fractionation could take place. The inclusion of various proteinase inhibitors (leupeptin and antipain) and phospholipase inhibitors (mepacrine and chelating agents) in the breakage buffers during amastigote lysis and the use of different breakage techniques were all without success. Putative amastigote glycosomal enzymes such as hexokinase and glucose phosphate isomerase exhibited no latency after alumina lysis, and hexokinase, a particulate enzyme (Table 11, pg. 88) which was recovered mainly in pellet 2 (P2) during promastigote differential centrifugation fractionation (Table 15), was spread throughout the 3 pellets after centrifugation (Table 16, pg. 99). If these "Megasomes" are indeed involved in the failure of the amastigote fractionation technique, then the use of Leishmania species that do not have such developed "Megasomes", for example L. donovani (Coombs and Pupkis 1984), may be more successful.

In this study as an alternative approach, L. m. mexicana amastigotes were treated with digitonin to lyse the parasite membranes and release soluble enzymes at different rates. Digitonin has been used to separate particulate and soluble components of rat liver cells (Zuurendonk and Tager 1974) and also to investigate metabolite compartmentation in isolated hepatocytes (Brocks et al. 1980); both based on the fact that the cholesterol content of plasma membranes is in general higher than that of intracellular membranes (Colbeau et al. 1971) and that digitonin specifically binds β-

hydroxysterols. Digitonin has also been used to investigate the activation of glycolytic enzymes in the bloodstream forms of T. brucei (Visser and Opperdoes 1980). The trypanosome plasma membrane was completely disrupted at 0.05mg digitonin/mg protein, as judged by the release of the cytosolic enzyme alanine aminotransferase, whereas the glycosomal membrane required considerably higher digitonin concentrations to fully activate the glycosomal enzyme glycerol-3-phosphate dehydrogenase (1-3mg digitonin/mg protein) (Visser and Opperdoes 1980). Digitonin was reported to be less useful as a method of cell disruption for T. brucei in fractionation studies (Oduro et al. 1980b). This was confirmed for L. m. mexicana promastigotes in this study, where the high concentrations of digitonin (400µg/mg protein) required for cell lysis of the majority of the cells was found to abolish all hexokinase latency, presumably an indication of damaged organelles. Digitonin was found, however, to be effective in enzyme release studies, as treatment of L. m. mexicana amastigotes with the detergent distinguished 3 subcellular compartments (Fig. 9 and 10, pgs. 103,104) and gives the first biochemical evidence to support the ultrastructural evidence for the presence of glycosomes in amastigotes (Tetley et al. 1983). The results presented support the suggestion that glycosomes may be present in all trypanosomatids (Taylor et al. 1980, Opperdoes 1982, Opperdoes 1984b).

The finding that the glycolytic enzymes hexokinase, glucose phosphate isomerase, phosphofructokinase, and glyceraldehyde-3-phosphate dehydrogenase of L. m. mexicana promastigotes banded at a density of between 1.215-1.220g/ml on sucrose density gradients (Fig. 7, pg. 100) indicates that promastigote glycosomes are of a lower density than the equivalent organelles in bloodstream and procyclic forms of T. brucei, which were reported to have densities of 1.250

and 1.228g/ml respectively (Opperdoes and Borst 1977, Opperdoes et al. 1981), and C. fasciculata (density 1.26g/ml, Cannata et al. 1982). The glycosomes of T. cruzi culture epimastigotes, however, were found to have a lower density (1.20g/ml, Cannata et al. 1982).

There was found to be a fairly disperse distribution of glycosomal enzymes on sucrose gradients in this study (Fig. 7) in comparison to the distribution reported for bloodstream and procyclic T. brucei (Opperdoes and Borst 1977, Opperdoes et al. 1981) and also T. cruzi (Cannata et al. 1982). Analysis of purified glycosomes of bloodstream T. brucei showed that the organelles were extremely homogeneous in size. The overall recovery for glycosome purification was 28% and it is possible that the purification technique could have selected a specific type of microbody, even though isolated glycosomes were similar in size to those analysed in situ (Opperdoes et al. 1984). When enzymes distributions were compared on sucrose gradients, however, T. brucei glycosomes certainly appear to be more homogeneous than those from L. m. mexicana promastigotes.

The mitochondrial marker oligomycin-sensitive ATPase, which is a particulate enzyme, banded at a density of 1.190g/ml (Fig. 8) and gave a sharp peak in comparison to the soluble putative mitochondrial enzymes, glutamate dehydrogenase, alanine aminotransferase and malic enzyme, all of which showed only small peaks at 1.190g/ml and a more disperse distribution. Presumably much of the soluble enzyme activities were lost from the matrix of the mitochondrion upon its breakage into vesicles.

The relatively low density of the glycosomes of L. m. mexicana promastigotes meant that they banded close to the peak of the mitochondrial vesicles on the sucrose gradient. Separation of these two organelles was achieved, however, by using a shallow sucrose

gradient (ρ = 1.15-1.24) and this showed that the main location of the enzymes PEP carboxykinase, hexokinase, phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase was in the glycosome, whereas malate dehydrogenase, glutamate dehydrogenase, malic enzyme and alanine aminotransferase had mitochondrial activity.

The finding of glyceraldehyde-3-phosphate dehydrogenase in the promastigote glycosome fractions suggests that the initial enzymes of glycolysis, up to and including glyceraldehyde-3-phosphate dehydrogenase, are located in the organelle. With phosphoglycerate kinase mostly, and pyruvate kinase totally, cytosolic it is likely that the last few steps of glycolysis occur within the cytosol. The recovery of approximately 50% of the glucose phosphate isomerase activity in the soluble fractions with both differential centrifugation and sucrose density gradients was probably due to enzyme leakage from damaged glycosomes, although the possibility that some of the early glycolytic enzymes are present in the cytosol of the parasite cannot be excluded.

Glucose-6-phosphate dehydrogenase was found to be a mainly cytosolic enzyme, but there was a small but significant activity associated with the glycosome (Fig. 7). Pyruvate kinase was not detected on the gradient, however, which indicates that the apparent glycosomal activity of glucose-6-phosphate dehydrogenase is unlikely to be caused by non-specific trapping in the organelle fractions. Glycosomal glucose-6-phosphate dehydrogenase could be important in obtaining glucose-6-phosphate produced by hexokinase within the glycosome for the pentose phosphate pathway, whereas the cytosolic enzyme may be involved in using the substrate produced from other sources, or possibly glycosomal glucose-6-phosphate that leaves the organelle. The release profile of glucose-6-phosphate dehydrogenase from amastigotes and promastigotes treated with increasing digitonin

concentrations (Fig. 9 and 11, pgs. 103,105) differ from those for glycosomal glucose phosphate isomerase, suggesting that the cytosol is the main location for glucose-6-phosphate dehydrogenase in both stages of the parasite. A small amount of glycosomal activity could not be detected with confidence using this method, although the slightly higher digitonin concentration required for the total release from promastigotes of glucose-6-phosphate dehydrogenase in comparison to pyruvate kinase (Fig. 11) was presumably a reflection of the presence of some enzyme in glycosomes. Amastigote pyruvate kinase activity is so low that such comparisons were not possible in the amastigote digitonin experiments.

Leishmania m. mexicana culture promastigotes have only low PEP carboxykinase and malate dehydrogenase activities in comparison to amastigotes, (Table 7). Promastigote PEP carboxykinase showed a glycosomal distribution on sucrose density gradients (Fig. 7), whereas malate dehydrogenase peaked at the density of the mitochondrial marker enzyme, oligomycin-sensitive ATPase (Fig. 8). A small amount of glycosomal malate dehydrogenase activity, however, cannot be discounted. This apparent difference in the main location of these two enzymes in promastigotes of L. m. mexicana is interesting as it contrasts with the situation found in T. brucei culture procyclic trypomastigotes, where both enzymes are mainly glycosomal (Opperdoes et al. 1981, Opperdoes and Cottem 1982), and T. cruzi culture epimastigotes (Cannata and Cazzulo 1984), where there is a dual mitochondrial and glycosomal distribution of malate dehydrogenase. Neither PEP carboxykinase nor malate dehydrogenase are detectable in the glycosomes of the bloodstream forms of T. brucei but are produced on transformation to the procyclic form and this is thought to be one of the adaptations of the parasite to life in the

midgut of the insect vector (Opperdoes et al. 1981, Opperdoes and Cottem 1982). There appear to be similar major differences in malate dehydrogenase with L. m. mexicana. The isoenzyme pattern for malate dehydrogenase shows marked differences between amastigotes and promastigotes of a L. m. mexicana clone. The 3 major bands in amastigote homogenates were also found in the pellet and to a much lesser extent in amastigote supernatant (bands c,d,e, in fig. 12, pg. 108). The latter supernatant activities were presumably particulate enzymes that were not removed in the 5min, 10,000g centrifugation step. There was only one major band (c) in the promastigote pellet fraction with also a minor band, presumably a soluble enzyme contaminant. The results suggest that the large increase in activity observed upon transformation of L. m. mexicana promastigotes to amastigotes is mainly due to the production of two particulate isoenzymes not found in promastigotes, enzymes possibly associated with the glycosome.

One of the explanations suggested for the changes that occur to glycosomes of T. brucei upon transformation between bloodstream and procyclic trypomastigotes was the maintenance of energy and redox balance (Opperdoes et al. 1981, Opperdoes and Cottem 1982). It was found with purified glycosomes from T. brucei procyclics that externally provided ATP was as efficient as PEP for promoting glycolysis (The ATP produced by PEP carboxykinase used in the initial steps of glycolysis), infering that the glycosome must be permeable to ATP (Broman et al. 1983). NAD^+ was found to stimulate glycerol-3-phosphate and 3-phosphoglycerate formation by isolated glycosomes so this cofactor may also be permeable to the glycosomal membrane (Broman et al. 1983), as has been suggested previously for NADH in bloodstream T. brucei glycosomes (Oduro et al. 1980a). The degree of damage to the glycosomes during the purification procedure is

difficult to assess and the permeability of these cofactors may be an artifact produced with damaged organelles. Nevertheless, the fact that glycosomal enzymes show high degrees of latency indicates that there is some substrate compartmentation involved within these organelles. It has been suggested, however, that the permeability barrier in T. brucei glycosomes is not absolute and a slow equilibration of glycosomal and cytosolic intermediates occur (Visser and Opperdoes 1980, Visser et al. 1981, Broman et al. 1983) (See section 1.4.3.1 of the introduction). If the same was true for Leishmania glycolytic intermediates and nucleotides, there may be no requirement to balance energy and redox levels within the glycosome as a distinct entity from the cytosolic pool. Anyway, the association with leishmanial glycosomes of several enzymes not involved in glycolysis, including enzymes involved in fatty-acid oxidation and plasmalogen biosynthesis (Hart and Opperdoes 1983), pyrimidine biosynthesis (Hammond et al. 1981) and purine salvage (Hassan and Coombs 1984), makes it impossible, as yet, to fully elucidate how energy and redox balance would be maintained even if these organelles did represent a self-contained energetic unit within the cell.

The results obtained from this study, together with enzyme distribution data from other workers (Coombs et al. 1982, Hammond and Gutteridge 1982, Hart and Opperdoes 1983, Hassan and Coombs 1984), has enabled the construction of theoretical schemes that represent the apparent relationship between the glycosome, mitochondrion and cytosol in Leishmania promastigotes and amastigotes (Figs. 27 and 28).

It is likely that in promastigotes glucose is catabolised within the glycosome as far as 3-phosphoglyceric acid (3-PGA) which crosses the glycosomal membrane into the cytosol (Fig 27). Cytosolic 3-PGA is

Fig. 27

Schematic representation of the compartmentation of metabolism in Leishmania promastigotes.

Major pathways are indicated with thick arrows (➡);

Minor pathways are indicated with thin arrows (→);

Movement of intermediates across membranes (major routes) (● ● ●);

Movement of intermediates across membranes (minor routes) (•••••••).

The pathways enclosed within a double line in the glycosome represent enzymes that are possibly associated with the outside of the glycosomal membrane.

To avoid confusion not all cytosolic or mitochondrial enzymes have been included in the scheme.

Key to the abbreviations: G-6-P, glucose-6-phosphate; F-6-P fructose-6-phosphate; 6-PG, 6-phosphogluconate; FDP, fructose-1,6-diphosphate; DHAP, dihydroxyacetone phosphate; 1,3-DPGA, 1,3-diphosphoglyceric acid; 3-PGA, 3-phosphoglyceric acid; G-3-P, glycerol-3-phosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetic acid; NEFA, nonesterified fatty acids; Hypoxanth, hypoxanthine; PRPP, phosphoribosylpyrophosphate; 2-PGA, 2-phosphoglyceric acid; Ala, alanine; Resp chain, respiratory chain.

Fig. 28

Schematic representation of the compartmentation of metabolism in Leishmania amastigotes.

Key as described in fig. 27.

Fig. 27

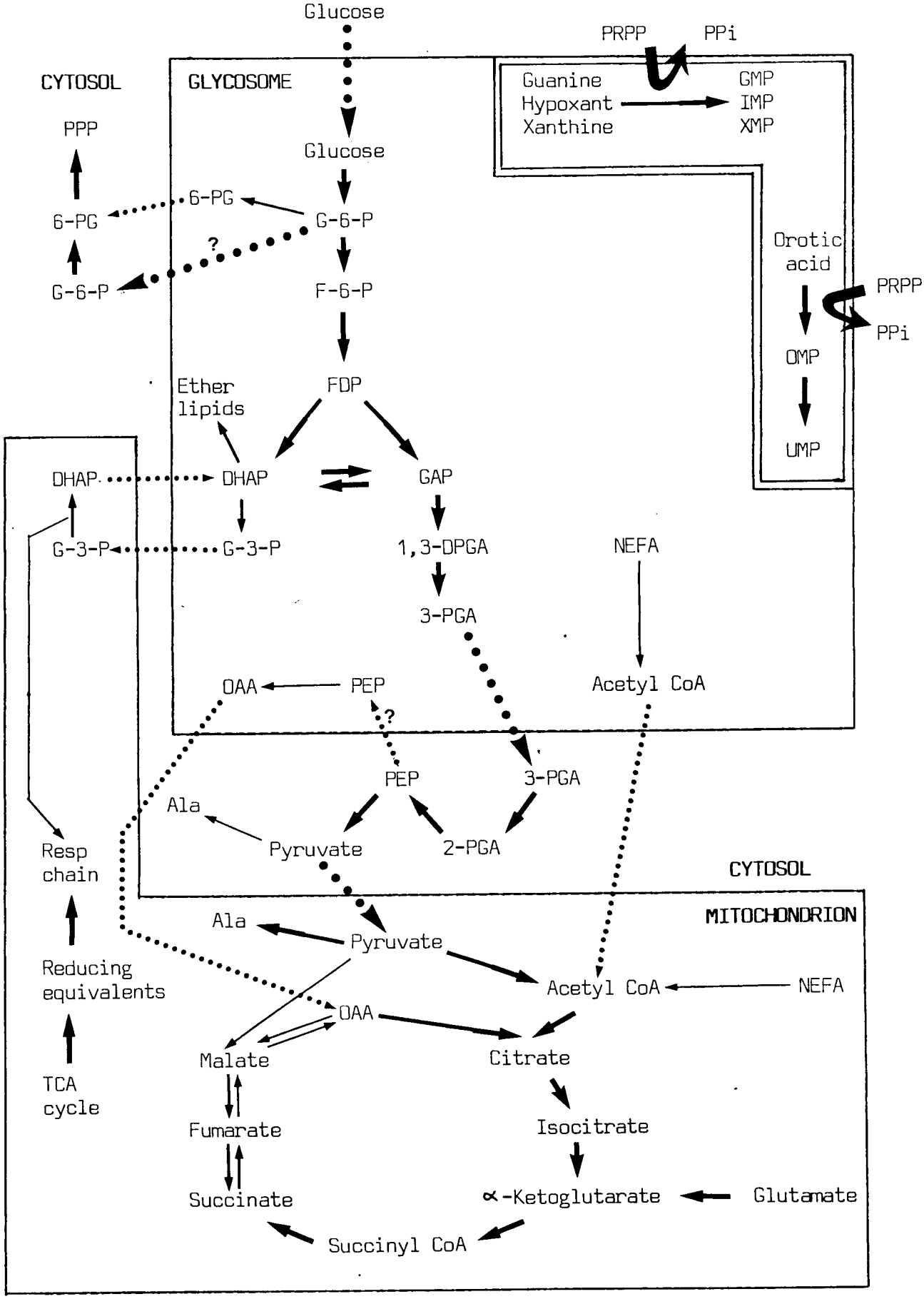
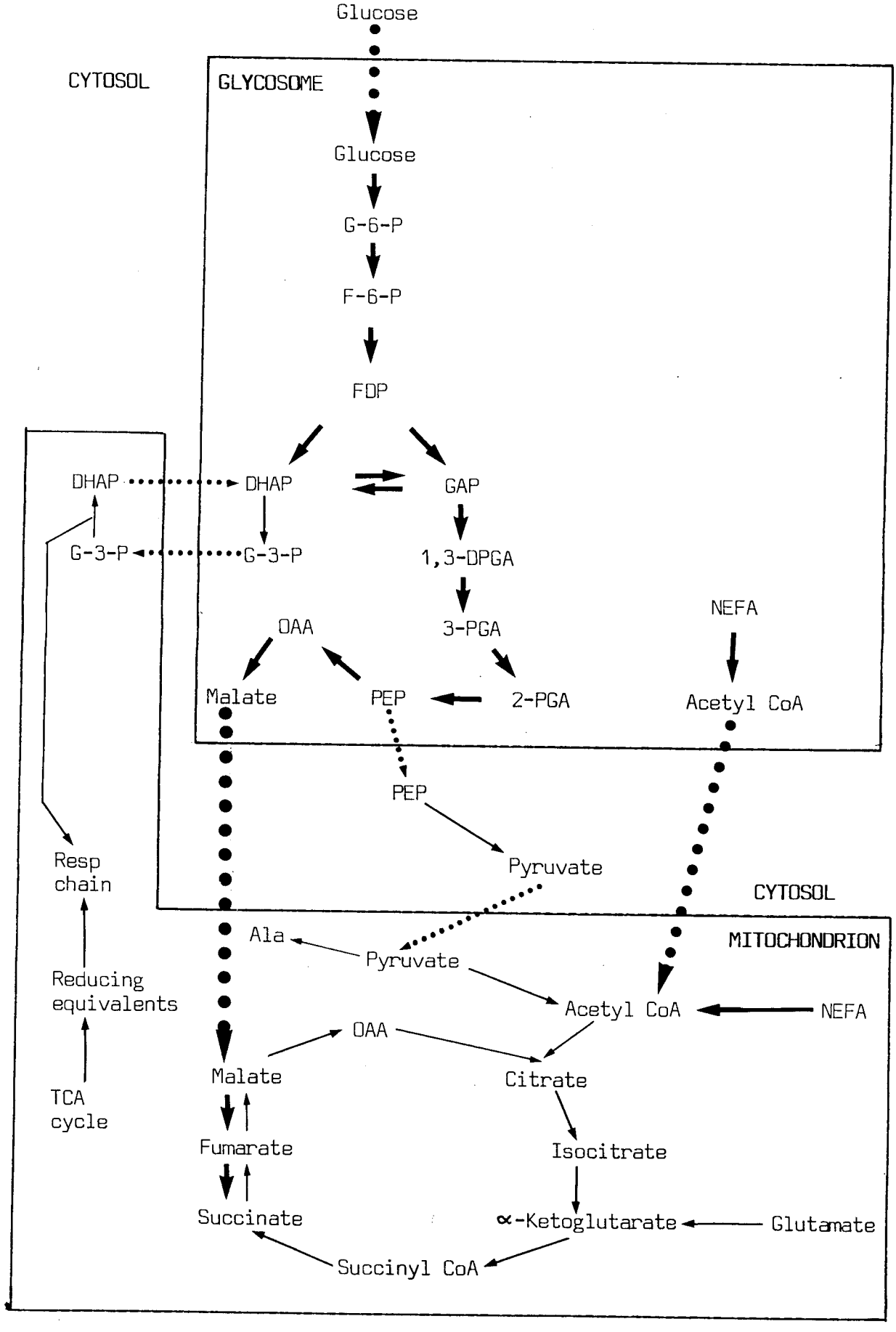

Glucose
PRPP
PPi
CYTOSOL
GLYCOSOME
Guanine
Hypoxant
Xanthine
GMP
IMP
XMP
PPP
Glucose
6-PG
6-PG
G-6-P
?
G-6-P
F-6-P
Orotic acid
PRPP
PPi
OMP
UMP
Ether lipids
FDP
DHAP
DHAP
GAP
G-3-P
G-3-P
1,3-DPGA
NEFA
3-PGA
OAA
PEP
?
Acetyl CoA
PEP
3-PGA
Ala
Resp chain
Pyruvate
2-PGA
CYTOSOL
MITOCHONDRION
Ala
Pyruvate
Reducing equivalents
Acetyl CoA
NEFA
OAA
Malate
Citrate
TCA cycle
Fumarate
Isocitrate
Succinate
∝-Ketoglutarate
Glutamate
Succinyl CoA

Fig. 28


Glucose
CYTOSOL
GLYCOSOME
Glucose
G-6-P
F-6-P
FDP
DHAP
DHAP
GAP
G-3-P
G-3-P
1,3-DPGA
NEFA
OAA
3-PGA
Malate
PEP
2-PGA
Acetyl CoA
PEP
Resp chain
Pyruvate
CYTOSOL
MITOCHONDRION
Ala
Pyruvate
Reducing equivalents
Acetyl CoA
NEFA
OAA
Malate
Citrate
TCA cycle
Fumarate
Isocitrate
Succinate
∝-Ketoglutarate
Glutamate
Succinyl CoA

then metabolised to PEP and further converted to pyruvate by pyruvate kinase. A small amount of PEP may cross back into the glycosome where it is converted to oxaloacetic acid by PEP carboxykinase. The low activity of PEP carboxykinase suggests that it has only a minor role in promastigote catabolism, being involved only in the metabolism of any PEP produced in, or which permeates into, the glycosome. It is possible that the small glycosomal activity of phosphoglycerate kinase may serve to regenerate some ATP within the glycosome for maintaining energy balance, and indeed PEP carboxykinase may also serve the same function. Pyruvate produced in the cytosol may either cross into the mitochondrion and enter the tricarboxylic acid cycle or be converted to alanine by transamination. The acetyl CoA produced by β-oxidation of fatty acids within the glycosome could also cross into the mitochondrion and enter the TCA cycle (Fig. 27).

The high PEP carboxykinase and malate dehydrogenase activities produced upon transformation of L. m. mexicana promastigotes to amastigotes are balanced by a concomitant reduction in the level of pyruvate kinase activity (Table 7, pg. 82). In addition, it appears from the digitonin release experiments (Fig. 9 and 10, pgs. 103,104) that there is considerable phosphoglycerate kinase activity within the amastigote glycosome. This contrasts with promastigotes with which it was found, albeit using a different technique, that less than 5% of promastigote phosphoglycerate kinase activity was recovered in the glycosomal fractions on sucrose gradients (Fig. 7, pg.100). A possible interpretation of these results is that glucose catabolism proceeds entirely within the amastigote glycosome as far as PEP, which is then carboxylated to oxaloacetic acid by PEP carboxykinase and reduced to malate by malate dehydrogenase within the glycosome (Fig. 28). As amastigotes of L. m. mexicana utilize

fatty acids at 10 times the rate of promastigotes (Hart and Coombs 1982), the β-oxidation of fatty acids has been suggested as a major pathway of amastigote catabolism. The intracellular location of the enzymes involved in β-oxidation, pyrimidine biosynthesis and purine salvage in amastigotes, however, has not been investigated, although the presence of these pathways in promastigote glycosomes implies that they may also be present in the glycosomes of amastigotes. It is possible therefore that the greatly elevated amastigote malate dehydrogenase and PEP carboxykinase activities (Table 7) may serve to reoxidize the additional glycosomal NADH produced by β-oxidation of fatty acids (Fig. 28). Some of the glycolytically produced NADH could also be reoxidized by glycerol-3-phosphate dehydrogenase; the glycerol-3-phosphate produced being oxidized by the respiratory chain in the mitochondrion. The ATP required for the first step of B-oxidation could be provided by glycosomal PEP carboxykinase. Unfortunately as many of these enzyme activities are largely particulate in the amastigote (Table 11, pg. 88), the location of the enzymes cannot be determined by the digitonin enzyme release experiments. Nevertheless, it is clear that the changes in the enzyme contributions of the glycosome that occur upon transformation from insect to mammalian stage are quite different for L. m. mexicana and T. brucei.

Glycolytic enzymes are found in the cytosol of eukaryotic and prokaryotic cells and it is generally assumed that the macromolecular components are freely dissolved within this cellular environment. The possible existence of a glycolytic complex with its inherent metabolic advantages, however, has been suggested in a number of systems (Green et al. 1965, Reed and Cox 1966, Mowbray and Mosses 1976). Studies on glycolytic components in skeletal muscle extracts

has shown the existence of a complex that is dependent on high protein concentrations, pH and ionic strength; conditions that have possible physiological significances (Clarke and Masters 1974). A multienzyme aggregate of all the enzymes of glycolysis was also detected in Escherichia coli (Mowbray and Moses 1976), and in the glycosomes of bloodstream form T. brucei (Oduro et al. 1980a). The clear advantage of such an arrangement is that high local concentrations of intermediates would be created in the microenvironment of the enzyme complex thus allowing an extremely high rate of glycolysis to take place. The particulate nature of a number of glycolytic enzymes within the glycosomes of Leishmania (Table 11, pg. 88) and other trypanosomatids suggests that the multienzyme complex may be a general feature of the trypanosomatids.

The presence of a multienzyme complex within the glycosome, the lack of allosteric control of glycosomal glycolytic enzymes, and the possible permeability barrier to metabolic intermediates produced outside the organelle, suggests that the control of glycolysis is very different in Leishmania than mammalian cells. It is likely that a certain level of control is maintained through the regulation of the uptake of substrates, e.g. glucose into the glycosomes, and also the transport of glycosomal end products such as 3-phosphoglycerate across the membrane.

The results presented suggest that the energy metabolism of L. m. mexicana amastigotes and promastigotes differ to some degree, however the full extent of the changes that occur in the enzyme content of leishmanial glycosomes during transformation and their functional significance are yet to be determined. Apparently the changes are less dramatic than those reported for T. brucei, but this correlates well with the findings of this and previous studies showing similarities in energy metabolism of the two forms of

Leishmania (Coombs et al. 1982, Hart and Coombs 1982). The detection of an enzyme activity in a parasite does not imply that it is involved in the metabolism of that form; it may simply remain from, or be a preadaptation to, another stage. This could be the situation with L. m. mexicana and confirmation of the pathways operating in vivo awaits further studies on the fate of substrates in each form.

4.4 PURIFICATION OF PARTICULATE PEP CARBOXYKINASE AND MALATE DEHYDROGENASE FROM LEISHMANIA M. MEXICANA AMASTIGOTES.

The particulate nature of malate dehydrogenase and PEP carboxykinase, their substrate and cofactor specificities and the location in glycosomes of the latter, demonstrates that there are significant differences between the leishmanial and host enzymes and similarities between PEP carboxykinase from Leishmania and other trypansomatids (Bacchi et al. 1970, Klein et al. 1975, Cataldi de Flombaum et al. 1977). These characteristics of the enzymes, together with their high activity in leishmanial amastigotes, makes them potential chemotherapeutic targets. For this reason, and the fact that PEP appears to be a key branch point in amastigote metabolism, particulate PEP carboxykinase and malate dehydrogenase were purified with the aim of characterising the enzymes in more detail. The described procedure also allowed the purification of particulate hexokinase, and it is probable that with a few modifications other particulate leishmanial enzymes also could be isolated.

In mammalian cells PEP carboxykinase is a soluble enzyme that is found in either the cytosol or mitochondrion (or both), depending on the species (Utter and Kolenbrander 1972). In trypanosomatids,

however, a particulate PEP carboxykinase has been demonstrated in glycosomes of C. fasciculata and T. cruzi epimastigotes (Cannata et al. 1982) as well as procyclic T. brucei (Opperdoes and Cottem 1982, Broman et al. 1983).

Most higher eukaryotic cells contain two malate dehydrogenase isoenzymes (Banaszak and Bradshaw 1975), the cytoplasmic form (s-MDH) and the mitochondrial form (m-MDH), which are involved in the malate shuttle and the TCA cycle. Some plants and free-living protozoa are known to have a third malate dehydrogenase isoenzyme, which is located in glyoxysome microbodies (mb-malate dehydrogenase) and is involved in the glyoxylate cycle (Rocha and Ting 1971, Walk and Hock 1976), whereas prokaryotes appear to have only one isoenzyme (Murphey et al. 1967a). A fourth cellular location for malate dehydrogenase has been found recently in the glycosomes of T. brucei and T. cruzi (Opperdoes et al. 1981, Cannata and Cazzulo 1984). Evidence from the fractionation studies (section 3.4) suggest that L. m. mexicana promastigotes possess s-MDH, m-MDH and possibly also a glycosomal enzyme. The amastigote total malate dehydrogenase activity is much higher than that of the promastigote (Table 7, pg. 82), largely due to an increase in particulate activity (Table 11, 88) which may possibly be associated with the glycosome. It is this isoenzyme that was purified in this study.

The m-MDH from pig heart has been shown not only to have soluble matrix activity but also a particulate activity strongly associated with the inner membrane of the mitochondrion that could be solubilised with high ionic strength (3M KCl) (Compte and Gautheron 1978). Particulate glycosomal enzymes, but not including malate dehydrogenase or PEP carboxykinase, of the bloodstream forms of T. brucei have been released from the membrane with high ionic

strength (Misset and Opperdoes 1983). The finding in this study that particulate malate dehydrogenase, PEP carboxykinase and hexokinase also were released from L. m. mexicana amastigote membranes with 500mM NaCl (Fig. 13a, pg. 110) suggests that the procedure may have broad applications in the purification of particulate glycosomal enzymes. In both this study on Leishmania and that on T. brucei (Misset and Opperdoes 1983), a Phenyl-Sepharose Cl-4B column was found to be a useful initial step in the purification of these highly hydrophobic glycosomal proteins. The presence of 10% ethylene glycol in all the buffers during the purification procedure had the disadvantage of slowing down flow rates from the columns, however it was found to be essential for maintaining malate dehydrogenase activity.

Metal chelate affinity chromatography (MCAC) was attempted in the purification procedure in an effort to separate malate dehydrogenase and PEP carboxykinase. This technique is based on the differing ability of sample proteins to bind heavy metal ions. The binding can be reversed by reducing the pH and increasing the ionic strength or using chelating agents such as EDTA which bind the metal ions. The technique has been used to fractionate serum proteins on Zn^{2+} and Cu^{2+} columns linked in tandem (Porath et al. 1975) and also to purify the enzyme nucleosidediphosphatase from rat liver on a single Cu^{2+} column (Ohkubo et al. 1980). PEP carboxykinase from L. m. mexicana amastigotes is manganese dependent and its binding to a Mn^{2+} loaded gel might be expected to achieve a good purification. Only a small percentage of the total protein was retained on the column, however, and all PEP carboxykinase and malate dehydrogenase activity was eluted with the initial loading buffer (Fig. 15b, pg. 117). Mn^{2+} has been reported to have a lower binding capacity to the

chelating Sepharose gel than Zn^{2+} (Porath et al. 1975), therefore a Zn^{2+} loaded column was attempted. Although this separated the sample into three protein peaks under different elution conditions (Fig. 15a) the procedure was not suitable for purification of these enzymes as both were inhibited by zinc ions.

The lack of success with MCAC prompted the use of a different affinity chromatography technique; 5' AMP-Sepharose which binds NAD^+-dependent dehydrogenases and ATP-dependent kinases. Cytosolic, mitochondrial and glyoxysomal isoenzymes of malate dehydrogenase from water melon cotyledons have been separated previously using affinity chromatography on a 5' AMP-Sepharose column, with the two particulate isoenzymes m-MDH and mb-MDH being bound whilst s-MDH was not (Walk and Hock 1976). m-MDH and s-MDH of Schistosoma mansoni have also been successfully separated and purified using 5' AMP-Sepharose (Rotmans 1978), s-MDH not binding whilst the bound m-MDH was eluted with a 0-200mM gradient of KCl or low NADH concentrations. Amastigote malate dehydrogenase showed two peaks of activity using 5' AMP-Sepharose with 7% of the activity eluted immediately in the presence of 200mM NaCl with the remaining 93% of bound activity eluted with 1M NaCl (Fig. 16, pg. 118). It is unclear whether the 2 peaks of malate dehydrogenase activity eluted from 5' AMP-Sepharose were due to the presence of different isoenzymes, possibly from the mitochondrial and glycosomal compartments, or whether there was a small contaminating cytosolic activity remaining from the initial centrifugation step. The latter suggestion, however, seems unlikely as the pellet (P1) from the first step of the purification was washed well. Leishmania m. mexicana malate dehydrogenase is unusual as it binds to the 5' AMP-Sepharose even in the presence of 200mM NaCl, a concentration of salt that usually dissociates bound enzymes from the column (Walk and Hock 1976, Rotmans 1978). This binding may have been

due to a hydrophobic interaction with the 6-carbon spacer arm which couples the 5' AMP ligand to the Sepharose gel, rather than the 5' AMP ligand binding to a substrate site. Rotmans (1978) found that the S. mansoni m-MDH which bound to the column was inhibited 75% by 20mM 5' AMP, whereas the non-binding s-MDH was unaffected. Neither of the Leishmania amastigote malate dehydrogenase enzymes eluted from the 5' AMP-Sepharose column, however, showed any inhibition by 3.5mM 5'AMP, the highest concentration tested.

The malate dehydrogenase which bound to the 5' AMP-Sepharose column, and was subsequently eluted with 1M NaCl, was shown to be homogeneous on SDS polyacrylamide gels (Fig. 17, pg. 119). Isoelectric focusing, however, showed one major and two minor malate dehydrogenase isoenzymes, the former of which banded mid-way between 2 of the major isoenzymes in amastigote homogenates (Fig. 12, pg. 108). The purified enzyme may have been modified in some way during the purification procedure so that it behaves differently during isoelectric focusing and it is unclear if the 2 minor bands are due to malate dehydrogenase isoenzymes which have co-purified or again one enzyme that has undergone some procedural modification.

The malate dehydrogenase isoenzymes m-MDH and s-MDH from a wide range of organisms have been found to have similar MWts of around 67,000, being dimeric with sub-units ranging from 32-35,000 (Murphey et al. 1967b, Banaszak and Bradshaw 1975) . The enzyme has either a dimeric or tetrameric structure, MWts 67,000 and 117,000, in prokaryotes (Murphey et al. 1967a), whilst both mitochondrial and glycosomal malate dehydrogenase from T.cruzi have been reported to have MWts of around 60,000 (Cannata and Cazzulo 1984). Purified particulate malate dehydrogenase from L. m. mexicana amastigotes appeared to have a MWt of 33,300 as determined by SDS-gel

electrophoresis (Fig. 17, pg. 119), whereas the enzyme that was not bound to the 5' AMP-Sepharose column was found to have a MWt of 39,800 from gel filtration (Fig. 18, pg. 120). In addition, malate dehydrogenase of L. m. mexicana amastigote crude homogenates containing all isoenzymes, including solubilized particulate malate dehydrogenase, eluted as a single peak (MWt 36,100) from Sephadex G100 (Fig. 19, pg. 122), similar to the MWt found for the purified particulate enzymes. These results suggest that, suprisingly, malate dehydrogenase isoenzymes occur as single polypeptides in L. m. mexicana. It is possible, however, that the conditions under which the Sephadex column was run in order to maintain full enzyme activity (0.12M triethanolomine buffer pH 7.5, 10% (v/v) ethylene glycol, 1M NaCl and 1mM DTT) caused the dissociation of the enzyme into sub-units which nevertheless retained their full catalytic potential.

The molecular weights of 65,000 and 63,100 determined for L.m mexicana amastigote PEP carboxykinase by the two methods used agree well with the MWts of 66,000 - 75,000 for PEP carboxykinase isolated from vertebrate sources (Utter and Kolenbrander 1972) and from parasitic helminths (Cornish et al. 1981, Wilkes et al. 1982). The lack of material did not allow any further characterisation of PEP carboxykinase in this study, however the technique allows the purification of the enzyme in sufficient quantities for investigations in the future.

There have been studies on the enzyme kinetics of malate dehydrogenase from a wide range of sources including human erythrocytes, ox heart, Neurospora crassa (Munkres and Richards 1965), Eschericia coli (Murphey et al. 1967a, S. mansoni (Rotmans 1978) as well as the parasitic protozoa Trichomonas gallinae (Dowda and Betterton 1974), Trypanosoma cruzi (Juan et al. 1976, Cannata and

Cazzulo 1984) and T. brucei (Falk et al. 1980). The K_m oxaloacetic acid of 39μM and the K_m NADH of 41μM for L. m. mexicana amastigote particulate malate dehydrogenase fall within the range of Michaelis constants found with these other organisms. Similarly the inhibition of L. m. mexicana malate dehydrogenase by oxaloacetate is a feature found with many malate dehydrogenase isoenzymes (Duproque and Kun 1969, Rotmans 1978). The concentration of oxaloacetate required to produce inhibition, however, was higher with the amastigote enzyme than for many m-MDH isoenzymes, with which the reaction procedes in the malate oxidation direction. The finding in this study that the activity of malate dehydrogenase in the direction of malate oxidation proceded optimally at high pH (8.5) has also been reported for other organisms (Munkres and Richards 1965). The high activity of oxaloacetate reduction at neutral pH, together with the observed high K_m values for malate and NAD^+ (3.6mM and 0.79mM, respectively) in comparison with many other organisms (Murphey et al. 1967b, Banasak and Bradshaw 1975) again suggests that the major physiological activity of L. m. mexicana amastigote particulate malate dehydrogenase activity is the reduction of oxaloacetic acid. The very high malate concentration required to inhibit oxaloacetic acid reduction suggests that such inhibition is not relevant to the in vivo situation, and the lack of any apparent regulation by glycolytic intermediates and nucleotides implies that the enzyme is not tightly regulated by allosteric control.

The precise role of malate dehydrogenase in L. m. mexicana amastigotes is still unclear, but its necessity is suggested by the extremely high malate dehydrogenase activity in this form of the parasite. Such an enzyme is a potential target for chemotherapeutic attack, the results presented here, however, show that the

particulate amastigote enzyme is similar in many respects to mammalian enzymes. Nevertheless, its apparent association with PEP carboxykinase and the glycosome, together with its activity as a single polypeptide chain, demonstrate that there are distinct differences from the mammalian enzymes. The methods developed in this study will allow a more detailed characterisation of purified amastigote enzymes which should clarify these differences further and help to elucidate the functional significance of these enzymes to the mammalian stage of this important human parasite.

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