A study of dihydrolipoamide dehydrogenase in the Trypanosomatidae

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A STUDY OF DIHYDROLIPOAMIDE DEHYDROGENASE IN THE TRYPANOSOMATIDAE

submitted by Helen Linda Long
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Dedicated to my father whose memory still inspires and to my mother and my brother for their invaluable support .

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ABSTRACT

Dihydrolipoamide dehydrogenase (DHlipDH) has been shown to be present in isolated plasma membrane fractions from mitochondrially inactive bloodstream *T.brucei* (Danson et al, 1987) and has been shown, by subcellular fractionation, to have a unique plasma membrane location in these mitochondrially inactive cells (Jackman et al, 1990).

Two synthetic peptide epitopes (DLD1 and DLD2) were chosen from an antigen prediction profile of bloodstream *T.brucei* DHlipDH. Both peptides were successfully synthesised by the F-moc solid phase method and used to raise polyclonal antisera. ELISA analysis confirmed that anti-DLD1 and anti-DLD2 were peptide specific. Anti-DLD1 antisera was shown to cross-react with procyclic *T. brucei* sonicate on western blot, forming bands in the MW range of 47-65 kD. This suggested some cross-reactivity of DLD1 antiserum with denatured *T.brucei* DHlipDH. Immunoprecipitation experiments showed some cross-reactivity of DLD1 antiserum with native *T. brucei* DHlipDH, although reactivity appeared weak. Immunofluorescence studies confirmed weak cross-reactivity of anti-DLD1 with native DHlipDH antigen, indicating that the raised antiserum was unsuitable for use in immunocytochemical localisation studies.

DHlipDH was assayed and partially characterised in sonicates of *Crithidia fasciculata* and *Phytomonas sp.* The discovery of DHlipDH in mitochondrially inactive *Phytomonas sp.*, in the absence of mitochondrial multienzyme complexes with which it is normally associated, was unexpected and an investigation of a possible plasma membrane location was undertaken by cell fractionation. Assays of potential enzyme organelle markers for use in subcellular fractionation studies were performed and their suitability discussed. Plasma membrane isolates were prepared from *Crithidia fasciculata* and *Phytomonas sp.* and fraction purity gauged by morphological examination under the electron microscope. A two fold increase in DHlipDH specific activity over homogenate was observed in an isolated plasma membrane fraction from *Crithidia fasciculata* , while a five-fold increase was observed in *Phytomonas sp.*

Preliminary evidence for an extramitochondrial DHlipDH in *Phytomonas sp.* is presented and the implications of a possible plasma membrane location discussed.

ABBREVIATIONS

2OGDHC	2-Oxoglutarate dehydrogenase complex
ABC	Avidin: Biotinylated enzyme complex
AMPS	Ammonium persulphate
ADP	Adenosine - 5'-diphosphate
ATP	Adenosine-5'-triphosphate
ATPase	Adenosine triphosphatase
AV-17	Acid Violet - 17
BCKDHC	Branched-chain 2-0x0 acid dehydrogenase complex
BSA	Bovine serum albumin
CNS	Central nervous system
DCCI	Dicyclohexylcarbodiimide
DDH_2O	Double distilled water
DHAP	Dihydroxyacetone phosphate
DHlip	Dihydrolipoamide
DHlipDH	Dihydrolipoamide dehydrogenase
DIEA	Diisopropylcarboiimide
DLD1	Synthetic peptide (1) derived from bloodstream *T.brucei* DHlipDH primary sequence
DLD2	Synthetic peptide (2) derived from bloodstream *T.brucei* DHlipDH primary sequence
DHlipDH	Dihydrolipoamide dehydrogenase
DMAP	4-dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulphoxide
DOC	deoxycholate
DMSO	Dimethylsulphoxide
DNA	Deoxyribonuclease
DTNB	5,5-dithio-bis-(2-nitobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
FAD	Flavin adenine dinucleotide
Fru(2,6)P2	Fructose 2,6-bisphosphate
GCS	Glycine Cleavage System
GSH	Glutathione
G-3-P	Glycerol-3-phosphate
G-6-P	Glucose-6-phosphate
HOBT	Hydroxybenzotriazole hydrate (HOBT)
HPLC	High performance liquid chromatography
ICDH	Isocitrate dehydrogenase
KLH	Keyhole limpet haemocyanin
MBS	Maleimidobenzoic acid Succinamide ester
mRNA	Messenger RNA
LS	Long slender
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)

OsO_4	Osmium tetroxide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDC	Pyruvate dehydrogenase complex
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
PK	Pyruvate kinase
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHAM	Salicylhydroxamic acid
SS	Short stumpy
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
TEA	Triethylamine
Tes	N-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid
TEMED	N,N,N', N-Tetramethylethylenediamine
TMB	Tetramethyl benzidine
Tris	Tris (hydroxymethyl)aminomethane
VSG	Variable surface glycoprotein

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CHAPTER 1

INTRODUCTION

1.1 The kinetoplastida

Organisms belonging to the order kinetoplastda are flagellated single-celled eukaryotic protozoa. Kinetoplastids are the most primitive eukaryotic group to contain mitochondria (Sogin, 1991). These cells are characterised by the possession of a unique organelle called the kinetoplast, a dense structure at the base of the flagellum that contains the DNA of the organism's single mitochondrion. Other features of kinetoplastids include a surface membrane which has been adapted in various ingenious ways to repel host defences, a cytoskeleton reinforcing the body surface, and a flagellar apparatus with associated flagellar pocket that represents an important site of traffic into and out of the parasite.

1.2. The Trypanosomatidae family

1.2.1 Introduction

The Trypanosomatidae family belongs to the order Kinetoplastida and consists of seven common genera of monoflagellates parasitic in invertebrates (leeches and arthropods) and vertebrates. The genera of the family Trypanosomatidae show a variety of developmental stages which are based mainly on the position of the kinetoplast relative to the nucleus, the degree of flagellar development and the position at which the flagellum emerges from the body (Hoare and Wallace, 1966) (Fig. 1). The following six distinct body types, each representing a genus, are recognised:

1. **The amastigote** (representative of the genus *Leishmania*) - a small rounded or oval body without a flagellum

2. **The promastigote** (representative of the genus *Leptomonas*)- characterised by the slender, spindle-shaped body with a free flagellum. The kinetoplast is near the

anterior end of the body. There is no undulating membrane The genus *Leptomonas* is morphologically indistinguishable from *Phytomonas* living in plants.

3. **The choanomastigote** (representative of the genus *Crithidia*) - small, flagellated body with broad end, a truncated anterior and a rounded posterior. The flagellum emerges from the funnel-shaped reservoir. There is no undulating membrane. The kinetoplast is anterior and lateral to the nucleus.The choanomastigote does not appear in the life cycle of any other genus..

4. **The opisthomastigote** (representative of the genus *Herpetomonas*) - characterised by the kinetoplastid being posterior to the nucleus and the axoneme runs through almost the entire length of the body. There is no undulating membrane.

5. **The epimastigote** (representative of the genus *Blastocrithidia*) - the kinetoplast is positioned near the anterior margin of the nucleus. A short undulating membrane is present.

6. **The trypomastigote** (representative of the genus *Trypanosoma*) - the kinetoplast is near the posterior end of the body and an undulating membrane runs the full length of the parasite. There may or may not be a free flagellum. Only in the genus *Trypanosoma* does a trypomastigote appear.

These stages are typical of the mature stage of the genus listed with it. The life cycles of these genera may include several of the morphological stages.

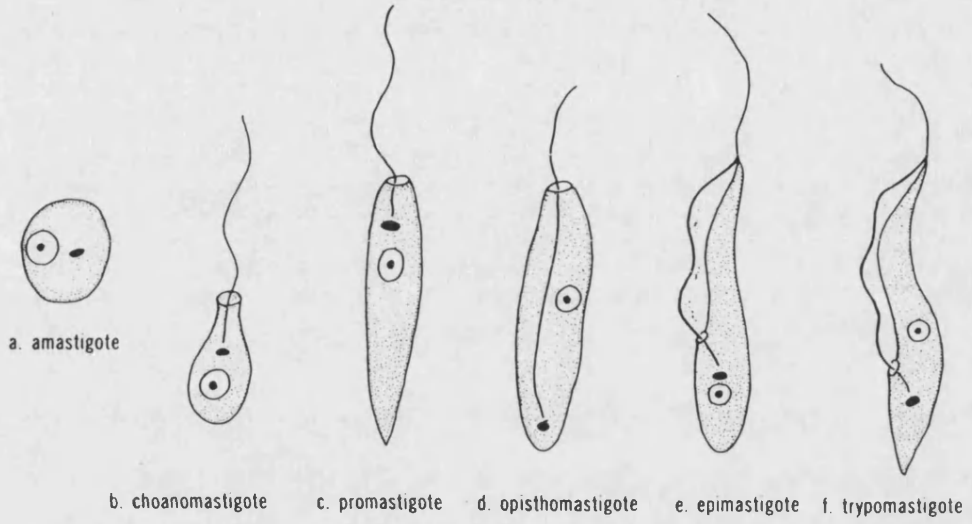


Figure 1: Developmental stages in the life-cycles of trypanosomatid flagellates (Modified from Hoare and Wallace, 1966).

1.2.2 Classification

The taxonomy of the Trypanosomatidae family is based on a combination of the morphological features and the life cycles of the genera (McGhee, 1968). There are six types of bodies (Fig. 1) and two basic types of life cycles.

The monogenetic cycle is completed in a single invertebrate host with the final parasite form being different and characteristic of the genus. Monogenetic Trypanosomatids include *Leptomonas, Herpetomonas, Crithidia,* and *Blastocrithidia.* In the digenetic cycle, there are two hosts, one an invertebrate and the other either a plant or a vertebrate. The genus *Phytomonas* completes its cycle in milkweed bugs and milkweed plants. Genera occurring in vertebrates include *Trypanosoma,* requiring leeches or bloodsucking insects as the invertebrate hosts and vertebrates of various kinds as the alternate host.

1.2.3 Phylogeny of the trypanosomatid flagellates

The family Trypanosomatidae is a diverse group of monogenetic and digenetic taxa (Vickerman, 1976; Molyneaux, 1983). Speculation on phylogenetic relationships within the Trypanosomatidae are largely centred around the ancestry of the digenetic parasites of mammals; the trypanosomes and leishmanias. Until recently, the history of

the kinetoplastids has been reconstructed from comparative studies on morphology, life cycles and host distribution. Novel techniques of molecular sequencing - especially of small subunit ribosomal RNA (SSU rRNA) genes - have now provided a means of assessing genetic distances between organisms.

Early speculation regarding the phylogeny of the trypanosomes was essentially divided between two very different view points. The first, favoured by Hoare (1972) saw the monogenetic trypanosomatids of non-bloodsucking insects as the progenitors of the digenetic parasites now transmitted by haematophagous insects. The transition to haematophagy allowed the insects to infect vertebrates, and the parasites to adapt to life in the bloodstream or to the intracellular habitat. The second theory, however, states that digenetic parasites originated from free living invaders and secondarily gave rise to monogenetic parasites (Wallace, 1966). Hence, the original trypanosomatids were parasites of vertebrates; later they invaded the blood and were transmitted by blood-sucking invertebrates.

The introduction of molecular sequencing techniques into phylogenetic studies has revealed some interesting patterns of phylogenetic descent. Comparisons of mitochondrial (kinetoplast) rRNAs (Vickerman, 1976) produced a tree that broadly confirmed Hoare's view and suggested that the genera *Crithidia*, *Leptomonas*, *Leishmania* and *Trypanosoma* diverged successively, with the digenetic life-style originating once before the separation of *Leishmania.* The principle problem with this study, however, was lack of use of an outgroup to root the tree and so the tree's direction of evolution was in doubt.

By using the free-living kinetolplastid *Bodo caudatus* as an outgroup in a comparison of both small and large ribosomal RNA gene sequences of several trypanosomatids, Fernandez et al (1993) constructed a very different tree in which *T.brucei* represented the most ancient lineage, then *T.cruzi.* The *Blastocrithidia* and *Phytomonas* lineage's branched off next after the trypanosomes, with *Crithidia* and *Leptomonas* some time later. *Leishmania* and *Endotrypanum* separated most recently of all. This appeared to indicate that the unrooted tree of Lake et al was infact upside down. Maslov et al (1994) published a very similar tree to Fernandez et al (1993) based on aligned 18S nuclear ribosomal RNA sequences and using *Euglena gracilis* as

an outgroup to root the tree. Evidence supporting this revised tree comes from a variety of other molecular comparisons (Fernandez et al, 1993), but in particular from comparative studies on RNA editing and on surface structure in different trypanosomatids. This was confirmed by Maslov et al (1996) who constructed a phylogenetic tree using maximum likelihood, maximum parsimony and evolutionary parsimony tree in which *T. brucei* and *T. cruzi* diverge before the lineage leading to *Phytomonas*, and then *Crithidia* and *Leishmania* (Fig. 2). The tree was rooted using rRNA sequences from two species from the suborder *Bodonina*. All methods showed that the mammalian parasite, *T.brucei*, constitutes the earliest divergent branch while the remaining trypanosomes formed a monophyletic group.

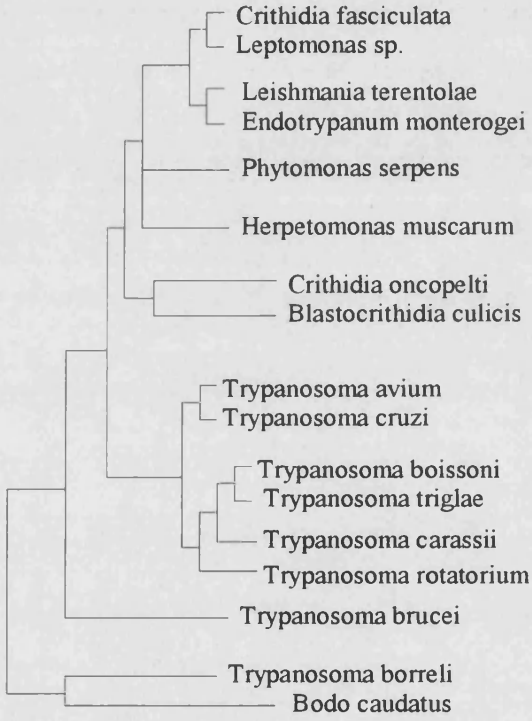


Figure 2: Majority consensus (50%) parsimony tree of Kinetoplastid species constructed from the SSU rRNA dataset (Maslov et al, 1996).

However, a recent report by Lukes et al (1997) presents evidence for the monophyly of trypanosomes. This is contrary to previous results in which trypanosomes were found to be paraphyletic with *T.brucei* representing the earliest diverging lineage. This work implies that the adaptation to invertebrate vectors plays a more important role in the trypanosome evolution than the adaptation to vertebrate hosts. Hence, the exact nature of the phylogenetic relationship among members of the family Trypanosomatidae is at present clearly open to contention.

1.2.4 Pathogenicity of Trypanosomatid flagellates

Digenetic members of the trypanosomatid family are being intensively studied as they are causative agents of serious and widespread diseases in man and animals. The most important trypanosomatids from the point of view of public health are 1) the leishmanias , causing several forms of leishmaniasis, some of which can be fatal (the visceral leishmaniasis or kala azar) and grossly disfigure (the mucocutaneous leishmaniasis or espundia) or be relatively mild, localised and in some cases self-healing (some forms of cutaneous leishmaniasis); 2) the African trypanosomes, which cause sleeping sickness in humans *(Trypanosoma brucei gambiense, T.b. rhodesiense*) and diseases of cattle (nagana: *T.b.brucei, T. congolense, T.vivax*) or of horses and camels (surra: *T. evansi*) that are of considerable economic importance ; 3) *Trypanosoma cruzi*, the agent of the American trypanosomiasis Chagas disease, which is endemic in most of Latin America. There are also trypanosomatid parasites of plants (the genus *Phytomonas,* which has plant juice-sucking insects as vectors) and trypanosomatids that infect only insects, such as those belonging to the genuses *Leptomonas* and *Crithidia.* In 1909, Lafont reported a trypanosomatid in the latex of *Euphorbia pilulifera* , calling it *Leptomonas davidi.*(Lafont, A., 1909). These observations were confirmed in the same year by Donovan (1909). Donovan proposed the creation of a new genus *Phytomonas* in the family Trypanosomatidae in order to differentiate plant from animal trypanosomatids.

1.2.4.1 An animal -infecting trypanosome : Pathogenicity of African trypanosomes (*T.brucei*)

African trypanosmes are digenetic parasites living alternately in the bloodstream and tissues of their host and the gut of their tsetse fly (*Glossina spp.*) vector. Epimastigotes, procyclic trypomastigotes, and metacyclic trypomastigotes are present in the tsetse-fly whereas long, slender and short, stumpy trypomastigotes are present in the bloodstream of the mammalian host (Fig.3). The long slender forms are replicative, as are the procyclic trypomastigotes and the epimastigotes present in the vector.

The genus Trypanosoma is split into two divisions (Stercoraria and Salivaria) that differ primarily in their course of development in their vectors. The first division, Stercoraria, consists of species in which development is completed in the terminal gut and where transmission is by faeces from the vector e.g. *T. cruzi*. The other division, Salivaria, is composed of species in which development is completed in the anterior part of the vector's digestive tract and where transmission is via vector saliva. The African trypanosomes belong to this division and are called salivarian trypanosomes.

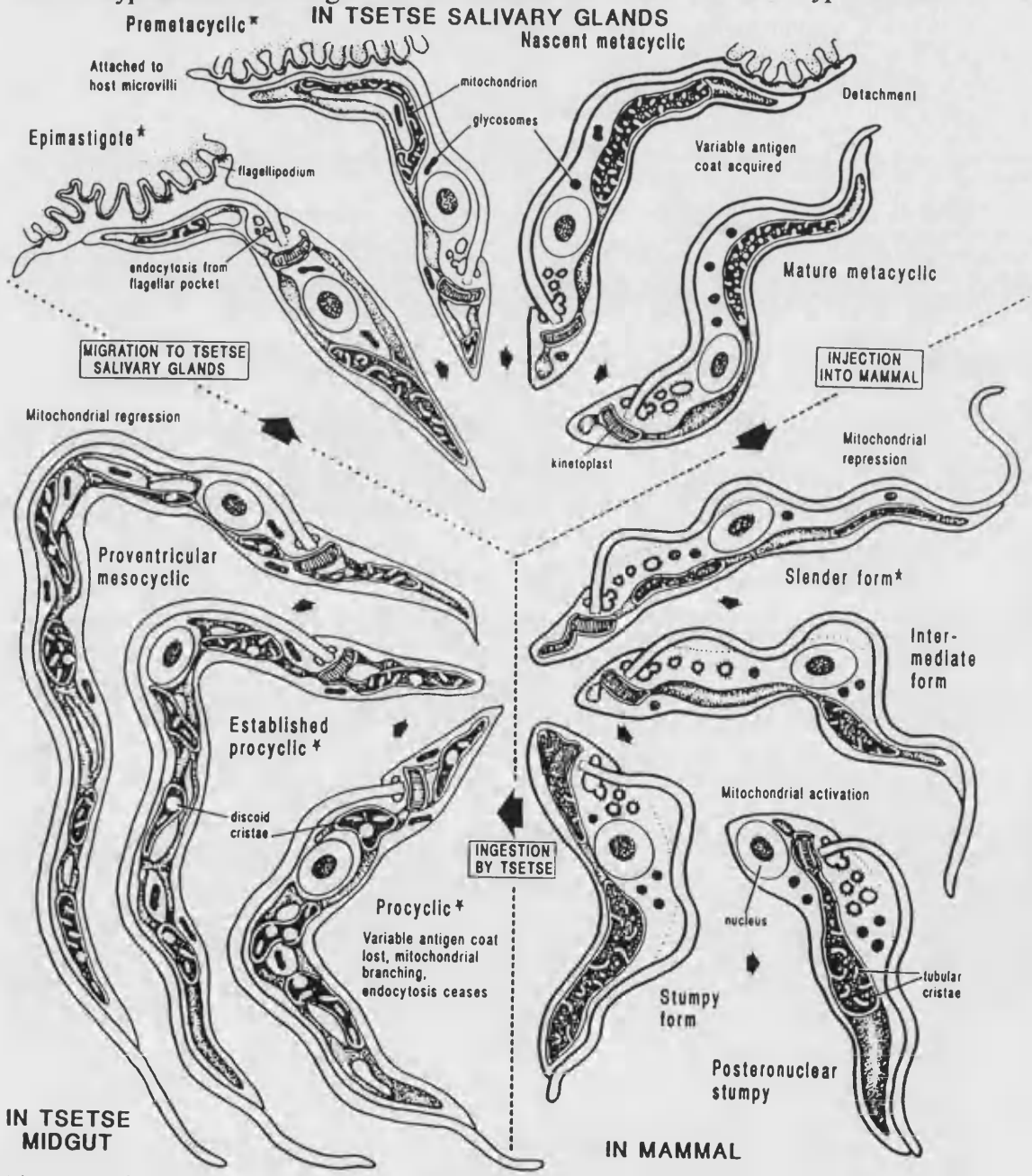


Figure 3: *T.brucei* - a schematic representation of developmental cycle in a mammal and in the tsetse fly vector. Stages with the variable antigen coat are shown on the right, uncoated forms on the left. The tubular mitochondrion is depicted partly in section to show changes in its cristae. The posteronuclear stumpy form is included as an example of a form produced in some stocks, but plays no essential part in the cycle. Division occurs in asterisked stages. (From 'Introduction to animal parasitology' by JD Smyth, adapted from Vickerman, 1985)

1.2.4.1.1 African trypanosomiasis

Although the number of human deaths occurring annually because of African sleeping sickness is relatively low, the African trypanosome is considered by the World Health Organisation to be one of the most important parasites affecting the people of the Third World (Trigg, 1979). The reasons for this are that the disease caused by this parasite is usually chronic and debilitating in humans and significantly decreases the availability of cattle for food. Up until 1979 there were some 10,000 new cases recorded each year (Barry and Emery, 1984). The disease has been on the increase in the last decade with more than 20,000 new cases reported each year, although the true incidence is likely to be much higher because of inadequate reporting. Recent estimates suggest that 50 million people are exposed to the risk of infection in Africa.

1.2.4.1.2 Human trypanosomiasis

There are two forms of human trypanosomiasis, West African or Gambian sleeping sickness, and East African or Rhodesian sleeping sickness (Greenwood , 1982). The trypanosomes causing these diseases are both subspecies of *T. brucei* ; *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. *T. b. gambiense* is distributed mostly in West and Central Africa, while *T. b. rhodesiense* is found mostly in Southern and East Africa. The parasites are indistinguishable morphologically. The infections are distinguished from each other by their locale and clinical course. If untreated, human sleeping sickness is inevitably fatal because of the invasion and destruction of the central nervous system. The duration of the illness ranges from weeks to months in *T. rhodesiense* infections and from months to years with *T. gambiense*.

The disease progresses through three stages: the initial stage in which the parasite is localised to the site of the tsetse fly bite ; the early or systemic stage in which the trypanosomes are widely distributed by the bloodstream throughout the body ; and the advanced or neurological stage in which the parasite invades the central nervous system.

1.2.4.1.3 Animal Trypanosomiasis

Closely related to human trypanosomiasis is nagana, trypanosomiasis in livestock. While the human disease is important because of the threat of epidemic outbreaks, animal trypanosomiasis presents a constant economic problem in Africa. This disease renders 4 million square miles of land in sub-Saharan Africa unfit for livestock production. In addition to denying the expanding human population this needed source of animal protein, the lack of cattle restricts crop production because there is a shortage of draught animals and animal manure.

The course of African trypanosomiasis in domestic livestock is similar to the early stages of the disease in humans. However, the two main livestock pathogens, *T. congolense* and *T.vivax* rarely invade the central nervous system. Thus while in the less common livestock infections with *T. b. brucei* CNS pathology can occur, in livestock trypanosomiasis it is the parasite-induced anaemia and cachexia leading to weakness and inability to forage for food that causes most deaths.

1.2.4.2 An insect-infecting trypanosome : Pathogenicity of Crithidia *(Crithidia fasciculata)*

Species of *Crithidia* are common parasites of the alimentary canal of many different types of insect. Although generally considered non-pathogenic, occasional deaths of their arthropod hosts have been attributed to *Crithidia sp. (*Molyneux and Ashford (1983)

Crithidia fasciculata occurs naturally in the intestines of mosquitoes, specifically in *Anopheles maculipennis* and *Culex incidens* mosquitoes (Whitfield, 1979), although the flagellates may be easily cultured *in vitro.* Choanomastigotes from *Crithidia fasciculata* have hence been widely used for metabolic studies as an innocuous model for human trypanosome pathogens. *Crithidia sp.* exists in the choanomastigote stage and what might be called a semiamastigote stage (McGhee and Cosgrove, 1980) Within the mosquito, the amastigote forms (3 to 4 μ long by 2 to 4 μ wide) of *Crithidia fasciculata* are attached to the mid and hindgut intestinal mucosa. The swimming choanomastigote form (free-living stage) (Clark, 1959) is 6 to 8μ long by 2 to 3μ wide.

1.2.4.3 A plant- infecting trypanosome : Pathogenicity of *Phytomonas sp.*

Phytomonas are fusiform promastigotes, characterised by a slender, elongated cell body twisted two or three times around their longitudinal axis.(Sanchez-Moreno et al, 1995) They measure 10 - 20μm in length, have a width of about 1.5 μm and the free (anterior) flagellum measures 10-15 μm.

Trypanosomatids of the genus *Phytomonas* are associated with diseases of coffee, coconut and oil palm, being transmitted to the plant host by feeding hemipteran insects. *Phytomonas* populations differ in the types of tissue or cell which they colonise (latex, phloem or fruit) (Dollet, 1994). In plants they parasitize the laticiferous tubes that contain the milky substance known as latex and the phloem and xylem of palm trees and other plants where they may exert a pathogenic effect (Stahel, 1931; Parthasarathy et al, 1976; Dollet et al, 1977; Dollet, 1984). Although initially considered not particularly damaging to most laticiferous plants, it has been shown that infection with intraphloemic flagellates can have devastating consequences on economically important plants occurring in Latin America, causing diseases such as hartrot of coconut (Parthasarathy et al, 1976.) marchitez sorprisiva of oil palm (Dollet et al, 1977); and phloem necrosis of coffee (Parthasarathy et al, 1976; Dollet et al, 1977; 1984)

Other plant trypanosomatids parasitize plants and fruits without inducing a pathological syndrome in the plant. The parasite has been observed in some varieties of garden vegetables and legumes, including beans and soybeans, although nothing is known about the pathogenicity on these crops.(Dollet et al, 1982 ; Jankevicius et al, 1988)

1.2.5 Life cycles of Trypanosomatid flagellates

The life cycle of parasites is frequently complex, often utilising a sequence of different hosts, with each host providing habitats which may vary markedly in their physico-chemical characteristics. Even within the same host, parasites may experience a wide range of environments and nutrients available for their episodes of extensive growth and differentiation. The metabolic resources required to sustain growth may be derived from endogenous host reserves or exogenous nutrients. It is obviously

essential that the different life cycle stages of a parasite are able to exploit the environment for the nutrients they require.

1.2.5.1 The life cycle of African trypanosomes (*T.brucei*)

The life cycle of *T.brucei* is the most widely studied of all the African trypanosomes. *T.b. brucei* has a digenetic life cycle, undergoing a cyclical transmission from mammal to mammal effected by their insect vector, the tsetse fly. Different developmental stages of the life-cycle are accompanied by markedly different morphological stages (Fig. 3). There are also corresponding biochemical adaptations. Most notable amongst these changes are those of the mitochondrion and the surface of the flagellate, which must undergo alterations in order for the parasite to survive in the two very different environments of the mammalian host and the insect vector (Vickerman 1965 and 1985).(see section 1.2.6.1 'Energy (carbohydrate) metabolism in *T. brucei*')

Infection of the mammalian host is initiated by injection into the skin of the metacyclic trypomastigote forms in the vectors saliva. These parasites differentiate into a pleomorphic population of bloodstream forms, ranging from the long slender (LS) form, which has a free flagellum at its anterior end, to the short stumpy (SS) form, which has no free flagellum (Vickerman 1965).

It is the LS form which is the most abundant when parasitaemia is rising as it is able to divide rapidly with a generation time of 4-6 hours. It is this form which evades the host immune system by antigenic variation of its surface coat (Tanner , Jenni , Hecker and Brun , 1980). Each cell is ensheathed in a surface coat composed of a matrix of a single glycoprotein (Cross, 1975). Parasitaemia occurs in waves, with each peak appearing to coincide with the expression of one specific antigenic variant. As trypanosomes bearing a particular variant antigen are eliminated from the population by the host immune system, a new variant type arises to take its place as the principal surface antigen (Boothroyd, 1985). The mitochondrion of the LS form is poorly developed as the parasite has a plentiful supply of blood glucose, and is therefore able to meet all of its energy requirements through glycolysis, thus obviating the need for a citric acid cycle.

During periods of low parasitaemia, the SS form predominates. SS forms are thought to be a pre-adaptation to life in the insect, mainly because of the state of its mitochondrion, which is intermediate between those of the LS and procyclic insect forms.With ingestion into the gut of the feeding tsetse fly, bloodstream form trypanosomes undergo differentiation to become parasites that can survive in the vector. The adapted parasite is known as the tsetse fly midgut form (Shapiro et al, 1986) or procyclic stage. This form of the parasite lacks the variable surface coat characteristic of bloodstream forms and is not infective for mammalian hosts. The uncoated trypanosomes successfully grown *in vitro* are considered analogous to this stage.

Some of the tsetse fly procyclic trypanosomes migrate back up to the fly mouth parts and salivary glands where they differentiate into the epimastigote stage (Hoare, 1970). This is the only time in the African trypanosome life cycle when the parasite assumes the characteristic epimastigote morphology seen in other members of the family Trypanosomatidae; in this form the kinetoplast is anterior to the nucleus. Epimastigotes in turn differentiate into metacyclic trypomastigotes. When inoculated into the new environment of the mammalian host (as the tsetse feeds) they transform into proliferating long slender bloodstream forms and establish the mammalian infection (Jenni and Brun, 1977 and Brun, 1977).

1.2.5.2 The Life-cycle of *Crithidia* (*Crithidia fasciculata)*

Crithidia exhibit a monogenetic life cycle, having only a single invertebrate host, the mosquito. *Anopheles maculipennis* and *Culex incidens* mosquitoes become infected by ingesting *Crithidia* amastigotes that have been deposited in faeces on flowers by previously feeding mosquitoes harbouring the infective parasite in their mid and hindgut intestinal mucosa (Clark et al, 1964; Brooker, 1971)

Infected mosquitoes flying or blown over marshes, pond, pools, or other aquatic habitats, contaminate them with their faeces and dead bodies. Choanomastigote forms so released may then be ingested by larval mosquitoes present in the aquatic environment..

1.2.5.3 The Life cycle of *Phytomonas sp*

Phytomonas resembles the monogenetic genus *Leptomonas* of insects and the digenetic *Leishmania* of mammals in having an amastigote and a promastigote in its life cycle (Sanchez-Moreno et al, 1995; Jankevicius et al, 1988). *Phytomonas* might hence be considered as a transitional form between the monogenetic species in invertebrates and the digenic ones, which have an alternation of hosts between insects and vertebrates.

The life cycle of *Phytomonas* is still not completely understood although it is generally assumed that parasites in the latex of infected plants, mostly in the form of promastigotes, are transmitted by feeding phytophagous insects (Dollet, 1984, 1987). When ingested by feeding insects, multiplication of the flagellates ceases temporarily and no growth takes place in the oesophagus and crop. Upon arriving in the pylorus and midgut, rapid growth begins, producing giant promastigotes. These large forms leave the digestive tract for the haemocoel, where growth continues. From here, they go into the salivary glands. In these organs, reproduction is resumed with the formation of a few amastigotes and numerous small promastigotes. Insects sucking latex from plants inject the parasites with the salivary secretions. Inside the plant, the parasites remain small, being 13.5 μ long by 2.5 μ wide, but they proliferate rapidly, forming both promastigotes and amastigotes. At first they are localised in the area of the bites but the infection soon spreads, becoming generalised, with the flagellates distributed throughout all parts of the plant.

1.2.6 Energy metabolism in Trypanosomatids

Trypanosomatids - *T. brucei* in particular - have been the subject of many studies during the last 15 years due to a number of morphological and biochemical peculiarities unique to this family. These include features such as branched respiratory chains with an unusual cytochrome c, the presence of a glycosome organelle and the aerobic fermentation of glucose.(Opperdoes, 1991). The glycosome is an unusual, non-DNA containing organelle that houses the enzymes of the glycolytic chain. which, unusually, are mostly absent from the cytosol.

Bloodstream trypomastigotes of *T. brucei* are among the eukaryotic cells with the highest rates of glucose consumption, at least 10-fold higher than that of their

mammalian host. However, most other trypanosomatids also present high rates of glucose utilisation irrespective of the carbohydrate contents in their natural habitat (Cazzulo, 1992). There is a good correlation between glucose consumption rates and environmental carbohydrate contents in bloodstream forms and insect procyclic forms of *T. brucei* , the latter being an order of magnitude lower (Opperdoes, 1987; Darling et al, 1988). On the other hand, the insect parasite *Crithidia fasciculata* , which lives in the mosquito's gut, an environment of low carbohydrate contents, presents nevertheless a rate of glucose consumption of the same order as that shown by bloodstream African trypanosomes (Cazzulo et al, 1988)

Another interesting feature of glucose utilisation by trypanosomatids is the lack of a 'Pasteur effect' (Cannata and Cazzulo, 1984; Darling et al, 1987). In most organisms the transition from anaerobiosis to aerobiosis is accompanied by a rapid and considerable decrease in the rate of glucose utilisation. This inhibition of glycolysis by oxygen (the Pasteur effect) is lacking in trypanosomatids which seem to be adapted to consume as much glucose as is able to enter the cell (Cazzulo, 1992). Present evidence suggests that subcellular compartmentation is the major factor involved in the regulation of glycolysis in trypanosomatids (Opperdoes, 1987). The high rate of glucose consumption seems to be due to the very high concentration of glycolytic enzymes and intermediates inside the glycosome (Opperdoes, 1986).

1.2.6.1 Energy (carbohydrate) metabolism in *Trypanosoma brucei*

The activities of the trypanosome mitochondrion are modulated during the developmental cycle to take advantage of the changing environmental conditions. While the bloodstream parasite can rely on an abundant supply of host-derived carbohydrates for its relatively inefficient aerobic metabolism, the parasite taken into the gut of the tsetse fly vector must become more efficient to survive in this environment which is poorer in carbohydrates (Vickerman, 1971).

To remain functional, all glycolytic systems must have an efficient system for the reoxidation of the NADH generated in the glyceraldehyde-3-phosphate dehydrogenase reaction (Fig. 4). Under aerobic conditions, this system is usually the respiratory chain.

1.2.6.1.1 Long slender (LS) bloodstream forms

LS mitochondria do not contain spectrally detectable cytochromes, have little or no NADH dehydrogenase, and lack several key enzymes of the citric acid cycle (pyruvate dehydrogenase , α- ketoglutarate dehydrogenase, citrate synthase, and succinate dehydrogenase) (Ryley, 1956; Vickerman, 1965; Bowman et al, 1972; Brown et al., 1973; Flynn and Bowman, 1973; Bienen et al, 1991). Consequently, glucose can only be metabolised by glycolysis as far as pyruvate which is then excreted into the surrounding medium (Fairlamb and Opperdoes,1986). Most of the enzymes of the glycolytic pathway and an NAD-dependent glycerol-3-phosphate dehydrogenase enzyme are localised in the glycosome (Fairlamb and Opperdoes,1986; Hannaert and Michels,1994) (Fig. 4)

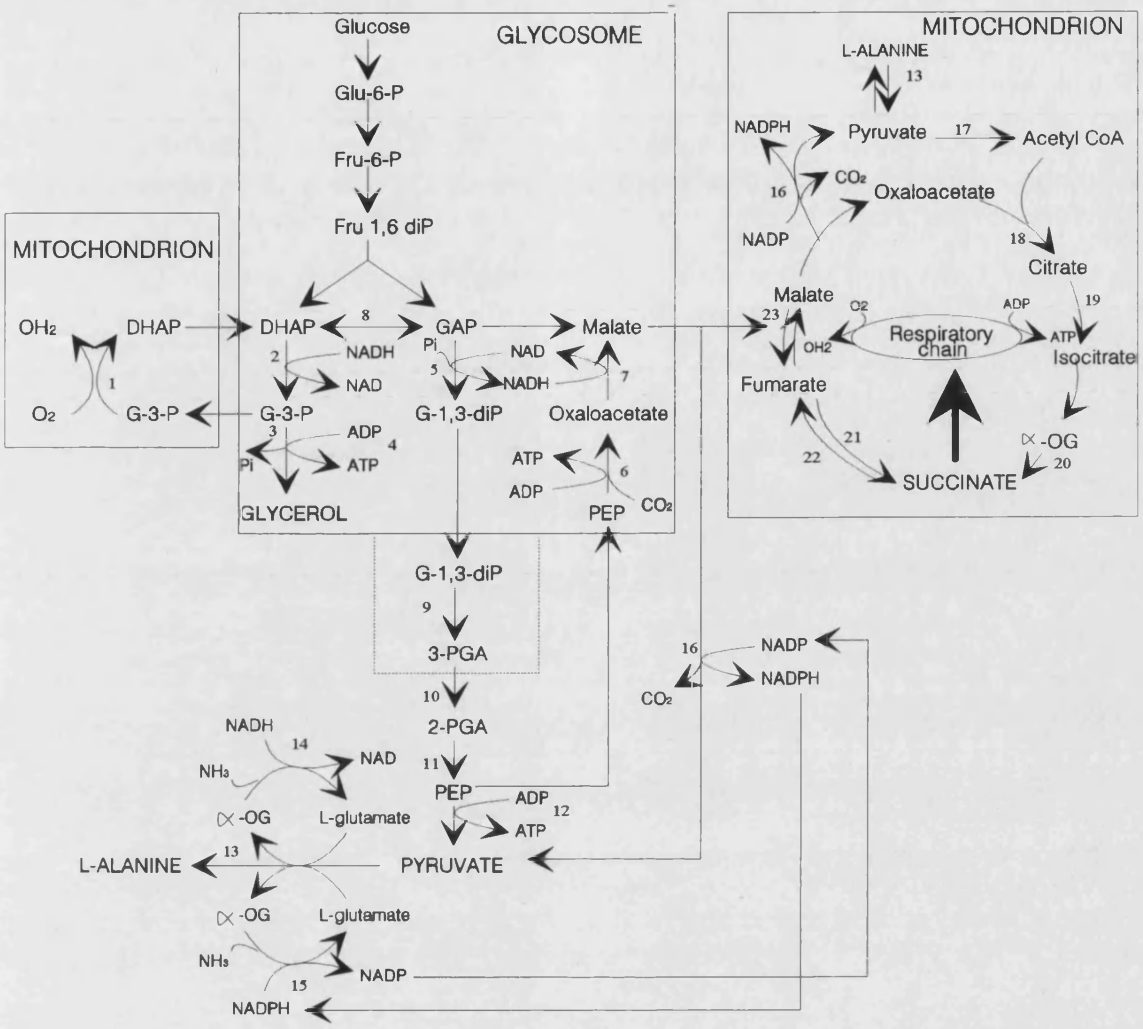


Figure 4. Subcellular compartmentation of the enzymes involved in glucose catabolism in *T.brucei*. Abbreviations of metabolites : Glu-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-diP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; GAP, glyceraldehyde-3-phosphate; G-1,3-diP, 1,3 diphosphoglycerate; 3-PGA, 3-phosphoglyerate; 2-PGA, 2-phosphoglycerate; G-3-P, glycerol-3-phosphate; ALA, L-alanine. G-1,3-diP and 3-PGA are shown inside a dotted box to indicate that enzyme (9) is present inside the glycosome of the bloodstream trypomastigote of *T.brucei* and in the cytosol of the procyclic trypomastigote. The mitochondrion on the left corresponds to the cristae-less tubular mitochondrion of bloodstream trypomastigotes; the mitochondrion on the right corresponds to the cristae-containing mitochondrion of procyclic trypomastigotes. The enzymes involved are: glycerol-3-phosphate oxidase (1); glycerol-3-phosphate dehydrogenase (2); glycerophosphate phosphatase (3); glycerol kinase (4); glyceraldehyde isomerase (8); phosphoglycerate kinase (9); phosphoglycerate mutase (10); enolase (11); pyruvate kinase (12); alanine aminotransferase (13); NAD-linked glutamate dehydrogenase (14); NADP-linked glutamate dehydrogenase (15); malic enzyme (16); pyruvate dehydrogenase complex (17); citrate synthase (18); aconitase (19); 2-oxoglutarate 3-phosphate dehydrogenase (5); PEP carboxykinase (6); malate dehydrogenase (7); triosephosphate dehydrogenase complex (20); fumarate reductase (21); succinate dehydrogenase (22); fumarase (23). The possible participation of both glutamate dehydrogenases in the formation of L-alanine linked to NADH reoxidation is indicated only for the cytosol, but the same pathways are possible inside the mitochondrion (Duschak and Cazzulo, 1991).

Diagram adapted from Cazzulo, 1992.

In the absence of tricarboxylic acid cycle enzymes and cytochromes, the bloodstream trypanosomes use a unique system in aerobiosis for the reoxidation of the NADH generated in the glyceraldehyde-3-phosphate dehydrogenase reaction. Regeneration of NAD^+ from NADH is required for continuous glycolysis, and this is mediated by a glycerol-3-phosphate shuttle to the mitochondrion. In the glycosome, glycerol-3-phosphate dehydrogenase reduces dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G-3-P) and regenerates NAD^+ from NADH. Glycerol-3-phosphate shuttles to the mitochondrion where it is oxidised to dihydroxyacetone phosphate by a novel cyanide insensitive L-glycerol-3-phosphate oxidase in which form it returns to the glycosome. This reoxidation allows the quantitative conversion of glucose to pyruvate and the maintenance of redox balance in the cell. The reducing equivalents delivered to the mitochondrion ultimately reduce molecular oxygen to water.

Under anaerobic conditions, or in the presence of glycerophosphate oxidase inhibitors e.g. salicylhydroxamic acid (SHAM) (Flynn and Bowman, 1973; Clarkson et al, 1981), glucose is converted to equimolar amounts of pyruvate and glycerol (Fairlamb and Opperdoes, 1986). Glycerol production seems to be accompanied by ATP synthesis by reversal of the glycerol kinase reaction.

1.2.6.1.2 Short stumpy bloodstream form

Later in the course of infection, a non-dividing short stumpy trypanosome form predominates. Like the long slender form, short stumpy form cells metabolise glucose to pyruvate and utilise the glycerophosphate oxidase system to regenerate NAD^+. Unlike the long slender form, short stumpy form cells are also capable of metabolising α-ketoglutarate to succinate (Vickerman, 1965; Bowman et al., 1972; Flynn and Bowman, 1973). The mitochondrion of these forms contains a more complete citric acid cycle in that pyruvate dehydrogenase and α-ketoglutarate dehydrogenase are present (Bowman et al., 1972; Flynn and Bowman, 1973). Cytochromes are not present in SS form cells, but inhibitor studies suggest the presence of a functional NADH dehydrogenase complex (Bowman et al., 1972; Flynn and Bowman, 1973; Bienen et al., 1991).

1.2.6.1.3 Insect (or procyclic form)

The transmission of the bloodstream form cells to a tsetse fly along with a bloodmeal induces the development of a cristate mitochondrion with functional cytochrome mediated electron transport and a complete citric acid cycle together with a shift to the use of proline as the preferred energy source (Brown et al., 1973). Proline, which is the major catabolisable substance in the fly's hemolymph (Fairlamb and Opperdoes, 1986), is used by the insect as an energy source for flight (Vickerman, 1985). There is partial attenuation of the glycerophosphate oxidase, which may still account for up to 30% of terminal oxidation in procyclics (Opperdoes, 1985 ; Fairlamb and Opperdoes, 1986). As indicated by the increased cyanide sensitivity of this form, the insect midgut procyclic form utilises a conventional cytochrome c oxidase as the terminal enzyme of electron transport (Hill, 1976; Bienen et al, 1983).

Procyclic cells divide rapidly in the fly midgut before migrating to the salivary glands of the fly. The salivary gland stages of the trypanosome are not well studied at the biochemical level, but it is believed that mitochondrial activity is repressed before the organisms become infective for a new mammalian host. This is based on the observation that the mitochondria of the infective metacyclic form have the unbranched, noncristate appearance that is characteristic of the bloodstream form. (Vickerman, 1985).

1.2.6.2 Energy (carbohydrate) metabolism in *Crithidia fasciculata*

In contrast to bloodstream *T. brucei (*and, more recently, *Phytomonas sp.)* , *Crithidia fasciculata,* together with all other studied trypanosomatids to date, seem to NADH deoxidise generated in the glyceraldehyde-3-phosphate dehydrogenase reaction, at least partially, through the respiratory chain. This oxidation is linked to phosphorylation which, as in all other known systems, is mediated by a mitochondrial oligomycin-sensitive Mg^{2+}-ATPase. However, oxidation through the respiratory chain does not seem to be enough to maintain the high levels of glycolysis attainable and glucose oxidation is incomplete, with the parasites producing, even in the presence of oxygen, reduced catabolites such as succinate and ethanol (Cazzulo, 1992). This type of metabolism, characterised by a nearly complete lack of Pasteur effect, has been called aerobic fermentation by von Brand (von Brand, 1979).

For *Crithidia fasciculata* the final products of aerobic catabolism are CO_2, succinate and ethanol with minor amounts of glycerol and acetate (Cazzulo et al, 1985, 1988). The final products of anaerobic glycolysis are mainly succinate, ethanol and glycerol with minor amounts of L-malate and acetate.(Cazzulo, 1988; De los Santos et al, 1985). The general outline of the pathways leading to the formation of end products succinate, ethanol and glycerol is depicted in Fig. 5 (over).

The degradation of glucose by *Crithidia fasciculata* follows the classical Embden-Meyerhof path of glucose degradation up to the level of phosphoenolpyruvate followed by its carboxylation to oxaloacetate. The latter is then converted into succinate by the action of malate dehydrogenase, fumarase and fumarate reductase. Glycerol is produced in small amounts and reflects the role of the glycerol-3-phosphate dehydrogenase system which is probably important as a shuttle to transport reducing equivalents into the mitochondrion for re-oxidation through the respiratory chain (Bacchi et al, 1968). The absence of L-alanine production (Cazzulo et al, 1988) appears not to be due to a lack of transaminase activity, but rather reflects the high effectiveness of the pathway for ethanol synthesis through the action of pyruvate decarboxylase and alcohol dehydrogenase (Cazzulo et al, 1988), which would leave little pyruvate available for transamination. Pyruvate kinase activity in *Crithidia fasciculata* appears to be very low, and the malic enzyme reaction is probably the major one involved in the formation of pyruvate necessary for ethanol production.

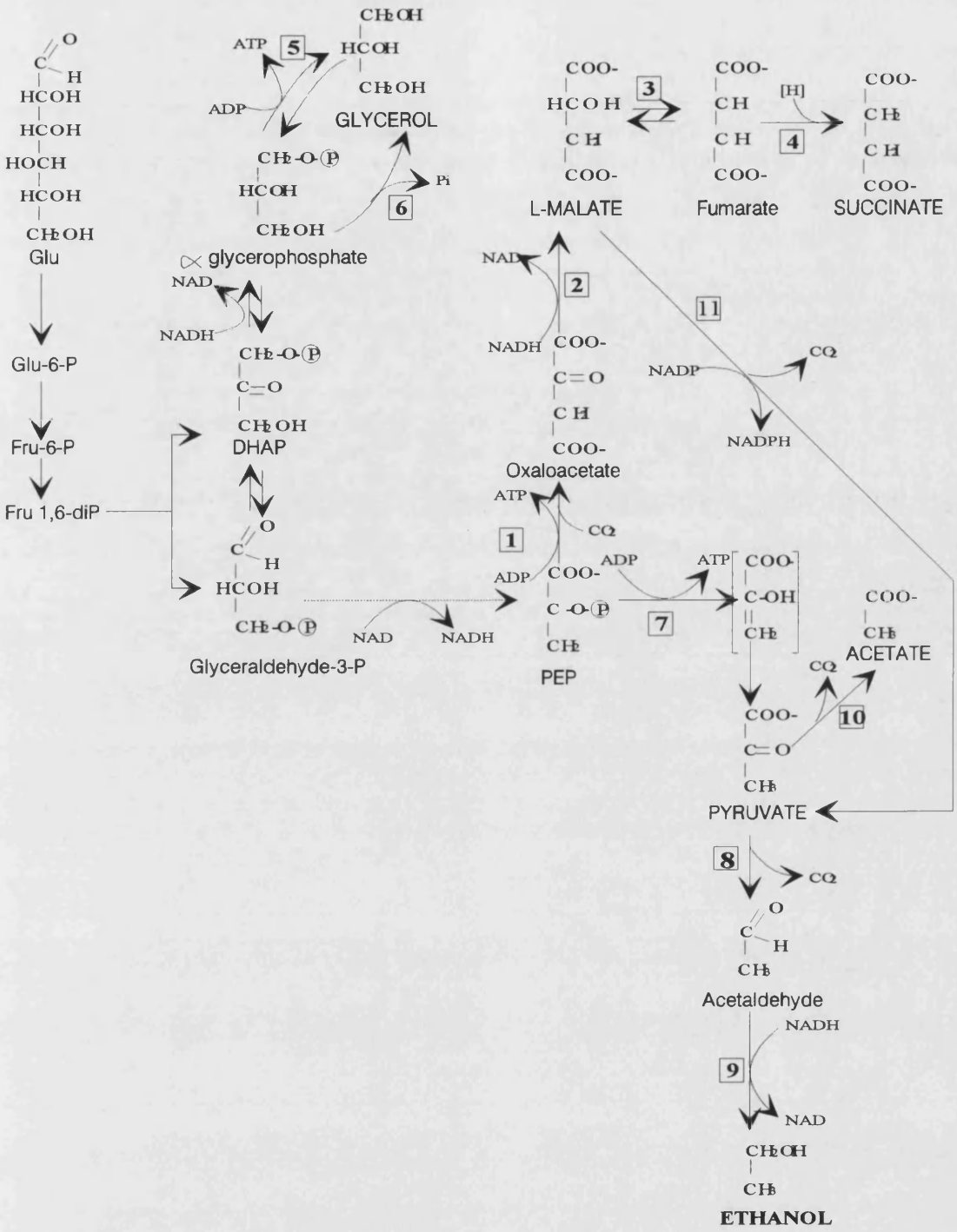


Figure 5 : Pathways for glucose fermentation by *Crithidia fasciculata* (Cazzulo, 1992). Glu-6-P, glucose-6-phosphate; Fru-6-P, fructose-6-phosphate; Fru-1,6-diP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate. The enzymes involved are: phosphoenol pyruvate carboxykinase (1); malate dehydrogenase (2); fumarase (3); fumarate reductase (4); glycerokinase (5); glycerophosphate phosphatase (6); pyruvate kinase (7); pyruvate decarboxylase (8); alcohol dehydrogenase (9); pyruvate dehydrogenase (10); malic enzyme (11).

1.2.6.3 *Phytomonas sp.*

In contrast to other trypanosomatids, little has been known about the biology of these protozoa until very recently, mainly due to the lack of an *in vitro* culture until 1984 when Dollet succeeded in establishing axenic cultures of parasites isolated from both the phloem and latex fluids of plants.(Dollet, 1984 ; Menara et al, 1988). This breakthrough has led to the study of carbohydrate metabolism of the typical promastigote stage of *Phytomonas*, with many metabolic studies being performed on culture promastigotes of *Phytomonas* from *Euphorbia characias.* More recently, such studies have been extended to a range of different *Phytomonas* isolates and it has been found that the energy catabolism follows the same general pattern in all species of *Phytomonas* studied to date (Fernandez-Becerra, 1997).

Phytomonas promastigote cells, isolated in the early stationary phase of growth, have been analysed for their capacity to utilise plant carbohydrates for their energy requirements and were found to contain a number of cellulose degrading enzymes including invertase (Sanchez-moreno et al, 1992). These flagellated protists, shown to utilise glucose, fructose and mannose as major energy substrates (Chaumont et al, 1994; Sanchez-Moreno et al, 1992) are also capable of utilising plant sucrose due to the presence of invertase. They have been shown to exhibit a glycolytic energy metabolism similar to *T.brucei* bloodstream forms, where these sugars are converted to pyruvate by aerobic fermentation according to the Embden-Meyerhof pathway. Citric-acid cycle activity, cytochromes and oxidative phosphorylation are absent in *Phytomonas sp.* and consequently oxygen consumption is insensitive to the classical inhibitors of mitochondrial respiration, such as antimycin and potassium cyanide. However, respiration is totally sensitive to salicylhydroxamic acid (SHAM), an inhibitor of the trypanosome glycerol-3-phosphate oxidase system, indicating that a mitochondrial glycerol-3-phosphate oxidase system, similar to the one described for bloodstream *T.brucei* (Opperdoes, 1987) is present and active in these organisms.

The absence of detectable cytochromes and the fact that respiration is not inhibited by the classical inhibitors of the mitochondrial respiratory chain in any strain

of *Phytomonas sp.* studied to date (Fernandez-Becerra et al, 1997) indicates that the glycolytic pathway serves as the major, if not the only, source of ATP within the cell.

1.3 Dihydrolipoamide dehydrogenase (DHlipDH) in bloodstream *T.brucei*

Dihydrolipoamide dehydrogenase (DHlipDH) is an essential component of the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, branched chain 2-oxoacid dehydrogenase and glycine cleavage system, which are multienzyme complexes located in mitochondria of most eukaryotic organisms. With the bloodstream form of *T.brucei* having a highly repressed mitochondrion containing no detectable 2-oxo acid dehydrogenase complexes, it was a surprise when Danson et al (1987) discovered DHlipDH in these cells. Further intrigue was added to this discovery when they found that this DHlipDH co-purified with bloodstream *T.brucei* plasma membranes.

A complete subcellular fractionation of *T.brucei*, and comparison with marker enzymes, performed by Jackman et al (1990) indicated that the DHlipDH was located over the entire inner surface of the plasma membrane of the bloodstream form only and was not located elsewhere in the bloodstream cell. Furthermore, a study of the latency of the enzyme suggests that it is located on the cytoplasmic surface of the plasma membrane (Jackman et al, 1990).

Interestingly, a DHlipDH in *E.coli* has been found to be functionally distinct from those of the pyruvate and 2-oxoglutarate dehydrogenase complexes and has been implicated in the binding-protein-dependent transport of galactose and maltose (Richarme, 1989) , suggesting a plasma membrane location.

These findings point to the possibility of a plasma membrane associated DHlipDH present in an evolutionary diverse range of species. Such a membrane associated DHlipDH may have an important function, possibly related to solute transport in and out of the trypanosome cell, and consequently be of therapeutic interest as a possible drug target.

1.3.1 Dihydrolipoamide dehydrogenase

The enzyme dihydrolipoamide dehydrogenase (DHlipDH) is a flavoprotein member of a group of disulphide oxidoreductase enzymes that include glutathione

reductase and trypanothione reductase. It employs FAD to catalyse the oxidation of dihydrolipoamide (DHlip) to lipoamide (lip) using NAD^+ as the final electron acceptor in the oxidation as shown in the reaction below:

$$\text{Dihydrolipoamide} + NAD^+ \longrightarrow \text{Lipoamide} + NADH + H^+$$

DHlipDH has been isolated from many species. It is a homodimeric enzyme, with subunit sizes varying from 49 Kda in *Pseudomonas putida* to 61 Kda in *Saccharomyces cervisiae.* Each active site consists of two binding sites which act as channels for NAD^+ and lipoamide, separated by the flavin moiety, and a redox-active disulphide bridge.

Various biochemical and spectroscopic studies have revealed that the reaction proceeds via a ping-pong mechanism in which the enzyme goes from an oxidised to a semi-reduced state, where in the latter the reactive disulphide bridge is open and one of the two cysteine residues forms a charge transfer complex with the flavin. NAD^+ accepts the electrons from this complex, and, in being reduced to NADH, re-oxidises the enzyme in preparation for another catalytic cycle (Fig.6). (Reviewed by Guest, 1978).

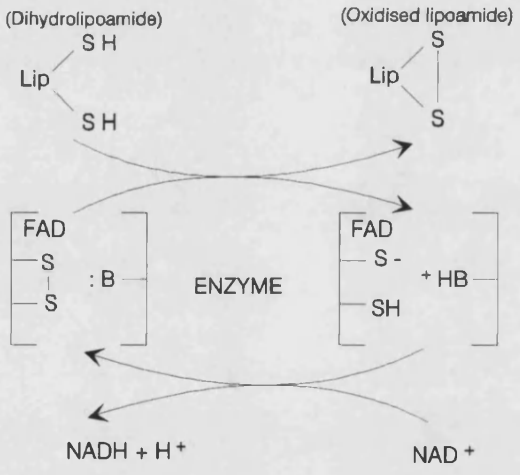


Figure 6 : NAD^+-dependent oxidation of dihydrolipoamide catalysed by dihydrolipoamide dehydrogenase. Catalysis proceeds via alternate oxidation and reduction of an intrachain disulphide bond and a base (B) on the enzyme. In the reduced enzyme, the electrons are shared between the active site dithiolate anion and a molecule of FAD (Danson, 1988).

1.3.2 Trypanothione reductase

Trypanothione reductase (TR) is a flavoenzyme which is of particular interest as a target molecule for antitrypanosomal therapy. Like other members of the disulphide

oxidoreductase class of flavoproteins that includes glutathione reductase, dihydrolipoamide dehydrogenase and mercury reductases (Williams et al, 1992), trypanothione reductase contains a cysteine disulphide bridge in the active site, which undergoes reduction by the pyridine dinucleotide co-factor as an obligatory step in the overall reaction mechanism (Shames et al, 1986).

TR has been found only in parasitic protozoa of the order Kinetoplastida where it is a key enzyme of the parasite's thiol metabolism, catalysing the NADPH-dependent reduction of glutathionylspermidine conjugates which serve as the parasites main thiols. TR and DHlipDH have a number of structural features in common, and exhibit an unusually high one-electron-reducing capacity (Krauth-siegel and Schoneck, 1995).

1.3.3 Role of DHlipDH in multienzyme complexes

DHlipDH performs its catalytic role as part of the pyruvate dehydrogenase complex (PDHC), 2-oxoglutarate dehydrogenase complex (2OGDHC), branched chain 2-oxoacid dehydrogenase complex (BCKDHC) and the glycine cleavage system (GCS) (Yeaman, 1989). All of these complexes have been found in prokaryotic systems, and are intramitochondrial in eukaryotes. They consist of multiple copies of several component enzymes, designated E1, E2 and E3. Each complex is situated at a key position in carbon and energy metabolism and they are of particular importance because the PDHC, 2OGDHC and BCKDHC catalyse irreversible catabolic steps (reviewed by Perham, 1991)

1.3.3.1 Pyruvate dehydrogenase complex

The pyruvate dehydrogenase complex is the most studied of the 2-oxo acid dehydrogenase complexes and catalyses the following enzyme reaction:

$$\text{Pyruvate} + \text{CoA} + \text{NAD}^+ \longrightarrow \text{Acetyl CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

The PDHC has a pivotal role in the utilisation of pyruvate by most cells, producing acetyl CoA for energy or biosynthesis. It consists of 3 catalytic components, E1, E2 and E3, which have similar properties in all species:

E1(pyruvate decarboxylase) catalyses the decarboxylation of pyruvate to a hydroxyethyl group followed by reductive acetylation of the lipoamide coenzyme of E2

E2 (dihydrolipoyl transacetylase) E2 hands the acetyl group, derived from pyruvate , to CoA using a swinging arm, with a lipoamide coenzyme bound to the end, which rotates among the catalytic centres of the 3 component enzymes (Guest, 1978; Perham, 1991)

E3 (dihydrolipoamide dehydrogenase) oxidises the reduced lipoamide coenzyme in preparation for receiving another acetyl group, and hands the reducing equivalents to NAD^+.

1.3.3.2 2-Oxoglutarate dehydrogenase complex

The 2-Oxoglutarate dehydrogenase complex is of the same basic structure as the PDHC, but is not regulated by phosphorylation. The 2OGDHC is part of the citric acid cycle of the cell, in which it catalyses the irreversible oxidative decarboxylation of 2-oxoglutarate to succinyl-CoA in a reaction analogous to that of the PDHC.

1.3.3.3 Branched-chain 2-oxoacid dehydrogenase complex

The BCKDHC catalyses the oxidative decarboxylation of branched-chain amino acids, the general reaction being:

$$\text{R-CO-COOH} + \text{Co A} + \text{NAD}^+ \longrightarrow \text{R-CO-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+$$

The eukaryotic BCKDHC is similar in structure to the PDHC and is also regulated by phosphorylation. Its metabolic importance is as the first committed step in branched- chain amino acid catabolism. Acyl CoA products are fed into the citric acid cycle through acetyl CoA or succinyl CoA. In oxidising the branched chain amino acids, it is able to prevent their otherwise toxic effects on the cell. The complex is found in eukaryotic systems and in those bacteria that utilise branched chain amino acids (e.g. *Pseudomonas putida*)

1.3.3.4 Glycine cleavage system

The glycine cleavage system (GCS) consists of four protein components: P-protein (pyridoxal-phospate containing glycine decarboxylase), H- protein (lipoyl

bearing aminomethyltransferase), T-protein (N^5, N^{10} methylene tetrahydrofolate synthetase) and L-protein (DHlipDH) (Kikuchi and Hiraga, 1982). It catalyses the pyridoxal phosphate-dependent decarboxylation of glycine:

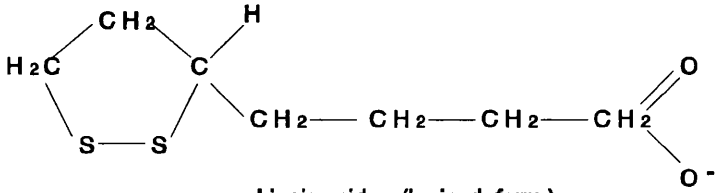
$$\text{glycine} + H_4\text{-folate} + NAD^+ \longrightarrow 5,10\text{-methylene-}H_4\text{-folate} + NH_3 + CO_2 + NADH + H^+$$

This complex is also capable of glycine synthesis in the reverse of the above reaction, and is in contrast to the 2-oxoacid dehydrogenase complexes which catalyse an irreversible reaction.

The GCS forms the major pathway for the catabolism of glycine and serine in vertebrates (Kikuchi and Hiraga, 1982) and for the breakdown of glycine by certain anaerobic glycine-utilising bacteria, including *Clostridium cylindrosporum* (Dietrichs and Andreesen, 1990)

1.3.4 Lipoic acid

Investigations into the role of DHlipDH in the bloodstream form of *T.b.brucei* have involved looking for its presumed substrate, lipoic acid, in these cells (Jackman et al, 1990)



Lipoic acid (Ionized form)

The presence of lipoic acid in any DHlipDH - containing cell would suggest that the enzyme may be performing an analogous role to that in the 2-oxo acid dehydrogenase complexes, in catalysing the interconversion of reduced and oxidised lipoic acid/lipoamide. If it were absent, then the enzyme would have a different substrate *in vivo*, and be able to catalyse the oxidation of dihydrolipoamide as a secondary reaction *in vitro*. The presence of lipoic acid in a cell would therefore seem to indicate that DHlipDH would use it as a substrate *in vivo*.

There are a number of different methods that have been developed for the quantitative determination of lipoic acid in biological systems. Following release of the

cofactor from protein by acid or base hydrolysis, lipoic acid has been assayed a) manometrically using *Streptococcus faecalis* (strain 10Cl) (Gunsalus and Razzell, 1957), b) Polarographically using the lipoic acid mutant of *Escherichia coli* K12, W1485*lip*2, (Herbert and Guest, 1968; 1970 and 1975) or by turbidimetric assay using *Escherichia coli* K12, W1485*lip*2 (Herbert and Guest, 1975).

1.4 Study aims

There is overwhelming evidence to suggest the existence of an unusual extramitochondrial DHlipDH in the mitochondrially repressed bloodstream *T. brucei.* No such investigation has been extended to Trypanosomatidae family members possessing a similarly repressed mitochondrion e.g. *Phytomonas sp.* and one aim of this study is to investigate the presence and location of DHlipDH in cultured *Phytomonas sp.* promastigote cells..

Jackman *et al* (1990) demonstrated substantially higher levels of DHlipDH in the procyclic form of *T.brucei* (13 - fold higher in the procyclic compared to the bloodstream form) which is almost certainly due to DHlipDH-containing multienzyme complexes present in the active mitochondrion, but they were unable to determine conclusively if any remained at the plasma membrane. Similarly, as would be expected in the mitochondrially active *Crithidia fasciculata,* there is evidence for at least one mitochondrially located DHlipDH in the form of the pyruvate dehydrogenase complex (Diaz and Komuniecki, 1995). However, no research has been done to investigate a possible plasma membrane location in *Crithidia fasciculata* .

It is hence the immediate aim of this study to investigate whether a plasma membrane DHlipDH occurs in procyclic *T. brucei* by immunolocalisation studies. Polyclonal antibodies to selected antigenic peptide sequences derived from the bloodstream *T.brucei* DHlipDH gene sequence (Else et al, 1993) are to be raised. Characterisation of raised polyclonal peptide antisera is to be achieved by western blots of procyclic *T. brucei* cell sonicates and specificity for native DHlipDH gauged by immunoprecipitation of DHlipDH activity from procyclic *T. brucei* cell sonicates. If the raising of a tightly binding peptide antisera specific for native DHlipDH is successful, preliminary immunofluoresence studies may be followed by more sensitive immunogold localisation studies to determine unequivocally whether a plasma mebrane

location exists in procyclic *T. brucei* cells. Such studies may then be extended to other members of the Trypanosomatid family, namely *Crithidia fasciculata* and *Phytomonas sp.*

If the raising of a native DHlipDH-specific peptide antiserum is unsuccessful, DHlipDH localisation studies may be carried out in *Crithidia fasciculata* and *Phytomonas sp.* using cell fractionation techniques. Isolated plasma membrane fractions may be assayed for DHlipDH activity and compared to cell homogenate DHlipDH activity in order to gauged any DHlipDH enrichment in plasma membrane fractions. Determination of the purity of plasma membrane fractions would be imperative for interpretation of any findings, and purity may be assessed by the measurement of 'marker' enzymes for other cell organelles, or else by morphological examination under the electron microscope.

CHAPTER 2

MATERIALS AND GENERAL METHODS

(A) MATERIALS

All chemicals used in this work were of analytical grade or the finest grade commercially obtainable.

2.1 CELL CULTURE

2.1.1 General cell culture

All solvents and chemicals used in cell culture work were obtained from BDH Chemicals Ltd., Poole, Dorset. HEPES was obtained from the Aldrich Chemical Co., Gillingham, Dorset. L-glutamine (200 mM), penicillin/streptomycin (5000 I.W./ml and 5000 mg/ml), phosphate buffered saline (Dulbecco's formula) tablets were obtained from Flow laboratories, Irvine, Scotland.

All chemicals used in preparation of SDM-79 medium were obtained from Sigma Chemical Co., Poole, Dorset except Dulbecco's modified Eagle medium powder, medium199, MEM amino acid solution (X50 concentration), MEM non-essential amino acids (X100 concentration), and Gentamycin which were obtained from Life technologies Ltd., Paisely, together with tissue culture medical flasks (25 cm^2). Bovine serum albumin (fraction V), triethanolamine, and haemin used in the preparation of *Crithidia fasciculata* culture medium were supplied by Sigma. Foetal calf serum was obtained from Globe Pharm, Esher, Surrey. Yeast extract, tween 80 and tryptone ('bacto-tryptone) were supplied by Difco. Grace medium (without haemolymph) used in preparation of *Phytomonas sp.* growth medium was supplied by Gibco.

2.1.2 Organisms

Established procyclic cultures of *T.brucei 427* were a gift from Dr. W. Gibson, Department of Pathology, University of Bristol. Cultures of *Phytomonas sp. (*isolated from *Euphorbia pinea)* and *Crithidia fasciculata* (clone H56) were a gift from A. M Page, Department of Biochemistry, Royal Holloway University of London.

2.2 PEPTIDE SYNTHESIS

All amino acid derivatives (table 1) and resin used for peptide synthesis were purchased from Milligen (manufactured by Cambridge Research Biochemical Ltd). Piperidine (98%), 4-dimethylaminopyridine (DMAP,99%), 1-hydroxybenzotriazole hydrate (HOBT), dicyclohexylcarbodiimide (DCCI), and t-amyl alcohol (99%) were supplied by Aldrich (Gillingham, Dorset)

Trifluoroacetic acid (TFA) was supplied by Fluorochem, and distilled at atmospheric pressure for use in HPLC solvents.

Freeze-drying of samples was achieved by solution in water, freezing in an acetone-solid CO_2 bath, and vacuum evaporation on an Edwards Modulyo freeze-dryer.

Table 1: The amino acid derivatives used in peptide synthesis

Ala	Fmoc-L-Ala-OPfp
Arg	Fmoc-L-Arg(Mtr)-OPfp
Asn	Fmoc-L-Asn-OPfp
Asp	Fmoc-L-Asp-(otBu)-OPfp
Cys	Fmoc-L-Cys(Trt)-OPfp
Glu	Fmoc-L-Glu(OtBu)-OPfp
Gly	Fmoc-L-Gly-OPfp
Ile	Fmoc-L-Ile-OPfp
Leu	Fmoc-L-Leu-OPfp
Lys	Fmoc-L-Lys(Boc)-OPfp
Thr	Fmoc-L-Thr(tBu)-ODhbt
Val	Fmoc-L-Val-Opfp

Abbreviations:

Boc: tert -butyloxycarbonyl.
tBu: tert -butyl.
Mtr: methoxytrimethylbenzene sulphonyl
OtBu: tert -butyl ester
ODhbt: 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazol ester
OPfp: pentafluorophenyl ester

A polydimethylacrylamide resin was used for the synthesis of peptide DLD1, while a polyethyleneglycol polystyrene resin was used for the synthesis of DLD2. Both resins were supplied by Millipore, UK.

2.3 PREPARATION OF ANTI-PEPTIDE ANTIBODIES

Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) and keyhole limpet haemocyanin (KLH) were supplied by Pierce (Chester). Sodium borohydride, 5,5'-

dithio-bis-(2-nitrobenzioic acid) (DTNB) and dithiothreitol (DTT) were supplied by Sigma.

2.4 ENZYME- LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme substrate, tetramethyl benzidine (TMB) was supplied by Sigma. Anti rabbit IgG-peroxidase was supplied by Amersham.

2.5 IMMUNOBLOTTING

Nitrocellulose strips were supplied by Millipore corporation, Bedford. Free peptide was synthesized according to sections 2.20 and 2.21 (The amino acid sequences of peptides DLD1 and DLD2 are detailed in section 2.20) and conjugated KLH-peptide was produced as described in section 2.18.2.

2.6 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS, N,N'methylenebisacrylamide, acrylamide, TEMED, ammonium persulphate (AMPS) and bromophenol blue were supplied by BDH chemicals, Poole, Dorset, UK. Molecular weight markers were obtained from Sigma.

2.7 IMMUNOLOGICAL DETECTION OF PROTEINS

Biotinylated goat(gt) anti-rabbit IgG was supplied by Sigma.

Vectastain ABC kit was supplied by Vector Labs, Peterborough . The kit utilizes a preformed Avidin:Biotinylated enzyme Complex (ABC) principle. Each kit is composed of biotinylated affinity purified primary antibody, Reagent A (Avidin DH solution) and Reagent B (biotinylated horseradish peroxidase).

2.8 IMMUNOPRECIPITATION

Goat anti-rabbit IgG, Protein A (non-viable Staphylococcus aureus) and polyethylene glycol (PEG) 6000 were supplied by Sigma. Microtitre wells (polystyrene) were supplied by Labsystems (Life sciences International), Hampshire. High purity triton X-100 was from Pierce and Warriner (UK), Chester. Dihydrolipoamide was prepared by Keith Jolly at the University of Bath Biochemistry Department.

2.9 IMMUNOFLUORESCENCE

Goat anti rabbit IgG-FITC conjugate, poly-L-lysine, rabbit anti-mouse IgG-FITC conjugate, normal rabbit serum and normal goat serum were supplied by Sigma. Vectorshield mounting fluid was supplied by Vector laboratories, Peterborough. ROD1 monoclonal antibody was kindly donated by Prof. Keith Gull, University of Manchester.

2.10 ISOLATION OF CRUDE PLASMA MEMBRANE FRACTIONS FROM *CRITHIDIA FASCICULATA* AND *PHYTOMONAS SP.*

Tris, EDTA (disodium ethylene diamine tetra-acetic acid), PMSF (phenylmethylsulphonylfluoride) and leupeptin (synthetic hemisulphate) were supplied by Sigma. Tris - EDTA buffer consisted of 0.5 M Tris-HCl and 0.1 M EDTA and pH adjusted to pH 7.8 at 4°C using sodium hydroxide. Leupeptin (2µg/ml) was dissolved in methanol and added to buffers with vigorous stirring, and PMSF (in 100 µl of dimethyl sulphoxide) added to warmed buffers, including sucrose solutions used in sucrose density gradient ultracentrifugation, with vigorous stirring to give a final concentration of 1 mM. Silicon carbide abrasive grain, -400 mesh was supplied by Aldrich Chemical Company, Inc. Analar sucrose was supplied by Fisons PLC. Sucrose solutions were prepared by dissolving sucrose in warmed Tris-EDTA buffer containing PMSF (1mM). All sucrose solutions were chilled on ice before use

2.10.1 Protein determination of isolated plasma membrane fractions

Plasma membrane protein concentration was found using a protein assay kit , supplied by Sigma (Cat. No. P5656), based on Peterson's modification of the micro-Lowry method (Peterson, 1977). Interference in the direct Lowry procedure is caused by commonly used chemicals including tris, EDTA and sucrose. The procedure with protein precipitation, which uses DOC (deoxycholate) and TCA (trichloroacetic acid), eliminates all these interferences. Lowry reagent solution (Cat. No. L1013) and Folin and Ciocalteu's Phenol reagent (Cat. No. F9252) were supplied by Sigma.

2.10.2 Marker enzyme assays to determine purity of plasma membrane fractions

DL-isocitrate, dihydroxyacetone phosphate, potassium chloride and magnesium chloride were supplied by Sigma Chemical Co. (Poole, Dorset). Phosphoenol pyruvate (PEP, monohexylamine salt) and disodium adenosine diphosphate (ADP), were purchased from BDH Chemical Co. Nicotinamide adenine dinucleotide (oxidised and reduced forms, NAD^+ and NADH respectively) and lactate dehydrogenase (pig heart, 300μ/mg protein, 5mg protein/ml) were supplied by Boehringer Mannheim, Germany. Analar sucrose and triethanolamine hydrochloride (TEA), were purchased from Fisons Plc., and Ultrapure Triton X-100 from Pierce Chemical Co., U.S.A. Dihydrolipoamide was a gift from Keith Jolly, Bath University, Bath, United Kingdom. Adenosine monophosphate, [^{3}H] labelled in the adenosine moiety (250μCi; specific activity 18.9 Ci/ mmole) was obtained from Amersham for use in the 5' nucleotidase assay. A stock solution was prepared by addition of 50 % ethanol to give a final volume of 2 ml, and was stored at -20°C. Piperazine, magnesium chloride hexahydrate and β-glycerophosphate were supplied by Sigma. Non-labelled AMP was supplied by Boehringer Mannheim.

2.10.3 Electron microscopical examination of plasma membrane fractions

Electron microscopy grade reagents (uranyl acetate, lead citrate) and copper grids were supplied by the University of Bath Electron Microscopy Unit. Epoxy resin (Taab premix, medium hardness) and osmium tetroxide (OsO_4) were supplied by Taab laboratories Ltd., Aldermaston, Reading, Berkshire. Glutaraldehyde and plastic truffs were supplied by Agar Aids Ltd.. Analar grade cacodylate (sodium dimethyl arsenic acid) was supplied by Sigma Chemical Co., Poole, Dorset. Thin sectioning was performed on a Reichert Om U3 ultramicrotome supplied by Reichert-Jung Ltd., Slough, Berkshire. Electron microscopy was carried out on a JEOL 100CX transmission electron microscope supplied by JEOL Ltd., Akichima, Tokyo, Japan).

(B) GENERAL METHODS

2.12 STERILE TECHNIQUE

All equipment such as bottles, tubes, Pasteur pipettes, plastic tips and solutions such as HEPES and PBS were sterilised by autoclaving at 15 lb/in^2 for 20 minutes. Freshly prepared medium was filter sterilised using Millipore 0.22 μM filter units. All experiments requiring sterile conditions were performed in a bench laminaflow hood.

2.13 PREPARATION OF CULTURE MEDIA

2.13.1 *T.brucei* culture medium

Procyclic forms of *T.b.brucei* were grown in SDM-79 medium (table 2).

Table 2: SDM-79 Medium for the growth of procyclic forms of *T.b.brucei*

(Brun and Shoenberger, 1979)

Ingredients	per litre
Dulbecco's modified Eagle medium powder	7.0 g
Medium 199 powder	2.0 g
MEM amino acid solution	8.0 ml
MEM non-essential amino acids	6.0 ml
Glucose	1.0 g
HEPES	8.0g
MOPS	5.0 g
NaHCO3	2.0g
L-Alanine	0.2 g
L-Arginine HCL	0.1 g
L-Methionine	0.07 g
L-Phenylalanine	0.08 g
L-Proline	0.6 g
L-Serine	0.06 g
Taurine	0.16 g
L-Threonine	0.35 g
L-Tyrosine	0.1 g
Adenosine	10 mg
Guanosine	10 mg
D(+)Glucosamine HCL	50 mg
Folic acid	4 mg
p-Aminobenzoic acid	2 mg
Biotin	0.2 mg
Penicillin solution	12 ml
Gentamycin	20 mg

All the ingredients were dissolved in 1/4 of the final volume, then both MEM amino acid solutions were added, and the pH adjusted with 4 M NaOH to pH 7.3. The medium was adjusted to final volume and filter sterilised with Millipore 0.22 μM filter units. To this solution was then added 2 ml of filter sterilised haemin solution (2.5 mg/ml in 50 mM NaOH) and the whole stored at -20°C in 100 ml quantities. Before use the medium was supplemented with 10% v/v sterile foetal calf serum (heat inactivated) and glutamine (10 mM).

2.13.2 *Phytomonas sp.* culture medium

Stock solutions of bovine serum (BSA fraction V), yeast extract, HEPES and antibiotic (0.3 g penicillin and 0.5 g streptomycin) were made up using double distilled water and then filter sterilised.

100 mls of growth medium was prepared aseptically as follows:

Grace's medium*(without haemolymph)	83 ml
BSA fraction V 1% w/v	5 ml
Yeast extract 1 % w/v	2.5 ml
Foetal calf serum 10% v/v	10 ml
HEPES (1M) 1% v/v	1 ml
Penicillin 500 IU/ml & Streptomycin 500 mg/ml	1 ml

The complete medium was stored at -20 °C.

* Ref. Grace, (1962)

2.13.3 *Crithidia fasciculata* culture medium (Shim and Fairlamb, 1988)

A culture medium was prepared by autoclave sterilising a mixture containing 5g yeast extract, 4g tryptone, 15g sucrose, 2.5g triethanolamine and 5g tween 80, made up to 1 litre with distilled water. The pH was adjusted to 8.00 if necessary. An antibiotic stock solution of 200 mg per ml streptomycin , plus a 2 mg per ml solution of haemin (with a drop of 880 ammonia), were made up in distilled water. Both antibiotic and haemin stocks were filter sterilised. A 1 ml aliquot of each stock solution was added to the medium, upon cooling after autoclaving, to give a final

concentrations of 0.2 mg per ml streptomycin and 2μg per ml haemin. The complete medium was stored at 4 °C.

2.14 IN VITRO CELL CULTIVATION

2.14.1 In Vitro Culture Of *T. brucei* Procyclic Trypomasigotes

Procyclic trypomastigotes were stored in liquid nitrogen under sterile conditions at a density of about 0.5 - 1 x 10^7 cells/ml in a 1.8 ml volume. When required, the tube containing the procyclics was thawed quickly and added to a culture flask containing 10 ml of warmed complete SDM-79 medium. The culture was incubated at 27°C in an incubator. Once in culture, they grew very quickly and reached a density of 2-3 x 10^7 cells /ml. They were then subcultured at a density of 1-5 x 10^5 cells/ml. The procyclics could be subcultured continuously for many months.

2.14.2 In vitro culture of *Phytomonas sp.* promastigotes

Frozen *Phytomonas sp.* isolates were prepared asceptically. 3 ml of culture (approximate density of 1 x 10^7 cells per ml) was spun for 10mins at 2000g and resuspended in 900 μl of fresh sterile medium. 100 μl of filtered DMSO was then added. Isolates were frozen for 48 hours at - 80 °C before being transferred to liquid nitrogen. When required, a tube containing the *Phytomonas sp.* isolate was thawed quickly and added to a culture flask containing 20 ml of warmed complete Graces medium. The culture was mixed gently and then incubated at 28 °C . The promastigotes grew quickly, reaching a maximum density of 1 - 1.4 x 10^7 cells per ml. This cell density signified the end of the parasites' log phase growth. They were then subcultured at a density of 1x 10^4 cells per ml and continually passaged into fresh medium on reaching a cell density of around 1x10^7 cells per ml (approx. every 5 days).

2.14.3 In vitro culture of *Crithidia fasciculata* choanomastigotes

Crithidia fasciculata cells at a density of 5 x 10^8 cells per ml were stored at 4°C for up to 4 weeks. They were then passaged into warmed complete medium in a ratio of 1:1000 of inoculum to medium. The culture was mixed gently and placed in an incubator at 28°C where growth was very rapid, achieving a density of around 5x10^8 cells per ml in about 48 hours. Cells were passaged typically every 48 hours.

2.15 PROTEIN ESTIMATION

The Bradford dye-binding method

Protein determination was carried out using Coomassie brilliant blue (G-250). 150 mg Coomassie G-250 was dissolved in 250 mls of 0.3M perchloric acid (PCA) and the reagent then filtered using whatman No. 1 filter paper. Assays were carried out in 3 ml cuvettes, to which 50µl sample, 0.95 mls phosphate buffer (pH 7.2) and 1 ml reagent were added. Protein content was assessed by measuring absorbance at 595 nm between 10 min and 1 hr after mixing by inversion. Cell samples were suspended in 200 µl PBS and 200 µL of 0.3M NaOH to lyse the cells prior to assay. A blank, of 1 ml dye reagent plus 1 ml PBS, was used in each assay. A standard curve, using BSA (stock concentration of 1 mg/ml) was set up for each assay, using BSA in the range of 5-50 µg/ml.

2.16 PEPTIDE SYNTHESIS

Two synthetic peptides, each of 15 amino acid residues (DLD1 and DLD2, corresponding to residues 130-144 and 228-242 of the bloodstream DHlipDH sequence respectively) were chosen (section 3.2) and synthesised as described below. The exact amino acid sequence of both peptides were as follows:

DLD1 (130-144)

130 **144**

Asn-Thr-Leu-Asn-Val-Lys-Gly-Ile-Asp-Gly-Lys-Asp-Glu-Ala-Ile

DLD2 (228-242)

228 **242**

Asp-Ala- Leu-Val-Gly-Ala-Leu-Lys-Arg-Asn-Gly-Glu-Asp-Glu

2.16.1 Assembly of peptide

The solid resin supports used (PepSyn-KA or PEG-PS) were commercially available with the C-terminal residue already attached via a benzyl ester linkage and the amino group protected by an Fmoc group. After solvation of the Fmoc protected C-terminal residue attached to the resin support with dimethylformamide (DMF), the reaction column was packed and the resin washed with DMF. The Fmoc-protecting group was removed by treatment with 20% piperidine for 9 minutes, followed by removal of piperidine and the protecting group reaction product with DMF. The next activated Fmoc-amino acid was dissolved in a solution of 5% w/v 1-hydroxy-benzotriazole (HOBT), 0.25% w/v Acid Violet-17 (AV-17), and 0.07% v/v diisopropylcarboiimide (DIEA), made up in DMF. The HOBT acted as a catalyst for the formation of a peptide bond between the activated ester group of the new Fmoc amino acid and the deprotected amine group of the amino acid linked to the soluble support. The AV-17 and the DIEA were included to check completion of the coupling reaction prior to deprotection by spectrophotometric monitoring of the synthetic cycles. The activated Fmoc amino acid was added to the polymer support and allowed to circulate through the reaction column. After subsequent washing away of excess reagent and co-products, the Fmoc protecting group of the new terminal amino acid was removed by treating with piperidine. This cycle was repeated for every amino acid in the chain. Elongation of the peptide chain was performed stepwise until the N-terminal amino acid was reached. Before cleavage of the peptides from the resins, both peptides were extended with two more amino acid residues using the same Fmoc technique. The additional amino acids were glycine, which was used as a spacer, and cysteine. The cysteine residue was added to the amino terminus of the peptides to allow coupling to a protein carrier.

After complete assembly of the extended peptide, final deprotection was carried out using 20% piperidine in DMF. The column was removed from the peptide synthesiser and the contents of the column (peptide-resin) were washed serially with 40ml each of t-amyl alcohol, acetic acid, t-amyl alcohol, dichloromethane and diethyl ether, after which the resin was dried using nitrogen gas.

2.16.2 Cleavage of the assembled peptide from resin and deprotection of side chain protecting groups

The processes of cleavage of peptide from the resin support and removal of side chain protecting groups were combined. The benzyl ester linkage of peptide to resin can be easily cleaved by 90% trifluoroacetic acid (TFA). However this acidic cleavage can lead to the generation of carbocations, which may react with electron-rich amino acid side chains. In order to reduce the potential risks these carbocations posed, carbonium ion scavengers were added to the cleavage reagent. Hence the cleavage reagent was made up of a mixture of 90% TFA, 5% thioanisole, 3% ethanedithiol, and 2% anisole under an atmosphere of nitrogen to prevent oxidation. The required amount of peptide-resin was weighed out and suspended in 1 ml freshly prepared cleavage reagent and allowed to react at room temperature.

In order to test the suitability of the chosen cleavage conditions, the cleavage was first performed on a 50 mg sample of the resin dissolved in 1 ml of cleavage reagent The process was monitored by removal of 200 µl samples at time intervals of 1 hr, 2hr and 4hr for DLD1 and 2 hr, 4hr and 8 hr for DLD2. All the peptidic components were isolated with two rounds of washing with petroleum ether, followed by two rounds of di-ethyl ether washing, and the resulting white precipitates were suspended in 500µl 5% acetonitrile + 1% TFA. Analytical HPLC was then performed on the samples.

Once the optimum conditions for the cleavage and deprotection had been elucidated, the process could be scaled up to a more significant quantity of the peptide-resin (approx. 1g) ie. a preparative run could be performed. The preliminary analytical HPLC run (see chapter 3) showed that DLD1 and DLD2 existed in both monomer and dimer forms and so had to be reduced into the monomer form using DDT (500 µg / ml for DLD1, 250 µg / ml for DLD2) overnight prior to the preparative purification by HPLC.

2.17 REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Analytical HPLC was carried out on a LKB Bromma Analytical HPLC under anaerobic conditions to ensure collection of peptide in the reduced form. Preparative HPLC was performed using a Millipore Waters system. Separations were effected on VYDAL C18 wide pore reversed phase columns (300Å pore size, 5µl particle size). Analytical runs were performed on columns 4.9mm by 25 cm at a flow rate of 0.7ml / min. Preparative HPLC was performed on columns 25mm by 25cm at a flow rate of 10 ml / min using a 0.1%TFA / acetonitrile solvent.

As with the cleavage / deprotection step, a trial run was initially performed to determine the acetonitrile : water ratio at which the peptide peak would elute from the HPLC column. Prior to loading the crude preparation onto the HPLC column, it was filtered to remove any insoluble particles that would disrupt the chromatography and the peptide was eluted from the column with a linear gradient (from 5% to 95%) of acetonitrile in 0.1% TFA. The preparatory HPLC was then run using all of the 5% acetonitrile solvated peptide and when the peak came off, fractions were collected every 20 seconds. Fractions from the start and finish of the peak and two from the middle of the peak were then re-analysed using the analytical HPLC. Clean fractions were pooled and then freeze dried under nitrogen overnight to remove TFA. Rotary evaporation under water pump vacuum without heating was then performed to remove acetonitrile. The dry purified peptide was stored at -20°C.

2.18 PREPARATION OF ANTI-PEPTIDE ANTIBODIES

The extended peptides were coupled to the carrier protein keyhole limpet haemocyanin (KLH).The carrier protein was activated with N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) and subsequently coupled to the peptide through its cysteine residue (Green et al, 1982).

For optimum peptide - carrier coupling to occur, it was essential that the peptide to be coupled had a fully reduced terminal cysteine group, so avoiding the tendency towards dimerisation which was observed in the preliminary HPLC run (chapter 3).This was achieved by purifying both peptides, following reduction with DTT, by HPLC under anaerobic conditions, i.e. using nitrogen purged reagents and performing all operations under nitrogen gas. (section 2.17)

2.18.1 Conjugation method

1 ml KLH was dialysed (20 mg/ml^{-1}) in 10 mM potassium phosphate pH 7.2 overnight against 20 ml 10 mM potassium phosphate pH 7.2 at 4°C. The concentration was adjusted to 16 mg KLH ml^{-1} prior to use. For each peptide, 4 mg KLH in 250µl of 10 mM potassium phosphate buffer (pH 7.2) was reacted with 0.7 mg MBS (dissolved in 50 -70 µl dry DMF) which was added dropwise with stirring for 30 min at room temperature. 300 µl of the reaction product was added to a characterised P-30 Biorad column (1.5 x 13 cm , void volume 3.2 mls), equilibrated with 50 mM sodium phosphate buffer, pH 6.0, to remove free (unreacted) MBS. The KLH - MBS conjugate was recovered by pooling fractions from the first peak of the column eluate (monitored by absorbance at 280nm) and then reacted with peptide (5 mg dissolved in 1 ml phosphate buffer pH 7.2). The pH was adjusted to pH 7-7.5 and the reaction was stirred for 3 hr at room temperature. Solid NaCl was added to a final concentration of 0.9%. The peptide conjugate was stored in 0.5 ml aliquots (1 mg peptide/ml) at - 20°C.

2.19 IMMUNISATION METHOD

A total of four rabbits were used, two for each peptide-carrier conjugate. Each conjugate aliquot (0.5 mls) was mixed 1:1 with an aluminium hydroxide based adjuvant (Pierce Imject Alum) by gently vortexing for 10 min. Rabbits were immunised subcutaneously with 1ml of immunogen (500 µg protein) at 5 sites both for the original injection and for all booster injections according to the following schedule:

day 0	1st injection
week 4	1st booster injection
week 8	2nd booster injection
week 16	3rd booster injection

A test ear-bleed of 10-15 ml was taken 10 days after each boost.

2.20 PREPARATION OF ANTISERA

Fresh rabbit blood was left for 4-5 hr at room temperature, then overnight at 4°C for clotting. The clots were removed (and any free serum collected) and then centrifuged at 2000g for 15 mins at 4°C. Serum so obtained was pooled with the free serum already collected and was given a second centrifuge at 11000g (10,000 rpm) for 15 mins. Serum was collected and stored in aliquots at -20°C.

2.21 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

A standard ELISA assay was performed based on the method of Engvall, (1980). Reagents for the assay were made up as follows:

(i) **Solutions:**

a) Coating buffer : 0.05M sodium carbonate buffer, pH 9.8

b) PBS / tween : PBS with 0.1 % v/v tween-20

c) Acetate buffer : 50 mM sodium acetate/citric acid pH 6

(ii) **Substrate:**

Stock solution: Tetramethyl benzidine (TMB) 10 mg/ml in dimethylsulphoxide (DMSO)

Enzyme substrate solution (freshly prepared prior to immediate use): 50 mM sodium acetate/citric acid pH6 + 0.1 mg/ml TMB (diluted from 10 mg/ml stock) + 0.006% hydrogen peroxide .

(iii) **Enzyme -antibody Conjugate:**

Anti - rabbit IgG-peroxidase. Stock conjugate was stored at -20°C as a 1:1 dilution of commercially obtained antibody in glycerol.

The anti-rabbit IgG peroxidase was used as a suspension in PBS / tween (10 mls of diluted stock antibody conjugate per plate).

In this assay, the antigen was immobilised by direct attachment to a solid plastic 96- well support. Wells were coated with a solution of peptide / protein (10µg protein / ml) in coating buffer (100µl per well) for 18 hrs at 4°C. The wells were washed at room temperature with three successive portions of PBS/tween and then each well

was blocked by incubation with 250-300µl of 1% (w/v) casein in PBS / tween for 1 hr at room temperature followed by two washes with PBS / tween. Serial dilutions of the test antisera in PBS / tween were performed (the highest dilution being 1/50, then 1/100, 1/200 and so on). The plates were incubated for 2 hrs at room temperature and then each well washed three times with PBS / tween. To each well was added 100 µl anti rabbit-IgG-peroxidase conjugate (stock diluted 1 in 1000 in PBS/tween) and the wells were left to incubate for 2 hrs at room temperature. After washing three times with PBS / tween and then twice with PBS, 100µl of the enzyme substrate (TMB) solution was added and the resulting colour allowed to develop for between 5-15 mins at room temperature. The reaction was stopped by adding 50µl of 1.84M sulphuric acid and the colour produced was read using a "Titertek Uniskan" microtitre plate reader with a 405nm filter.

2.22 PREPARATION OF CELLS FOR ENZYME ASSAY

Two 10 -15 ml aliquots of cell suspension were removed into 15 ml falcon tubes and cooled to 4°C on ice for 5 minutes. The cells were then pelleted by centrifugation at 2000 g for 10 minutes. The supernatant was removed and the cells were washed by alternately suspending and pelleting in fresh PBS using a bench microfuge. After washing in PBS was complete, each pellet was suspended in 1ml PBS and the pellets combined in one tube. The number of cells per ml was determined using a haemocytometer and 1 ml aliquots typically containing between $1x10^7$ and $1x\ 10^8$ cells in PBS were placed in plastic eppendorf tubes for sonication.

2.23 PREPARATION OF SONICATED CELL LYSATE

Sonic extracts of procyclic *T.brucei*, *Crithidia fasciculata* and *Phytomonas sp.* were prepared by sonicating cells in eppendorf microfuge tubes for 3 x 30 seconds at 40w using a 3mm probe. Sonication tubes were incubated at 4°C to minimise heating effects, and each 30 second sonication was separated by a 30 second time period to allow maintainance of the temperature at 4°C. Cellular debris, observed as a small insoluble pellet, was removed by microfuging for 5 minutes at 10,000g and the supernatant removed and used for enzyme assay.

2.24 IMMUNOBLOTTING

2.24.1 Blot preparation

Nitrocellulose paper was cut into strips of 4cm by 10cm. The strips were washed by floating in approximately 20 ml of distilled water and then dried at room temperature. The strips were spotted with 5µl of protein (see table 3) and allowed to dry for 30 minutes at room temperature.

Dot No.	*Protein*	*Amount. protein per dot*
1	KLH-peptide	1µg
2	Free peptide	10µg
3	Sonicated cell lysate	5×10^6 cells
4	KLH	1µg

Table 3: Protein antigens used in immunoblot experiments for testing cross-reactivity of raised immune and preimmune sera. Sonicated cell lysates were prepared as detailed in section 2.24

Strips were washed by floating in 20 ml PBS and then incubated with blocking agent (10 % v/v foetal calf serum in PBS/tween) for 2 hrs at room temperature with gentle agitaton. The strips were washed in 20 ml PBS/tween and then incubated with antiserum/pre-immune serum by immersing in 20 mls of serum (diluted 1/100 in PBS/ tween) at 4°C overnight with gentle agitation.. Controls received fresh PBS/tween only. Antibody binding was detected as described below.

2.24.2 Immunological detection of protein blots

The method of detection employed is described by Towbin et al, 1979.

The method used labeled antibodies to increase the size of the immune complex , leading to increased sensitivity. In this case, biotinylated horseradish peroxidase was mixed with avidin (Hsu et al, 1981; Kendall et al, 1983). Because avidin is tetravalent, the biotinylated enzyme and avidin can associate to form large biotin binding enzymatically active complexes. These complexes can then be used to bind to the antigen through a biotinylated anti-immunoglobulin antibody.

Washed electrophoretic blots were soaked in blocking agent (PBS containing 1% v/v Tween-20 and 10% v/v foetal calf serum) for 1 hr at room temperature to saturate additional protein binding sites. The blots were then rinsed in PBS/Tween-20 and incubated with probing antibody (immune serum from rabbits immunised with synthetic peptide DLD1, diluted 1/100 in 0.1 % v/v tween 20/PBS) and then left to incubate overnight at 4° C with constant agitation. Controls were performed using preimmune rabbit serum similarly diluted.

Following this, the strips were washed four times in 20 ml 0.05% PBS/Tween-20, to remove non-specifically bound antibodies or serum proteins, and were then incubated in a recommended (Sigma) dilution (1 in 500 in PBS/tween) of biotinylated anti-rabbit IgG. In parallel with this, the avidin (reagent A) and biotinylated horseradish peroxidase (reagent B) were each dilured 1/500 in PBS/tween and incubated together in a ratio of 1:1 for 1 hour according to Vectastain kit instructions.

Strips were incubated in 20 mls of the resulting avidin-biotinylated Horse radish peroxidase for 1 hr at room temperature. The strips were then washed four times (for 5 min) in PBS / Tween-20 and then once in PBS.

2.25.3 Staining procedure

4-Chloro-1-Naphthol (0.3% (w/v)) in methanol was diluted with PBS, to give a final concentration of 0.06% w/v. 30% hydrogen peroxide (0.5 µl/ml) was added and strips were incubated in the substrate solution. A positive result was indicated by the development of an insoluble blue product spot in the exact location of the origional antigen spot. This was clearly visible within 30 mins of substrate incubation. Colour development was terminated by several washes in 20 ml distilled water followed by 20 ml PBS. Finally, the nitrocellulose pieces were dried between filter papers.

2.25 WESTERN (PROTEIN) BLOTTING

2.25.1 SDS Polyacrylamide Gel Electrophoresis

2.25.1.1 Resolving and stacking gels: Slab gels were prepared by using a vertical slab gel apparatus (Biorad Proteam TMN). The chemical compositions of the

resolving and stacking gels used in the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) are listed in table 3:

Table 4: Chemical compositions of resolving and stacking gels

Chemicals	Resolving gel	Stacking gel (ml)
Acrylamide stock (25% (w/v) in H_2O; 24.4% acrylamide in H_20, 0.6% (w/v) bis acrylamide in H_20	(ml) 16	2
1M Tris/HCl, pH 8.8	15	-
1M Tris/HCl, pH6.8	-	- 1.25
distilled H_2O	7	1.25 6.3

6.3

The above reagents were mixed in the ratios described and then degassed and the following reagents were then added:

	Resolving gel (ml)	Stacking gel (ml)
10% SDS (w/v) in H_2O	0.4	0.1
N,N,N',N'-Tetramethyl-ethylene diamine (TEMED) in H_2O	0.025	0.01
1.5% (w/v) ammonium-persulphate in H_2O	0.9	0.35

The addition of ammonium persulphate and TEMED resulted in polymerisation of the gels.

2.25.1.2 Electrophoresis buffer for SDS-PAGE, contained 0.13 M glycine, 0.025M Tris and 0.1% (w/v) SDS at a final pH 8.3. Approximately 2.5L of buffer were used to run each gel.

2.25.1.3 Sample buffer : 0.125 M Tris-HCl pH 6.8 containing 50% w/v glycerol, 10% (w/v) SDS, 10% (w/v) 2-mecaptoethanol and 0.01% (w/v) bromophenol blue. Samples were diluted in sample buffer and then boiled for 2 minutes to ensure complete denaturation.

2.25.1.4 Protein standards for SDS gels

1. 150 µl sonicated cell lysate (containing $4x10^6$ cells) per lane (section 2.4)
2. Protein MW markers (total of 60 µg protein per lane):

Carbonic anhydrase	29kD
Egg albumin	45kD
BSA	66kD
Phosphorylase B	97.4 kD
Galactosidase	116kD
Myosin	205kD

2.25.2 Running of SDS-PAGE

SDS gels (stacking gel (5% w/v polyacrylamide), resolving gel (10% w/v acrylamide) were prepared according to the method of Laemmli (1970)

Protein samples were prepared in sample buffer and boiled as detailed in section 2.26.1.3 and then loaded onto the stacking gel and run at 70 volts (15 mA/gel) until the bromophenol blue dye bands reached the resolving gel. The voltage was then increased to 170 volts (30mA/gel) for the remainder of the run ie. until the bromophenol blue reached the bottom of the gel.

2.25.3 Electroblotting

Electrophoresis was used to transfer the proteins from the polyacrylamide gels to nitrocellulose sheets (Tsang et al., 1983)

The gel was placed on top of a sheet of filter paper (Whatman, 3 mm) already saturated in blotting buffer (25 mM Tris buffer, pH8.3, containing 192 mM glycine and methanol to a final volume of 20%). A nitrocellulose sheet, which was also saturated in blotting buffer, was then placed on top of the gel, followed by a second filter paper sheet. This sandwich was placed between two sponge pads and supported between parallel electrodes in a reservoir tank filled with blotting buffer (Bio Rad transblot vertical tank). The sandwich was positioned so that the nitrocellulose sheet was

between the gel and the anode. A current of 30 volts (0.1 amps) was passed through overnight at 4 °C , resulting in the transfer of proteins from gel to nitrocellulose sheet.

2.25.4 Staining of proteins on nitrocellulose paper using Ponceau S stain

Following the transfer of proteins, the nitrocellulose sheet was stained with Ponceau S to facilitate visualisation of the transferred protein bands (Klein et al , 1995). Electrophoretic blots were washed with PBS, pH 7.3 for 15 mins, then rinsed twice with PBS and stained in Ponceau S (0.1% (w/v) in trichloroacetic acid) to visualise the transferred bands which appeared within 20 mins and the nitrocellulose sheet was cut into appropriate sections. The nitocellulose blots were then quickly washed four times with 20 ml PBS (approximately 5 mins each wash) to remove the stain. Immunological detection of protein blots was performed as detailed in section 2.24.2.

2.26 IMMUNOFLUORESCENCE

The entire procedure was carried out employing sterile technique and using sterilised materials and was based on the method detailed by Sherwin et al (1987).

13 mm diameter glass coverslips were placed in plastic welled plates. The coverslips were prepared by coating with poly-L-lysine solution (5 μg/ml in 100 mM borate buffer, pH 8.2) for one and a half hours at room temperature. *T.b.brucei* procyclic cells, grown to a cell density of $3x10^6$ cells / ml were harvested by centrifugation at 2000g in a bench centrifuge for 3 minutes. Cells were washed twice in PBS by low speed centrifugation and the cells suspended in PBS to give a final cell density of 3×10^6 cells/ml PBS. 250 μl of the trypanoso`mal cell suspension was added to each well containing a polylysine-coated coverslip and the cells were allowed to settle for 1 hr at 25 °C.

The cells, now bound to the coverslips, were then fixed in methanol at -20 °C for 1 hr, after which they were rehydrated by washing three times in PBS (0.5 mls per well). Cells were blocked using 0.5 mls 10% v/v FCS in PBS/tween per well for 1 hr at room temperature to reduce any non-specific serum protein binding and then incubated with various dilutions of antiserum / preimmune serum (in PBS/tween) for 1 hr at 26 °C. The coverslip-containing wells were then washed three times with PBS/tween (10

mins per wash). Secondary antibody, goat antirabbit-FITC conjugate , diluted to an appropriate working dilution (determined to be 1/100) in PBS/tween was added to the cells (250 µl per well) for 1 hr at room temperature. Slides were washed three times in PBS (10 mins per wash) before adding 10 µl of Vectorshield mounting fluid. Coverslips were then viewed using an inverted fluorescent microscope.

2.27 ISOLATION OF CRUDE PLASMA MEMBRANE FRACTIONS FROM *CRITHIDIA FASCICULATA* AND *PHYTOMONAS SP.*

Many of the techniques that are used for the isolation and characterisation of plasma membranes have been reviewed by DePierre and Karnovsky (1973). The most critical part of the preparative procedure has been found to be at the cell homogenisation step (Voorheis et al, 1979) where it was important to lyse cells while retaining the microtubule-pellicular membrane association characteristic of trypanosomatid flagellates. Various methods of trypanosomal cell disruption have been reported in the literature, although no one type of method has been shown to be satisfactory. For example osmotic lysis yielded resealed 'ghosts' (Voorheis et al, 1979) with many trapped organelles that made further fractionation difficult, while even mild sonication gave rise to small vesicles that lacked the characteristic morphological feature (microtubular array) of plasma membranes in trypanosomes. The homogenisation procedure employed in this study to prepare plasma membrane sheets used a combination of two different techniques, osmotic stress and mild mechanical force. The procedure involved cell swelling followed by manual grinding with silicon carbide in a pestle and mortar as described by Toner and Weber (1972).

2.27.1 General procedure

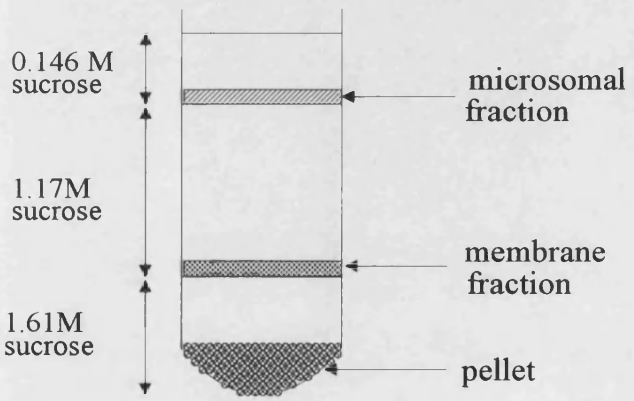
The plasma membrane isolation process employed in this study was based on that developed by Dwyer et al (1980) and Voorheis et al (1979) and culminated in the isolation of a plasma membrane band by discontinuous density-gradient ultracentrifugation

C. fasciculata and *Phytomonas sp.* cells, at a cell density of 1.5×10^8 cells per ml, were rapidly chilled on ice and then harvested by centrifuging for 10 minutes at

1000g. Cells were washed twice in ice cold PBS , centrifuging for 8 minutes at 1600g between washes. The washed, pelleted cells were weighed and resuspended in 1 ml chilled isolation buffer (tris-EDTA buffer (pH 7.8) containing leupeptin (2μg/ml) and PMSF (1mM)) and then transferred to a chilled mortar. Chilled silicon carbide abrasive grain was added to the mortar (approximately 60:40 gm carbide/ gm wet weight) and the cell paste was ground manually using a chilled pestle until light microscopic examination showed that approximately 90% or more of the cells were lysed (Opperdoes et al, 1977). The suspension was diluted with 5 ml chilled isolation buffer and spun for 3 minutes at 120 g to remove the carbide. The supernatant was carefully removed and centrifuged for a further 10 minutes at 4,000 g at 4 °C. The pelleted material was suspended in 6 ml 0.146 M sucrose solution (dissolved in tris-EDTA containing 1 mM PMSF) and the pellet disrupted by 2 passages through a 22 gauge needle. The suspension was then overlaid onto a discontinuous sucrose gradient consisting of a 1.61 M sucrose cushion (5ml) and a 1.17M sucrose layer (15 ml), and spun in a Beckman L5-50B ultracentrifuge at 27,000 rpm (4 °C), for 2 hours using an SW27 rotor (Dwyer, 1980)

A dense 'plasma membrane' band formed at the 1.61 M / 1.17M interface (Fig. 7) and was collected by careful removal of gradient layers using a pipette. This was diluted to approximately 10% (v/v) sucrose with Tris-EDTA buffer and ultracentrifuged for a further 2 hours at 27,000 rpm. The resulting pelleted membranes were washed twice in PBS by centrifugation as described above and finally suspended in 1 ml PBS for analysis.

Fig. 7 Isolation of plasma membrane fraction on discontinuous sucrose gradient



2.27.2 Protein determination of isolated plasma membrane fractions

Protein was determined by Peterson's modification of the micro-Lowry method (Peterson, 1977).1 ml deoxycholate solution (1.5 mg per ml in double distilled water) (see materials, section 2.10.1) was added to tubes containing protein standard solutions or protein sample diluted to 1 ml with double distilled water. Tubes were mixed well and allowed to stand for 10 mins at room temperature. Precipitation of the trypanosomal proteins with 7.2% (w/v) trichloroacetic acid was followed by the resuspension of the precipitated pellet in 1 ml Lowry reagent solution (see materials, section 2.10.1). After a 20 min incubation at room temperature, 0.5 ml Folin and Ciocalteu's Phenol reagent was added to each tube. Colour was allowed to develop for thirty minutes and absorbance readings taken at 750 nm. Readings were completed within 30 mins. Bovine serum albumin (Fraction V, Sigma) was used as a protein standard and sample protein concentrations determined from a standard calibration curve performed each assay.

2.27.3 Enzyme Assays

2.27.3.1 Dihydrolipoamide dehydrogenase (DHlipDH)

DHlipDH was assayed by following the oxidation of dihydrolipoamide to lipoamide, with the concurrent reduction of NAD^+ to NADH, resulting in an increase in absorbance at 340 nm.

$$\text{dihydrolipoamide} + NAD^+ \xrightarrow[\text{dehydrogenase}]{\text{dihydrolipoamide}} \text{lipoamide} + NADH + H^+$$

The assay took place in a 1 ml cuvette containing 50 mM potassium phosphate (pH 7.0) , 2 mM EDTA , 0.14 mM dihydrolipoamide, 1 mM NAD^+ and 0.1% triton x-100. The assay was started with the addition of 5 - 100 µl enzyme-containing sonicated cell lysate (depending on the particular experiment) and progress was monitored by the increase in absorbance at 340 nm.

2.27.3.1.1 'No dihydrolipoamide' controls

Control assays were routinely performed in the absence of dihydrolipoamide substrate. Any observed increase in absorbace at 340nm, due to endogenous enzyme activity, was subtracted from experimental rates.

2.27.3.1.2 Alcohol dehydrogenase control

Alcohol dehydrogenase activity was assayed for in 50 mM potassium phosphate buffer pH 7, 2mM EDTA, 1mM NAD and 20µl of absolute ethanol in a final volume of 1 ml.

2.27.3.2 NADH oxidising activity

A NADH oxidase assay was performed on both *Phytomonas sp.* and *Crithidia fasciculata* cell sonicates to ensure that any enzyme activity, observed as a decrease in A_{340}, was due to the activity of the enzyme of interest as opposed to endogenous NADH oxidase activity. The assay was carried out by the addition of sonicated cell extract to a 1ml cuvette containing 50 mM potassium phosphate (pH 7.0) , 2mM EDTA and 0.14 mM NADH. Any endogenous NADH oxidase activity was detected by a decrease in absorbance at 340 nm.

2.27.3.3 Marker enzyme assays to determine purity of plasma membrane fractions

Fractions taken from the plasma membrane isolation procedure were originally to be assayed for organelle marker enzymes to determine the purity of the plasma membrane fraction. The enzymes to be assayed were NAD-dependent isocitrate dehydrogenase (mitochondrial marker), NAD-dependent glycerol - 3 - phosphate dehydrogenase (glycosomal marker), pyruvate kinase (cytosolic marker) and 5'nucleotidase (plasma membrane marker).

Preliminary enzyme assays were performed on *Phytomonas sp.* and *Crithidia fasciculata* cell sonicates to determine the suitability of each enzyme assay as an organelle marker in these cell types.

All assays were carried out at 30°C, in the presence and absence of 0.1 % (w/v) Triton X-100 and initiated by addition of sample to the reagents at the concentrations below. All samples were assayed for background and endogenous rates, which were subtracted where necessary. Control NADH oxidase assays were performed where indicated.

A unit of enzyme activity is defined as that amount that catalyses the conversion of 1 µmole of substrate per minute at 30 °C .

2.27.3.3.1 NAD-dependent Isocitrate dehydrogenase (ICDH)

Isocitrate was reduced by the activity of ICDH in the presence of NAD^+ producing 2-oxoglutarate and NADH. The subsequent rise in absorbance at 340 nm allowed the reaction to be followed.

$$\text{isocitrate} + NAD^+ \xrightarrow[\text{dehydrogenase}]{\text{isocitrate}} \text{2-oxoglutarate} + NADH + H^+$$

100 µl sample was added to a 1 ml cuvette containing 0.2 mM NAD, 5 mM DL-isocitrate and made up to 1 ml with TME buffer (20 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA) pH 8.

2.27.3.3.2 NAD - dependent glycerol - 3 - phosphate dehydrogenase (G-3-P DH) (Panes,1988)

Dihydroxyacetone phosphate (DHAP) was reduced by the activity of G-3-P DH in the presence of NADH producing α-glycerol phosphate and NAD^+. The subsequent loss in absorbance at 340 nm allows the reaction to be followed.

$$DHAP + NADH + H^+ \xrightarrow[\text{dehydrogenase}]{\text{L-glycerol-3-phosphate}} \text{glycerol phosphate} + NAD$$

5-50 µl sample were added to a 1 ml cuvette containing 0.14 mM NADH, 1 mM dihydroxyacetone phosphate and made up to 1 ml with 50 mM TEA (pH 7.6).

2.27.3.3.3 Pyruvate kinase (Bergemeyer, 1974)

Pyruvate released by the activity of pyruvate kinase was reduced to lactate by lactate dehydrogenase, whilst NADH was oxidised to NAD^+. The reaction was followed by the loss in absorbance at 340nm.

$$\text{Phosphoenolpyruvate} + ADP \xrightarrow[\text{kinase}]{\text{pyruvate}} \text{pyruvate} + ATP$$

$$\text{pyruvate} + NADH + H^+ \xrightarrow[\text{dehydrogenase}]{\text{lactate}} \text{lactate} + NAD$$

5 - 25 µl sample was added to a 1 ml cuvette containing 0.5 mM PEP, 5 mM ADP, 100mM potassium chloride, 5 mM magnesium chloride, 0.14 mM NADH, 50µg lactate dehydrogenase and made up to 1 ml with 50 mM TEA (pH 7.6).

2.27.3.3.4 5' nucleotidase assay

5' nucleotidase activity of *Crithidia fasciculata* whole cell sonicate and isolated plasma membrane was determined using tracer amounts of radio-labeled 5'-AMP in an assay method adapted from that of Avruch and Wallach (1971). Following incubation with reaction mixture containing [^{3}H]AMP, hydrolysis of the radiolabeled nucleotide was estimated by measuring released radiolabeled adenosine following addition to the reaction mixture of $ZnSO_4$ and $Ba(OH)_2$. The reaction was carried out at pH 9.0 to inhibit acid phosphatase activity, while the incorporation of β-glycerophosphate, an alkaline phosphatase substrate, into control assays provided a control by which the extent of any nonspecific alkaline phosphatase hydrolysis of AMP could be gauged

Working substrate solution was freshly prepared each assay and comprised 60 mM piperazine - HCl buffer (pH 9.0) containing 0.1 % Triton X-100, 24 mM magnesium chloride hexahydrate, 12 mM β-glycerophosphate (to inhibit non-specific alkaline phosphatases), 0.12 mM non-labelled AMP (Boehringer Mannheim) and 1.3 µl stock [^{3}H] AMP solution per ml working substrate solution.

Sonicated cell extract (10µl) was added to 0.5 mls of working substrate solution for various time periods (see individual experiments in results section) at 37°C. The reaction was stopped by addition of 0.2 ml of 0.15M zinc sulphate. Samples were vortexed and the tubes placed on ice. Three 10 µl samples were taken and counted in optiphase (for determination of a total cpm value). 0.5 mls of 0.15 M barium hydroxide solution was added and the samples vortexed again. After standing on ice for 30 minutes, the tubes were microfuged at 13,000 rpm at 4°C for 10 minutes. Three 10 µl aliquots of supernatant (containing the reaction product, free [^{3}H] adenosine) were taken and [^{3}H] counted in optiphase as previously described. The release of adenosine was calculated in relation to the measured [^{3}H]activity of the working substrate solution.

Various modifications of this basic procedure were carried out as detailed in the results section.

2.27.4 Preparation of plasma membranes for examination by electron microscopy

The trypanosome plasma membranes were fixed with both osmium tetroxide and glutaraldehyde. Osmium tetroxide was used primarily to bind lipid double bonds and hence cross-link neighbouring lipid molecules, but also to fix proteins via sulphydryl groups and side chain amino groups.

Glutaraldehyde was used for more efficient fixation of the plasma membrane proteins via side chain amino groups. More importantly glutaraldehyde preserved microtubules (Weakley, 1981) important for plasma membrane identification.

Lead acetate was used to increase the contrast of the plasma membranes by binding the reduced osmium and also sulphydryl, tyrosyl, carboxyl (anion form) and ionised phosphate groups. Uranyl acetate was used to bind phosphate and carboxyl groups and was particularly useful in visualising the plasma membranes (Hayat, 1981). Cacodylate buffer replaced phosphate buffer to prevent precipitation of lead citrate stain.

The washed plasma membrane pellet, obtained following sucrose density gradient centrifugation, was immediately fixed in 4% glutaraldehyde overnight and then processed according to the schedule in Fig.8 (over) prior to examination under the electron microscope. When required, plasma membrane sheets were collected at 7,500g, 30 seconds, throughout the procedure. Thin sections were stained 45 minutes in uranyl acetate, followed by 4 minutes in lead citrate.

2.27.5 Determination of the Michaelis Menten constant and maximum velocity of the plasma membrane associated DHlipDH in *Phytomonas sp.*

Cuvettes were prepared containing 50 mM potassium phosphate buffer (pH 7.2) with excess NAD (10 mM) , 0.1 % triton and various concentrations of dihydrolipoamide (DHlip) (0.5 - 0.05 mM). 50 µl of sonicated cell extract was added to each cuvette (each containing a different DHlip concentration) to give a final volume of 1ml. Assays was performed in duplicate at 30°C as detailed previously.

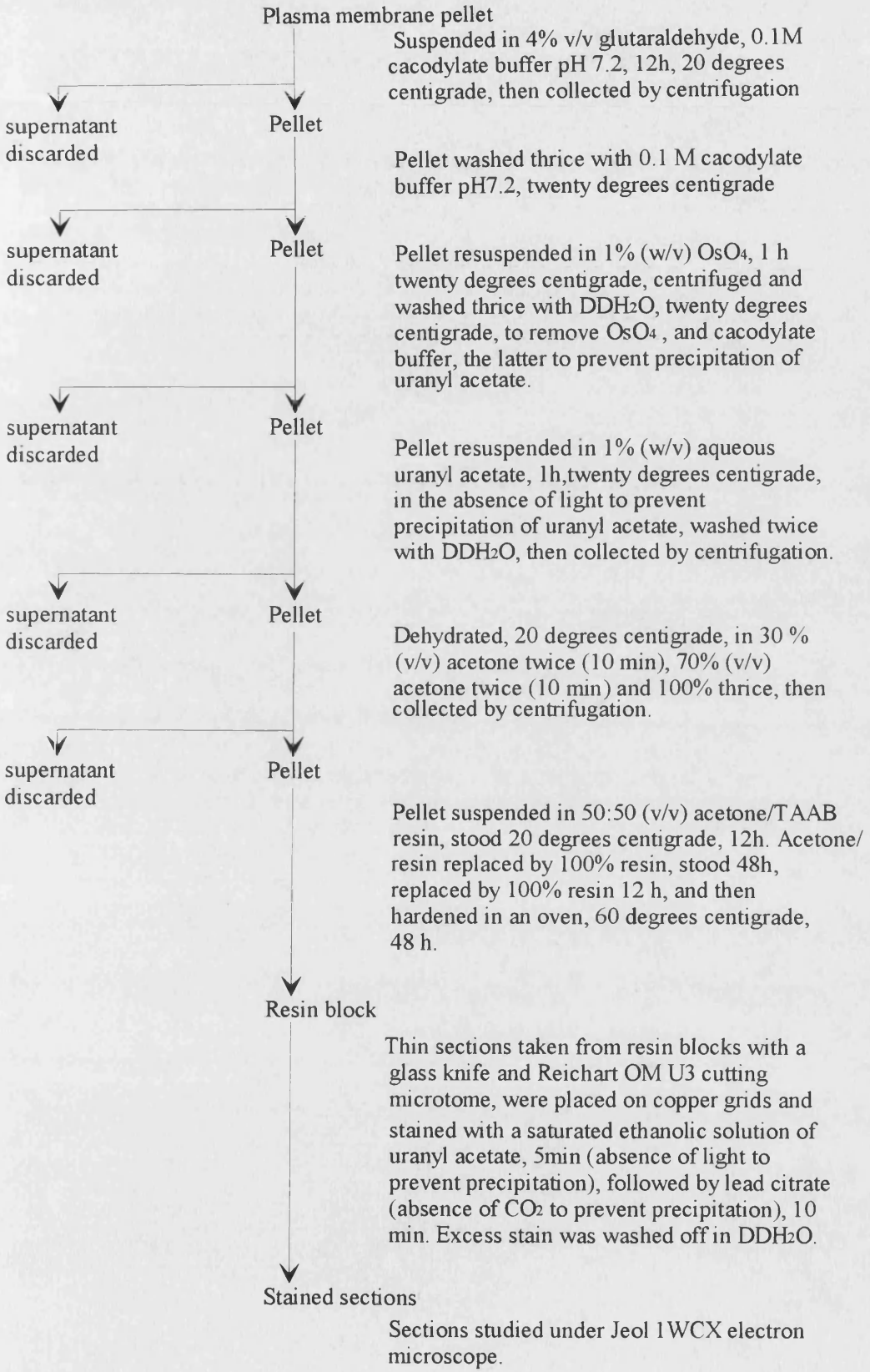


Figure 8: Processing of *Crithidia fasciculata* and *Phytomonas sp.* plasma membrane isolates prior to electron microscopic examination

CHAPTER 3

CHAPTER 3 (A): SELECTION AND SYNTHESIS OF PEPTIDE ANTIGENS

3.1 Prediction Of The Epitopes Of DHlipDH

3.1.1 Introduction

Recently, as more DNA sequences and their corresponding protein sequences have become known, synthetic peptides have been used to prepare antibodies specific for previously uncharacterised proteins. Peptides are normally synthesised using the solid-phase techniques pioneered by Merrifield (1963).The synthetic peptides are purified and coupled to carrier proteins, and these conjugates are then used to immunise animals. Peptide-carrier conjugates seldom fail to elicit a response because of tolerance. Consequently, the peptides can usually be seen as epitopes, and high-titred antisera commonly are prepared. Characteristically, these antibodies will bind well to denatured proteins, but may or may not recognise the native protein.

Assays that need or benefit from anti-native antibodies will succeed only when the peptide sequence is displayed on the surface of the native molecule in a conformation similar to the peptide-carrier conjugate. Therefore, the successful production of anti-peptide antibodies is often determined by the ability to predict the location of certain peptide sequences in the three-dimensional structure of the protein.

3.1.1.1 Choosing the appropriate peptide sequence

Much work has been undertaken to find a predictive approach for locating the sites on a protein which are antigenically important. Following correlation studies of known protein antigenic structures (found by experimental means) with various physical parameters, using computer analysis, selection of peptides may now be based on the use of algorithms that predict potential antigenic sites. These predictive methods are, in turn, based on predictions of hydrophilicity, surface probability, flexibility, and secondary structure.

3.1.1.1.1 Hydrophilicity

Early work suggested that peptides containing hydrophilic amino acids (Hopp and Woods 1981,1983; Kyte and Doolittle 1982) and proline residues were more likely to be exposed on the surface of the native protein than other sequences, and many peptides have been prepared using these criteria Each amino acid has been assigned a numerical value (hydrophilicity index) based on the polarity of its side chain with the data largely coming from the solvent parameters of Levitt, 1976. Repetitively

averaging these values for overlapping sets of 6 residues along the chain and plotting them against the residue number of the leading residue gives a hydrophilicity profile, where a positive value indicates an above average hydrophilicity.

However, although hydrophilicity is required, it is not sufficient to predict the surface location of a particular sequence. Many strongly hydrophilic amino acid sequences are buried in water pockets or form inter- or intramolecular bonds and are thus excluded from interactions with anti-native antibodies

3.1.1.1.2 Surface probability (accessibility)

Molecular surface calculations can be performed on a protein of known structure by mathematically rolling a solvent water molecule over the van de Waal's surface of the protein (Hubbard and Ivatt, 1981). Scales of accessibility are based on the study of proteins of known three-dimensional structure, and are constructed by measuring the accessible surface of all the residues in a number of proteins.

3.1.1.1.3 Side-chain Flexibility

In view of the observed link between antigenicity and segmental mobility (Westhof et al, 1984), Karplus and Shultz developed a method for predicting mobility of protein segments on the basis of the known temperature B factors of the α-carbons of 31 proteins of known structure. Amino acids could be separated into 2 classes comprising 10 flexible and 10 rigid residues. The rigid residues (Ala, Leu,His,Val,Tyr,Ile,Phe,Cys,Trp, and Met) possessed average B values lower than 1. The authors derived three scales for the amino acids called , BNORM0,BNORM1, and BNORM2 corresponding to different degrees of rigidity in the neighbouring residues. BNORM0 is the scale that applies when none of the neighbouring residues is rigid; BNORM1 is the scale applying when one neighbouring residue is rigid while BNORM2 applies when the two neighbours are rigid. As a result, the prediction takes into account not only the propensity of single residues to rigidity, but also the flexible nature of a stretch of residues.

3.1.1.1.4 Secondary structure

Scales of secondary structure are based on the prediction of turns and loops obtained from statistical analysis of proteins of known structure. Since the knowledge of the secondary structure of a protein is very useful for predicting antigenicity, a number of algorithms for predicting secondary structure have been applied to the prediction of epitopes.

The core of proteins usually contains a combination of helices and sheets, which are hydrophobic and invariably non antigenic. In contrast, loops and turns are

accessible and hydrophilic which are two characteristics of antigenic regions (Rose et al 1985). Loops are also flexible, as judged from the blurred electron density maps often observed around those regions.

Two main algorithms for predicting two-dimensional structure exist, namely those proposed by Fasman (1989) and Garnier et al (Garnier et al 1978). Tests made to assess the success rate of those two prediction algorithms show that, at most, 55-70% of two-dimensional elements are successfully predicted.(Kabsch and Sander 1983; Fasman, 1989; Rooman and Wodak 1988).

The premise is that an antibody raised against a peptide from a surface area or a flexible area of the protein is more likely to cross-react with the native protein. Thus , the objective is to choose a region of the protein that is:

1. surface exposed, e.g. a hydrophilic region
2. conformationally flexible relative to the rest of the structure e.g., a loop region or a region predicted to form a β-turn.

3.1.2 Results

A variety of predictive methods, developed by the authors quoted, have been used in this work to determine potential antigenic and immunogenic peptide sequences of bloodstream *T. brucei* DHlipDH. All prediction profiles for DHlipDH were obtained using PREDICT7, a programme developed by Carmenes et al (1989). This programme analyses hydrophilicity, surface probability, side chain flexibility, and secondary structure.(Fig. 9)

3.1.2.1 Hydrophilicity

It can be seen from the results shown in Fig.9 that the top seven peaks of hydrophilicity occur at residues 56-66, 94-99, 115-122, 138-142 , 236-240 , 337-340 and 375-382. It should be noted that, according to Hopp and Woods, only the highest peak of hydrophilicity was invariably found to correlate with antigenic sites in the model protein system they used. For bloodstream trypanosomal DHlipDH, the highest peak of hydrophilicity occurs between residues 236-240 (Fig. 9).

3.1.2.2 Surface probability (accessibility)

Seven major peaks are observed for surface probability occurring at residues 93-100, 137-142, 180-184 ,234-242, 286-288 ,335-338 , and 413-417.

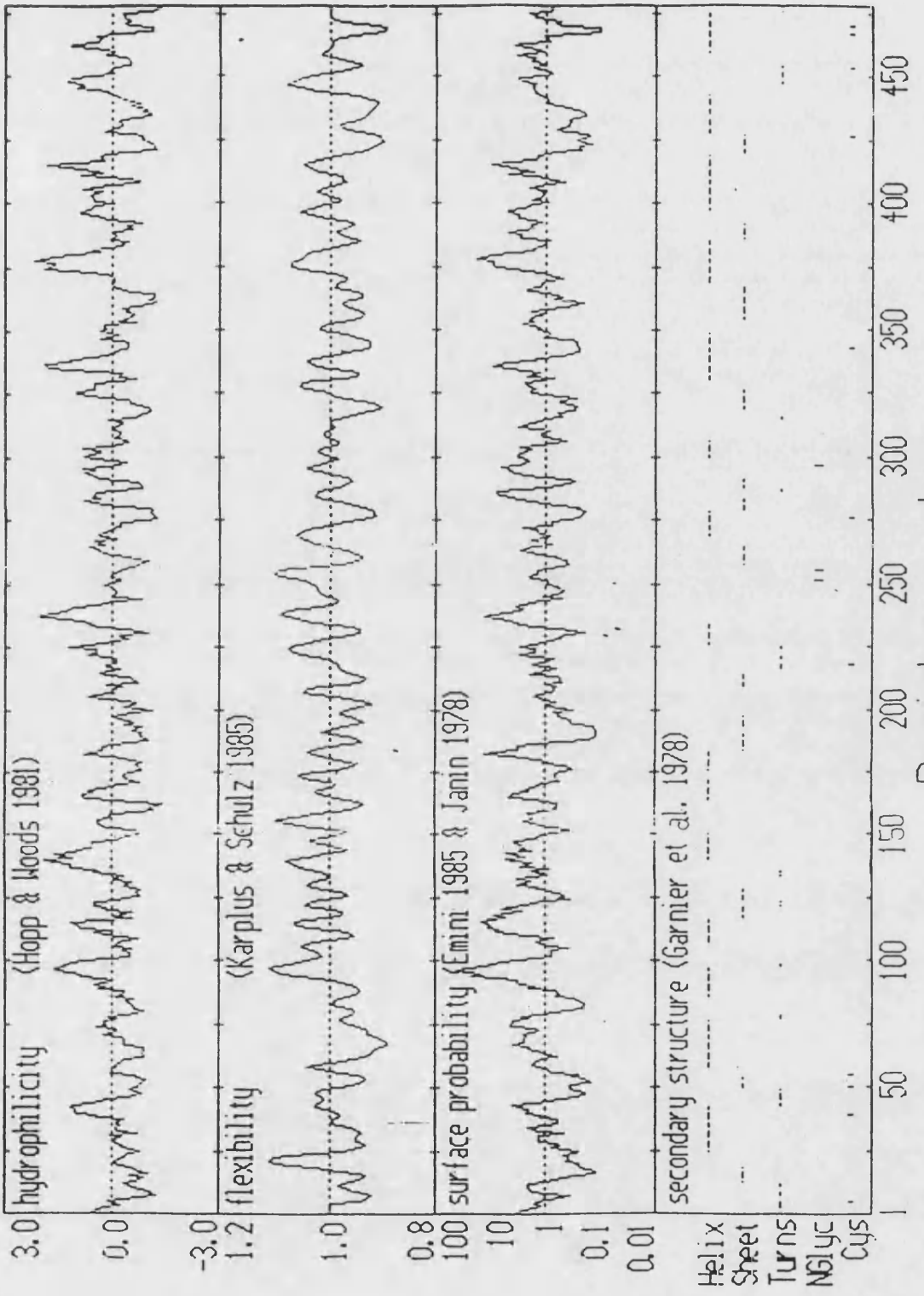


Figure 9: A prediction profile for potential antigenic peptide sequences from bloodstream *T.brucei* DHlipDH determined by author using b.s *T. brucei* DHlipDH primary sequence data (Else et al, 1993) and PREDICT7, a programme developed by Carmenes et al (1989).

3.1.2.3 Side chain flexibility

The scale of Karplus and Shultz was used to construct a flexibility profile (Fig. 9). Six major peaks are observed for side chain flexibility occurring at residues 19-22, 95-98, 137-141, 155-158, 235-241, 254-257, 375-379 and 447-449.

3.1.2.4 Secondary structure

Because turns and loops are both accessible and hydrophilic, two characteristics of antigenic regions, and are very often flexible, it was decided to predict regions of the primary sequence corresponding to a turn conformation according to Garnier et al (1978). Nine regions are predicted to have a turn conformation, namely residues 1-10, 43-49 , 77-82 , 98-104, 133-136, 216-225, 236-240, 286-289, and 448-459

3.1.3 Conclusions

Four different predictive approaches, developed by the referenced authors, have been applied in this work, using the PREDICT7 programme, to compute the likely antigenic sites present on bloodstream *T.brucei* DHlipDH.

Predictions made by all four methods suggest that a region contained within residues 228-242 is likely to be an antigenic site. One further sequence (residues 130-144) is predicted by methods a, b and c, and is in a different region of the DHlipDH molecule..

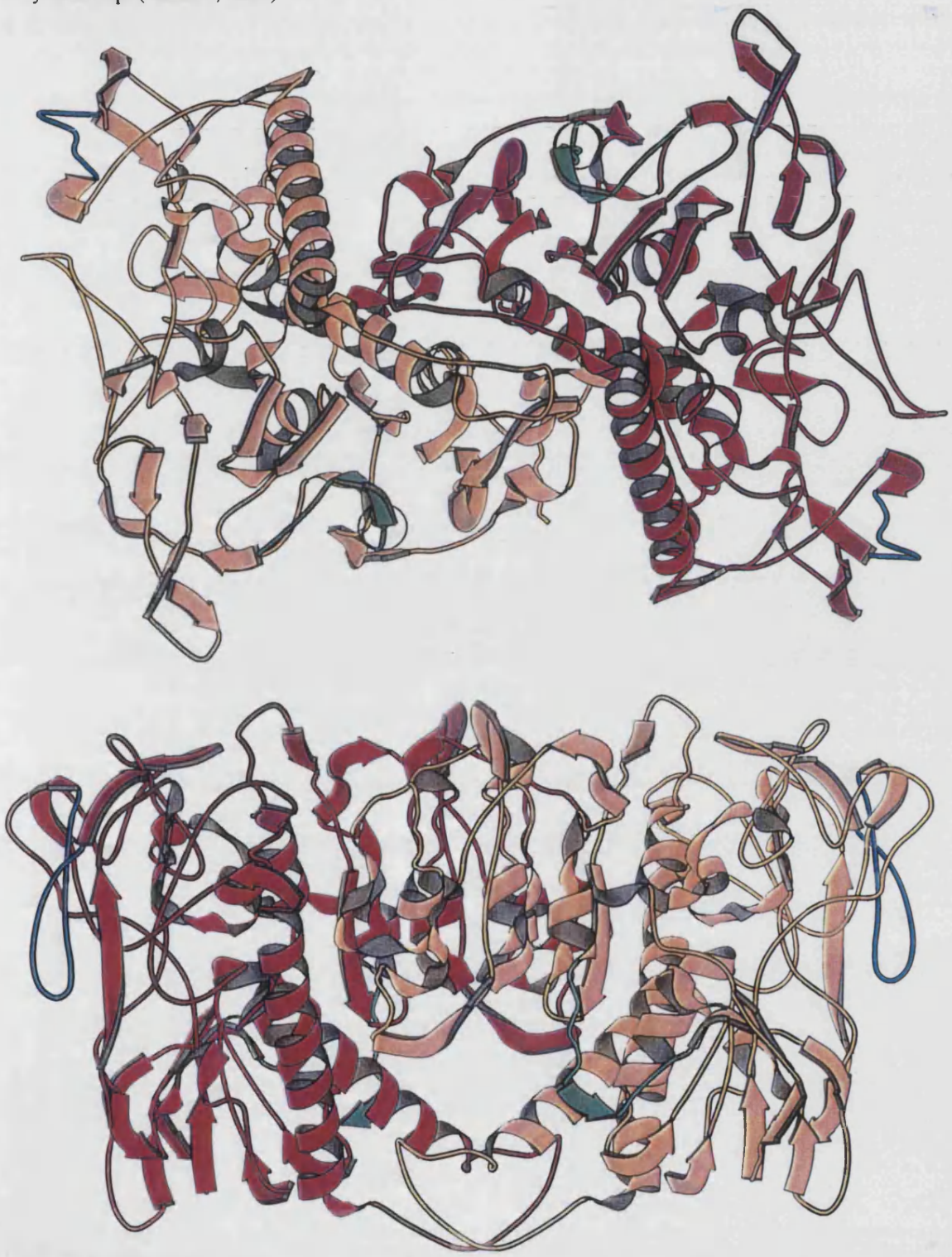
The positions of both peptides were checked on a crystal model of *Pseudomonas fluorescens* DHlipDH by Dr. Rupert Russell, Department of Biology and Biochemistry, University of Bath.. This was the most accurate model available with a sequence identity of around 47% and a sequence similarity of 68%. Both sequences corresponded to surface loops on the crystal structure, so confirming their predicted position. Sequence alignments were performed by Dr. Russell to ensure that the two sequences were not homologous with trypanothione reductase (a very closely related enzyme in the cytosol of *T. brucei*) or rabbit mitochondrial DHlipDH (a high sequence identity may mean that rabbits would fail to raise an immune response). No significant identity was found for either peptide.

At a later date, a homology model of bloodstream *T.brucei*, constructed by Simon Wagstaff, was made available. This homology model confirmed a surface location for both peptide sequences (Fig. 10), so strengthening the possibility of both sequences occurring on the surface of the native DHlipDH protein.

Figure 10

Orthogonal views of the homology model of bloodstream *T.b.brucei* DHlipDH

Constructed by Simon Wagstaff, Department of Biology and Biochemistry, University of Bath. Putative epitopes DLD1 and DLD2 are highlighted in blue and green respectively. Figure is generated by molscript (Kraulis, 1991)



Both peptides were synthesised, purified and used as both antigens and immunogens in this study. They were delegated short names (DLD1 and DLD2), as detailed previously (section 2.16)

3.2 Peptide Synthesis

3.2.1 Introduction

Peptide synthesis was performed on a Milligen 9050 Pepsynthesiser, using the fluorenylmethoxycarbomyl-polyamide solid phase approach to peptide synthesis developed by Atherton and Sheppard (1987). The principle is the same as that first proposed by Merrifield (1963) i.e. that a growing peptide chain is extended while it is attached to a stable solid particle to which it remains attached throughout all the synthetic steps.

3.2.2 Results

3.2.2.1 Synthesis

Both peptides, DLD1 (residue no. 130-144) and DLD2 (residue no.228-242) were successfully synthesised (as detailed in section 2.16) and the yield of peptide estimated to be the final weight of resin after all the synthetic steps minus the initial weight of the resin used. Therefore for the 1g of resin used initially for both peptides, DLD1 yielded a final mass of 0.8g and DLD2 yielded a final mass of 0.76g.

3.2.2.2 HPLC analysis and purification

A small scale trial cleavage and deprotection was performed on a 50 mg sample of peptide / resin prior to full scale preparation. The progress of the reaction was monitored by taking 200 µl aliquots of sample at various time intervals (see section 2.16.2). The peptide component was isolated and 100 µl used for analytical reverse-phase HPLC chromatography on a LKB Bromma Analytical HPLC.

3.2.2.2.1 Analytical trace for DLD1 (Fig. 11)

The trace shows the presence, after one hour cleavage and deprotection, of three main peaks, eluting at 26%, 36% and 44% acetonitrile respectively (Fig. 11 (a)). There are some minor peaks occurring at low acetonitrile concentrations (between 12 -18%) and these are probably due to peptide that is not fully assembled. The first major peak at 26% acetonitrile corresponds to the fully assembled peptide. The peak at 36% elutes at a higher acetonitrile concentration (and is therefore more hydrophobic in nature) and decreases with incubation time, being at its lowest after 4 hrs incubation (Fig.11 (b), Fig.11 (c)). This suggests that this peak is probably caused by peptide still with hydrophobic deprotection groups attached. The peak decreases in quantity with time as the protection groups are finally cleaved and removed by scavengers, leaving the fully deprotected peptide after 4 hrs incubation with cleavage reagent (Fig. 11 (c)).

The peak at 44% acetonitrile increases with time until it is present in equal concentration to the peak at 26% (fully assembled peptide) indicating the possibility of dimerisation. This is confirmed by the trace of incubation with DTT overnight (Fig.11(d)). The peak at 44% acetonitrile is completely absent while the peak at 26% is considerably larger, confirming the peak at 44% to be a peptide dimer which is reduced to the monomer form (26%) following reduction with DTT.

3.2.2.2.2 Analytical trace for DLD2 (Fig. 12)

The trace shows the presence, after two hours cleavage and deprotection, of three main peaks, eluting at 27%, 36% and 45% acetonitrile (Fig.12 (a)). As before, the peak at 36% is eluted at a higher acetonitrile concentration and also decreases in size with time suggesting that this peak corresponds to peptide still with hydrophobic deprotection groups attached. The peak is completely absent in the sample taken after 8 hrs (Fig 12 (c)), indicating that full cleavage of protection groups occurs after 8 hrs incubation with cleavage reagent. The peak at 45% acetonitrile remains fairly constant throughout the incubation and is due to an impurity. The peak eluted at 27% acetonitrile does not diminish but increases with time strongly indicating that this peak corresponds to the fully deprotected peptide. The trace (Figs 12 (a) - (c)) clearly shows the emergence of a second peak (eluted at 34% acetonitrile) with time until eventually after eight hours it is present at the same level as the first peak. This indicates the possibility of peptide dimerisation and this is confirmed by the trace of crude peptide incubated with DTT for 8 hrs (Fig. 12 (d)). Only the peak at 27% acetonitrile is observed while the peak at 34% acetonitrile is completely absent, indicating that it has been fully reduced by the DTT. This shows that even at extremely low pH the peptide is able to exist in both a monomer and a dimer form.

3.2.3 Conclusions

DLD1 has an optimum cleavage time of 4 hrs while DLD2 has an optimum cleavage time of 8 hrs. Both peptides are capable of rapid dimerisation and consequently reduction of the peptides to their monomer forms using DTT was essential before a larger scale preparative run could be undertaken.

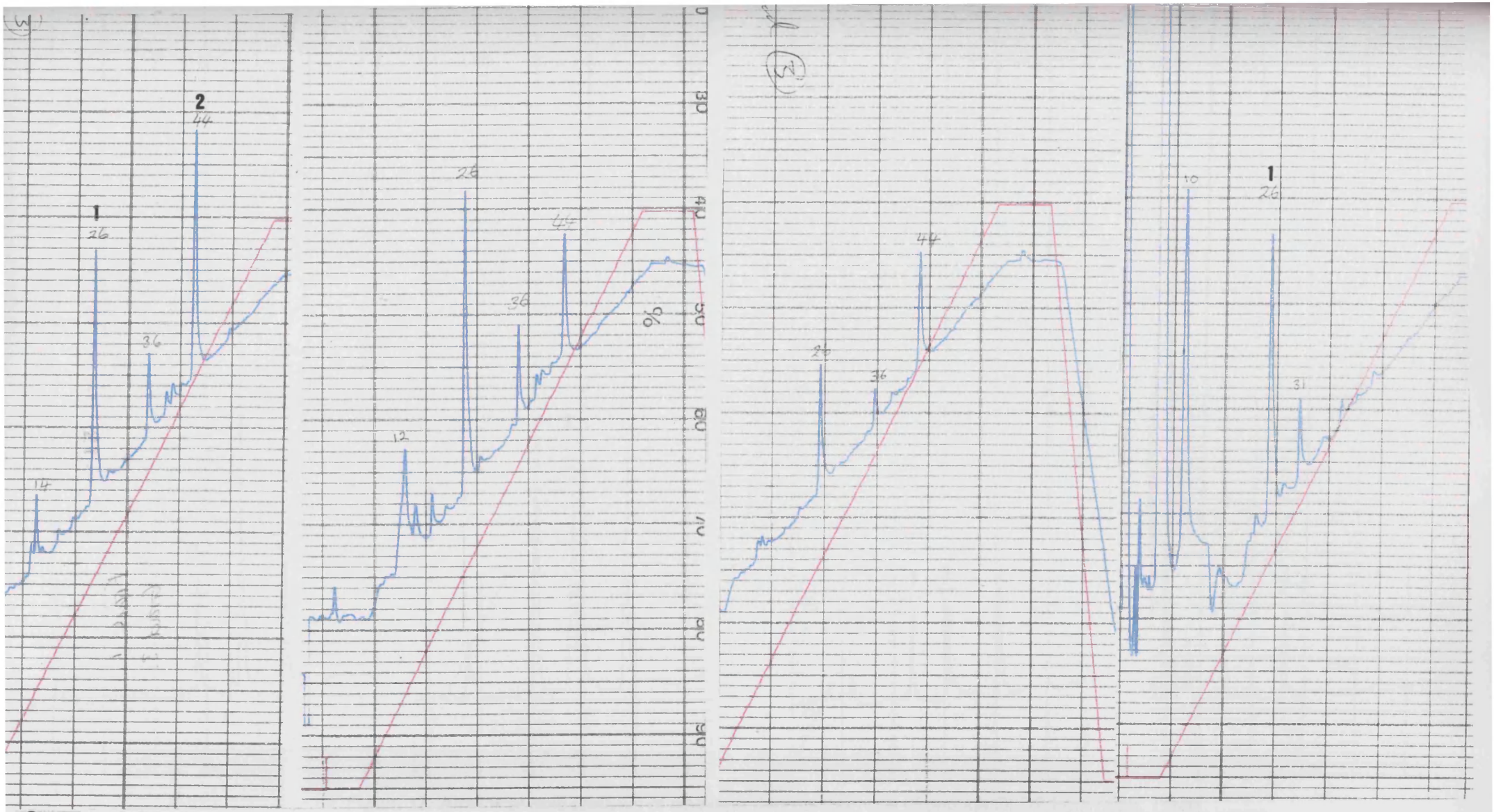


Figure 11 : Analytical hplc analysis of cleavage deprotection trial of peptide DLD1 from resin. Trace A is the sample obtained 1 hr from the start of the trial, trace B = 2 hrs from the start, trace c = 4 hrs from the start and trace D is the sample obtained folowing incubation with DTT overnight The peak eluted at 26% acetonitrile (peak 1) corresponds to the fully assembled and deprotected peptide. The peak at 44% acetonitrile (peak 2) corresponds to the peptide dimer which is reduced to the monomer form (peak 1 at 26% acetonitrile) following reduction by DTT (trace D).

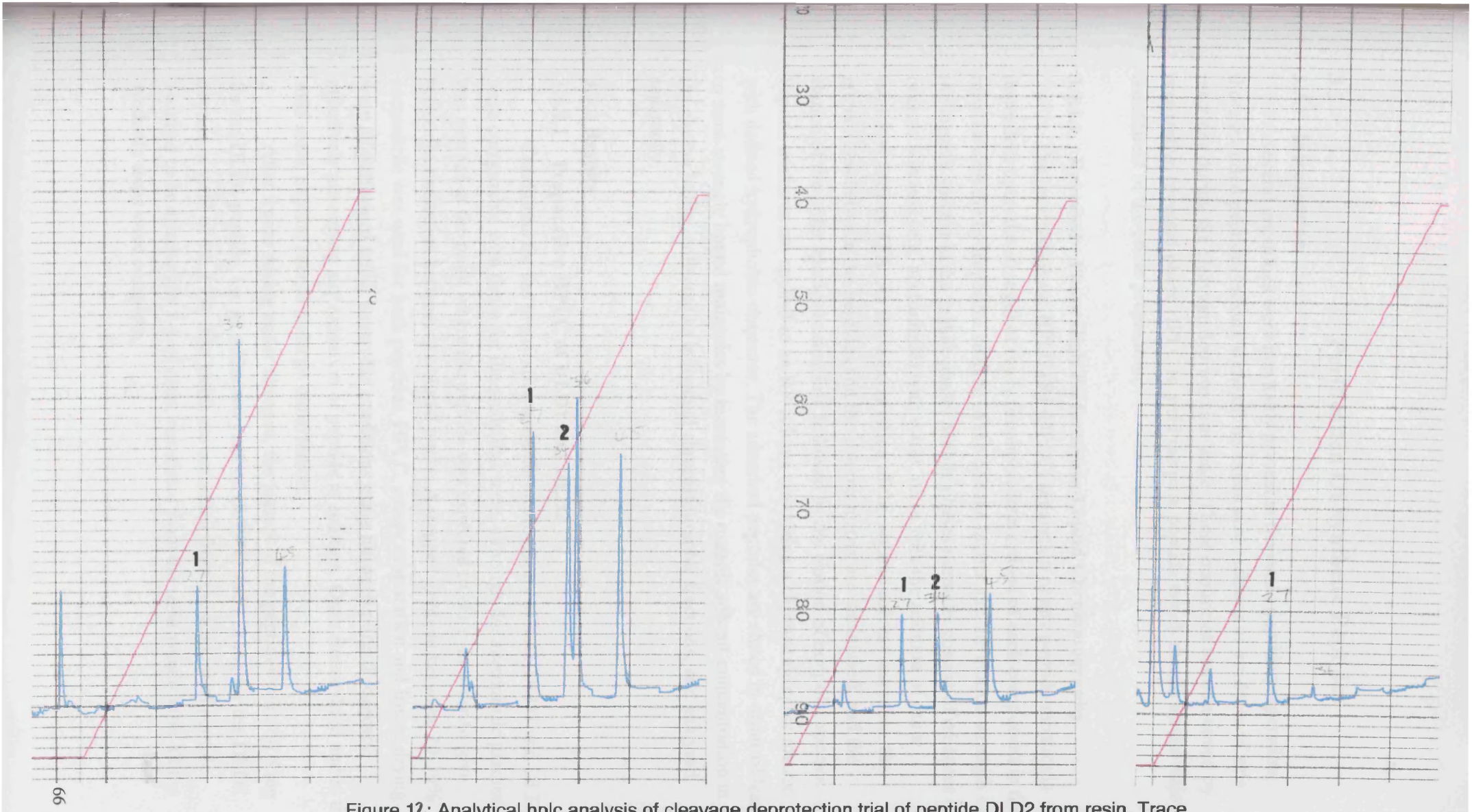


Figure 12: Analytical hplc analysis of cleavage deprotection trial of peptide DLD2 from resin. Trace A is the sample obtained 2 hrs from the start of the trial, trace B = 4 hrs from the start, trace c = 8 hrs from the start and trace D is the sample obtained folowing incubation with DTT overnight The peak eluted at 27% acetonitrile (peak 1) corresponds to the fully assembled and deprotected peptide. The emerging peak at 34% acetonitrile with time (peak 2) corresponds to the peptide dimer as confirmed by its absence following reduction by DTT (trace D)

3.3 Purification Of Synthetic Peptides

3.3.1 Introduction

Several impurities were inevitably present in the crude peptide preparation; these included peptides lacking some of the amino acid residues or products of side reactions during the cleavage deprotection stage. These impurities were removed by preparative reversed phase HPLC to yield the pure peptide which should be the major constituent of the crude preparation.

3.3.1.1 Reversed - Phase High Performance Liquid Chromatography.

The reversed-phase HPLC (RP-HPLC) separation of any peptide mixture is dependent upon the strength of the hydrophobic interactions of each component in the mixture with the hydrophobic surface of the column matrix and the elution strength of the organic solvent in the mobile phase. Mobile phases normally consist of mixtures of organic solvents (e.g. acetonitrile) with water, often with the addition of other modifiers such as TFA. As the concentration of the organic solvent increases, the interactions between the peptides and the column matrix are diminished, and the elution of the polar species occurs first followed by the elution of nonpolar species. Peptide mixtures are applied to an RP column containing a chromatographic matrix with defined hydrophobic character. The adsorbed peptides are eluted in order of least to most strongly bound molecules by increasing the organic solvent concentration in the elution buffer, collected as individual chromatographic fractions, and analysed separately.

3.3.2 Results

3.3.2.1 Preparative HPLC of DLD1 and DLD2

Conditions for the preparative scale chromatography of both DLD1 and DLD2 were comparable with those for the analytical scale, although the acetonitrile gradient was stepped at the point where the peptide was expected to elute, in order to give better discrimination between the eluted peaks. A stepped gradient between 20-30% acetonitrile was used for both peptides. HPLC, rotary evaporation and freeze drying were all performed under anaerobic conditions using nitrogen purged reagents, effectively preventing any oxidation of peptide in solution. Once freeze dried and in the solid state, peptide cannot undergo dimerisation.

After freeze drying under nitrogen, the masses of the peptides were 66.8 mg for the DLD1 peptide, i.e. an estimated yield of 18.79%, and 113 mg for the DLD2 peptide, a yield of 32..17%. The yields are estimated from the amount of peptide assumed to be attached to 1 g of resin, calculated from the resin weight after all the synthetic steps were complete.

3.4 General Summary

Using various predictive parameters, two peptide sequences corresponding to residues 130 - 144 and 228 - 242 (obtained from the primary sequence of the cloned bloodstream DHlipDH gene) were chosen in this study as probable surface epitopes on the native protein. These two synthetic peptides (DLD1 and DLD2), each of 15 amino acid residues, were successfully synthesised by the F-moc solid phase method . Before cleavage of the peptides from the resins, both peptides were extended with two more amino acid residues : glycine, which was used as a spacer, and cysteine. The cysteine residue was added to the amino terminus of the peptides to allow coupling to a protein carrier. The extended synthetic peptides were then cleaved from the resin and purified by reversed phase HPLC under anaerobic conditions to yield peptides in the reduced monomeric form.

CHAPTER 3 (B) PREPARATION AND CHARACTERISATION OF ANTI-PEPTIDE ANTIBODIES

3.5 Peptide coupling to carrier protein

3.5.1 Introduction

The extended, reduced peptides were coupled to the carrier protein keyhole limpet haemocyanin (KLH) using N-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) as a crosslinker. The carrier protein was first activated with MBS and subsequently coupled to the peptide through its cysteine residue (Green et al, 1982).

Because of high susceptibility to air oxidation, it was essential to have peptides in their reduced monomer form prior to use in order to achieve maximum coupling. Peptide reduction was achieved by carrying out the HPLC preparative step under anaerobic conditions as detailed above. The percentage of reduced peptide was determined by dissolving 1 mg of solid peptide into acetate buffer pH5 and testing 100µl aliquots with 100µl DTNB (Ellmans reagent) in 2 ml sodium phosphate buffer pH8. The % reduced peptide was determined by dividing the concentration of thiols present by the expected thiol concentration assuming complete reduction. DLD1 was found to be 62.7% reduced while DLD2 was 95.8% reduced.

3.5.2 Results

3.5.2.1 Preparation of activated KLH

Following activation of KLH with MBS (section 2.18.1), the reaction product was added to a P-30 Biorad column to remove any unreacted MBS. 1 ml fractions

were collected and monitored at 280 nm and absorbancies were plotted to give an elution profile (Fig. 13).

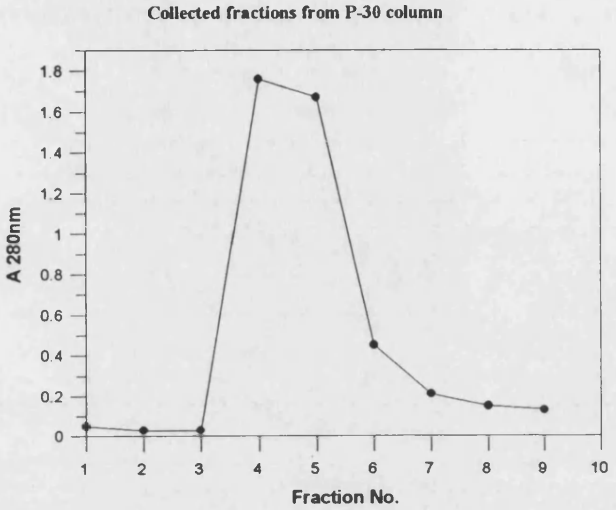


Figure 13: Elution profile of activated KLH. 1ml fractions of activated KLH were collected from a P-30 column. Protein content was determined by measuring absorbancies at 280nm and plotted against fraction number to give an elution profile.

KLH - MBS conjugate was recovered by pooling fractions 4, 5 and 6 from the first peak of the column eluate, giving 3 mls of activated KLH.

The activated KLH was reacted with 1 ml of a 5 mg / ml peptide solution in phosphate buffer pH 7.2. and the pH adjusted to pH 7.4 using sodium hydroxide. The reaction mixture was left continually stirring at room temperature for 3 hrs and then frozen at -20°C into 0.5 ml aliquots (1 mg peptide/ml) to be used for rabbit immunisation. (section 2.19)

3.6 Anti-peptide antibody characterisation using enzyme-linked immunosorbent assay (ELISA)

3.6.1 Method

ELISA was used to screen for specificity and evaluate the antibody titre of collected antiserum samples. Four rabbits (No.s 142-145) were immunised with 0.5 mg peptide (rabbits 142 and 143 were immunised with DLD1 while 144 and 145 were immmunised with DLD2) and the first bleeds were taken 4 weeks later. All subsequent boosts and bleeds were performed at 4 week intervals.

Rabbit immune serum was tested for reactivity with the peptide antigen and carrier protein (KLH) using the ELISA method (see methods, section 2.21) The serum was also tested for cross-reactivity with the second unrelated peptide.

3.6.2 Results

The results were plotted as graphs of the optical density at 450nm against serial dilution, and tabulated as the fold-dilution of serum which gave 50% of the maximum absorbance at 450 nm. (table 6).

Table 6 : A summary of the antibody titres obtained for anti-DHD1 (rabbits 142 and 143) and anti-DHD2 (rabbits 144 and 145) serum.

RABBIT No.	BLEED 1	BLEED 2	BLEED 3	BLEED 4
142	3200	12800	12800	1600
143	1600	1600	12400	1800
144	800	1600	800	800
145	1600	3200	3200	3200

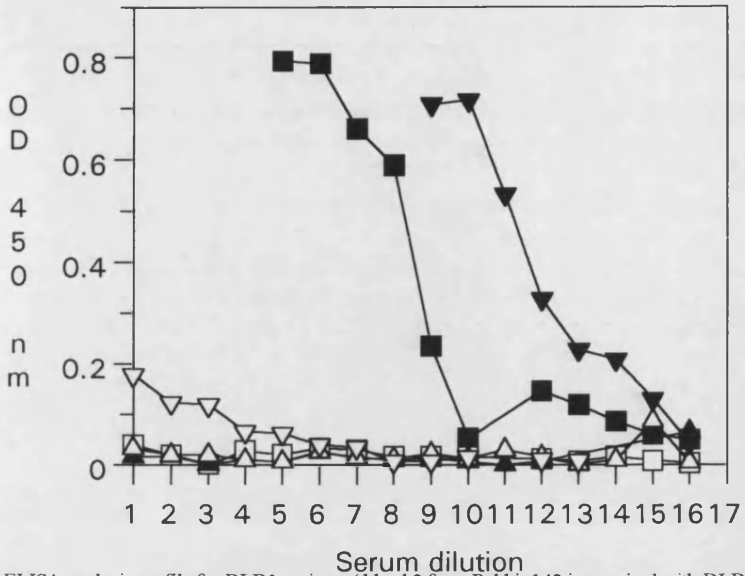
The antibody titre is expressed as the fold-dilution of immune serum which gave 50% of the maximum absorbance at 450 nm

From these findings, the following bleeds were selected as anti-peptide sera for use in all subsequent studies:

- **Anti DLD1 :** Rabbit 142 - bleed 2
- **Anti DLD2 :** Rabbit 145 - bleed 2

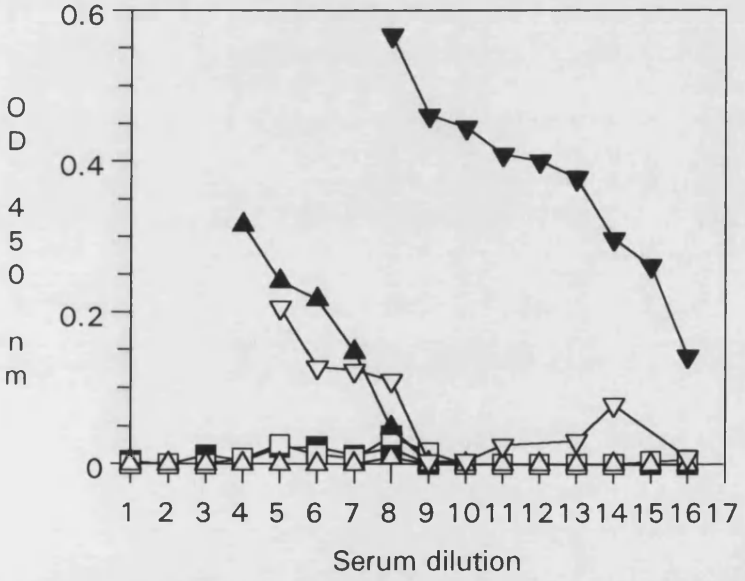
Elisa analysis profiles for each of these bleeds, presented below (Figs 14 and 15) show that each antiserum exhibits a significant anti-peptide antibody response compared to pre-immune response. There is no cross-reactivity between peptides indicating that the raised antibodies are peptide-specific.

Fig 14. BLEED (2) PROFILE FOR RABBIT 142 (immunised with peptide DLD1)



ELISA analysis profile for DLD1 antisera (bleed 2 from Rabbit 142 immunised with DLD1 peptide antigen).The profile shows strong specific cross-reactivity with peptide DLD1 compared to the pre-immune serum response while no crossreactivity is observed with peptide DLD2.

Fig 15. BLEED (2) PROFILE FOR RABBIT 145 (immunised with peptide DLD2)



ELISA analysis profile for DLD2 antisera (bleed 2 from Rabbit 145 immunised with DLD2 peptide antigen).The profile shows strong specific cross-reactivity with peptide DLD2 compared to the pre-immune serum response while no crossreactivity is observed with peptide DLD1.

Key for Figs. 14 and 15

- DLD1
- DLD1 Pre-immune
- DLD2
- DLD2 Pre-immune
- KLH
- KLH Pre-immune

CHAPTER 4

IMMUNOBLOTTING

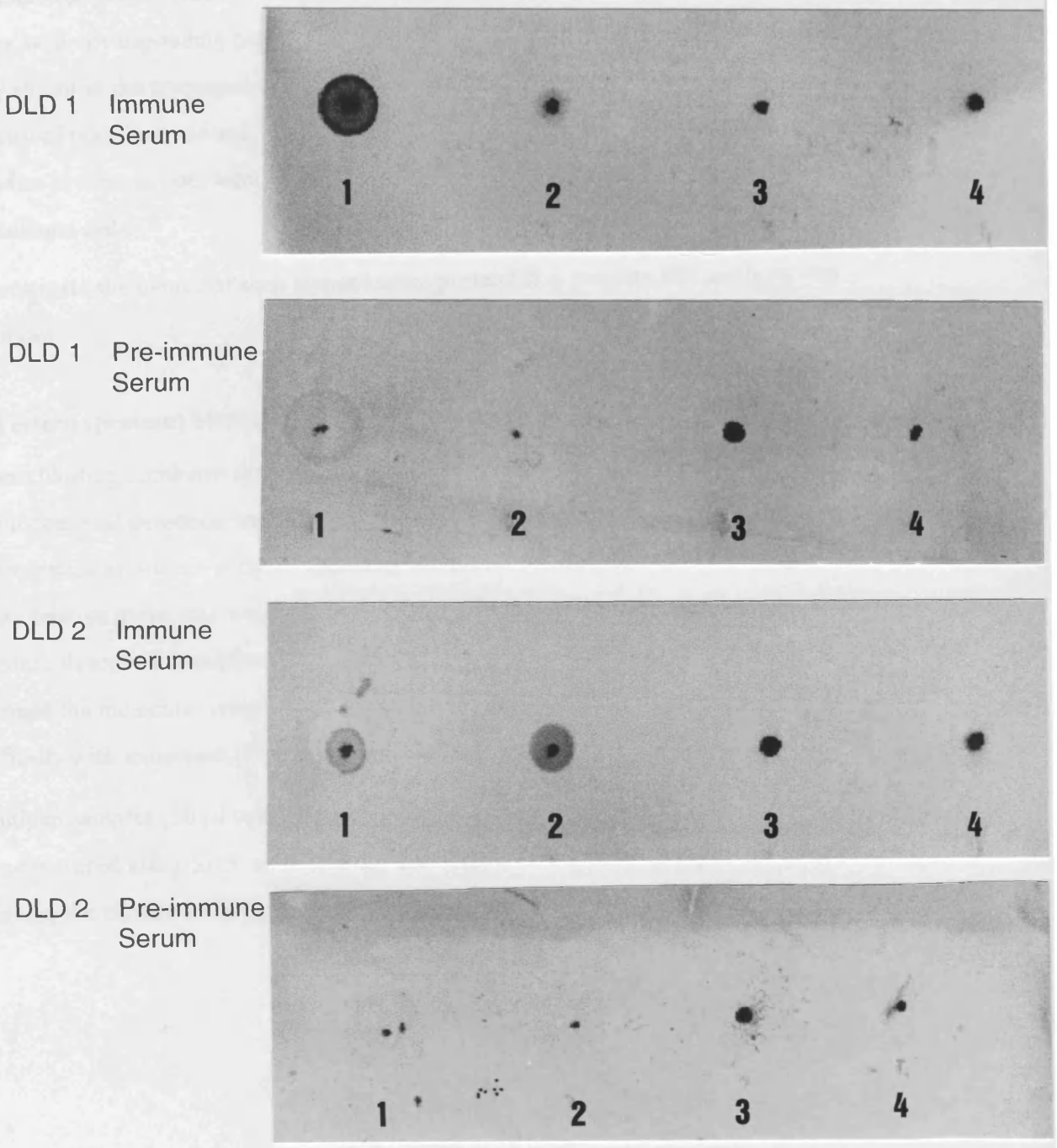
4.1 Dot Immunobinding

A dot immunobinding assay (methods section 2.24) was performed to confirm the previously obtained ELISA results (chapter 3, section 3.6) which indicated significant differences in peptide antigen binding between immune and preimmune sera. (Fig. 16). Additionally the assay was used as a preliminary investigation of antibody binding to trypanosomal proteins present in freshly sonicated procyclic *T. brucei* cell lysate.

4.1.1 Result

See Fig. 16 (over)

The blots show definitive binding to both KLH-peptide and free peptide with both anti-DLD1 and anti- DLD2 sera (as indicated by a strong blue colouration) which is absent with the corresponding pre-immune sera. Both antisera and pre-immune sera show some binding to free KLH, the binding being stronger for antisera, as would be expected from the ELISA results. Antisera and pre-immune sera also bind to sonicated trypanosome cell lysate apparantly with the same intensity.



dot 1: KLH-peptide (1µg)
dot 2: Free peptide (10µg)
dot 3: Cell sonicate (5 x 10^6 cells)
dot 4: KLH (1 µg)

Figure 16: Dot immunoblot of DLD1 and DLD2 immune/preimmune sera with a conjugated peptide antigen (KLH-peptide), free peptide antigen, *T.brucei* procyclic cell sonicate and KLH.

4.1.2 Conclusion

This result verifies previous ELISA results in that it shows cross-reactivity of specific antisera with corresponding peptide antigen for both anti-DLD1 and anti-DLD2 that is clearly absent in the corresponding pre-immune sera. In addition, the observed reactivity of both immune and pre-immune sera with cell lysate indicates that there are antibodies present in both which cross-react with native protein(s) present in sonicated trypanosome cells.

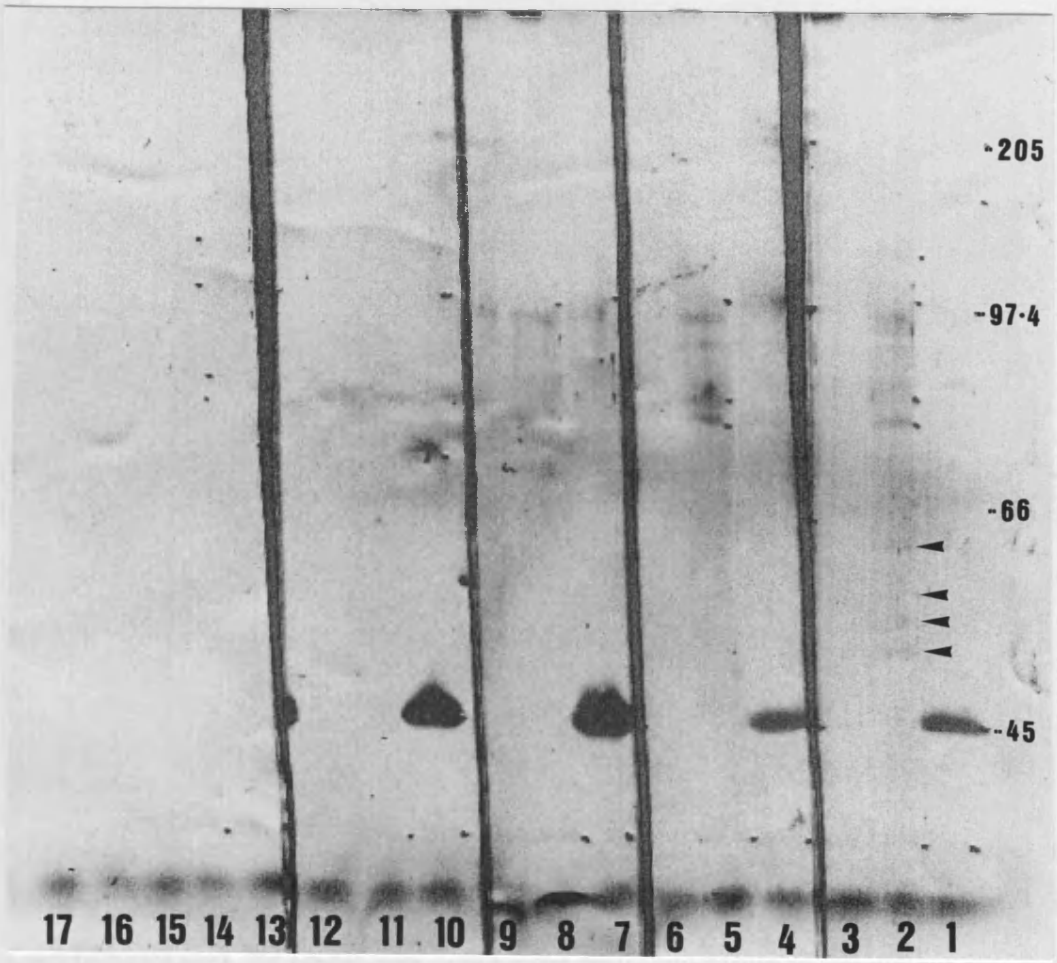
To investigate the nature of such trypanosome protein(s), a western blot analysis was carried out.

4.2 Western (protein) blotting

Western blotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection and may be used to determine a number of important characteristics of protein antigens including the presence and quantity of an antigen and the relative molecular weight of the polypeptide chain. The western blotting procedure described in section 2.25 was used to confirm the above result, and to determine the molecular weight of any trypanosomal proteins which cross-reacted specifically with antiserum.(Fig.17)

Antigen samples (50 µl sonicated cell lysate (containing $4x10^6$ cells per lane) were denatured using SDS, so increasing the number of exposed epitopes and hence increasing the chance of DHlipDH antibody binding

4.2.2 Result



Wells 1, 4, 7, 10, 13 - Molecular weight markers

Wells 2, 5, 8, 11, 14 - cell sonicate

Figure 17 : Western blot of procyclic *T.brucei* cell lysate with DLD1 and DLD2 immune/pre-immune serum. Cross-reactivity of both DLD1 and DLD2 immune/ pre-immune is observed with trypanosomal protein bands between 66 and 97.4kD. Four additional bands are observed with anti-DLD1 immune serum only, occurring in the MW range of 47-65 kD.

A calibration curve for the migration of molecular weight markers was plotted (Fig. 16) and used to determine the molecular weights of bands of interest.

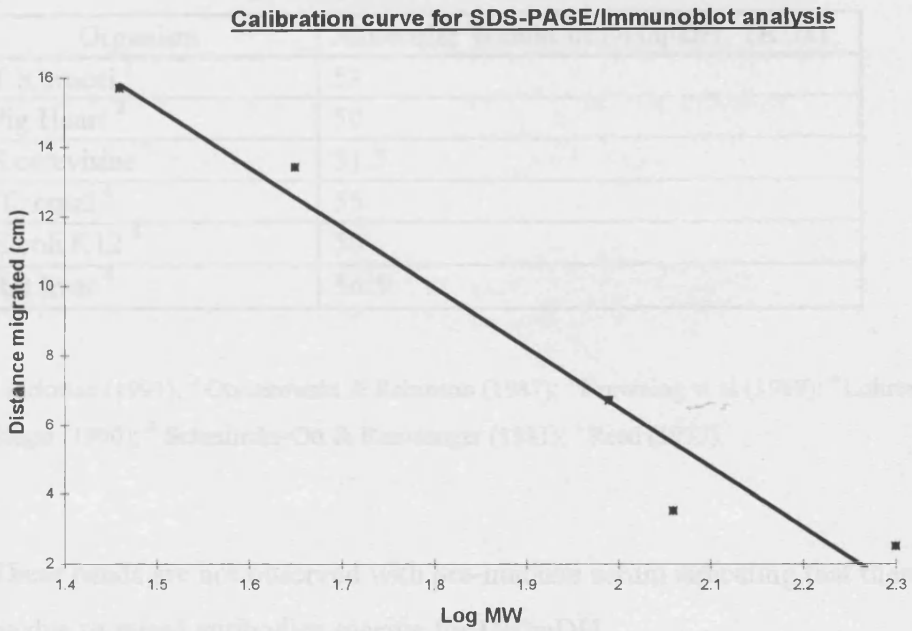


Figure 18: 60 µg per lane of protein molecular weight markers (table 5, section 2.25.1.4) were run on western blots together with samples. The migrated distances were used to plot a calibration curve (above) from which sample band molecular weights were calculated.

4.2.3 Conclusion

Western blots of procyclic *T. brucei* cell lysate with DLD1 and DLD2 immune/pre-immune serum were performed twice and the result presented as Fig. 17 was found to be repeatable. There appear to be antibodies (IgG) present in both immune and pre-immune sera for DHD1 that cross-react with numerous trypanosomal proteins in the MW range 66-97.4 kD. This is also the case for antiserum raised against peptide 2 (DLD2). As whole sera contain the entire repertoire of circulating antibodies found in the pre-immunised/immunised animal at the time the serum was collected, it is likely that whole serum may contain antibodies that recognise spurious trypanosomal antigens. This may explain the generally high level of reactivity of both pre-immune and immune sera with trypanosomal sonicate.

However, additional bands are observed with anti-DHD1 immune serum only, occurring in the MW range of 47 - 65 kD (ie. 47.9, 52.3, 56.2 and 64.6 kD). These

are comparable with previously determined subunit molecular weights for DHlipDH from various species as detailed in table 7 (below).

Table 7: Molecular weights of DHlipDH from various organisms

Organism	Molecular Weight of DHlipDH (KDa)
T.b.brucei [1]	53
Pig Heart [2]	50
S.cerevisiae [3]	51.5
T. cruzi [4]	55
E.coli K12 [5]	56
Rat liver [6]	56.5

[1] Jackman (1991); [2] Otulakowski & Robinson (1987); [3] Browning et al (1988); [4] Lohrer & Krauth-Siegal (1990); [5] Schmincke-Ott & Bisswanger (1981); [6] Reed (1973)

These bands are not observed with pre-immune serum indicating that these bands may be due to raised antibodies specific for DHlipDH.

If this is indeed the case and antiserum raised for the DLD1 epitope also cross-reacts with the denatured enzyme, the question of cross-reactivity with intact native enzyme still remains. This was investigated by a series of immunoprecipitation experiments as detailed in chapter 5.

CHAPTER 5

IMMUNOPRECIPITATION

5.1 Introduction

Immunoprecipitation is the immunochemical method of choice to isolate specific proteins and was employed in this study to investigate raised peptide antisera (DLD1 and DLD2) binding to native DHlipDH in sonicated procyclic *T. brucei*. The procedure has three main stages:

1. Lysis of the cells to release the antigen
2. Formation of the antibody-antigen complexes
3. Purification of the immune complexes

Formation of antigen-antibody immune complexes, on incubation with specific antiserum, was detected by measuring the corresponding decrease in DHlipDH activity compared to controls incubated with PBS only. All assays were performed in duplicate.

Section 1

5.2 Direct inhibition of enzyme activity

Neither peptide antisera DLD1 or DLD2 were raised to peptide sequences occurring at the catalytic active site of the DHlipDH enzyme, as determined by the homology model presented in Fig. 10 (chapter 3). However, because of the polyclonal nature of the antisera and also for thoroughness of investigation, it was decided to determine whether there was any direct cross-reactivity of antiserum with the enzyme's active site which would be observed as a direct inhibition of enzyme activity

5.2.1 Method

Frozen cell pellets (6×10^7 cells in 0.5 mls PBS) were thawed and sonicated on ice as detailed in section 2.23. 30µl samples of freshly sonicated trypanosome cell lysate were incubated with equal volumes of DLD1 and DLD2 immune/pre-immune serum or PBS (control) on ice for two time intervals, 6 hr and 24 hours (Figs 19 and 20). 20µl from each sample were assayed for DHlipDH enzyme activity (see methods, section 2.27.3.1) **5.2.2 Results**

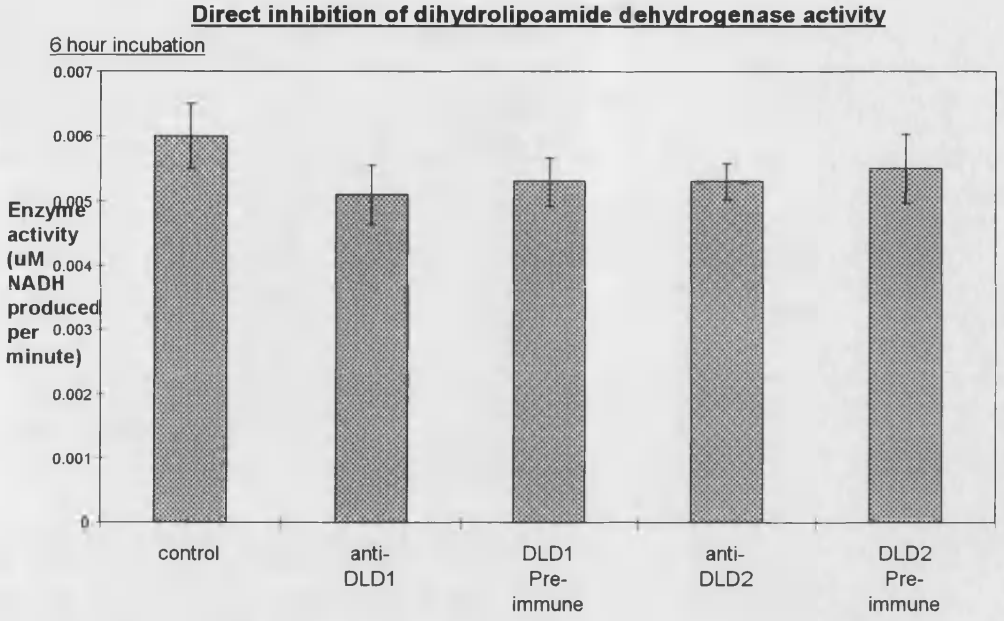


Figure 19: 6 x 10^7 procyclic *T.brucei* cells were sonicated in 0.5 mls PBS. 30 µl samples were incubated for 6 hours on ice with equal volumes of DLD1/DLD2 immune and preimmune sera. Incubation with PBS was carried out as a control. 20µl samples were assayed for DHlipDH activity.

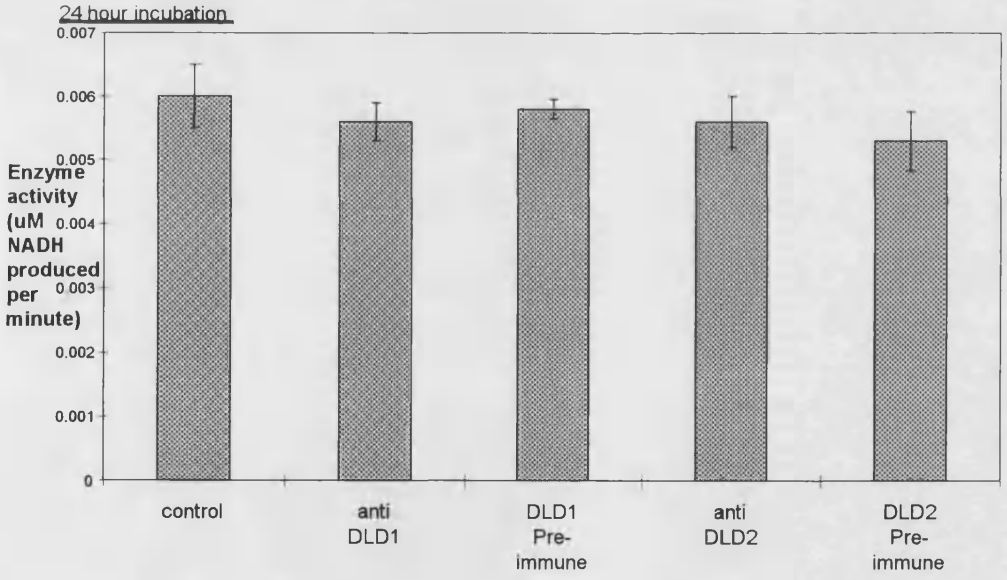


Figure 20: 6 x 10^7 procyclic *T.brucei* cells were sonicated in 0.5 mls PBS. 30 µl samples were incubated for 24 hours on ice with equal volumes of DLD1/DLD2 immune and preimmune sera. Incubation with PBS was carried out as a control. 20µl samples were assayed for DHlipDH activity

5.2.3 Conclusion

There is no significant reduction in enzyme activity for either the 6 hr or 24 hr incubation, indicating that any DHlipDH-specific antibodies present do not bind to the enzyme's active site. Antibody binding to other parts of the dimeric enzyme were investigated as detailed in section 2 (below).

Section 2

5.3: Indirect immunoprecipitation of enzyme activity : Adsorption of immune complexes using goat anti-rabbit IgG

Any immune complexes, formed by polyclonal rabbit antiserum binding to DHlipDH, can theoretically be bound to by anti-rabbit immunoglobulin. It was assumed that any raised anti-DHlipDH antibody would be IgG in nature and so immobilised goat anti-rabbit IgG was used to adsorb out any formed immune complexes.

5.3.1 Adsorption of immune complexes with various concentrations of goat anti-rabbit IgG

5.3.1.1 Method

Microtitre wells were coated with 100µl of 10µg/ml goat anti-rabbit IgG diluted in coating buffer (see section 2.21) at 4°C overnight. Control wells containing no goat anti-rabbit IgG were set up also.Wells were washed three times with PBS/tween. Equal volumes of cell lysate and anti-DLD1 immune serum were incubated for 24 hrs at 4°C. 40 µl of incubated lysate/antibody samples were added to each well and left for 6 hrs at room temperature before being assayed for DHlipDH activity (Fig. 21).

5.3.1.2 Results

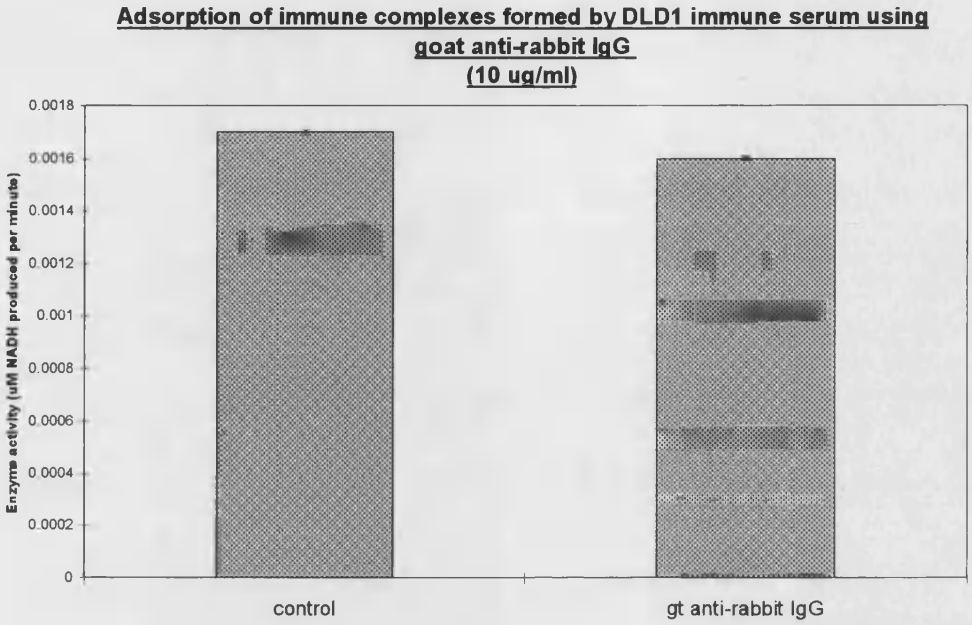


Figure 21: Microtitre wells were coated with 100µl of 10 ug/ml gt antirabbit IgG in coating buffer at 4 °C overnight. Control wells containing no gt antirabbit IgG were also set up. Equal volumes of sonicated cell lysate and anti-DLD1 immune serum were incubated for 24 hours at 4 °C *T*. 40µl of this incubated lysate/immune serum were added to each well and left for 6 hours at room temperature before being assayed for DHlipDH activity.

There appears to be a 5% reduction in enzyme activity. Although small, the reduction appears to be real as indicated by very low standard deviations (see error bars) obtainined in each duplicate assay. The experiment was repeated using 100 µg/ml of gt anti-rabbit IgG in an attempt to improve upon this slight reduction (Fig 22).

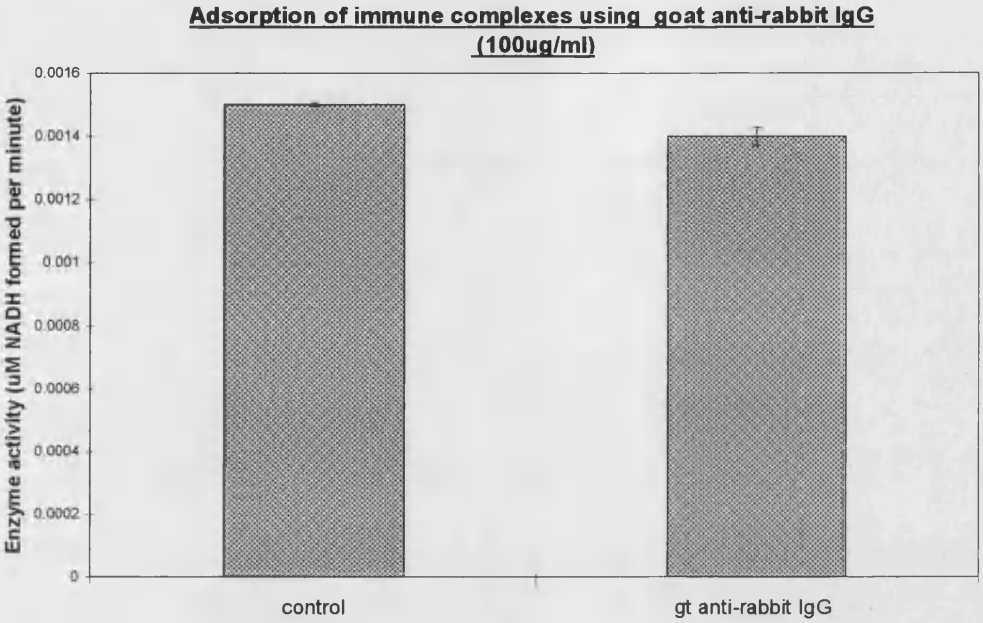


Figure 22: Microtitre wells were coated with 100µl of 100 ug/ml gt antirabbit IgG in coating buffer at 4 °C overnight. Control wells containing no gt antirabbit IgG were also set up. Equal volumes of sonicated cell lysate and anti-DLD1 immune serum were incubated for 24 hours at 4 °C. 40µl of this incubated lysate/immune serum were added to each well and left for 6 hours at room temperature before being assayed for DHlipDH activity.

Reduction of enzyme activity appears to improve very slightly, with an 8% reduction being observed.

5.3.1.3 Conclusions

Adsorption of immune complexes with immobilised goat anti-rabbit IgG resulted in a possible very small reduction in enzyme activity. However, this reduction is so small that immune complex formation is open to question.

5.3.2 Adsorption with successive goat anti-rabbit IgG

A possibility remained that the amount of goat anti-rabbit IgG coating the well surface was insufficient to bind all formed immune complexes. To determine if this was the case, incubated supernatants were exposed to a succession of wells, each coated with gt anti-rabbit IgG (10µg/well)

5.3.2.1 Method

Five wells were coated with goat anti-rabbit IgG (10 µg/well) overnight at 4°C. A series of five uncoated control wells were also set up. Equal volumes (50µl) l of immune serum (anti-DHD1 / anti-DHD2) and freshly sonicated cell lysate were incubated together overnight at 4°C. The samples were diluted with 100µl PBS and mixed thoroughly. Samples (200 µl) were added successively to each series of wells (coated and uncoated) and were left to incubate for 3 hrs at room temperature per well before being transferred to the next. After incubation in the final well, 20µl samples were taken and assayed for enzyme activity (Fig. 23).

5.3.2.2 Results

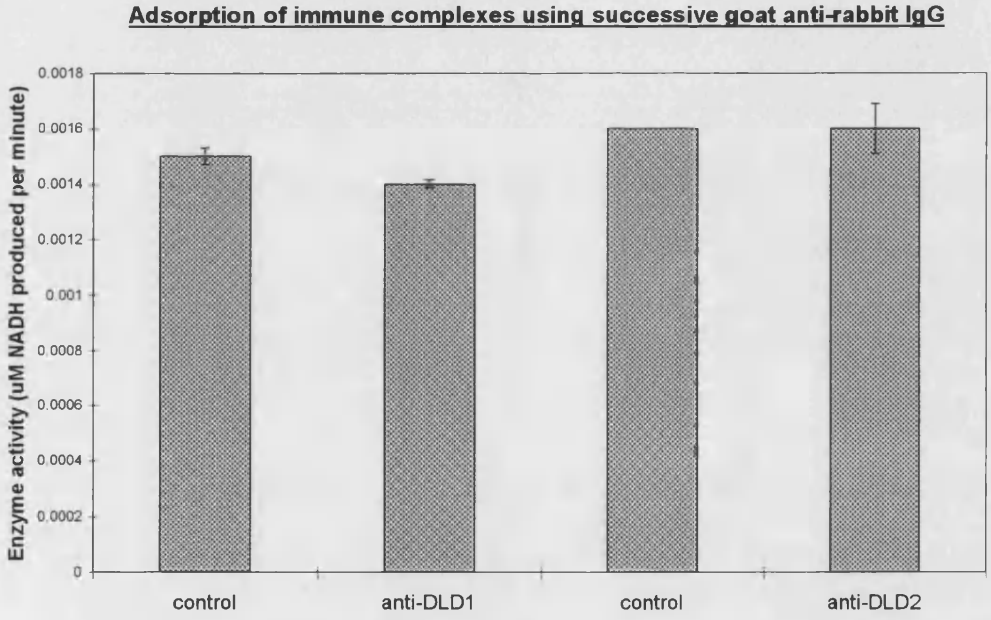


Figure 23 : Five microtitre wells were coated with 100µl of 10 ug/ml gt anti rabbit IgG in coating buffer at 4 °C overnight. Five control wells containing no gt antirabbit IgG were also set up. Equal volumes of sonicated cell lysate and anti-DLD1 immune serum were incubated overnight at 4 °C. 200µl of this incubated lysate/immune serum were added successively to each series of wells (coated and uncoated) and left for 3 hours at room temperature per well before transference to the next. 20 µl samples were taken following incubation in final well and assayed for DHlipDH activity.

There is a 11% reduction in enzyme activity compared to control for anti-DHD1 immune serum while there is no significant reduction in activity with anti-DHD2 immune serum. This is in agreement with western blot results shown previously (section 4.2.2) which indicate that anti-DLD1 contains antibodies specific for *T. brucei*

DHlipDH while anti-DLD2 seems to contain no such DHlipDH-specific antibodies. Although small, this reduction in DHlipDH activity appears to be significant and may be a real effect of specific anti-DHlipDH antibody binding. However, a greater reduction in activity was desired for this to be conclusive and further investigations were carried out using PEG and Protein A from the Cowan strain of *Staphylococcus aureus* to adsorb any immune complexes formed.

Section 3

5.4 Immunoprecipitation of immune complexes using polyethylene glycol (PEG)

PEG precipitates proteins in proportion to their molecular size and concentration. Thus free IgG is soluble in 2% w/v PEG, but is insoluble when part of an immune complex.

5.4.1 Method

Equal volumes of sonicated cell lysate were incubated with DLD1/DLD2 immune serum or PBS (control) overnight at 4°C. A polyethylene glycol 6000 (PEG) solution (20% w/v in saline solution) was adjusted to the working concentration by mixing 6 ml of 20 % PEG with 3 ml 0.2 M EDTA (pH 7.6) and 1 ml saline (0.15M). This was then added to the incubated sample to give a final PEG concentration of 2%. Samples were mixed and left overnight at 4°C. Samples were centriguged at 20,000 rpm for 30 mins at 4°C and 20µl of each sample was assayed for enzyme activity (Fig. 24).

5.4.2 Results

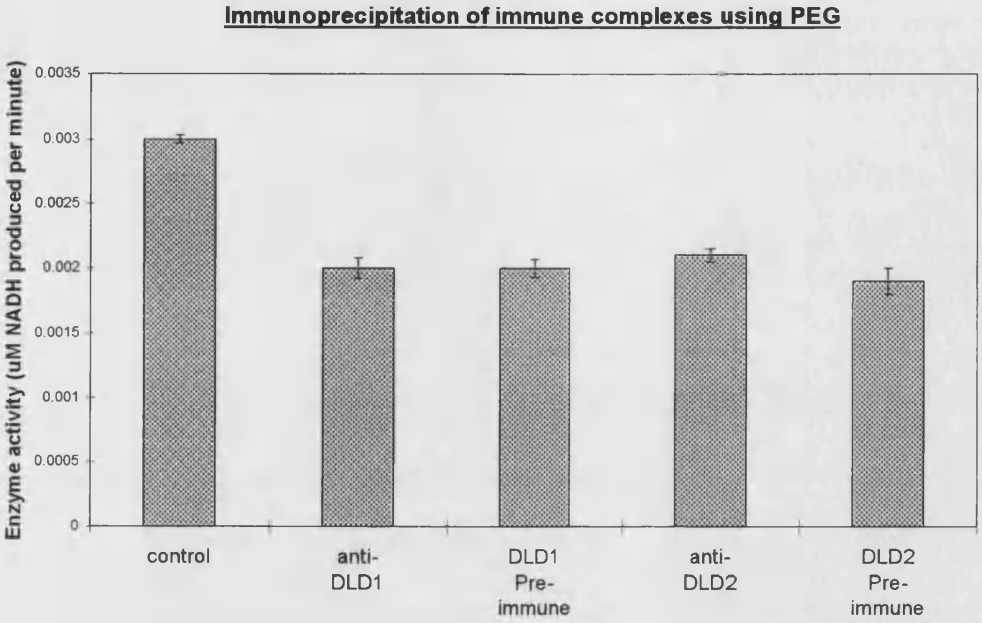


Figure 24: Equal volumes of sonicated lysate and DLD1/DLD2 antiserum (or PBS for control) were incubated overnight at 4°C. PEG solution was added to a final PEG concentration of 2%. Samples were mixed and left overnight at 4°C . Samples were centrifuged and 20µl of each sample assayed for DHlipDH activity.

Although there is considerable reduction in enyme activity, compared to the PBS control, for both anti-DLD1 and anti-DLD2 antisera, the effect does not appear to be immune serum specific ie. large reductions in enzyme activity were observed following incubation with the corresponding pre-immune sera.

5.4.3 Conclusions

Polyclonal antibodies are often used in immunoprecipitations as they tend to produce stable multivalent interactions. However, their use also tends to yield high nonspecific backgrounds as multiple interactions that lead to forming large complexes are more apt to trap or bind nonspecific proteins. It may be possible that the DHlipDH is still associated with the mitochondrial multienzyme complex in which it is normally found together with enzymes E1 and E2. These large enzyme complexes, rather than any specific DHlipDH immune complexes, are perhaps directly precipitated out by the PEG so explaining the unexpected pre-immune serum results obtained above.

Alternatively, it may be that antibodies are present in both pre-immune and immune sera which are cross-reactive with trypanosomal proteins, but not necessarily specific for DHlipDH. This would be in agreement with the western blot result (section 4.2.2). Such antibodies may cause large complex formation which could trap free DHlipDH enzyme molecules, so bringing about non specific precipitation of DHlipDH activity

To overcome the problem of non-specific large complex precipitation by PEG, it was decided to try an IgG specific precipitating compound, *Staphylococcus aureus* Protein A. This would ensure that any observable reduction in enzyme activity was due to precipitation of immune complexes as opposed to general large complexes.

The problem of non-specific precipitation of DHlipDH activity was tackled by the inclusion of a preclearing step using non-immune, commercially available rabbit serum ('neutral serum'). If rabbit serum naturally contains strong cross-reactive antibodies, as appears to be the case, addition of commercially obtained rabbit serum to the lysate will result in large non-DHlipDH immune complex formation. Such complexes may then be removed by centrifugation, prior to treatment with DHlipDH-specific antiserum.

PEG precipitates large complexes only, hence if a single DHlipDH specific IgG antibody molecule is bound to the enzyme, it is unlikely that it will be precipitated. Protein A from *Staphylococcus aureus* binds to and precipitates all IgG molecules, and so maximal immunoprecipitation would be expected.

Section 4

5.5 Immunoprecipitation using *Staphylococcus aureus* Protein A

The protein A incorporated into the cell wall of some strains of *Staphylococcus aureus* bacteria has the property of binding strongly and specifically to the Fc portion of certain mammalian Ig classes and subclasses, especially IgG (Kronvall et al., 1970 a,b ; Goding, 1978). The bacteria provide a solid phase that may be easily manipulated. Because the binding site on the antibody is found in the Fc region, the interaction with protein A does not change the ability of the antibody to combine with the antigen.

Protein A, a 42 kDa polypeptide is bifunctional, allowing multimeric complexes to be formed

The problem of non-specific precipitation of DHlipDH activity (as observed with preimmune sera in PEG immunoprecipitation) was tackled by including a preclearing step as detailed below.

5.5.1 Method

Preclearing the lysate

Non-specific binding was lessened by 'pre-clearing' freshly prepared procyclic *T. brucei* sonicate with commercially obtained neutral rabbit serum. This will contain any general cross-reactive antibodies present normally in rabbit serum. Any large non-specific complexes so formed were removed by centrifugation prior to addition of DHlipDH-specific antibodies. Control precipitations with pre-immune serum were carried out, as were absolute controls containing PBS.

0.5 mls of freshly sonicated cell lysate was pre cleared by the addition of 25µl normal rabbit serum with incubation for 1 hr on ice. A washed pellet of 250 µl *Staphylococcus aureus* protein A (10 % solution, washed in sterile PBS) was added to the lysate and incubated for 30 mins at 4°C. Protein A and any complexed rabbit IgG molecules were removed by centrifugation for 15 mins at 4°C.

Various volumes of test serum (5 , 50 and 100µl of anti-DHD1 immune and pre-immune (or PBS for control)), were added to 100 µl of the lysate for one hour on ice to allow formation of any immune complexes. 50µl of a suspended pre-washed *Staphylococcus aureus* Protein A pellet in PBS was added and incubated for 30 mins on ice to allow adsorption of antibody (and hence any formed immune complexes). Any adsorbed complexes were removed by centrifugation for 10 minutes at 10,000 g in a bench centrifuge. The remaining supernatant was removed and 20µl samples assayed for DHlipDH activity (Fig. 25). All assays were performed in duplicate.

5.5.2 Results

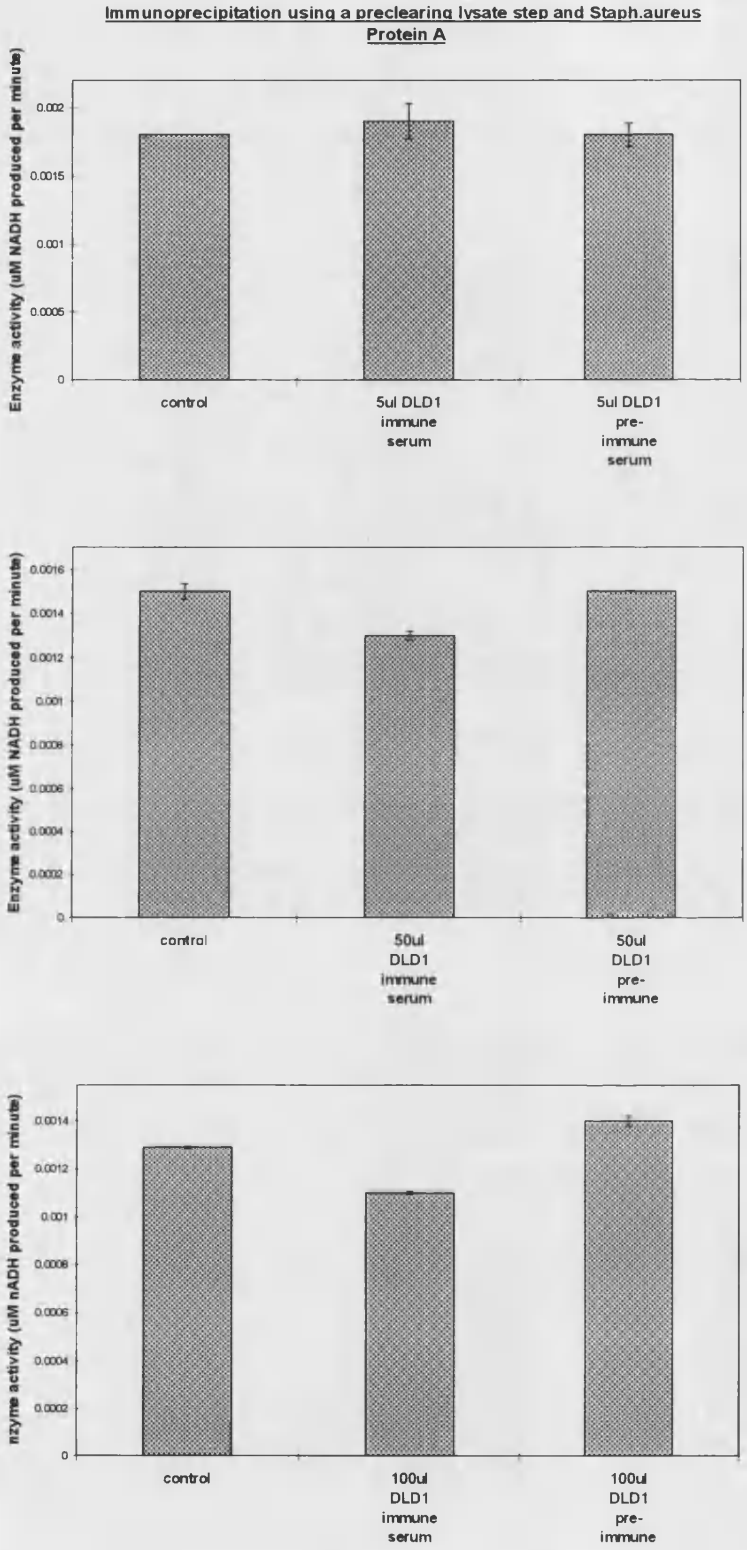


Figure 25: 5µl , 50µl and 100µl aliquots of DLD1 immune/preimmune (or PBS for control) were added to 100µl procyclic *T.brucei* cell lysate (previously cleared with neutral rabbit serum) for 1 hr on ice. 50µl of *Staph. aureus* Protein A suspension was added and left to incubate for 30 minutes on ice. Adsorbed immune complexes were removed by centrifugation at 10,000g for 10 minutes. Samples of the supernatant (20µl) were assayed for DHlipDH activity.

Treatment with pre-immune serum resulted in no significant difference in enzyme activity from the PBS control, whereas incubation with immune serum resulted in a significant reduction in enzyme activity. A 17% reduction in enzyme activity was observed, compared to control, after incubation with 50µl anti-DHD1 immune serum, while 100µl immune serum resulted in a reduction of 15 %. No significant decrease in activity was observed on treating with 5µl immune serum.

In an attempt to maximise this observed decrease in enzyme activity further, incubation with 50µl DLD1/DLD2 test serum (or PBS) was performed followed by the protein A immune complex adsorption step which was repeated three times, adding fresh immune/preimmune serum (50µl) and fresh protein A (50µl) with each repeat step (Figs. 26 and 27) This experiment was repeated twice to verify the result. Anti-DLD2 (and DLD2 preimmune serum) was included as a negative control.

In experiment 1 (Fig. 26), a 26% reduction in DHlipDH activity was observed with DLD1 immune serum compared to control, while no significant reduction in activity was observed with DLD1 pre-immune serum. Similarly, experiment 2 (Fig. 27) resulted in a 25% reduction in DHlipDH activity with DLD1 immune serum while no significant reduction was observed following incubation with DLD1 pre-immune serum. No significant reduction in activity was observed in either experiments for DLD2 immune or preimmune serum.

Figure 26 Experiment 1

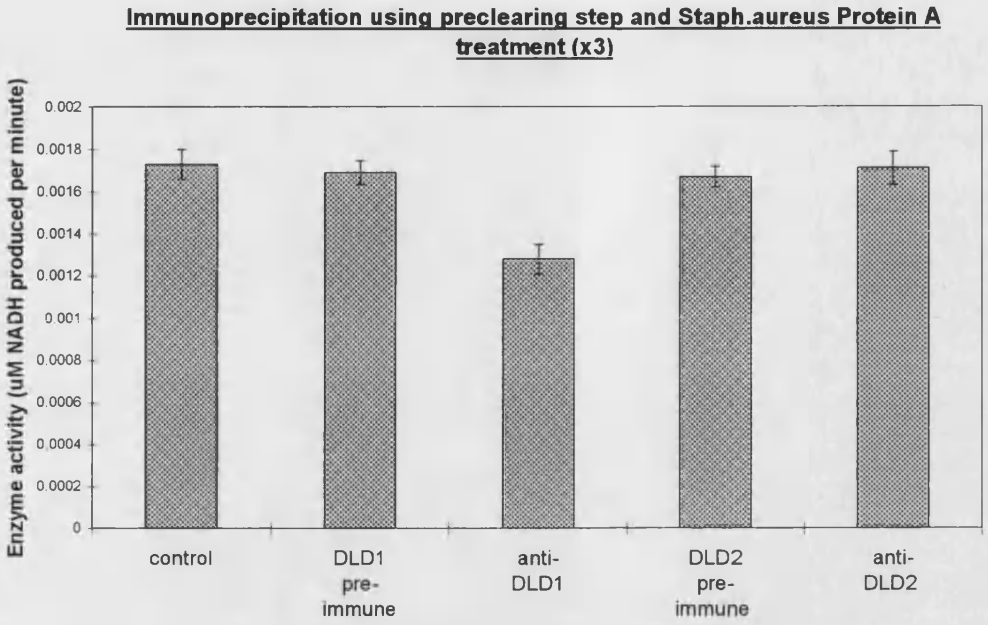
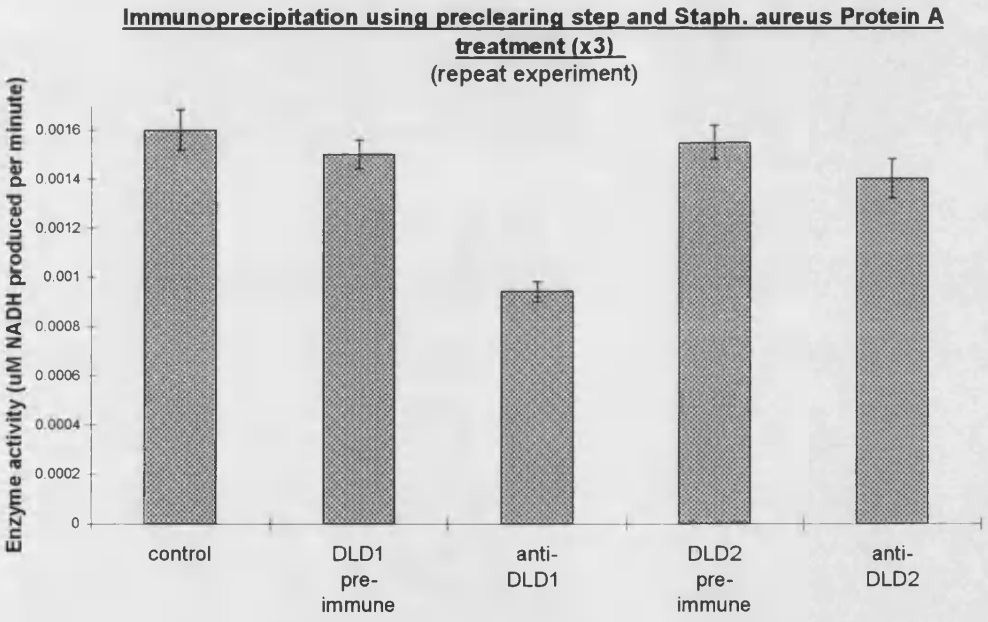


Figure 27 Experiment 2



Figures 26 and 27: 50µl of test serum (DLD1/DLD2 immune and preimmune serum) or PBS (control) were added to 100µl of *T.brucei* cell lysate (previously cleared with neutral rabbit serum) for 1 hour on ice. 50µl of *Staph.. aureus* Protein A suspension was added and left to incubate for 30 minutes on ice. Adsorbed immune complexes were removed by centrifugation for 10 minutes at 10,000g. This adsorption step was repeated three times, adding fresh immune/preimmune serum (50µl) and fresh protein A (50 µl) with each repeat step. Samples (20µl) of supernatant were assayed for DHlipDH activity.

5.5.3 Conclusion

This result shows a significant, reproducible large reduction in DHlipDH activity on treatment of trypanosomal cell lysate with anti-DHD1 immune serum. The absence of any reduction in activity with DLD1 pre-immune sera indicates that non-specific precipitation of enzyme activity is elucidated by the preclearing step using neutral rabbit serum. These results show the presence of a DHlipDH-specific antibody in antiserum raised against peptide 1 (DLD1) and absence of such an antibody in antiserum raised against peptide 2 (DLD2) and hence verify immunoblot results presented previously (section 4.2.2).

For use in immunogold localisation studies, an antibody has to be both strongly specific for the native enzyme and tightly binding to withstand the rigorous washing procedures inherent in this method. It was therefore decided to proceed with preliminary immunofluorescence studies to decide if anti-DLD1 immune serum was suitable for use in immunogold labelling localisation experiments.

CHAPTER 6

IMMUNOFLUORESCENCE STUDIES

6.1 Introduction

6.1.1 Indirect immunofluorescence

Indirect immunofluorescence was employed as the method of choice to determine if the raised antiserum bound specifically to internal structures in intact trypanosome cells. Immunofluoresence analysis entails the use of a fluorescent-labelled antibody to localize antigens in intact permeabilised cells. Indirect immunofluorescence involves the use of a "middle layer" of non-fluorescent primary antibody (antiserum) followed, after washing, with fluorescent-labelled reagent (secondary antibody). This reagent is usually an antibody directed against the middle

layer. The indirect assay is very sensitive since the layering of reagents increases the total number of determinants available to the fluorescent reagent, and the total number of fluorochromes bound to the specimen is subsequently increased. However, the indirect method is difficult to control, and the increases in background may be a poor-trade off for the increased sensitivity.

6.1.2 Controls

The heterogenous and undefined nature of polyclonal antisera makes the choice of controls in immunocytochemical experiments critical. Adequate controls, both positive and negative, were hence performed to ensure validity of any observed staining. Negative controls included the use of pre-immune serum, obtained from the original rabbit prior to immunisation with peptide antigen and used at an equivalent dilution to immune sera, in all experiments. An additional negative control involving omission of the middle primary antibody layer (ie. using PBS buffer instead of immune serum) was carried out in all experiments to ensure that the fluorescent tracer (secondary antibody) in the final layer was specific for binding to the middle layer only, and not to other components of the specimen. In addition, a positive control using the mouse monoclonal antibody ROD1, which has been shown to specifically bind to the paraflagellar rod in *T.brucei* (Woods et al, 1989), was performed to ensure the validity

of the immunofluoresence procedure. Finally, the assay was performed with various concentrations of primary and secondary antiserum in an effort to "dilute out" potential non-specific staining.

The inclusion of a blocking step, in which blocking agents such as BSA or a commercially available neutral serum such as foetal calf serum (FCS) are normally used, was employed to reduce background staining due to non-specific binding of both primary and secondary antisera. Surfactants such as Tween further reduce non-specific background staining. Pre-incubation of cells with 10% FCS in PBS/tween (PBS with 0.1 % v/v tween-20) was used as a blocking step in all experiments.

6.2 Results

6.2.1 Section 1: Initial conditions

6.2.1.1 Method

A 1/15 dilution of immune/pre-immune serum for both DLD1 and DLD2 was used as a starting primary antibody serum dilution. A control of undiluted ROD1 (a fully characterised monoclonal antibody) was also used to test that the system was working. Normally, commercial immunoconjugates (secondary antibody) work at a 1:100 dilution, although this is a very general starting concentration. Hence, a dilution series of secondary antibody was performed. Secondary antibody, goat anti-rabbit-FITC, was used at 1/50, 1/100, 1/500 ,1/1000 and 1/10,000 dilutions and added to all wells previously incubated with primary antibody (immune or pre-immune) including a control well treated with no primary antibody. Rabbit antimouse-FITC was used at a range of dilutions from 1/50 through to 1/10,000 in wells previously incubated with ROD1. A control well treated with no primary antibody was also incubated with rabbit antimouse-FITC.

6.2.1.2 Result

Cells incubated with ROD1 as primary antibody showed good immunofluoresence with 1/50 rabbit antimouse-FITC. Staining diminished with successive dilution of secondary antibody, giving a weak signal at 1/100 dilution and no signal at higher dilutions. Staining appeared as a thin line down the length of the

trypanosome cell, corresponding to specific staining of the paraflagellar rod. No immunofluoresence was observed in the absence of primary antibody (negative control). Strong immunofluorescence over the entire trypanosome cell was observed with both DLD1 and DLD2 preimmune and immune serum at both 1/50 and 1/100 dilutions of secondary antibody, while 1/500 and 1/1000 dilutions resulted in increasingly weaker immunofluoresence. No immunofluoresence was observed at 1/10,000 dilutions of secondary antibody. No immunofluoresence was observed with the negative control in the absence of primary antibody.

6.2.1.3 Conclusions

The distinctive fluorescent staining observed after treatment with ROD1 was as expected, being similar to staining observed by Woods et al (1989) and confirmed that the immunofluoresence procedure employed was a viable one. The negative control showed that the flouresence staining was due to primary antibody binding and that no cross-reactivity of antimouse secondary antibody with trypanosomal antigen had occurred.

The very strong uniform immunofluorescence observed for DLD1 and DLD2 immune and pre-immune serum was thought to be due to the very low primary antiserum dilution (1/15) used, resulting in high background immunofluoresence. The good immunofluoresence obtained with 1/100 dilution of anti-rabbit-FITC, together with the negative control, indicated that 1/100 dilution of secondary antibody was a reasonable working dilution. This dilution of secondary antibody was used in all subsequent experiments.

6.2.2 Section 2: Reduction of non specific background staining

Excessive background non-specific antibody interactions arising from spurious antibody activities in the serum are a common problem in cell staining with polyclonal sera. Because such antibodies will normally not account for the major activities in the serum, their binding can often be reduced below the levels of detection by careful titration of the polyclonal antibodies

6.3.2.1 Dilution of primary antibody

6.2.2.1.1 Method

a) The procedure was repeated as described in "general method" for both immune and preimmune serum for DLD1 and DLD2 except that various primary antibody dilutions were performed (1/10,1/00, 1/1000, 1/10,000) in an effort to dilute out any non specific background binding.

b) The experiment was repeated using a range of dilutions (1/100 - 1/1000) for anti-DLD1 and 1/50-1/100 for anti-DLD2.

6.2.2.1.2 Result

see over (Figs 28 and 29)

Good immunofluorescene staining was observed for DLD1 immune and preimmune serum (Fig.28). Although a general staining of the whole trypanosome cell was evident, areas of intense nuclear staining were evident for both immune and preimmune serum. A similar staining pattern, with intense nuclear staining, was observed for DLD2 immune and preimmune serum (Fig. 29).

6.2.2.1.3 Conclusions

a)Good immunofluorescence staining was observed at 1/10 , 1/50 and 1/100 dilutions of DLD1 (Fig. 28 (a)) while staining was noticably weaker at 1/1000 dilutions and absent at 1/2000. Good staining was observed for DLD2 at 1/10 and 1/50 while becoming weak at 1/100 (Fig. 29 (a)) and absent at 1/1000. Surprisingly, pre-immune and immune sera (for both DLD1 and DLD2) were found to stain the whole cell with approximately the same intensity (as observed by eye; Fig.s 28 (b) and 29(b)) at each

dilution, indicating that attempts to reduce any nonspecific background staining by dilution of primary antibody had failed.

b)Optimal fluorescence was found at 1/100 dilution of DLD1, while 1/50 gave optimal fluorescence for DLD2. However, there was no noticeable difference between antiserum and preimmune serum at these dilutions.

Although dilution of primary antibody failed to resolve the problem of background immunofluoresence staining, there is a clear difference in staining intensity between the two antisera. Incubation with DLD 1 immune serum (1/100 dulution) results in much higher fluorescence than incubation with DLD 2 immmune serum (1/100 dilution), appearing to support previous results (western blot, 4.2.2; immunoprecipitation, 5.3.2.2) that indicate the presence of a DHlipDH-specific antibody present in DLD1 immune serum only.

Attempts to reduce background staining were investigated further.

Figure 28 (a) Anti-DLD1 immune serum
1/100 dilution

As viewed under phase contrast
light microscope

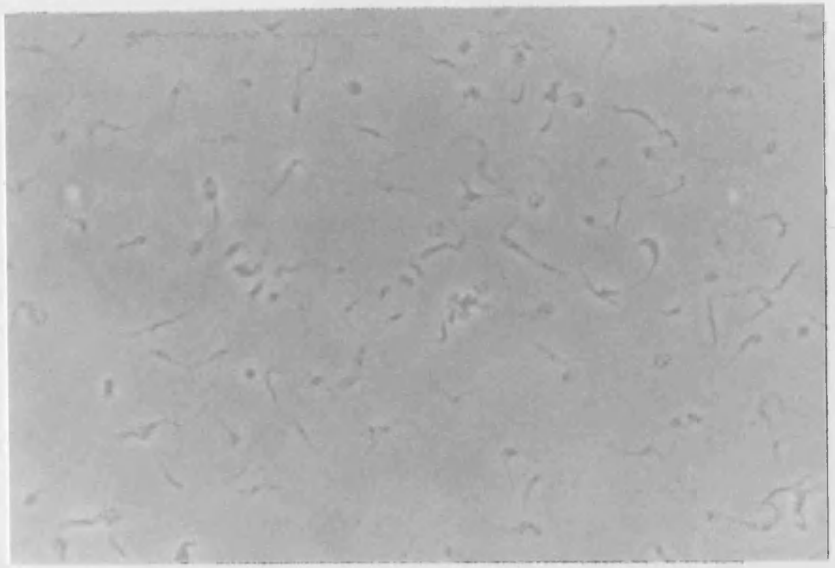
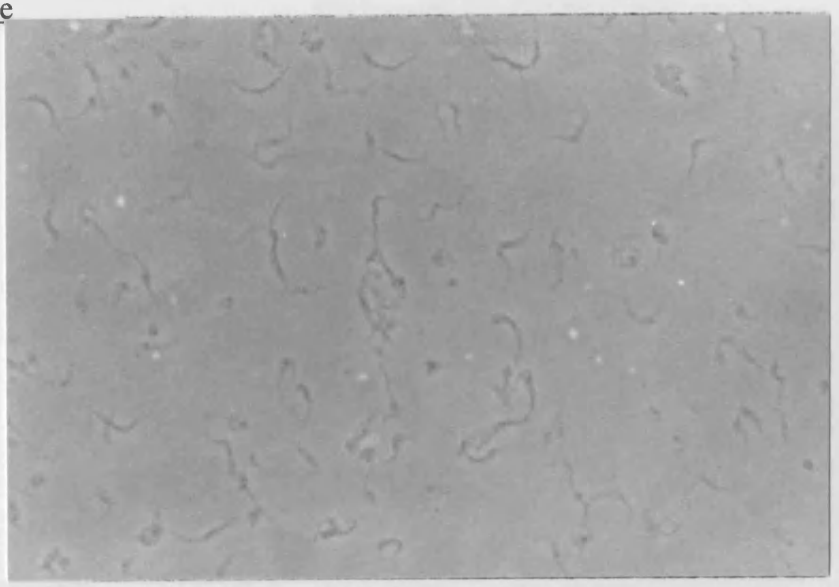
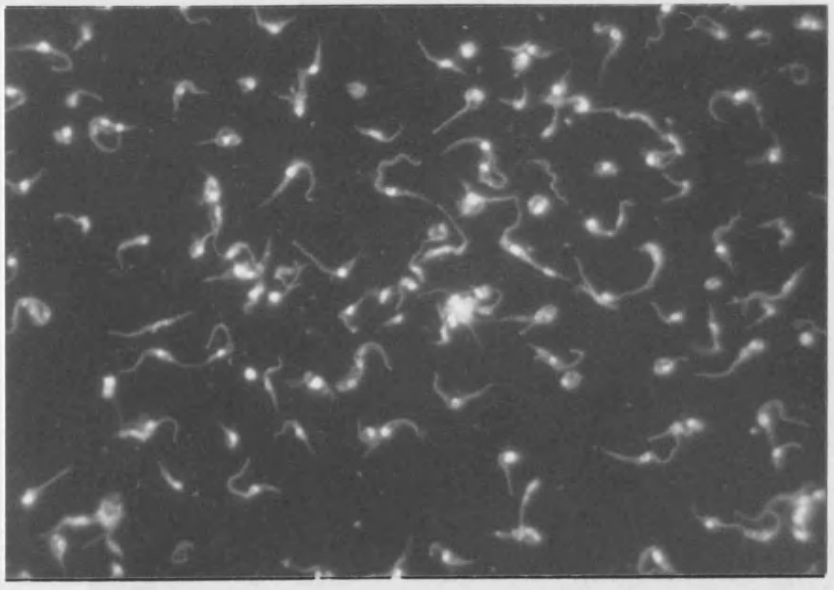


Figure 28 (b) Pre-immune serum
1/100 dilution



As viewed under fluorescent
microscope



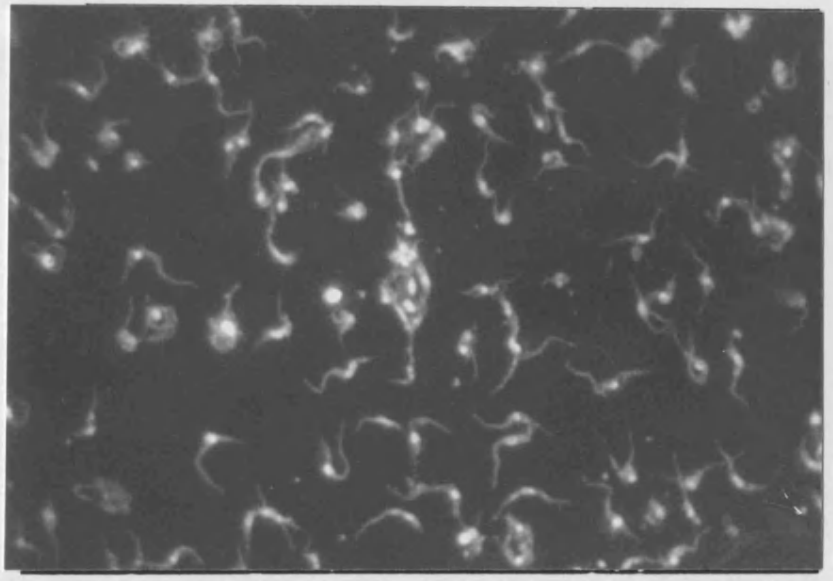


Figure 29 (a) Anti-DLD2 immune serum
Antiserum 1/50 dilution

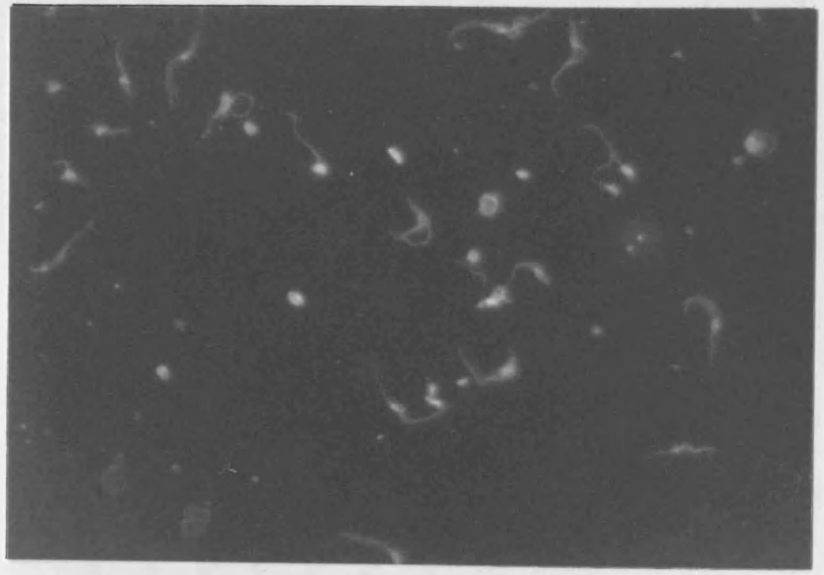
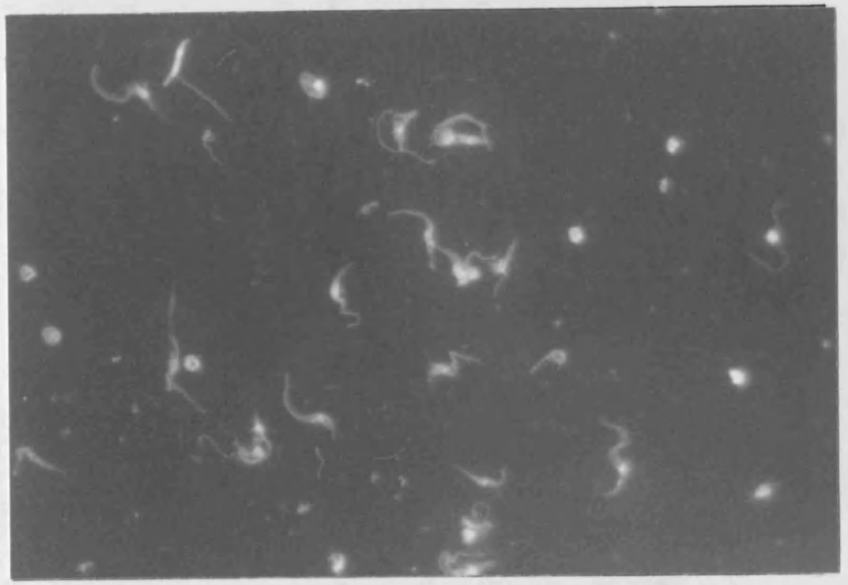
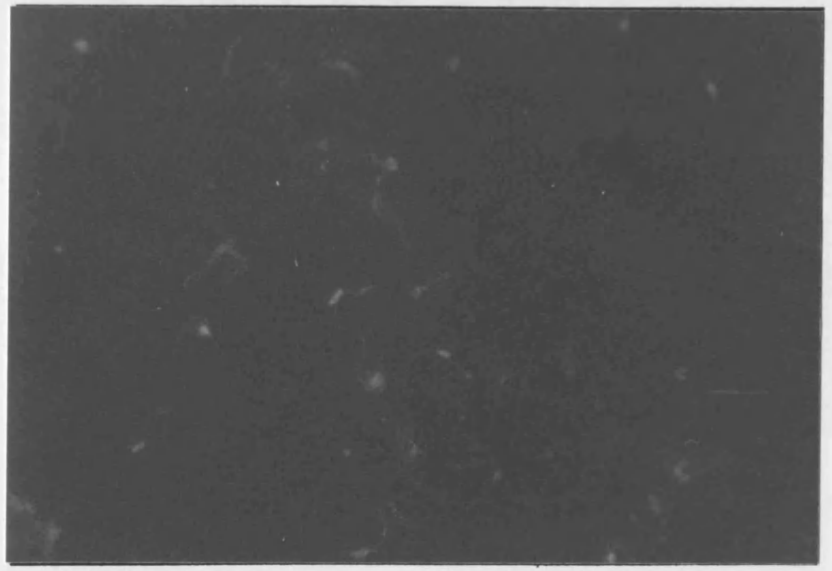


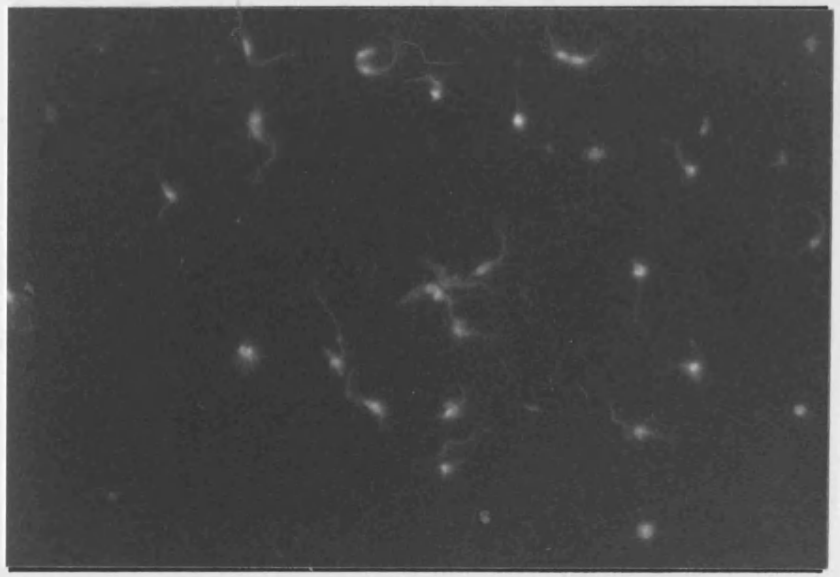
Figure 29 (b) Pre-immune serum
Pre-immune serum 1/50 dilution



Antiserum 1/100 dilution



Pre-immune serum 1/100 dilution



6.2.2.2 Use of neutral serum to reduce non specific background staining.

Commercially obtained serum (or 'neutral' serum) was used in an attempt to remove non-specific background staining. Cells were pre-incubated with blocking buffer containing neutral serum (1-10%) prior to labelling with primary antibody.

6.2.2.2.1 The use of commercially obtained neutral rabbit serum in both blocking buffer and primary antibody dilution

6.2.2.2.1.1 Method

a) Procedure was as described in section 2.26 except that blocking was performed with 10% FCS in PBS-tween overnight and various dilutions (1-10%) of neutral rabbit serum were added to the blocking buffer. Wells were incubated with 1/100 dilution of DLD1 immune/ preimmune serum and 1/50 dilution of DLD2 immune / pre-immune serum. A negative control in the absence of primary antibody was performed.

6.2.2.2.1.2 Result

Although strong immunofluorescence was observed both for DLD1 and DLD2, again no difference between immune and pre-immune sera was evident. On repeating the experiment , using 5% rabbit serum in blocking buffer and using 1/100, 1/200, 1/500, 1/1000 dilutions of both DLD1 and DLD2 (immune and preimmune) primary antibody, the same result was obtained ie. no observable difference in fluoresence between immune and pre-immune sera. Interestingly, there was no obvious sequential lowering in fluorescence intensity with primary antibody dilution as previously observed (section 6.3.2.1) and strong fluorescence was detected at dilutions as low as 1/1000 for both immune and pre-immune sera. The negative control (no primary antibody) performed following overnight incubation with blocking buffer containing rabbit serum resulted in a marked uniform immunofluorescence.

6.2.2.2.1.3 Conclusions

Not only is treatment with the normal rabbit serum having no effect in reducing background staining and creating an observable difference between immune and

preimmune sera, but appears to contribute to background immunofluoresence.This was confirmed by the negative control which showed uniform immunofluoresence following preincubation with blocking buffer containing neutral rabbit serum. This confirms the presence of strong cross-reactive antibody(s) in commercially obtained rabbit serum as indicated in previous immunoprecipitation experiments (chapter 5 section 5.5). Indeed, cross-reactive antibodies appear to be a common occurrence in rabbit serum in general (Keith Gull, personal communication).

6.2.2.2.2 Use of commercially obtained neutral goat serum in both blocking buffer and primary anitbody dilution

6.2.2.2.2.1 Method

Procedure was followed as described above (section 6.3.2.2.1.1) except that neutral rabbit serum was replaced by neutral goat serum. Cells were preincubated overnight in blocking buffer containing 1-10% normal goat serum prior to labelling with a 1 /100 - 1/2000 dilution of DLD1 primary antibody (immune and pre-immune) or 1/50 - 1/1000 dilution DLD2 (immune and preimmune serum). The use of goat serum as blocking antibody ensures that there is no recognition by secondary antibody as the blocking serum is from the same animal species as that used to make the secondary antibody. A negative control in the absence of primary antibody was performed.

6.2.2.2.2.2 Results

Uniform immunofluorescence was observed with both DLD1 and DLD2 at all concentrations of goat serum used in blocking buffer. No difference between immune and preimmune serum was observed in either case. However, contrary to results using neutral rabbit serum, on repeating the experiment using 5% goat serum in blocking buffer and using 1/100, 1/200, 1/500 and 1/1000 dilutions of both DLD1 and DLD2, staining became weaker with increased dilution. However, no difference between immune and preimmune staining was observed at any dilution. The negative control (no primary antibody) resulted in no observable immunofluorescence.

6.2.2.2.2.3 Conclusions

Hence, although not able to reduce background staining, preincubation with goat serum does not result in additional non specific binding of secondary antibody as observed with neutral rabbit serum.

6.2.2.3 Investigation of incubation time and temperature to reduce non specific background staining

Many antibody solutions contain a small proportion of antibodies that will bind non specifically to certain antigens (e.g. to charged residues) in a cell. Such non specific interactions may be reduced by shortening incubation times and/or lowering temperature.

6.2.2.3.1 1 hr primary antibody incubation at different temperatures for neutral rabbit/goat serum

6.2.2.3.1.1 Method

A series of wells were blocked overnight with 10% FCS in PBS/Tween and then treated with normal rabbit serum (1/10, 1/100, 1/1000 and 1/10,000 dilutions) while a further series were treated similarly with commercially obtained goat serum. (These wells were treated with rabbit anti goat-FITC as secondary antibody). Primary antibody incubation was carried out for a) 1 hour at 26 °C and b) for 1 hour at 4 °C

6.2.2.3.1.2 Results

a) 1 hour at 26 °C (Fig. 30)

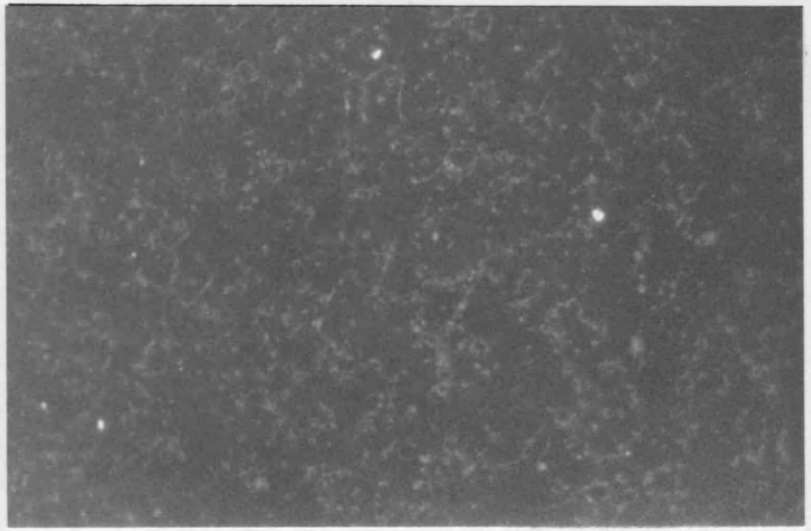
Good immunofluorescence was observed for normal rabbit serum up to 1/1000 dilution (Fig. 30 (a)). No immunofluorescence was observed with normal goat serum.

b) 1 hour at 4 °C (Fig. 31)

No immunofluorescence was observed for either rabbit or goat serum.

Figure 30

(a) 1 hr incubation (26 degrees) with neutral rabbit serum
(1/1000 dilution)

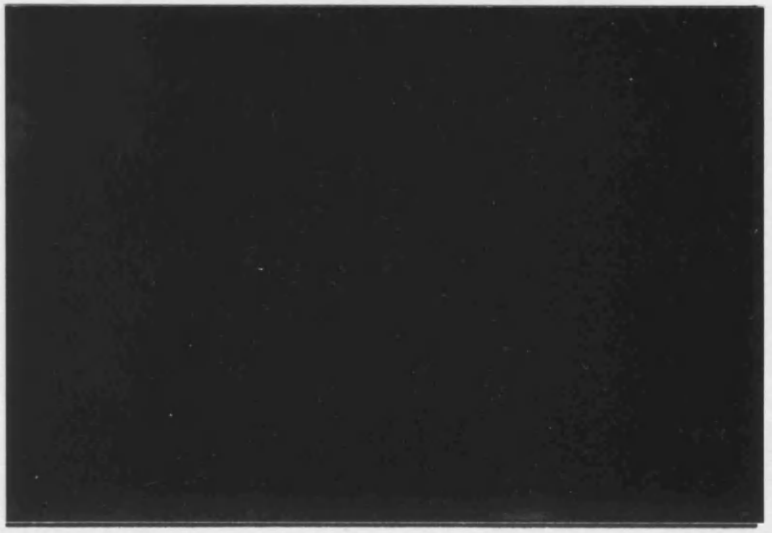


(b) 1 hr incubation (26 degrees) with neutral goat serum
(1/10 dilution)

Figure 31

(a) 1 hour incubation (4 degrees) with neutral rabbit serum
(1/10 dilution)

(b) 1 hr incubation (4 degrees) with neutral goat serum
1/10 dilution



6.2.2.3.1.3 Conclusion

This clearly shows that no background binding from neutral serum of either species occurs on incubation for one hour on ice. These conditions are therefore favourable for the removal of background binding

6.2.2.3.2 Primary antibody incubation for 1 hr (4°C) for immune / preimmune sera

6.2.2.3.2.1 Method

The general method was followed as described above, with wells being blocked overnight in 10% FCS in PBS/tween. However, primary antibody, anti-DLD1 immune and pre-immune sera (1/50, 1/100, 1/200 and 1/500 dilutions) were incubated with cells for 1 hr on ice before incubation with secondary antibody.

6.2.2.3.2.2 Result

No immunofluorescence was observed at any of the dilutions.

6.2.2.3.2.3 Conclusions

Incubation for 1hr on ice is not sufficient for primary antibody binding of test immune serum and is hence not a viable solution for the reduction of background staining.

The failure of both dilutions of primary antibody (section 6.3.2.1) and the use of neutral goat goat serum (section 6.3.2.2.2) to reduce background immunofluorescence suggests that observed staining is not due to non-specific binding of primary or secondary antibody, but rather that the rabbit polyclonal antiserum, and rabbit serum in general, contains strong cross-reactive antibodies which may mask the reactivity of raised DHlipDH-specific antibodies. Non-specific labeling of this type may best be avoided by affinity purification of the antibody

6.2.3 Section 3 Preliminary to affinity purification : adsorption of serum IgG component by *Staph. aureus* protein A

The strong, seemingly specific, staining of pre-immune serum was investigated further to determine if the observed reactivity was due to IgG or other serum antibodies

including IgM and IgA. The IgG content of the pre-immune serum was precipitated out using Staphylococcus aureus containing Protein A (see immunoprecipitation method, Chapter 5, 5.5 section 5) to observe if any staining occurred using IgG deficient pre-immune serum.

6.2.4.1 Method

Pre-treatment of immune serum

150 µl of pre-immune serum from rabbit 142 was incubated with a pellet of 1ml 10% *Staph. aureus* (washed twice in sterile PBS) for 30 mins on ice. The suspension was centrifuged and the supernatant removed and used as described below.

The method was followed as previously described ("general method.") except that 1/50, 1/100,1/200 and 1/500 dilutions of DHD1 immune, pre-immune and treated pre-immune sera were incubated in wells for I hr at 26°C before adding diluted goat antirabbit-FITC (1/100 dilution).

6.2.3.2 Result

Fig. 32 (See over)

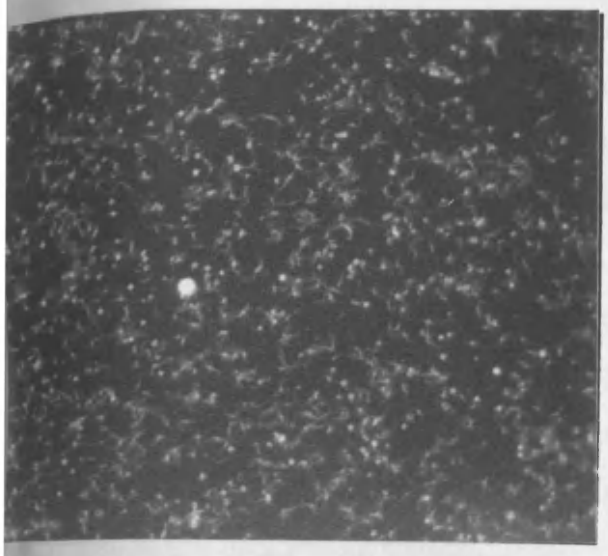
Immunofluorescence staining occurred with all three primary antibody samples. Fluorescence intensity was dilution dependent between 1/50 to 1/200. No fluorescence was detected at 1/500 for any of the samples. There was no difference in intensity between immune and pre-immune serum (in accordance with previous findings), however " treated " (ie. IgG-deficient) preimmune serum appeared to show a slight reduction in flourescence intensity. Even with "treated " serum, however, background staining was still prevalent, indicating the strong presence of trypanosome-specific non-IgG antibodies.

6.2.3.3 Conclusion

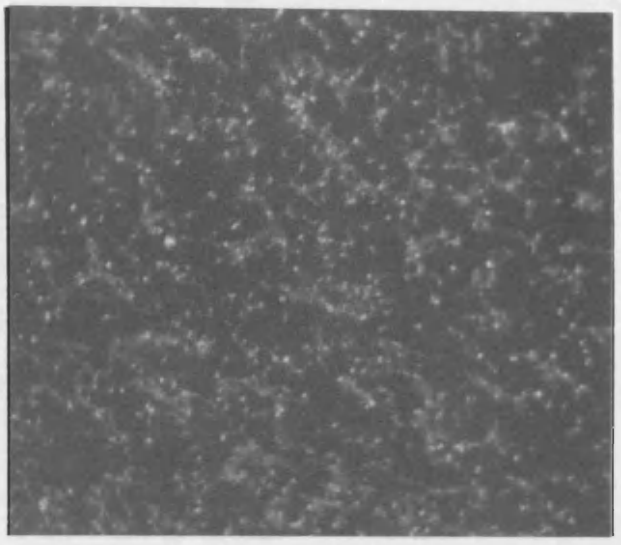
The high background staining for all three samples (immune, preimmune and treated) indicates the presence of cross-reactive antibodies other than IgG types which are masking any specific IgG-DHlipDH interaction.

Figure 32
Comparison of immune and treated pre-immune serum

DLD1 Immune serum
1/50 dilution

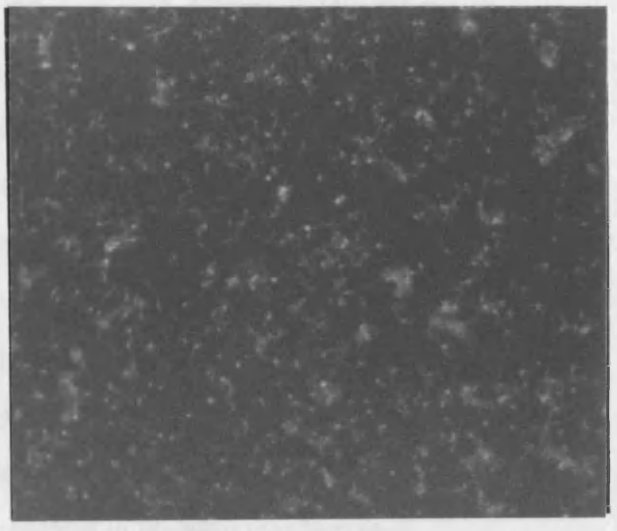


Treated pre-immune serum
1/50 dilution

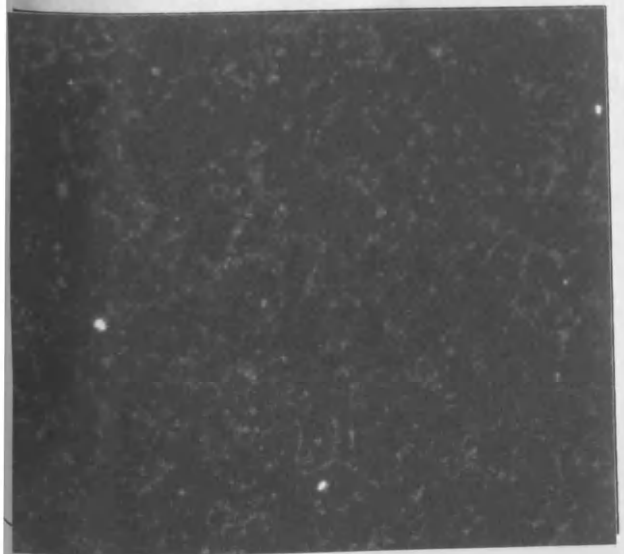


1/100 dilution

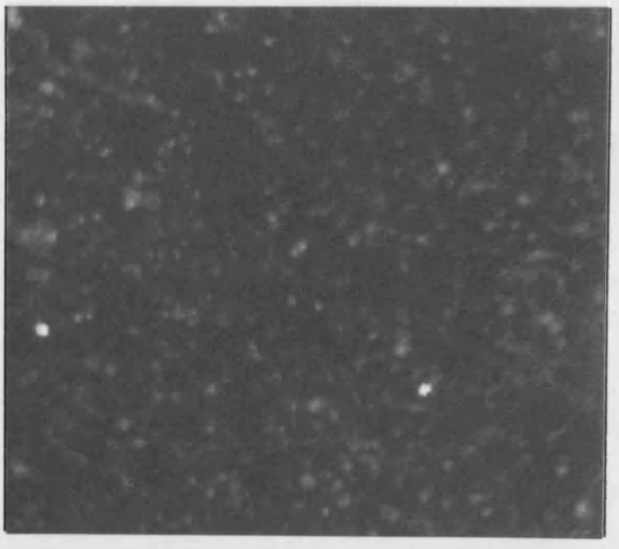
1/100 dilution



1/200 dilution



1/200 dilution



6.3 Discussion

Although satisfactory cell staining experiments may be conducted using polyclonal antibodies, their use requires careful control. The results described in this section show that strong specific background staining is present and this staining may be resposible for masking any DHlipDH-specific binding. All attempts to reduce specific background staining , including diluting out of any specific spurious antibodies (6.3.2.1) and attempts to reduce non specific binding of primary or secondary antibodies using neutral rabbit serum and goat serum (6.3.2.2.1) were unsuccessful. Similarly, attempts to reduce non specific background staining by reducing temperature and incubation time (6.3.3) were also unsuccessful. Staining observed following the removal (or at least reduction) of the IgG fraction of preimmune serum using protein A (6.3.4) indicates that antibodies other than IgG molecules (probably IgM or IgA) are involved in this background binding.

Affinity purification of both DLD1 and DLD2, using synthetic peptides DLD1 and DLD2, was carried out at Keith Gull's laboratory, University of Manchester, by Kit Tyler. The purified antibodies were tested for cross-reactivity in *T. brucei* bloodstream, stumpy and procyclic cells. DLD2 antibodies appeared not to cross-react at all with any cell type, while DLD1 antibodies were found to weakly bind to the mitochondrion of procyclic and stumpy cell and no crossreactivity was observed with bloodstream cells (Tyler, personal communication). This result was verified by Tyler et al (1997) using polyclonal antibody to *T. cruzi* DHlipDH provided by Lohrer and Krauth-Siegel (1990). Immunofluorescence studies, performed by Tyler et al using Lohrer and Krauth-Siegel's polyclonal antiserum, resulted in staining that localised DHlipDH to the mitochondrion of the stumpy and procyclic forms, while failing to detect the protein in the slender form. Hence although it seems that at least one of the antisera raised (antiDLD1) was active, binding was weak and hence unlikely to withstand the rigorous procedures involved in immunogold localisation studies. It was therefore decided to abandon plans for immunolocalisation of DHlipDH in *T.brucei* using peptide antisera.

Peptide antisera suffer, in general, from one major drawback- an antiserum with a high titre against the free peptide will not necessarily have a high affinity for the native protein (Beesley, 1993). Although the peptide sequence can be chosen to include regions which are likely to be antigenic determinants, it is impossible to predict with certainty that the selected region will be at the surface of the native protein and therefore accessible to antibodies, or if this region of the native protein is in a similar conformation to that of the peptide.

Although immunolocalisation of DHlipDH in other members of the Trypanosomatidae family using DHlipDH-specific antibodies was hence no longer an option, it was decided to continue localisation studies of DHlipDH in other trypanosomes, specifically *C.fasciculata* and *Phytomonas sp.* using cell fractionation techniques. The presence of DHlipDH in both cells was established by assaying cell lysates, and enzyme localisation investigated further by analysis of plasma membrane isolates obtained following fractionation of both *C.fasciculata* and *Phytomonas sp.*

CHAPTER 7

INVESTIGATION OF DHLIPDH IN *Crithidia fasciculata* AND *Phytomonas sp.*

7.1 Introduction

There are no reports in the literature to date concerning investigations of DHlipDH in either *Crithidia fasciculata* or *Phytomonas sp.* Verification of the presence of DHlipDH in both cell types was therefore an essential pre-requisite to any localisation studies of the enzyme.

7.2 DHlipDH in *Phytomonas sp.* and *Crithidia fasciculata* cell sonicates

7.2.1 Methods

A number of control enzyme assays were perfomed to ensure that any rise in absorbance observed at 340 nm was due to DHlipDH activity.

7.2.1.1 Alcohol dehydrogenase

Assay for NAD-dependent alcohol dehydrogenase in *Phytomonas sp.* and *Crithidia fasciculata* cell sonicates under conditions suitable for DHlipDH assay, as detailed in methods section 2.28.3.1, resulted in no observed increase in absorbance at 340 nm over a ten minute assay period.

7.2.1.2 'No substrate' controls

'No substrate' controls were performed routinely (methods section 2.28.3.1.) and any observed endogenous activity subtracted from experimental results.

7.2.1.3 NADH oxidising activity

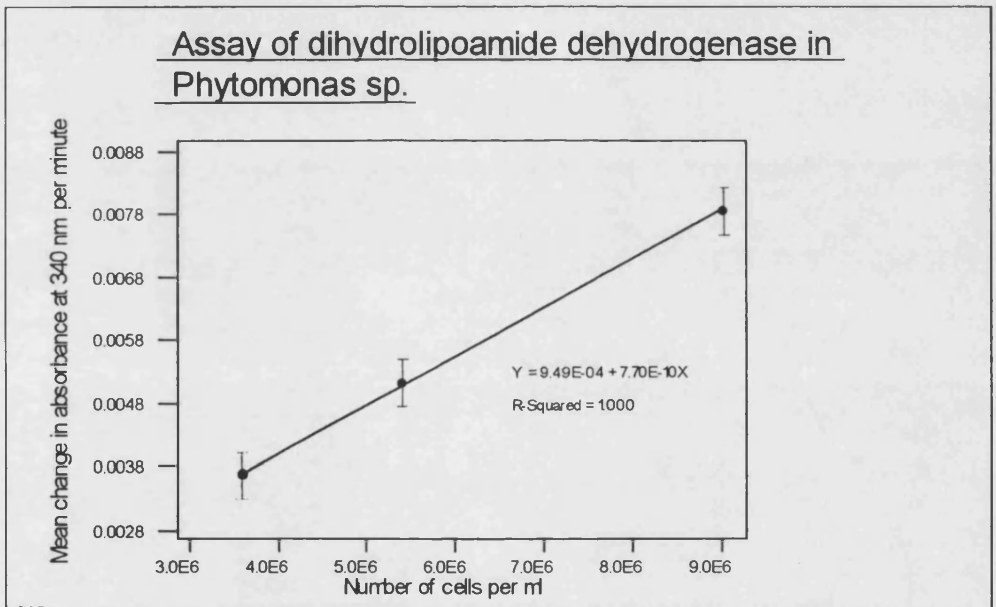
Phytomonas sp. sonicates exhibited some endogenous NADH oxidising activity, probably due to the presence of NADH oxidase, observed as a decrease in absorbance at 340 nm when assayed.(see methods section 2.28.3.2.). Hence, it was recognised that assays measuring NADH formation in *Phytomonas sp.* including the assay for DHlipDH would not reflect full enzyme activity. *Crithidia fasciculata* cell sonicates exhibited no NADH oxidase activity.

7.2.2 Results

7.2.2.1 Assay of DHlipDH in *Phytomonas sp.*

2.25 x 10^8 cells were sonicated in 1 ml PBS. 20µl, 30µl and 50µl aliquots were assayed as described in methods section 2.28.3.1. A good linear relation between activity and sonicate volume (Fig. 33) was obtained. Protein assays were performed by standard coomassie blue assay, and the mean specific activity was determined to be 0.06 µmol /min/mg protein. Because of the endogenous NADH oxidising activity in *Phytomonas sp.* sonicate (previously mentioned), it may be suggested that this is not the absolute specific activity but rather the minimum specific activity.

Figure 33



2.25E8 cells were sonicated in 800ul PBS. 20, 30 and 50 ul aliquots of sonicate were taken and assayed for DHlipDH activity. Protein determinations were carried out by coomassie blue protein assay, using BSA as a protein standard. mean specific actvity was calculated to be 0.06 um / min / mg protein

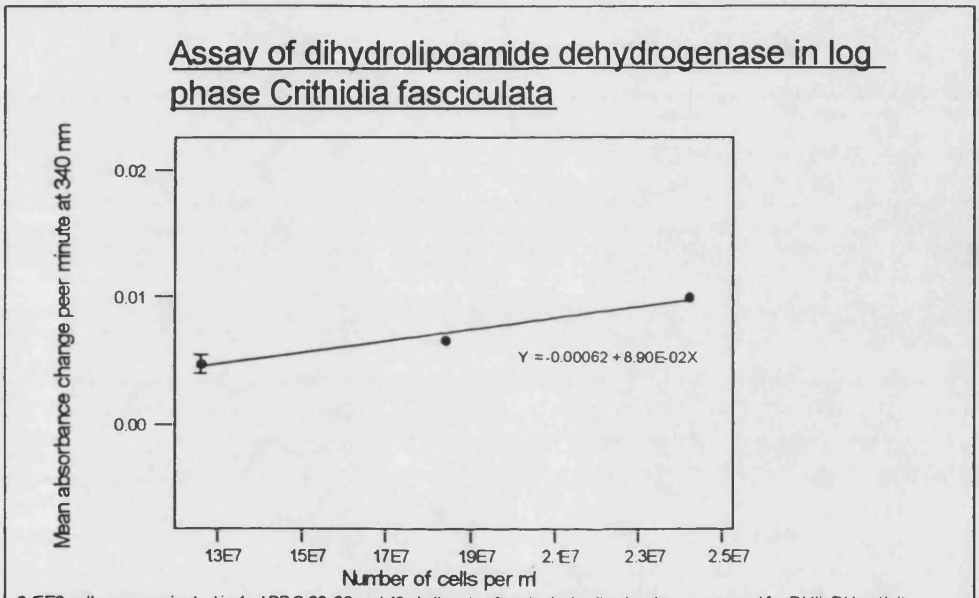
7.2.2.2 Assay of DHlipDH in *Crithidia fasciculata*

DHlipDH activity was assayed in both logarithmic (log) and stationary growth phase cells of *Crithidia fasciculata.*

7.2.2.2.1 Assay of DHlipDH in log phase *Crithidia fasciculata*

6.15 x 10^8 cells were sonicated in 1ml PBS. 20 µl, 30µl and 40 µl sonicate aliquots were assayed for dihydrolipoamide dehydrogenase activity. A good linear relation between activity and sonicate volume (Fig. 34) was observed. Protein assays were performed by standard coomassie blue assay, and the mean specific activity was determined to be 0.29 µmol /min/mg protein.

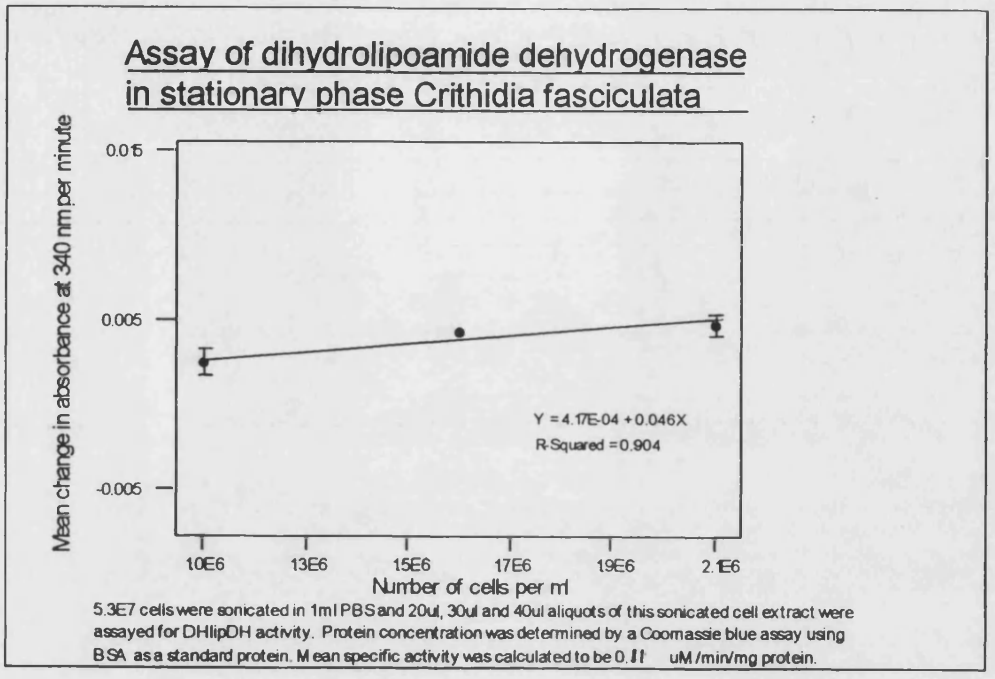
Figure 34



7.2.2.2.2 Assay of DHlipDH in stationary phase *Crithidia fasciculata*

5.3 x 10^7 cells were sonicated in 1ml PBS. 20µl, 30µl, and 40µl sonicate aliquots were assayed for DHlipDH activity. A good linear plot was obtained between activity and sonicate volume (Fig. 35). Protein assays were performed by standard coomassie blue assay, and the mean specific activity was determined to be 0.11 µmol /min/mg protein.

Figure 35



7.2.3 Summary

Table 8: Specific activities in cell free extracts :Comparison of DHlipDH-specific activities (in µM of NADH produced/minute/mg protein) from *T.brucei*, *C.fasciculata* and *Phytomonas sp.*

Trypanosome species	Specific activity µmol/min/mg protein
Phytomonas sp.	0.06
Log *Crithidia fasciculata*	0.29
Stationary *Crithidia fasciculata*	0.11
T.b. brucei bloodstream *	0.03
T.b. brucei procyclics*	0.4

* Taken from Cook et al, 1990.

7.2.4 Conclusions

The presence of DHlipDH in mitochondrially active log phase *Crithidia fasciculata* is 5 times the level in mitochondrially repressed *Phytomonas sp.* This is comparable to

DHlipDH activity in *T.b. brucei* where Cook et al (1990) reported increased levels of DHlipDH in mitochondrially active procyclic forms ten times those measured in mitochondrially inactive bloodstream forms. Both results may be accounted for by the activity of DHlipDH-containing mitochondrial 2-oxo acid dehydrogenase complexes which are absent in mitochondrially repressed forms, although it is impossible to say whether the increase in mitochondrially active forms is due entirely to these complexes.

The difference in DHlipDH levels between log and stationary phase *Crithidia fasciculata* is an interesting one. Differences in enzyme activity may be a consequence of differing metabolic rates, with log phase cells being the most metabolically active stage. Rapid glycolysis in dividing logarithmic cells would result in excretion of carboxylic acids e.g. acetic, pyruvic and succinic acid which may cause acidification of the media. Acidification tends to slow and then terminate growth of Crithidial cells in traditional media (Shim and Fairlamb, 1988), so explaining the observed metabolic inactivity of stationary phase *Crithidia fasciculata.*

Interestingly, Page and Lagnado (1998) found that *Crithidia fasciculata* , under certain conditions, form large bundles of trans-cytoplasmic filaments that are distinct from both microtubules, the only major cytoskeletal element identified to date in the cell body of trypanosomes, and actin microfilaments. These filaments were never seen in log-phase cells or lower density stationary-phase cultures of *Crithidia fasciculata.* Thus, filament formation appears to be linked to conditions of limited nutrient availability, such as are associated with dense stationary-phase cultures of the parasite (Shim and Fairlamb, 1988). Changes in DHlipDH activity between log- and stationary- phase *Crithidia fasciculata* may hence be a biochemical reflection of the general cell growth cycle.

7.2.5 Future work

Future work should focus on assays for the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched chain 2-oxo acid dehydrogenase complexes in both cell types. Such assays are essential to consolidate further the findings of Sanchez-Moreno et al (1991) and Fernandez Beccerra et al (1997) who reported that *Phytomonas sp.* were incapable of oxidising 2-oxoglutarate and pyruvate and had fully repressed mitochondria that were similar to bloodstream *T. brucei* mitochondria. DHlipDH -

containing 2-oxo acid dehydrogenase complexes are intra-mitochondrial in other eukaryotes, and therefore their presence might be expected in mitochondrially-active cells such as *Crithidia fasciculata* where both ∝-ketoglutarate and pyruvate dehydrogenase complex activity have been reported (Diaz and Komuniecki, 1995).

7.3 Determination of DHlipDH Km^{app} and $Vmax^{app}$ in *Phytomonas sp.* cell sonicate. (see methods section 2.27.5)

7.3.1 Method

DHlipDH assays were performed on crude *Phytomonas sp.* cell sonicates (obtained by sonicating 1 x 10^8 cells in 1 ml PBS as described in methods section 2.23) in order to investigate the kinetic properties of Phytomonas DHlipDH. NAD^+ was present at a constant high concentration (10 mM) while varying concentrations of dihydrolipoamide (from 0.5 mM to 0.05 mM) were assayed in the presence of 50 µl of *Phytomonas sp.* cell sonicate. The concentration of the dihydrolipoamide was determined by titration with 5,5'-dithiobis-(2-nitrobenzoic acid).

7.3.2 Result

An increase in DHlipDH activity with increasing DHlip substrate concentration was observed. This result is presented as a Hanes-Woolf plot (Fig. 36).

The plot shows the close fitting of all the points to a straight line, and indicates that that the kinetics of the reaction are steady state. The kinetic parameters Km^{app} and $Vmax^{app}$ were determined from direct linear plot analysis of the data using 'Enzpak' software and found to be 0.23 +/- 0.03mM and $3.6x10^{-3}$ +/- 0.001 s^{-1} respectively.

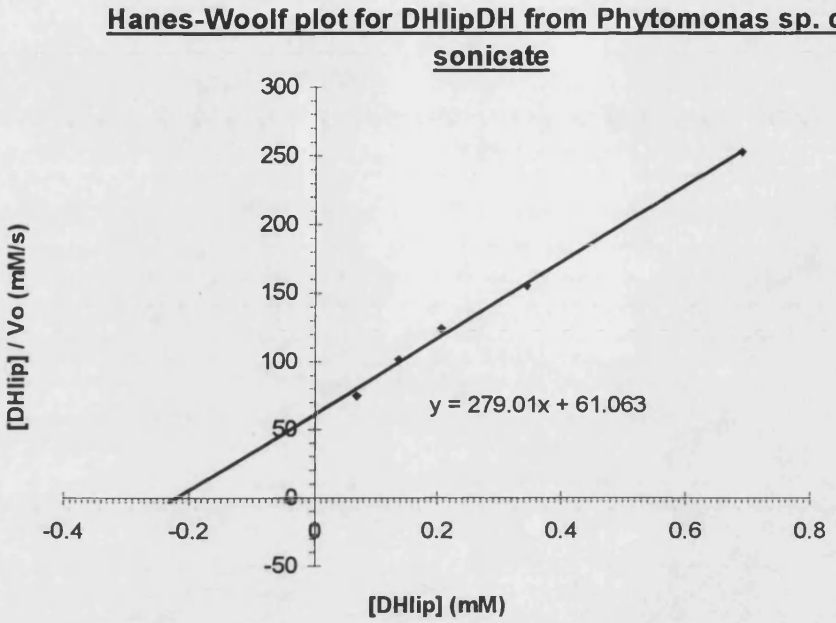


Figure 36: The kinetics of DHlipDH, represented as a Hanes-Woolf plot as DHlip concentration is varied at a constant NAD^+ concentration of 10mM.

Table 9: Comparison of DHlipDH specific activity in various Trypanosome strains

Trypanosome strain	Activity nmol/min/mg protein
T.b.rhodesiense (bloodstream) [a]	280
T.b.gambiense (bloodstream) [a]	115
T.b.brucei (bloodstream) [b]	29
T.b.brucei (bloodstream) [a]	30
T.evansi (bloodstream) [a]	45
T.b.rhodesiense (procyclic) [a]	350
T.b.gambiense(procyclic) [a]	740
T.b.brucei (procyclic) [a]	400
T.b. brucei (procyclic) [c]	400
C. fasciculata (choanomastigote) [d]	300
Phytomonas sp. (promastigote)	61

[a] Else et al 1994 [b]Danson et al(1987) [c]Cook et al (1990) [d]Andy McClennan, Bath University.

7.3.3 Conclusion

All three *T. brucei* species in the above table show higher levels of activity in procyclic than in bloodstream forms. This difference varied between species, being approximately 13 fold with *T.b. brucei,* 6-fold with *T.b.gambiense* and 1.25-fold with *T.b.rhodesiense.* Comparison of the mitochondrially active *Crithidia fasciculata* with the inactive *Phytomonas sp.* renders an analogous situation, with *Crithidia fasciculata* DHlipDH levels 5 times those observed in *Phytomonas sp.*

The level of DHlipDH in sonic extracts of *Phytomonas sp.* (0.06 μmol/min/mg protein) is twice the level of DHlipDH found in bloodstream *T. brucei* sonicates (0.03 μmol/min/mg protein) as determined by Danson et al (1987). The level compares favourably, however, with that found in *Halobacterium halobium* (Danson et al, 1986) which also lacks the 2-oxo acid dehydrogenase complexes and with some cyanobacterium which lack 2 oxoacid dehydrogenase complexes. While filamentous cyanobacterium *Anabaena sp.*, lacking pyruvate and 2 oxoglutarate dehydrogenase activities, contains DHlipDH with specific activity 190μmol /min/mg protein (Serrano,1992), DHlipDH from unicellular cyanobacterium *Synechoccus sp.* has a specific activity of 0.05-0.08 μmol/min/mg protein (Serrano et al,1992) while DHlipDH in another cyanobacterium, *Synechocystis*, was found to have a specific activity of 0.075 μmol /min/mg protein (Engels et al, 1997).

The level of DHlipDH in *Crithidia fasciculata* (0.3μmol/min/mg protein) is comparable with the mitochondrially active *T.brucei* procyclic (0.4μmol/min/mg protein). The specific activity is 3-fold higher than in the epimastigotes of *T. cruzi* (Lohrer & Krauth-Siegal, 1990) and in *Saccharomyces cerevisiae (*Wren and Massey, 1965), which possess active citric acid cycles, and 5-fold higher than the activity in *Halobacterium halobium* (Danson et al, 1986) which does not have the 2-oxo acid dehydrogenase complexes.

CHAPTER 8

ENZYME MARKERS FOR THE DETERMINATION OF PLASMA MEMBRANE PREPARATION PURITY

Prior to isolation and investigation of trypanosomal plasma membranes, it was a necessary to be able to determine the purity of the isolated plasma membrane fractions. Contamination of plasma-membrane preparations may occur in three ways. First, particles such as mitochondria may co-sediment with the fraction. Second, in the case of large sheets of plasma membrane, particles may be trapped inside the membrane and will be released only if the membrane is fragmented. Third, proteins or small vesicles may adhere to the membrane, often by electrostatic interaction.

The purity of plasma-membrane preparations may be assessed in two main ways;

1. by morphological examination, with either the light or electron microscope
2. by measurement of 'marker' enzymes for other cell organelles

Four enzyme markers, specific to various trypanosomal organelles, were chosen for use in determining plasma membrane purity following cell fractionation of *Crithidia fasciculata* and *Phytomonas sp.* cells. The chosen marker enzymes were:

- NAD-dependent isocitrate dehydrogenase (mitochondrial marker),
- NAD-dependent glycerol - 3 - phosphate dehydrogenase (glycosomal marker),
- pyruvate kinase (cytosolic marker)
- 5'nucleotidase (plasma membrane marker).

Preliminary enzyme assays were performed on cell sonicates to determine the suitability of each enzyme assay in *Crithidia fasciculata* and *Phytomonas sp.* Catalytic activity was assayed spectrophotometrically at 30 °C using a Perkin Elmer Lambda Bio or Lambda 11 spectrophotometer. Initial enzyme rates were calculated using the Perkin-Elmer Computer Spectroscopy Software (PECSS) version 4.31 supplied with the instrument. All assays were performed in duplicate unless otherwise stated.

8.1 NADH oxidising activity

While *Crithidia fasciculata* sonicates exhibited no endogenous NADH oxidising activity, *Phytomonas sp.* sonicates showed a decrease in absorbance at 340 nm when assayed (see methods section 2.28.3.2). Routine 'no substrate' controls to determine endogenous NADH oxidase activity in *Phytomonas sp.* (for each marker enzyme assay measuring NAD^+ formation) were therefore essential. All such rates were routinely subtracted from experimental rates. It was recognised that assays measuring NADH formation would not reflect full enzyme activity.
Routine 'no substrate' controls were performed as negative controls in *Crithidia fasciculata.*
Endogenous controls, in the absence of cell sonicate, were performed in all assays and subtracted from experimental rates where appropriate.

8.2 Isocitrate dehydrogenase

Isocitrate dehydrogenase catalyses the nicotinamide nucleotide-dependent oxidative decarboxylation of isocitrate to 2-oxoglutarate. Eukaryotes contain both NAD- and NADP- specific isocitrate dehydrogenases; the NAD-linked enzyme is confined to mitochondria and is allosterically regulated in line with its regulatory role of the energy-yielding citric acid cycle (Colman, 1983; Plaut and Gabriel, 1983) while the NADP-linked enzyme is found both in the mitochondria and the cytoplasm and is not allosterically regulated.

8.2.1 Assay of NAD-dependent isocitrate dehydrogenase in *Phytomonas sp*

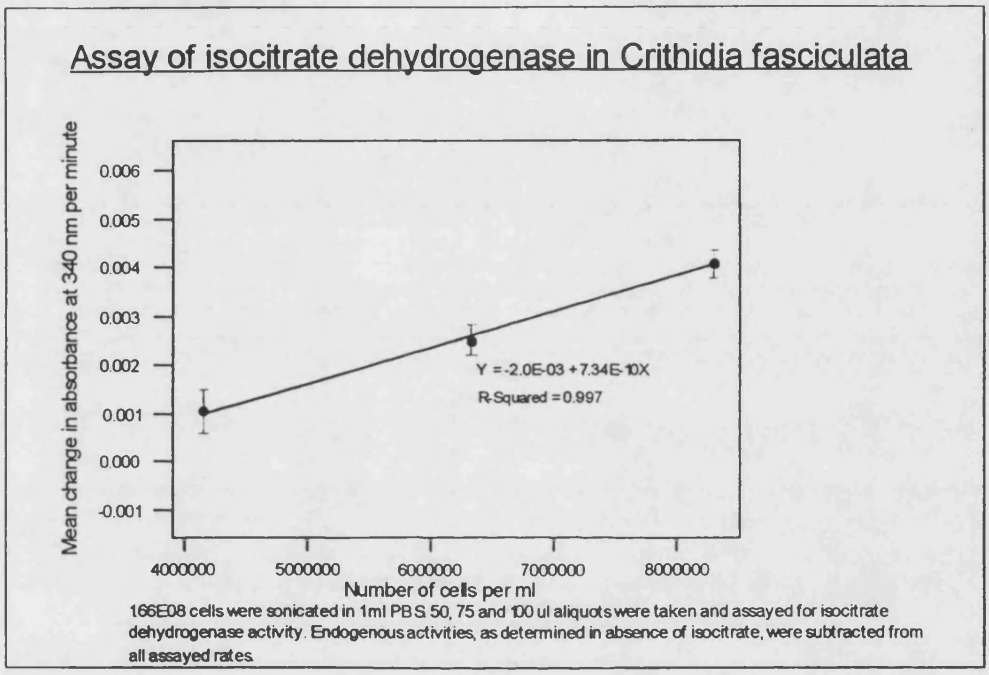
2.25×10^8 cells were sonicated in PBS and assayed as described previously (methods section 2.28.3.1). No isocitrate dehydrogenase activity was observed.

8.2.2 Assay of NAD-dependent isocitrate dehydrogenase in *Crithidia fasciculata*

1.66×10^8 cells were sonicated in 1 ml PBS. 50µl , 75µl and 100 µl aliquots were taken and assayed for isocitrate dehydrogenase activity as detailed in methods section 2.28.3.1. Blank rates in the absence of substrate were deducted from all observed initial rates (and all controls) which were then plotted giving a good linear

plot (Fig. 37). Control assays were performed in the absence of NAD^+ and in the absence of Mg^{2+} (no magnesium was added to assay buffer and 0.5 mM EGTA was added to buffer to bind any Mg^{2+} in the cell sonicate). Both controls were negative, confirming that the observed activity was specific to isocitrate dehydrogenase.

Figure 37



8.2.3 Conclusions

The absence of isocitrate dehydrogenase in *Phytomonas sp* is in keeping with its biochemical similarity to bloodstream *T. brucei*. Both have repressed mitochondrial activity (Durieux et al, 1991; Sanchez-moreno et al, 1995) and hence lack enzymes involved in the mitochondrial TCA cycle. The TCA cycle enzyme NAD^+-isocitrate dehydrogenase is not detectable in bloodstream *T. brucei* trypomastigotes (Duriex et al 1991) but is detectable in the procyclic form which, like *Crithidia fasciculata* , has a fully functional mitochondrial TCA cycle. Interestingly, NAD-dependent isocitrate dehydrogenase is not found in the insect trypanosome *Herpetomonas muscarum ingenoplastis* or *T. cruzi* (Francisco -Javier et al, 1988) where the presence of mitochondrial NADP-dependent isocitrate dehydrogenase was observed.

8.3 NAD^+-dependent glycerol-3-phosphate dehydrogenase

NAD^+- linked glycerol-3-phosphate dehydrogenase catalyses the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate and vice-versa within the glycosome where it is firmly particle bound. (Opperdoes and Nwagwu, 1980). Its main function is to guarantee the reoxidation of the glycolytically produced NADH by the glyceraldehyde-3-phosphate dehydrogenase reaction.

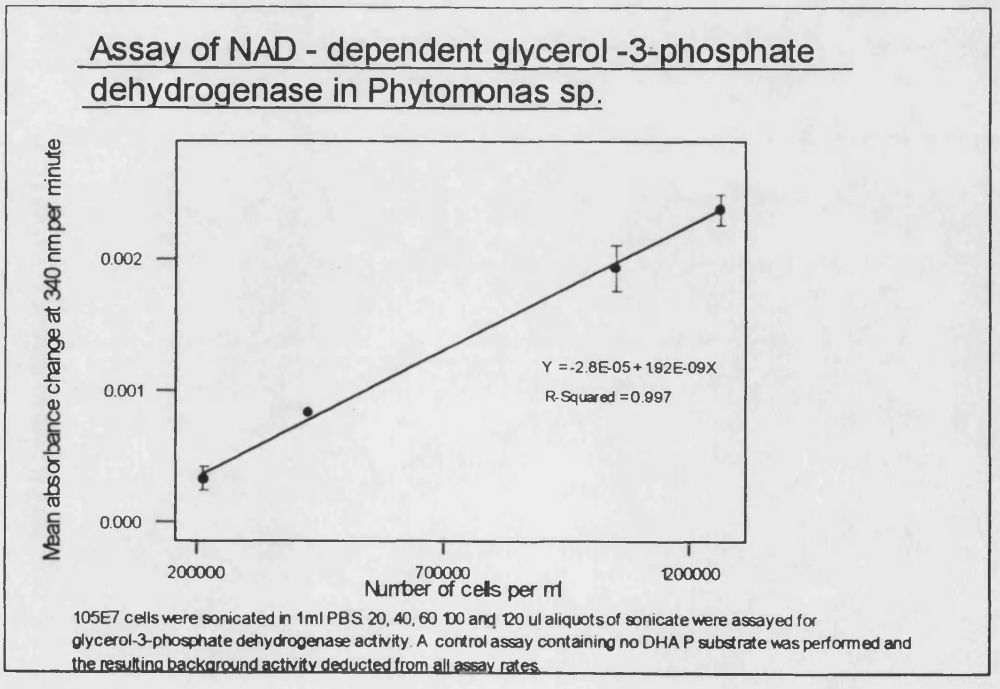
8.3.1 Assay of NAD^+-dependent glycerol-3-phosphate dehydrogenase in *Phytomonas sp.*

1.05×10^7 cells were sonicated in 1 ml PBS. 20µl , 40µl ,60µl ,100µl and 120µl aliquots were assayed for glycerol-3-phosphate dehydrogenase activity as detailed in methods section 2.28.3.2. Control assays were performed in the absence of DHAP. The decrease in absorbance, following oxidation of NADH, was measured at 340 nm.

Control assays resulted in a comparatively small decrease in absorbance at 340 nm compared to experimental rates. This is probably due to NADH oxidase activity which has been previously reported to be present in *Phytomonas sp* cell sonicate. These rates were subtracted from experimental values to give a linear plot of activity with sonicate volume (Fig. 38).

Results

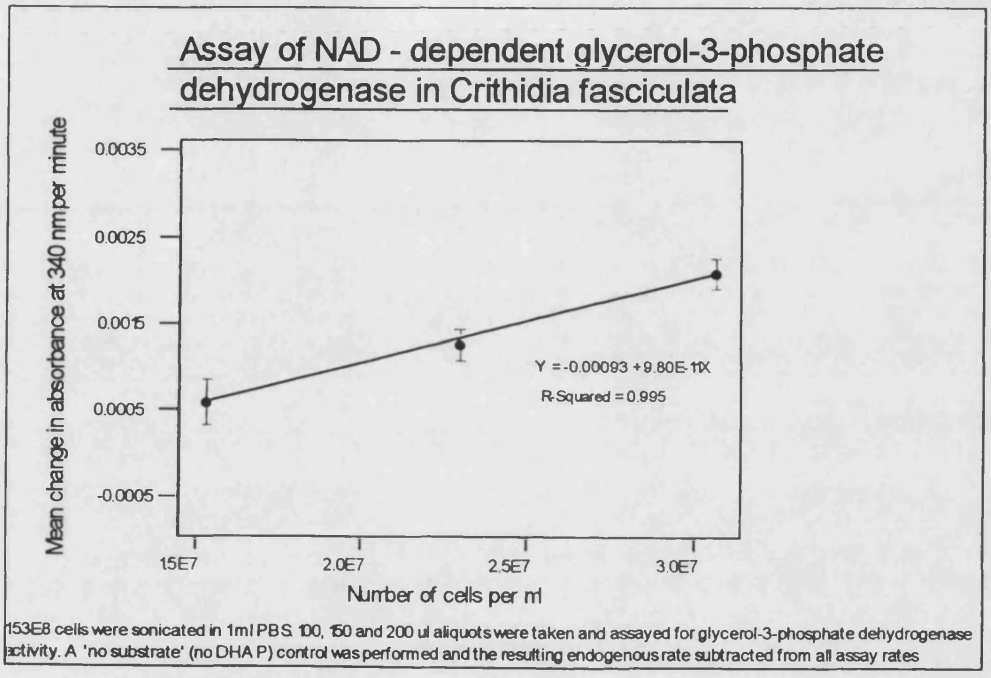
Figure 38



8.3.2 Glycerol-3-phosphate dehydrogenase in *Crithidia fasciculata*

1.53 x 10^8 cells were sonicated in 1 ml PBS. 100µl, 150µl and 200µl aliquots were assayed as described in methods section 2.28.3.2. Control assays, in the absence of DHAP, were performed, resulting in no change in absorbance at 340 nm.

Figure 39



8.3.3 Conclusions

These results confirm the presence of the glycosomal marker glycerol-3-phosphate dehydrogenase in both *Crithidia fasciculata* and *Phytomonas sp* cell sonicates. This is in accordance to previous reports which have detected glycerol-3 phosphate dehydrogenase in both *Phytomonas sp* and *Crithidia sp*, and went on to localise this activity to glycosomes (Sanchez-moreno et al, 1992).

These findings may be compared to Opperdoes' findings (Opperdoes et al, 1981) where both bloodstream and procyclic forms of *T. brucei* were found to possess glycerol-3-phosphate activity, which was only slightly reduced in the bloodstream form compared to procyclic forms. Glycerol-3-phosphate dehydrogenase (G3P) functions as part of the G3P:DHAP shuttle which is essential for aerobic metabolism of bloodstream form *T. brucei*, but is also operational in procylic cells (Opperdoes, 1987).

Glycerol-3-phosphate activity is sufficiently high in *Phytomonas sp* to be detectable after subtraction of background NADH oxidase activity, so confirming previously reported high levels of glycerol-3-phosphate dehydrogenase in a number of *Phytomonas sp.* isolates (Fernandez-Becerra et al, 1997).

Glycerol-3-phosphate dehydrogenase was hence found to be a suitable glycosomal enzyme marker in cell fractionations of both *Phytomonas sp* and *Crithidia fasciculata*

8.4 Pyruvate Kinase

Pyruvate kinases have been isolated from many organisms, including bacteria, protists, fungi, plants and animals (Kayne, 1973) In almost all cases, the enzyme is a tetramer composed of identical subunits with a molecular mass of approximately 60 kDa (Kayne, 1973) In prokaryotes and lower eukaryotes, generally one form of pyruvate kinase is found which is allosterically controlled (Kayne 1973). This is also the case in trypanosomes, where the role of the enzyme in metabolism is crucial.

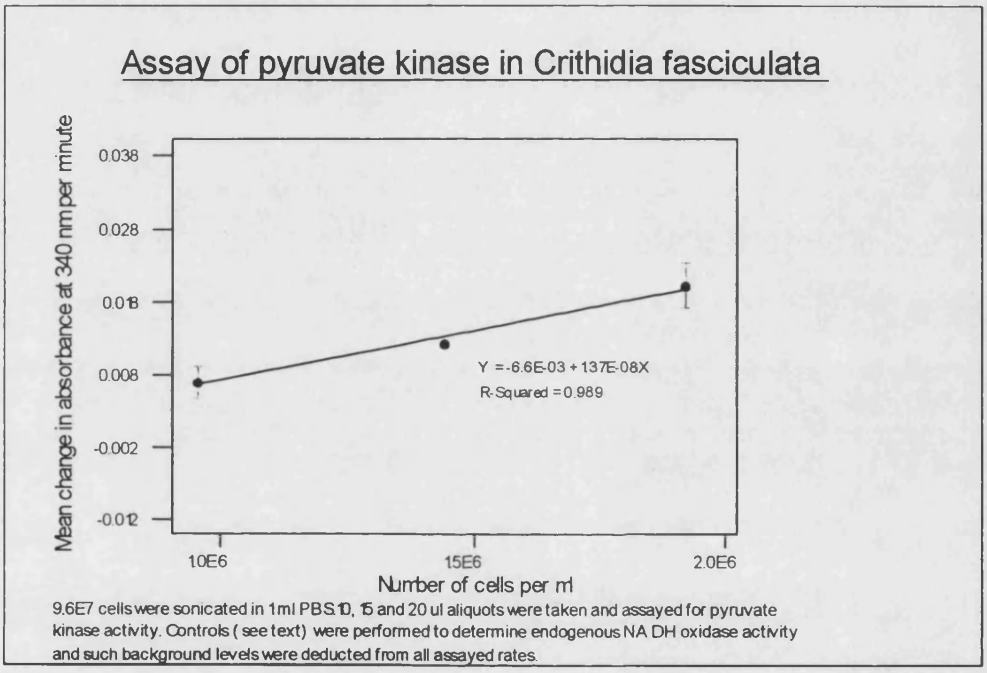
8.4.1 Pyruvate kinase in *Phytomonas sp.*

3.35×10^7 cells were sonicated in 0.5 ml PBS. 40µl, 50µl and 60 µl aliquots were taken and assayed as described previously (methods section 2.28.3.3). Experimental rates, detected by a decrease in absorbance at 340nm, were observed which were sonicate volume-dependent and which appeared to be due to pyruvate kinase activity.(data not shown for the reasons below). However, 'no substrate' controls gave much higher decreases in A_{340nm} than expected and were not significantly different from the experimental rates. This was confirmed by a second control experiment, in the absence of lactate dehydrogenase, where similar initial rates were observed.(data not shown). The observed experimental rates were therefore likely not to be attributable to pyruvate kinase activity, but were more likely the result of NADH oxidase activity inherent in *Phytomonas sp* sonicate.

8.4.2 Pyruvate kinase in *Crithidia fasciculata*

9.6 x 10^7 cells were sonicated in 1 ml PBS and assayed as described previously (methods section). Control assays were performed in the absence of PEP substrate and in the absence of sonicate as for *Phytomonas sp* (above). Both control assays gave similarly very low assay rates which were deducted from experimental rates, giving a good linear plot (Fig. 40)

Figure 40



8.4.3 Conclusion

It would be expected that pyruvate kinase activity is present in *Phytomonas sp.* considering its important role in glycolysis and especially considering the biochemical similarity to bloodstream *T.brucei* where pyruvate kinase is an abundant enzyme of the cytosol, appearing to comprise 1.6% of the total cytosolic protein (Barnard et al, 1988). Additionally, it would be expected that pyruvate kinase would be present in significant amounts as compared to *Crithidia fasciculata* which is less dependent on glycolysis for energy. Barnard et al (1994) have shown that pyruvate kinase levels are higher in procyclic *T. brucei* than bloodstream forms and that both are detectable in the absence of activators, as in this assay.

However, the observed experimental rates for *Phytomonas sp* appear to be the result of NADH oxidase activity present in the cell sonicate.as determined by performing control assays in the absence of PEP substrate. A second control in which lactate dehydrogenase was omitted from assays resulted in the same high rates, so confirming the activity of NADH oxidase. In a similar control experiment using sonicate from bloodstream *T. brucei*, Van Schaftingen (Van Schaftingen et al, 1985) failed to detect any NADH oxidase activity in *T. brucei* cells. Phytomonas therefore appears to have an unusual NADH oxidase activity which seems to mask the effect of any pyruvate kinase present. Pyruvate kinase, therefore, is an unsatisfactory enzyme marker for use in fractionation studies of *Phytomonas sp*

8.5 5' nucleotidase

Phosphomonoesterases specific for nucleotides ie. nucleotidases, are known from a wide variety of eukaryotic and prokaryotic sources (Drummond and Yamamoto, 1971). 5' nucleotidase activity has been reported from a wide variety of cell types, and it is generally considered as a constituent of, and a useful marker for, plasma membranes (De Pierre and Karnovsky, 1973; Riordan and Forstner, 1978; Trams and Lauter, 1974; Evans, 1978) although some intracellular activity has been demonstrated in membranes of the endoplasmic reticulum (Widnell, 1972) and in the Golgi apparatus (Farquhar et al, 1974), and a soluble form of the enzyme has been reported in certain tissues (Naito et al, 1974). The enzyme is also found in association with the cell surface membrane of various eukaryotic and prokaryotic microorganisms (Bengis-garber and Kushner, 1981; Armant et al, 1980).

5' nucleotidase activity has been investigated in some trypanosomatids. The enzyme has been found present in Leishmania (Gottlieb and Dwyer 1981; 1983; Konigk et al, 1978) and bloodstream forms of *T.brucei* (Voorheis et al, 1979), while Morgado Diaz et al (1996) confirmed the presence of 5'-nucleotidase in the plasma membrane of *T. foetus* (Queiroz et al, 1991; Morgado Diaz et al, 1996). Hunt and Ellar (1974) reported a very low level of 5'-nucleotidase in *Leptomonas collosoma.* Gottlieb and Dwyer(1983) confirmed the presence of 5'nucleotidase in *L donovani* while clearly differentiating the 5' nucleotidase from the non-specific membrane-bound acid phosphatase of the promastigotes.

3'-nucleotidase activities have been localised to the surface membrane of several members of the protozoan family Trypanosomatidae. These members include species of Leishmania (Steiger and Steiger, 1977; Marr and Berens, 1983; Gottlieb and Dwyer,1983), African trypanosomes (Gottlieb and Dwyer, 1988) and Crithidia spp. (Dwyer and Gottlieb (1983 ;1984;1985). However, 3'nucleotidase does not occur in all trypanosomatids for it was not detected in *T. cruzi* (Gottlieb et al 1986) and has been reported absent from other protozoal parasites such as *Babesia divergens* (Hassan and Coombs, 1987a), *Acanthamoeba species* (Hassan and Coombs, 1987b) and *T.vaginalis* (Miller and Lindstead (1983).

5'Nucleotidase appears to be generally more widely distributed in protozoal parasites (Hassan and Coombs (1987) and so it was decided to assay for 5'nucleotidase as a potential plasma membrane marker in both *Crithidia fasciculata* and *Phytomonas sp.*

8.5.1 General assay of 5'nucleotidase in *Crithidia fasciculata*

Hydrolysis of [^{3}H]AMP by 5'nucleotidase was measured in both cell lysates and isolated plasma membrane fractions from *Crithidia fasciculata.* Typically, 50 µl aliquots of sample (see individual results section) were incubated with 0.5 ml of a 'working substrate solution' which contained [^{3}H]AMP, 12mM glycerophosphate and 0.12mM cold AMP in a 60 mM piperazine - HCl buffer (pH 9.0) as detailed in methods section 2.28.3.5. The reaction was allowed to run at 37°C for various time periods (as detailed in individual results sections), at the end of which time 0.2 mls of 0.15M zinc sulphate ($ZnSO_4$) was added. Samples were vortexed and placed on ice. 0.5 mls of 0.15M barium hydroxide ($Ba(OH)_2$) was then added and left to incubate for 30 minutes on ice. These reagents served to both stop the reaction and to separate unreacted substrate from the product, [^{3}H]adenosine , which remained in solution. The tubes were microfuged at 13,000 rpm (4 °C) for 10 minutes and 10 µl aliquots of supernatant were removed for counting.

Controls were performed for each assay in which substrate and enzyme sonicate/ plasma membrane fraction was absent. These values were subtracted as background from the experimental samples. The release of adenosine was calculated in relation to the measured [^{3}H] activity of the working substrate solution.

Various modifications of this general assay procedure were carried out as detailed in individual results sections.

8.5.2 Verification of experimental conditions

Before any assays could be performed, some initial experimental conditions needed to be characterised. The first of these was the determination of the percentage precipitation of [^{3}H]AMP by $Ba(OH)_2$ in the absence of 5'nucleotidase enzyme.

8.5.2.1 Initial experimental conditions

8.5.2.1.1 Determination of % precipitation of [^{3}H]AMP

Recovery of [^{3}H]adenosine and precipitation of AMP were ascertained by performing a mock 5'nucleotidase assay (methods section 2.28.3.5) in the absence of cell sonicate. The procedure was followed as described above (section 9.5.1). 3 x 10µl supernatant samples were taken both before and after incubation with barium hydroxide and counted for [^{3}H]adenosine. Counts were adjusted to take the change in volume into consideration, and the percentage of [^{3}H] adenosine in the supernatant following precipitation with $Ba(OH)_2$ was calculated to be 15.4%. This means that barium hydroxide maximally precipitates 85% of [^{3}H] AMP under these assay conditions.

8.5.2.1.2 Determination of optimal Ba $(OH)_2$ incubation time for maximum [^{3}H]AMP precipitation

The procedure was repeated as described before (section 9.5.2.1.1) except that samples were incubated with $Ba(OH)_2$ for various time periods in an attempt to improve upon the percentage of [^{3}H]AMP precipitated at this step. 3 x 10µl samples of supernatant were taken before and after the addition of $Ba(OH)_2$ for five incubation periods in the range of 30 to 150 minutes and counted for [^{3}H]adenosine (Fig. 41).

8.5.2.1.2.1 Results

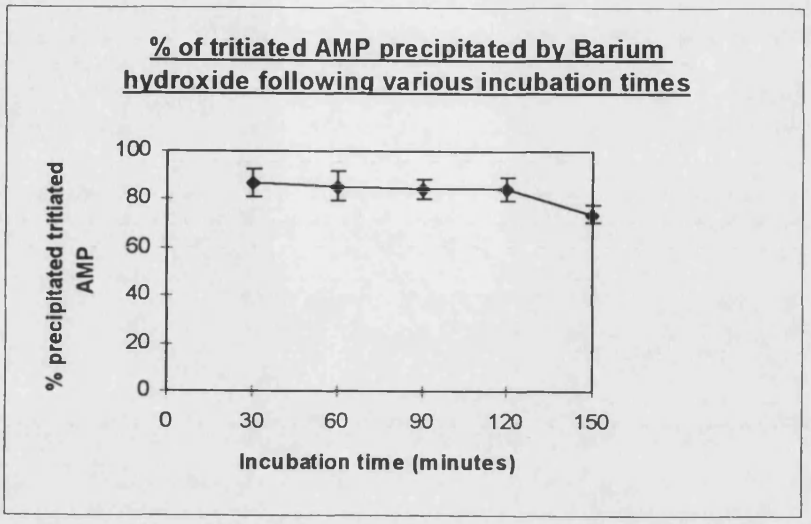


Figure 41 : 'Mock' 5'nucleotidase assays were performed in the absence of enzyme as described above in section 9.5.1.Various barium hydroxide incubation times (0 - 150 minutes) were tested in an attempt to maximise precipitation of [^{3}H] AMP. 3 x 10µl supernatant samples were taken both before and after incubation with 0.5 ml Ba $(OH)_2$ (0.15M) and counted for [^{3}H]adenosine.

No significant differences in % [^{3}H]AMP precipitated with $Ba(OH)_2$ was observed between incubations times.

8.5.2.1.2.2 Conclusion

There is no advantage to be gained by extending $Ba(OH)_2$ precipitation time beyond the 30 minutes detailed in the original method. 30 minutes is hence an appropriate precipitation time and was used in all subsequent experiments.

8.5.3 5' Nucleotidase assays (see methods section 2.27.3.5)

All assays were performed in triplicate (unless otherwise stated). Cell sonication (methods section 2.24) was performed in a stainless steel vessel at 4 °C to minimise 5'nucleotidase degradation with heat.

8.5.3.1 Time course for 5' nucleotidase assay

2.3 x 10^7 *Crithidia fasciculata* cells were freshly harvested, washed and sonicated (methods sections 2.2 & 2.24) in 1 ml PBS.. 10µl of sonicated extract was added to 0.5 mls of working substrate solution and left to incubate for various time periods between 0 and 60 minutes. Reactions were ended by the addition of 0.5mls

$ZnSO_4$ and [^{3}H]adenosine product was precipitated using $Ba(OH)_2$ and assayed as described previously. 'No glycerophosphate' control assays were performed simultaneously using 0.5 ml working substrate solution deficient in 12mM glycerophosphate.

8.5.3.1.1 Results

No significant increase in [^{3}H] adenosine product was observed with time in either the presence or absence of β-glycerophosphate, an inhibitor of non-specific alkaline phosphatase (data not shown). The experiment was repeated with 50µl sonicate aliquots and the same result obtained (Fig. 42).

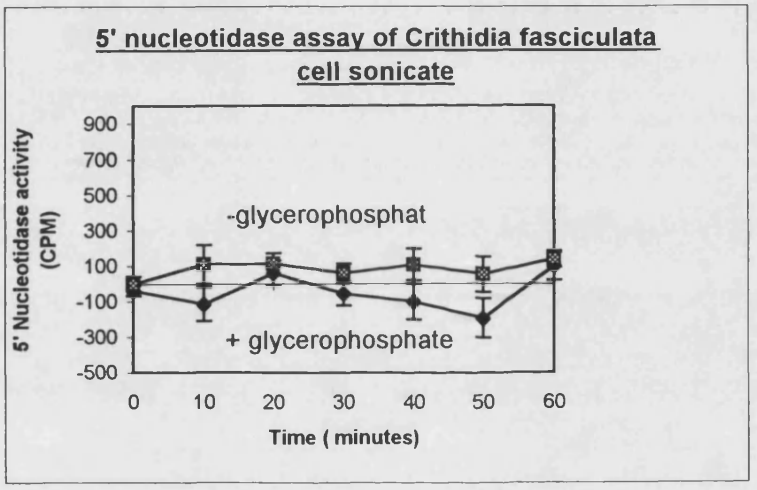


Figure 42: 2.3 x 10^7 *Crithidia fasciculata* cells were freshly harvested, washed and sonicated in 1 ml PBS. 50 µl of sonicated extract was added to 0.5 mls of working substrate solution mixture and left to incubate for various time periods between 0 and 60 minutes before the reaction was stopped with 0.2 mls of $ZnSO_4$ (O.15M). Precipitation and counting of [^{3}H] adenosine product was performed as detailed previously. Control assays for alkaline phosphatase were performed simultaneously in the absence of glycerophosphate using 0.5 mls working substrate solution deficient in 12mM glycerophosphate.

8.5.3.1.2 Conclusion

This result may indicate the absence of 5'nucleotidase activity in *Crithidia fasciculata*. Alternatively, the amount of cold AMP present in the assay (in the working substrate solution) may be too high and may be masking any hydrolysis of trace [^{3}H]AMP by 5'nucleotidase.

8.5.3.2 Investigation of possible masking effect of cold AMP.

The working substrate solution was modified in an attempt to overcome possible masking of enzyme activity. Cold AMP was omitted and a lower dilution of

stock [^{3}H]AMP (1µl stock [^{3}H]AMP (250µCi; specific activity 18.9 Ci/ mmole) in 10 mls reaction mixture) was used in an attempt to increase assay sensitivity.

1x10^8 *Crithidia fasciculata* cells were harvested, washed and sonicated in 1 ml PBS (section 2.23 & 2.24). 100µl aliquots were incubated with 0.5 mls of modified working substrate solution in both the presence and absence of 12mM glycerophosphate for various time intervals (10 - 60 minutes). The reaction was stopped by the addition of $ZnSO_4$ and the assay procedure followed as before (methods section 2.28.3.5).

8.5.3.2.1 Results

Again, no rise in [^{3}H]adenosine formed with incubation time (Fig. 43) was observed, with no significant difference in the hydrolysis of [^{3}H]AMP observed in the presence and absence of glycerophosphate.

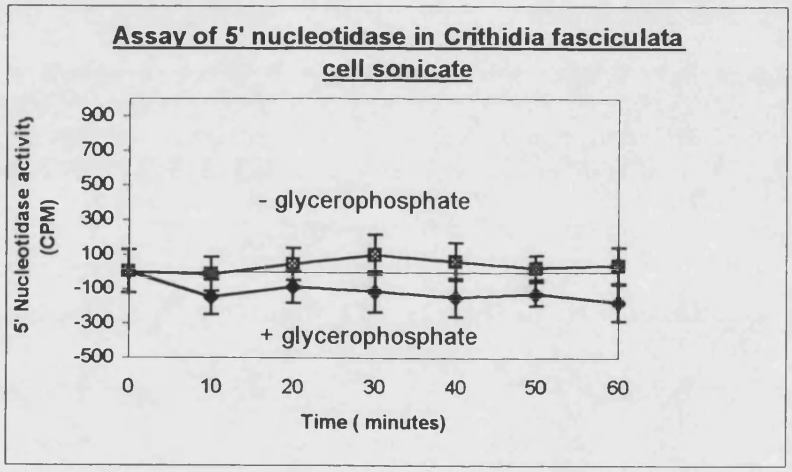


Figure 43: 1x10^8 *Crithidia .fasciculata* cells were harvested, washed and sonicated in 1 ml PBS 100 µl aliquots of cell sonicate were assayed both in the presence and absence of 12mM glycerophosphate using modified working substrate solution which was deficient in cold AMP and contained a higher concentration of [^{3}H]AMP in an attempt to increase assay sensitivity. The reaction was stopped by the addition of 0.2 mls $ZnSO_4$ (0.15M) and the formation of [^{3}H]adenosine product assayed as described previously.

8.5.3.2.2 Conclusions

Even in the absence of cold AMP, no significant hydrolysis of [^{3}H]AMP was observed over the hour time course. This suggests that either 5'nucleotidase activity is absent in *Crithidia fasciculata* cell sonicate, or is present in such low amounts as to be undetectable after one hour. To investigate the latter possibility further, a 36 hour time course was performed.

8.5.3.3 36 hour time course for *Crithidia fasciculata* sonicate

A 36 hr time course experiment was carried out using modified working substrate solution (see section 9.5.3.2.1) in order to see if any 5'nucleotidase activity could be detected over a prolonged time period (Fig. 44). 1.2 x 10^8 *Crithidia fasciculata* cells were harvested, washed and then sonicated in 1 ml PBS. 50µl of sonicate were assayed in 0.5 mls modified working substrate solution both with and without 12mM glycerophosphate. The reaction was terminated by the addition of $ZnSO_4$ and unreacted substrate separated from reaction product by precipitation using $Ba(OH)_2$ as previously described

8.5.3.3.1 Results

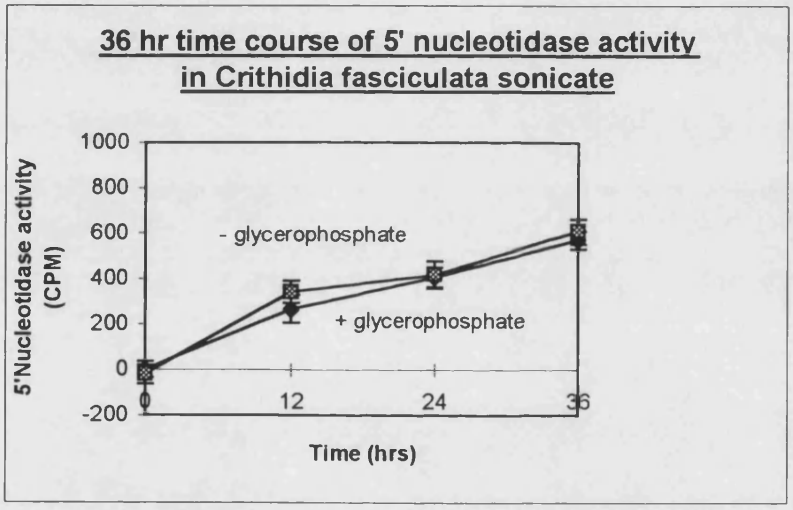


Figure 44: 1.2 x 10^8 *Crithidia fasciculata* cells were harvested, washed and then sonicated in 1 ml PBS. 50µl of sonicate were assayed in 0.5 mls modified working substrate in the presence and absence of 12mM glycerophosphate over a 36 hour time course. The reaction was terminated by the addition of $ZnSO_4$ and unreacted substrate separated from reaction product by precipitation using $Ba(OH)_2$ as previously described.

An increase in [^{3}H]adenosine formed with time was observed, which indicates 5'nucleotidase activity in the cell sonicate. There was no significant difference between reactions carried out in the presence and absence of glycerophosphate, so confirming that the observed activity is due to 5'nucleotidase and not a result of nonspecific alkaline phosphatase activity.

8.5.3.3.2 Conclusions

The time - dependent increase in [^{3}H]adenosine formed following incubation with *Crithidia fasciculata* sonicate indicates the presence of a small amount of 5'nucleotidase activity. However, the activity appears to be so low as to render the use of this enzyme as a membrane marker impractical and unreliable as a quantitative assay.

8.5.3.4 36 hour time course for isolated Crithidia fasciculata isolated plasma membrane fraction (see Chapter 9)

The above experiment was repeated using 50 µl of isolated *Crithidia fasciculata* plasma membrane instead of cell lysate (Fig. 42). The plasma membrane fraction from *Crithidia fasciculata* was isolated as detailed in Chapter 9 and methods section 2.28. Isolated plasma membrane was washed and suspended in 0.5 ml PBS prior to assay. 50µl of plasma membrane isolate was incubated with 0.5mls of modified working substrate solution (section 9.5.3.2) in the presence and absence of 12mM glycerophosphate over a 36hr timecourse. The reaction was stopped by the addition of $ZnSO_4$ and the assay carried out in the usual was as described previously.

8.5.3.4.1 Results

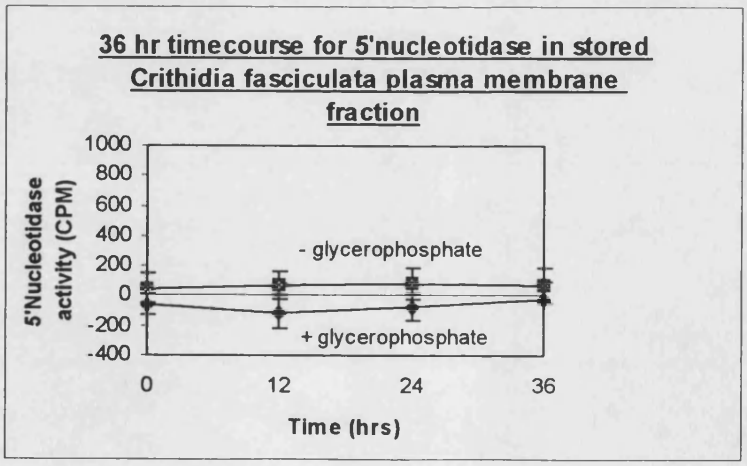


Figure 45: 50 µl of *Crithidia fasciculata* plasma membrane isolate, obtained following cell fractionation (see chapter 9), was assayed for 5'nucleotidase activity over a 36 hour time course. The isolate was incubated with 0.5mls of modified working substrate solution (section 9.5.3.2) in the presence and absence of 12mM glycerophosphate over a 36hr timecourse. The reaction was stopped by the addition of $ZnSO_4$ and the assay carried out in the usual was as described previously.

There appears to be no significant 5'nucleotidase activity in the plasma membrane fraction with time. As in the cell sonicate time course, there is no significant difference in activity between reactions carried out in the presence and absence of glycerophosphate, indicating the absence of non specific alkaline phosphatase in the isolated fraction.

8.5.3.4.2 Conclusions

There appears to be no 5'nucleotidase activity in the plasma membrane fraction. This is an unusual finding, considering the activity observed for the cell sonicate, as 5'nucleotidase is a common plasma membrane enzyme.

It is possible that 5'nucleotidase may be present in other parts of the cell other than the plasma membrane. 5'nucleotidase activity has been demonstrated in membranes of the endoplasmic reticulum (Widnell, 1972) and in the Golgi apparatus (Farquhar et al, 1974) of some cells, and a soluble form of the enzyme has been reported in certain tissues (Naito et al, 1974). Hassan and Coombs (1987) reported that 5'nucleotidase in *L.mexicana mexicana* occurs both membrane bound and in the cell matrix. A proportionally high level of cell matrix 5'nucleotidase activity compared to plasma membrane-located enzyme might explain the activity observed in cell sonicate and the apparent absence in the plasma membrane isolate.

8.5.4 Discussion

It appears that there is a very low 5'nucleotidase activity present in *Crithidia fasciculata* cell sonicate, detectable after a minimum 12 hours reaction time under optimally sensitive assay conditions. Although it is possible that the 5'nucleotidase does not have a plasma membrane location and is located in other organelle membranes, this is unlikely as in previous studies of trypanosomal 5'nucleotidase, the enzyme, if detected at all, has been shown to have a plasma membrane location (Morgado Diaz et al, 1996; Gottlieb and Dwyer,1983; Voorheis et al, 1979; Hunt and Ellar,1974). It is possible that a weak association with the plasma membrane, as suggested by Voorheis et al (1979) in *T. brucei*, may result in detachment of the enzyme during homogenisation. The enzyme has been found to be discontinuously distributed over the external surface of *Leishmania donovani* (Dwyer and Gottlieb,

1984) by means of a GPI anchor which renders the enzyme highly labile and this may be the case for *Crithidia fasciculata.* A weak plasma membrane association was also found for 3'nucleotidase in procyclic *T. brucei.* (Cook et al,1990), rendering the assay of nucleotidase in fractionation studies to be relatively inaccurate.

Hence, owing to the generally very low activity observed in cell sonicate experiments together with the possibility of considerable loss during fractionation experiments, it was decided to abandon 5'nucleotidase as a plasma membrane marker for use in fractionation studies.

8.5.5 Future work

Trypanosomes are dependent on an exogenous supply of preformed purines because they are incapable of de novo purine synthesis (Kidder and Dutta, 1958) and it has been widely reported in the literature that 5'nucleotidase (and 3'nucleotidase) expression in trypanosomes may vary dramatically depending on the culture medium. Nucleotidase activity has been observed to increase dramatically in some *Crithidia sp.* in the absence of purines and/or phosphate in the culture medium (Dwyer and Gottlieb, 1984 ; Gottlieb and Zlotnick, 1987). In *Crithidia fasciculata,* the level of 5'nucleotidase has been found to increase 5 fold when grown in medium containing low adenosine (i.e. under conditions of purine starvation) as compared to cells grown in adenosine-enriched medium (Gottlieb, 1985). The striking induction of 5'nucleotidase activity in response to clearly defined and easily manipulated nutrient signals may hence be explored further in order to gain higher 5'nucleotidase activity in *Crithidia fasciculata* cells for use in fractionation studies.

It has been reported in the literature that 5'nucleotidase activity varies depending on the growth stage of *Crithidia fasciculata,* with nucleotidase activity observed to be twice as high in late log-phase cells compared to early log- phase cells (Gottlieb, 1985). Furthermore, it has been reported that the level of 3'nucleotidase in *Leishmania donovani* does not remain constant throughout the growth curve, but is transiently elevated during the lag- and early log- phases of growth (Sacci et al, 1990). Cells used in the experiments detailed in this thesis may have been harvested during a phase of the growth cycle in which low levels of 5'nucleotidase were exhibited. Future

work involving cells from different phases of the growth curve should hence be performed in an attempt to maximise 5'nucleotidase activity in cells used for assay.

8.6 General conclusions

While isocitrate dehydrogenase, glycerol-3-phosphate dehydrogenase and pyruvate kinase appear to be suitable enzyme markers for mitochondrial, glycosomal and cytosolic contamination in plasma membrane isolates from *Crithidia fasciculata,* 5'nucleotidase was found to be an unsuitable plasma membrane marker. Only glycerol-3-phosphate dehydrogenase was found to be a suitable enzyme marker in *Phytomonas sp.*, with isocitrate dehydrogenase activity found to be absent and pyruvate kinase un-assayable due to masking by endogenous NADH oxidase activity. Time constraints did not permit an exhaustive investigation of enzyme markers for both cell types and it was decided to assess plasma membrane isolate purity for both cells by morphological examination of isolates by electron microscopy. Although non-quantitative, this method may be used effectively to determine whether plasma membrane enrichment has occurred following the isolation procedure.

CHAPTER 9
INVESTIGATION OF A PLASMA MEMBRANE LOCATION FOR DHLIPDH IN *CRITHIDIA FASCICULATA* AND *PHYTOMONAS SP*

9.1 Plasma membrane preparations of *Phytomonas sp.* and *Crithidia fasciculata*

A method of plasma membrane preparation, based on the procedure described by Dwyer (1980) who reported the isolation of highly enriched (> 95% purity) fractions of pellicular membrane-microtubule complexes from *L donovani* promastigotes, was used to obtain plasma membrane - enriched isolates for both *Phytomonas sp.* and *Crithidia fasciculata* (methods section 2.27; Fig. 43). Cells were grown to a cell density of 1.5 x 10^8 cells per ml for *Crithidia fasciculata* and 4 x 10^7 cells per ml for *Phytomonas sp.*, and harvested by centrifugation at 4°C (methods section 2.23). Washed , pelleted cells were resuspended in 1 ml ice cold swelling buffer (tris-EDTA buffer (pH 7.8) containing leupeptin (2μg/ml) and PMSF (1mM)) and transferred to a chilled mortar. Chilled silicon carbide abrasive grain was added to the mortar (60:40 gm carbide/ gm wet weight) and the cell paste ground manually using a chilled pestle. The suspension was diluted with 5 ml chilled isolation buffer and spun for 3 minutes at 120g to remove the carbide (see Fig. 43). The supernatant was removed and centrifuged a further 10 minutes at 4,000 g at 4°C. The pelleted material was suspended in 6ml 0.146 M sucrose solution (dissolved in tris-EDTA containing 1 mM PMSF) and the pellet disrupted by 2 passages through a 22 gauge needle. The suspension was overlaid onto a discontinuous sucrose gradient consisting of a 1.61 M sucrose cushion (5 ml) and a 1.17M sucrose layer (15ml), and spun in a Beckman L5-50B ultracentrifuge at 27,000 rpm (4 °C), for 2 hours using an SW27 rotor (Dwyer, 1980).

Following sucrose gradient centrifugation, a dense white band on the discontinuous sucrose gradient (corresponding to a plasma membrane enriched fraction) at the interface between 1.17M and 1.6M sucrose, was obtained. Fig. 46 shows a photograph of this band. A small amount of white material was observed at the interface between the 0.146M and 1.17M sucrose layers and corresponds to a very small microsomal fraction. The dark pellet at the bottom of the gradient was found to contain mainly unbroken cells and flagella, as observed under a light microscope. The

'plasma membrane' band was collected by careful removal of gradient layers using a pipette and then diluted with Tris-EDTA buffer before ultracentrifuging for a further 2 hours at 27,000 rpm. The resulting pelleted membranes were washed twice in PBS by centrifugation as described above and finally suspended in 1 ml PBS for analysis.

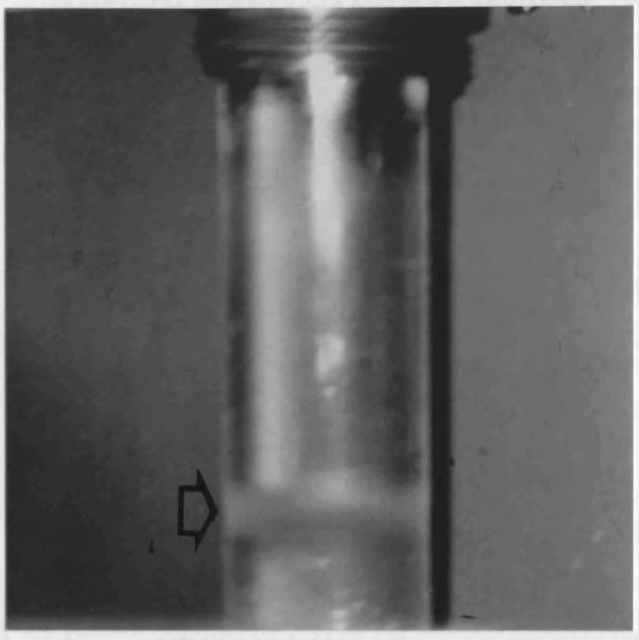


Figure 46 : Isolation of a plasma membrane fraction, observed as a dense white band at the interface between 1.17M and 1.6M sucrose, on a discontinuous sucrose gradient.

9.2 Investigation of DHlipDH activity during plasma membrane preparation

0.5 ml fractions taken from stages during the purification of the trypanosome plasma membranes were stored on ice until tested for protein content and DHlipDH activity. Fractions assayed were the homogenate (H), the supernatant (S1), the pellet in suspension (P1) and the final plasma membrane enriched pellet (P 2) (Fig. 47). 30 µl aliquots were taken from each fraction and DHlipDH was assayed as described in methods 2.27.3.1. NADH oxidase activity was measured as a control in each fraction (methods section 2.27.3.2). To provide some correction for the depression of measured NADH in the DHlipDH assay, the measured rate of any NADH oxidase was added to observed rates.

Total protein content and corrected DHlipDH activity was determined for all isolated fractions. Total enzyme activities are expressed in µ moles NADH formed per minute. Specific activities were determined and are expressed as µ moles of NADH formed /per minute/ per mg of protein. Tables 10 and 11 summarise the specific

activities, total activities and percentage of the total enzyme activity, taking the homogenate value as the 100% value. Figs 48 and 49 show bar charts of total enzyme activity and specific activities for easy comparison.

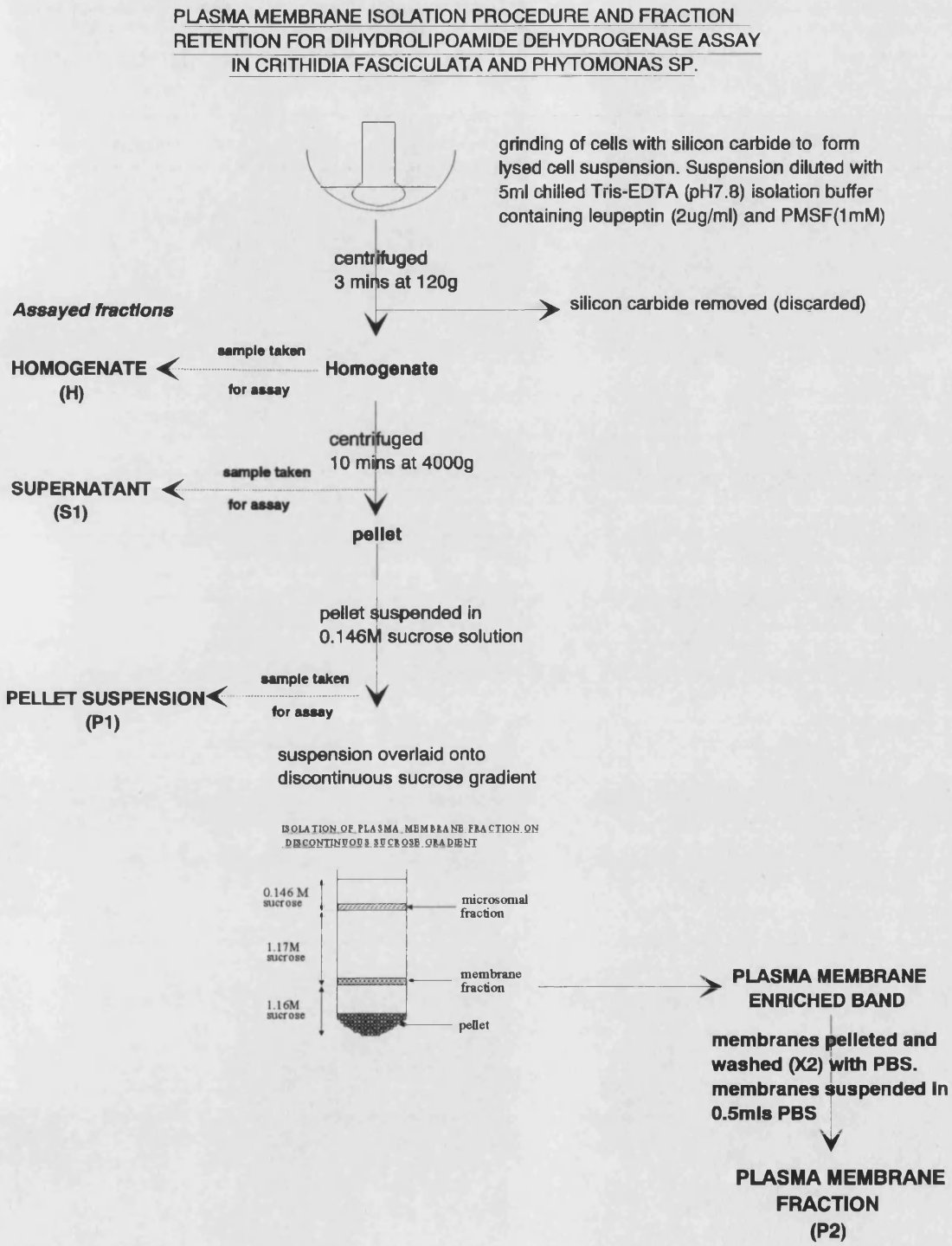


Figure 47: Plasma membrane isolation procedure used to isolate plasma membrane fractions in both *Crithidia fasciculata* and *Phytomonas sp.* Fractions were taken and assayed for protein content and DHlipDH activity at various stages of the isolation procedure as denoted in the schedule.

Table 10 (a) Total protein content for *Crithidia fasciculata* fractions

sample	H	S1	P1	P2
Total protein content (mg/ml)		1.075	0.644	0.322
% total homogenate protein	1.79			18
	100	60.1	36	

Table 10 (b) Dihydrolipoamide dehydrogenase activity in *Crithidia fasciculata*

Fraction	*Total activity*	*% total activity*	*specific activity* $M/min^{-1}/mg\ protein^{-1}$
H	0.49	100	0.273
S1	0.26	54	0.24
P1	-	-	-
P2	0.193	39.3	0.6

Table 11 (a) Total protein content for *Phytomonas sp.* fractions

Sample	H	S1	P1	P2
Total protein content (mg/ml)				0.196
% total homogenate protein	2.8	1.2	1.54	
	100	42.9	55	7

Table 11 (b) Dihydrolipoamide dehydrogenase activity in *Phytomonas sp.*

Fraction	*Total activity*	*% total activity*	*specific activity* $M/min^{-1}/mg\ protein^{-1}$
H	0.22	100	0.079
S1	0.022	10	0..0018
P1	-	-	-
P2	0.08	36.4	0.41

Figure 48: Comparison of total DHlipDH activity in *Crithidia fasciculata* and *Phytomonas sp.* fractions

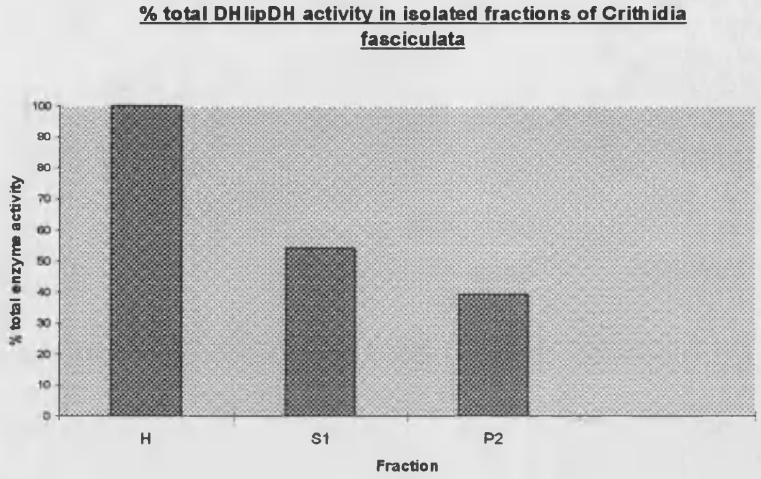


Figure 48(a) 500 mls of *Crithidia fasciculata* cell suspension with a cell density of 1.5 x 10^8cells per ml were rapidly chilled on ice and then harvested by centrifuging for 10 minutes at 2,000 g. Cells were washed twice in ice cold PBS. The washed , pelleted cells were weighed and resuspended in 1 ml Tris-EDTA isolation buffer (pH 7.8) containing leupeptin (2μg/ml) and PMSF (1mM), and transferred to a chilled mortar. Silicon carbide abrasive grain was added to mortar (approx. 60:40 ratio of grain to cell wet weight) and the cell paste ground manually using a chilled pestle. Fractionation procedure was carried out as detailed in methods section 2.27 and isolated fractions (see fig. 47) were assayed for DHlipDH activity as described previously (methods section 2.27.3.1)

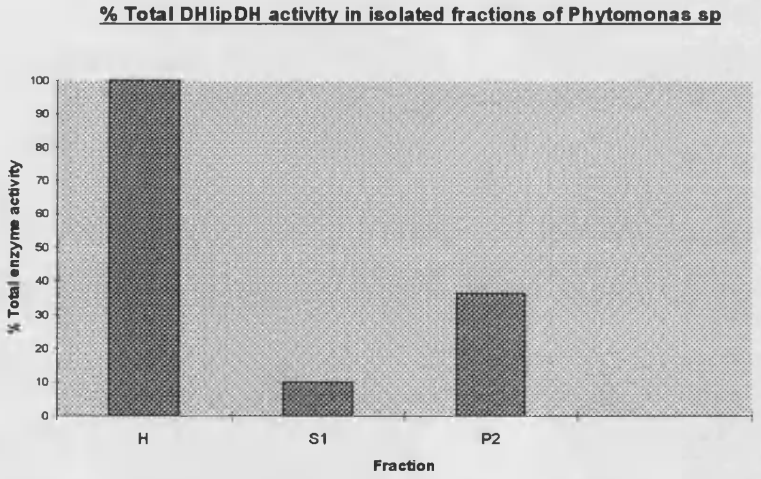


Figure 48(b) 2,500 mls of *Phytomonas sp.* cell suspension with a cell density of 4 x 10^7 cells per ml were rapidly chilled on ice and then harvested by centrifuging for 10 minutes at 2,000 g. Cells were washed twice in ice cold PBS. The washed , pelleted cells were weighed and resuspended in 1 ml Tris-EDTA isolation buffer (pH 7.8) containing leupeptin (2μg/ml) and PMSF (1mM), and transferred to a chilled mortar. Silicon carbide abrasive grain was added to mortar (approx. 60:40 ratio of grain to cell wet weight) and the cell paste ground manually using a chilled pestle. Fractionation procedure was carried out as detailed in methods section 2.27 and isolated fractions (see fig 47) were assayed for DHlipDH activity as described previously (methods section 2.27.3.1)

Figure 49: Comparison of DHlipDH specific activity in *Crithidia fasciculata* and *Phytomonas sp.* fractions

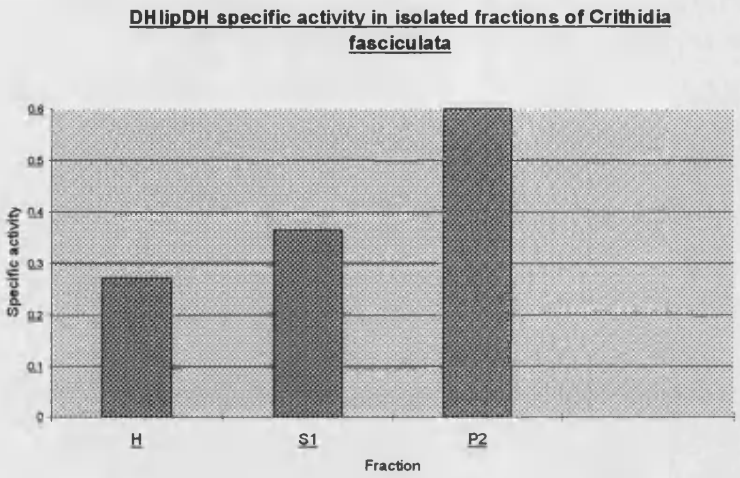


Figure 49 (a): Total DHlipDH activities were determined for each isolated fraction (Fig. 48 (a); table 10(b)). Protein determinations were performed using a modified Lowry method (methods section 2.27.2, table 10(a)) and DHlipDH specific activity for each fraction calculated.

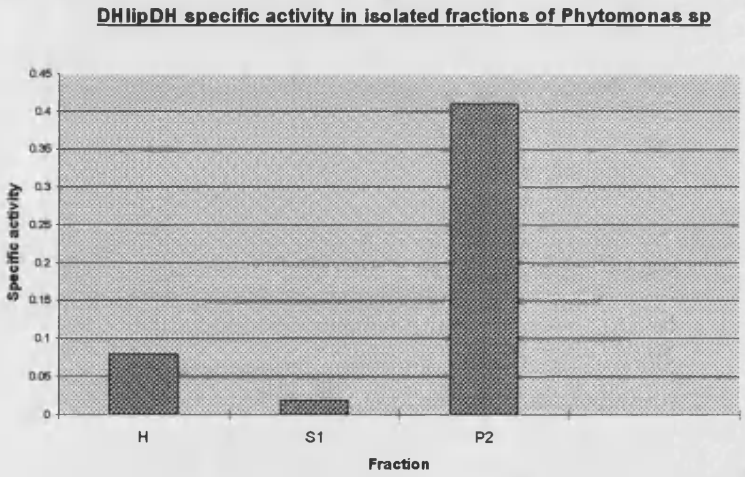


Figure 49(b): Total DHlipDH activities were determined for each isolated fraction (Fig. 48(b); table 11(b)). Protein determinations were performed using a modified Lowry method (methods section 2.27.2, table 11(a)) and DHlipDH specific activity for each fraction calculated.

9.2.1 DHlipDH activity in *Crithidia fasciculata*

The specific activity of DHlipDH in *C.fasciculata* cell homogenate was found to be 0.273 $\mu M/min^{-1}/mg\ protein^{-1}$ and was comparable to the specific activity of $0.29\mu M/min^{-1}/mg\ protein^{-1}$ previously determined for the cell sonicate (section

7.1.3.1). 54% of the total DHlipDH activity was found in the supernatant, S1, following centrifugation of homogenate. The specific activity in the supernatant was decreased to 88% of the homogenate value. However, the isolated plasma membrane-enriched fraction, after washing and pelleting to form P2, had a total activity 40% of the homogenate value. The specific activity of the final plasma membrane pellet, P2, was 0.6μM . This signified a 2 fold increase in specific activity over the homogenate, indicating some retention of enzyme activity in the plasma membrane-enriched fraction

9.2.2 Dihydrolipoamide dehydrogenase activity in *Phytomonas sp.*

The specific activity of DHlipDH in *Phytomonas sp.* cell homogenate was found to be 0.079 $\mu M/min^{-1}/mg\ protein^{-1}$ and was comparable to the specific activity of $0.06\mu M/min^{-1}/mg\ protein^{-1}$ previously determined for the cell sonicate (section 7.1.2). 10 % of the total enzyme activity was found in the supernatant (S1) following centrifugation of homogenate. This is approximately 40% less than that observed for *Crithidia fasciculata.* The specific activity in the supernatant was decreased to 23% of the homogenate value. 36.4 % of the total homogenate activity was observed in the plasma membrane pellet, with a high specific activity 5.2 times the homogenate activity. This result shows that the plasma membrane enriched fraction is also enriched with DHlipDH, suggesting some degree of copurification of DHlipDH with *Phytomonas sp.* plasma membrane.

Fig. 50 (a) and (b) are electron micrographs of the final plasma membrane fraction of *Crithidia fasciculata* , while Fig. 51 (a) and (b) are micrographs of the plasma membrane fraction of *Phytomonas sp.* Both clearly show cross sections through the pellicular microtubule array which is characteristically attached to the plasma membrane in trypanosomes (Vickerman and Preston (1976)). Hence the presence of these structures is definitive proof of plasma membrane enrichment in isolated plasma membrane fractions from both *Crithidia fasciculata* and *Phytomonas sp.* .

Figure 50 (a)

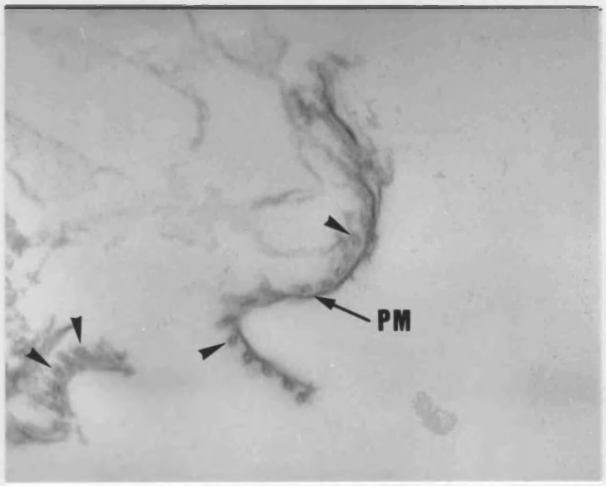


Figure 50 (b)

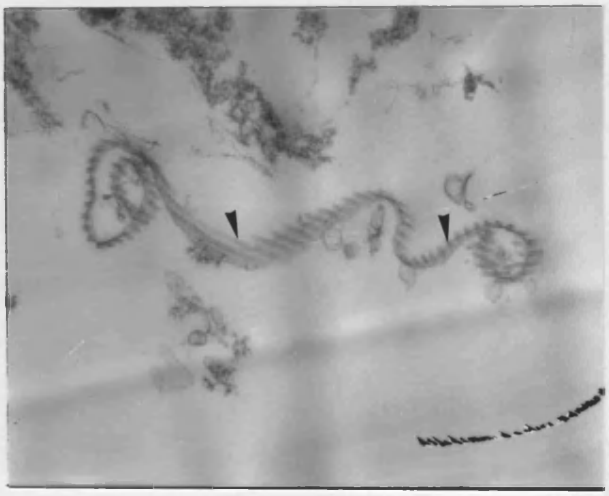


Figure 50 (a and b): electron micrographs of plasma membrane-enriched fractions isolated from *Crithida fasciculata* by sucrose gradient ultracentrifugation. Arrow heads indicate microtubules characteristically attached to the plasma membrane (PM).

Figure 51 (a)

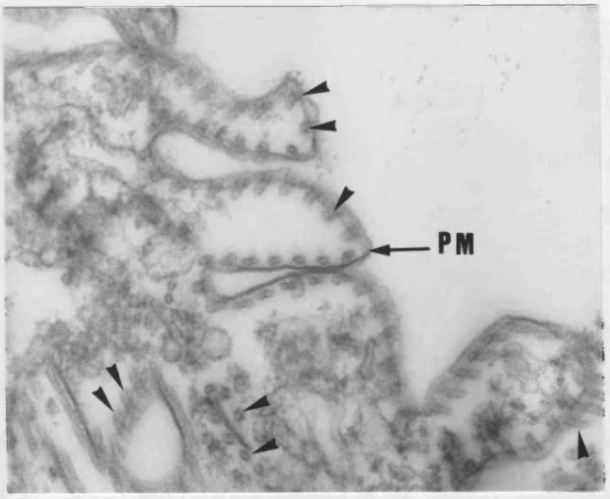


Figure 51 (b)

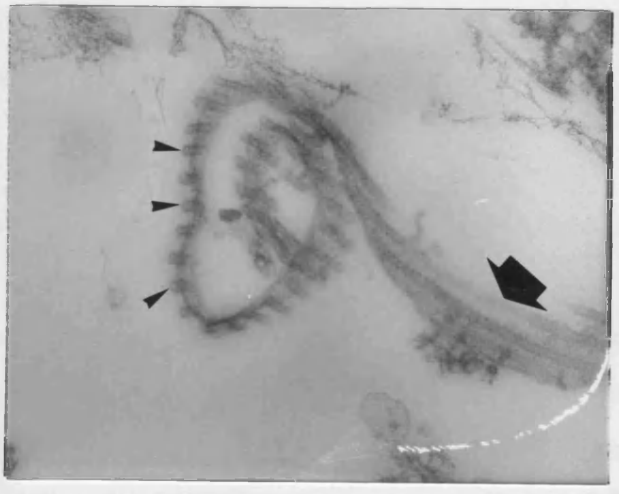


Figure 51 (a and b): electron micrographs of plasma membrane-enriched fractions isolated from *Phytomonas sp* by sucrose gradient ultracentrifugation. Arrow heads indicate microtubules characteristically attached to the plasma membrane (PM). Fig. 51 (b) shows microtubules, in both transverse (arrow head) and longitudinal (large arrow) section.

9.2.3 Mitochondrial contamination of plasma membrane fractions

9.2.3.1 Indicated by NADH oxidase in *Phytomonas sp.*

NADH oxidase activity was found to be absent in the isolated *Crithidia fasciculata* plasma membrane fraction but present in low levels in *Phytomonas sp.* plasma membrane fraction. The presence of NADH oxidase may be an indication of contamination by membranes from other organelles in the plasma membrane preparation. Such membranes may be mitochondrial, or possibly glycosomal (Conroy, 1988; Jackman 1991)

In the method of Voorheis (1979) (used by Danson et al (1987)) to purify bloodstream plasma membranes, and the method described by Dwyer (1980) used in this work, there is no way of determining whether DHlipDH is present at a plasma membrane location only within the cell. This is important as the possibility of contamination of the plasma membrane preparation with other cellular membranes, e.g. mitochondrial membranes, is a very real one. There is substantial evidence for the absence of functional citric acid cycle enzymes in *Phytomonas sp.* , with mitochondria incapable of oxidising pyruvate, succinate, 2-oxoglutarate, malate or proline (Fernandez-Beccerra et al, 1997; Sanchez-Moreno et al, 1992). However, residual amounts of citric acid cycle enzymes have been detected in the inactive bloodstream *T. brucei* promitochondrion by Jenkins et al (1988) and Durieux et al (1991) and a mitochondrial location of DHlipDH cannot be entirely dismissed. Hence it may be possible that the observed DHlipDH recovery with Phytomonas plasma membranes could be a result of contamination or adventitious adherence.

9.2.3.2 Indicated by NAD -dependent isocitrate dehydrogenase in *Crithidia fasciculata*

NAD-dependent isocitrate dehydrogenase, a previously characterised mitochondrial marker (chapter 8, section 8.2), was assayed during the fractionation of *Crithidia fasciculata* to determine the extent of any mitochondrial contamination in the plasma membrane preparation. Isocitrate dehydrogenase, together with DHlipDH, activity was measured in the unfractionated homogenate and isolated plasma membrane pellet and specific activities determined (Fig. 52). There was a four fold

decrease in isocitrate dehydrogenase specific activity , from 0.029 U/mg protein^{-1} in homogenate to 0.007 U/mg protein^{-1} in the isolated plasma membrane fraction, indicating a significant though low level of mitochondrial contamination in the isolated plasma membrane fraction.

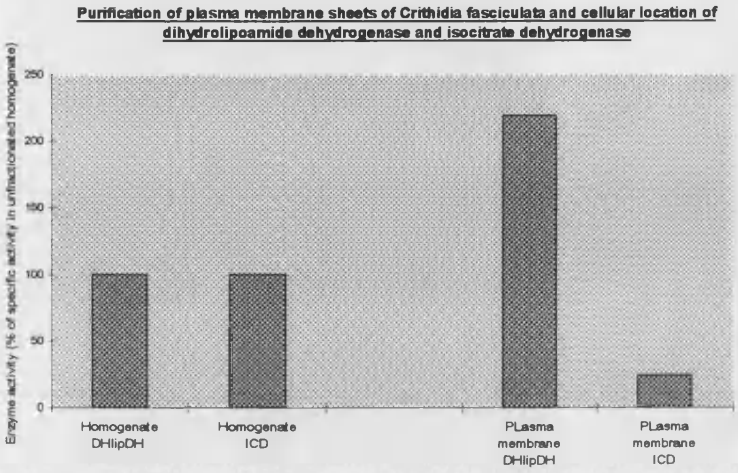


Figure 52: Plasma membrane sheets from *Crithidia fasciculata* were purified, and the enzymic activities of DHlipDH and isocitrate dehydrogenase were assayed, as described in methods section 2.28.3. The enzymic activities are expressed as a percentage of their specific activity in the unfractionated cell homogenate (DHlipDH, 0.273 μM/NADH produced min^{-1}/mg protein^{-1}; isocitrate dehydrogenase 0.029 μM/NADH produced min^{-1}/mg protein^{-1})

9.3 Discussion

The DHlipDH of the bloodstream form of *T. brucei* was first identified and localised to the plasma membrane of the organism by Danson et al (1987) using the method of Voorheis et al (1979) to purify plasma membranes. Contrary to the method described here, cells, swollen to turgidity, were homogenised with a tight-fitting Dounce homogeniser and then treated with leupeptin and DNAase before being subjected to sucrose-density-gradient centrifugation (Voorheis et al., 1979). A prominent dense band was extracted from the gradient and examination by electron microscopy revealed it to be enriched with plasma membranes, as evidenced by their characteristic subpellicular microtubular array. Enzymatic analysis of these membrane fractions indicated that they contained the majority of the DHlipDH (only 10% being found in the initial supernatant), up to 60% of a plasma membrane marker (the ouabain-sensitive $Na^+ + K^+$ stimulated ATPase), and a negligible amount of malate dehydrogenase contamination (<2%) (Danson et al, 1987). Further work by Jackman

et al (1990), based on full subcellular fractionation studies, reported that DHlipDH was located over the entire surface of the plasma membrane, including the flagellar pocket region in bloodstream forms. Jackman et al performed a subcellular fractionation according to the method of Gbenle et al, 1986. Cells were homogenised with silicon carbide, and subjected to differential centrifugation followed by isopycnic centrifugation of the post-large-granule extract on a linear sucrose gradient in which the plasma membrane migrated to a characteristic density. Fractions were taken and assayed for DHlipDH together with marker enzymes α-glucosidase (plasma membrane), acid phosphatase (flagellar pocket), 3'nucleotidase (plasma membrane), isocitrate dehydrogenase (mitochondria), α-mannosidase (lysosomes), hexokinase (glycosomes) and adenylate cyclase (flagellar pocket).

Both *Crithidia fasciculata* and *Phytomonas sp.* have specific activities in the plasma membrane fractions which are higher than the homogenate value, initially indicating some copurification of DHlipDH with plasma membrane in both cell types. This retention of enzyme activity in plasma membrane fraction appears to be more pronounced in *Phytomonas sp.* , where specific activity is over five times that of the homogenate value. Interestingly, only 10% of total DHlipDH activity was lost in the supernatant in *Phytomonas sp.* (as compared to a considerable 54% in *Crithidia fasciculata*) which compares directly with the 10 % loss found in the initial supernatant found by Danson et al (1987) in their preparation of bloodstream *T. brucei* plasma membrane preparations.

The two fold increase in specific activity over homogenate observed in the plasma membrane fraction of *Crithidia fasciculata* appears to indicate some copurification of DHlipDH with plasma membrane. However, in view of the mitochondrial contamination of the plasma membrane fraction (section 9.2.3.3), it is possible that the observed increase in activity is due to mitochondrial DHlipDH, which is certainly present in the mitochondrially active *Crithidia fasciculata.* Cook et al (1990) reported the possible presence of DHlipDH in the plasma membrane of procyclic *T. brucei* following kinetic analysis of DHlipDH in sonicate and plasma membrane isolates. However, a full subcellular fractionation study performed by

Jackman (1991) indicated the probable absence of an extra mitochondrial DHlipDH in procyclic *T. brucei,* although a plasma membrane location could not be entirely ruled out. Hence, although there is a possibility of an extra-mitochondrial DHlipDH in *Crithidia fasciculata* , it is unlikely and further investigations are essential.

The five fold increase in DHlipDH in *Phytomonas sp.* is comparable to the findings of Danson et al (1987) where the specific activity of *T. brucei* DHlipDH was 8-fold greater in plasma membrane preparations than that observed in the cell lysate. This increase in specific activity compares with reported values of 15-fold for ouabain-sensitive $Na^+ + K^+$-stimulated ATPase and 5-fold for adenylate cyclase in blood stream *T. brucei* (Voorheis et al, 1979). However, owing to the unavailability of a suitable mitochondrial enzyme marker, it was impossible to determine the degree, if any, of mitochondrial contamination in *Phytomonas sp.* However, the presence of NADH oxidase in the plasma membrane isolate may indicate some mitochondrial contamination although the considerable five-fold increase does seem to suggest the presence of an extra-mitochondrial plasma membrane DHlipDH.

One obvious difference between *Crithidia fasciculata* and *Phytomonas sp.* in the results presented above is the notable loss of DHlipDH in supernatant (S1) in *Crithidia fasciculata* as compared to *Phytomonas sp.* (Figs 48 and 49). This may be explained by suggesting that rupturing of the highly developed *Crithidia fasciculata* mitochondria during grinding with silicon carbide could easily take place, and enzymes present in the mitochondrial matrix could leak out. DHlipDH may be free in the mitochondrial matrix of *Crithidia fasciculata* and therefore able to leak out.

9.4 Future work

Future work should focus on the elucidation of reliable organelle enzyme markers which could be used in a full subcellular fractionation study of both *Phytomonas sp.* and *Crithidia fasciculata* , similar to that performed by Jackman et al (1990) on bloodstream *T. brucei,* in which all cellular locations of DHlipDH could be investigated. Assays for the pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched chain 2-oxo acid dehydrogenase complexes should also be performed, as suggested in Chapter 7 (section 7.1.6) as should an investigation of the presence of a glycine cleavage system in both cell types.

A plasma membrane location of DHlipDH could be investigated further by latency studies on whole cells to determine if the enzyme is located at the cytoplasmic or external face of the plasma membrane, as described by Steiger et al (1980). Assays for the presence of lipoic acid (Jackman et al, 1991) is another line of future investigation. Lipoic acid is a coenzyme for pyruvate dehydrogenase, α ketoglutarate dehydrogenase, branched chain-ketoacid dehydrogenase, and the glycine cleavage system. DHlipDH is an essential component of all these complexes and hence confirmation of the presence or absence of lipoic acid would be advantageous, especially in *Phytomonas sp.* where the possibility exists of a novel and possibly uncomplexed plasma membrane enzyme.

9.5 Summary

The discovery of DHlipDH in mitochondrially inactive *Phytomonas sp.*, and the possible plasma membrane location, is an exciting one and is comparable to the findings of Danson et al (1987) and Jackman et al (1991) of DHlipDH in the mitochondrially inactive bloodstream form of *T. brucei.* Their findings were important as it was the first and only known example of an extra-mitochondrial eukaryotic DHlipDH. The results reported here suggest that the previously reported presence of DHlipDH in mitochondrially inactive bloodstream *T. brucei* , and the unusual plasma membrane location, may be common to *Phytomonas sp.* and possibly other members of the Trypanosomatid family.

CHAPTER 10

GENERAL DISCUSSION

The work presented here in this thesis was inspired by the findings of Danson et al (1987) who reported the presence of dihydrolipoamide dehydrogenase (DHlipDH) in the bloodstream form of *T.brucei* in the absence of pyruvate (PDHC), 2-oxoglutarate, and branched chain 2-oxoacid dehydrogenase complexes (BCOADC). They also presented evidence for an unusual plasma membrane location, suggesting that DHlipDH might have a cellular function in addition to its established role in these dehydrogenase complexes. The finding of a plasma membrane associated DHlipDH was very exciting as it was the first reported incidence of an extra-mitochondrial DHlipDH in a eukaryotic cell.

Jackman et al (1991) continued investigations into the nature and localisation of DHlipDH in both bloodstream and procyclic forms of *T.brucei*. Using subcellular fractionation techniques and latency studies where DHlipDH activity was measured in the presence and absence of 0.1% triton X-100 according to the method described by Gbenle et al (Gbenle et al, 1986)., they confirmed that mitochondrially-inactive bloodstream *T.brucei* possessed a DHlipDH which was solely located in loose association over the inner surface of the plasma membrane. The mitochondrially-active procyclic form was shown to possess mitochondrial DHipDH, which would be expected owing to the presence of 2-oxo acid dehydrogenase complexes, although it was not possible to determine if there was also a plasma membrane location using the methods employed.

The aim of this study has been to determine unequivocally if a plasma membrane DHlipDH occurs in procyclic *T.brucei* cells and to extend the investigation of DHlipDH to other members of the Trypanosomatid family in which mitochondria may be either active or repressed, namely *Crithidia fasciculata* and *Phytomonas sp.* respectively.

Attempts to utilise DHlipDH-specific polyclonal antibodies, raised using synthetic peptide sequences, in immunolocalisation experiments proved largely unsuccessful owing to weak antibody binding to both the denatured enzyme, as seen in western blots, and the native DHlipDH protein as found in immunofluorescence

experiments (chapter 6). However, although unable to show conclusively whether DHlipDH was to be found in the plasma membrane of procyclic *T.brucei* cells by immunochemical means, this project presents convincing preliminary evidence, based on subcellular fractionation experiments, for an unusual plasma membrane associated DHlipDH in *Phytomonas sp.*

10.1 DHlipDH in members of the subgenus Trypanozoon

DHlipDH activity has been detected in both bloodstream and procyclic forms of *T.b.brucei, T.b.rhodesiense ,T.b.gambiense* and *T.evansi* (Else et al, 1994) while antibodies to *T.cruzi* DHlipDH, provided by Krauth-Siegel (Lohrer & Krauth-Siegel, 1990) have been shown to cross-react with a band of Mr 55 000, corresponding to DHlipDH, in western blots of all bloodstream and procyclic cell extracts (Else et al, 1994).

Tyler et al (1997) used the same polyclonal antibody to screen for DHlipDH in the differentiation of mitochondrially repressed slender *T.b.brucei* bloodstream to partially mitochondrially active stumpy forms. Western blots showed weak crossreactivity with bloodstream forms while pronounced reactivity was observed with stumpy and procyclic forms. Immunofluorescence, using Krauth Siegel's antibody, and NAD-diaphorase assays conclusively localised the enzyme to the mitochondrion of stumpy and procyclic forms while showing no plasma membrane localisation of DHlipDH in bloodstream forms. These results directly contradict the findings of Danson et al (1987) and Jackman et al (1991). Tyler et al's western blot results, using the DHlipDH-specific antibodies that they went on to use in their immunofluorescence experiments, clearly show a cross-reactive band with long slender *T. brucei* cell homogenates that corresponds to DHlipDH. They went on to report that there was an increase in DHlipDH expression in the stumpy form and procyclic form relative to the slender form, so confirming the unusual presence of DHlipDH in long slender *T. brucei* cells. It is most likely that their failure to detect a plasma membrane location of DHlipDH in long slender *T. brucei* using immunofluorescence is due to the insensitivity of this localisation method for the small amounts of DHlipDH that would be expected to be present at the trypanosome plasma membrane. It was for this reason that in the work presented here, very sensitive immunogold localisation techniques

were originally the method of choice for the investigation of a plasma membrane associated DHlipDH in procyclic *T. brucei* cells (see section 1.4, study aims).

It may also be possible that during cell fixation and immunofluorescence studies, as described by Sherwin et al (1987) and used in this work and by Tyler et al, a loosely associated plasma-membrane DHlipDH may have been lost during stringent washing steps.

Cellular localisation studies in *T.cruzi* epimastigotes (Portela and Stopopani, 1991) showed no evidence for association of DHlipDH with the plasma membrane and the authors suggested that all *T.cruzi* DHlipDH was present in the mitochondrion. However, *T.cruzi* differs from *T.brucei* in that there is a functional mitochondrion present in all stages of the parasite's life cycle. *T.cruzi* DHlipDH is therefore probably a component of the 2-oxoacid dehydrogenase complexes (Fairlamb 1982;1989) and as such would be expected to have a mitochondrial localisation.

10.2 Genetics of trypanosomal DHlipDH

In *T.brucei*, it appears that both the plasma-membrane and mitochondrial forms are encoded by the same gene and that there is little if any difference between the two proteins (Else at al, 1994). DNA probes have revealed only a single gene and the N-termini of both proteins are identical and correspond to the cloned gene sequence, but with a nine amino acid truncation. The enzymes are kinetically indistinguishable and have identical subunit molecular sizes on SDS-PAGE and identical native molecular sizes by gel filtration, (Else at al, 1994).

10.3 Intracellular mitochondrial targeting of DHlipDH in trypanosomes

The majority of constituent trypanosomal polypeptides are encoded in the nucleus and are synthesised on free ribosomes in the cytosol (De Marcucci et al, 1988), with kinetoplast DNA encoding only cytochromes, ATPase and a few other polypeptides (Simpson, 1987). In all mitochondrially-active trypanosomes, as in all other eukaryotes, DHlipDH is coded in the nucleus and then targeted to the mitochondrion by means of a protein signal peptide (Kim et al, 1991). N-terminal peptide extensions, which are removed during translocation, have been demonstrated for the mammalian PDHC and OGDHC components, with pre-E3 having an extra 15-20 amino acids in

each case (De Marcucci et al, 1988). The N-terminal extension to the pre-E3 is cleaved by a specific matrix protease during or shortly after entry into the mitochondrial matrix (Hunter and Lindsay, 1986). The dimeric DHlipDH then interacts with the E2 or protein X components of the 2-oxo acid dehydrogenase complexes.

A nine amino acid N-terminal pre-sequence determined for *T.brucei* DHlipDH contains two positively-charged amino acids and none that are hydroxylated (Else et al, 1994) (table 12). DHlipDH has also been characterised (Lohrer and krauth-Siegel, 1990), and the gene cloned and sequenced (Schoneck et al, 1997) in *T.cruzi* epimastigotes.

Similar to *T.brucei*, the DNA-derived amino acid sequence of *T.cruzi* DHlipDH shows an N-terminal extension of nine residues which contains basic residues and does not contain acidic amino acids. The *T.cruzi* terminal sequence, which is absent from the protein, shows a high degree of similarity with both the *T.brucei* presequence and other mitochondrial targeting sequences of proteins from other eukaryotes including the trypanosome *Crithidia fasciculata* (table 12). At the protein level, the *T.cruzi* enzyme shows 81% identity with the *T.brucei* enzyme (Schoneck et al, 1997)

Table 12: N-terminal nonapeptide of mitochondrial proteins from Kinetoplastida

Species	Sequences
	-9 -1
T.cruzi DHlipDH [a]	M F R R C A V K L
T.brucei DHlipDH [a]	M F R R C F P I F
C.fasciculata Pol B [b]	M F R R T F L T R
C.fasciculata p16 [c]	M L R F V P R R L
C.fasciculata p17 [c]	M L R R S P T L L
C.fasciculata p18 [c]	M L R R T V S N F

[a] Else et al (1994)
[b] mitPol b, mitochondrial DNA polymerase B (Torri and Englund, 1995)
[c] p16-p18, kinetoplast-DNA-associated proteins (Xu and Ray, 1993)

A general three-amino-acid motif common to leader peptides of mitochondrial proteins has been identified by Hendrick et al, (1989). Such sequences are cleaved off in a 2-step process: an Arg-10 (counting upstream from the N-terminus of the mature

protein), a hydrophobic residue (usually Phe or Leu) at position -8, and a Ser, Thr, or Gly at position -5. A first cleavage event, N-terminal to residue -8, results in an intermediate with an N-terminal octapeptide. Removal of this peptide in the second cleavage step yields the mature protein. The trypanosomal presequences are similar to this intermediate octapeptide. They also possess a small, mostly hydrophilic residue at position -5 and Phe or Leu at position -8. In addition, the trypanosomal precursor proteins show two conserved Arg residues at the positions -6 and -7. The mitochondrially-targeted Rieske iron-sulphur proteins from *T.brucei* and *Crithidia fasciculata* (Priest and Hajduk, 1995) have nine amino acid presequences which contain this motif twice: once within the presequences itself and then secondly upstream from the mature protein. Furthermore, it has recently been shown that that the N-terminal presequence of *T.brucei* DHlipDH mediates the import of dihydrofolate reductase as fusion protein into mitochondria of *Leishmania tarentolae*, *T.brucei* and yeast (Hauser et al, 1996).

The presence of DHlipDH in bloodstream *T.b.brucei*, *T.b.rhodesiense*, *T.b.gambiense* and *T.evansi* , all of which were shown to lack mitochondrial 2-oxoglutarate and pyruvate dehydrogenase enzyme complexes (Else et al, 1994) indicate that the DHlipDH of bloodstream trypanosomes is the result of normal expression and is not the result of faulty or leaky expression dependent on the strain of organism studied. However, whether the presence of an extramitochondrial DHlipDH in bloodstream forms is due to its expression not being fully suppressed and/or to diminished mitochondrial import or if the enzyme exerts a specific function remains unknown. Interestingly, the pre-sequence appears to be processed identically in both bloodstream and procyclic forms, even though the enzyme is not thought to be mitochondrially targeted in the bloodstream form. Targeting to the plasma membrane is reasonably well understood for secretory and integral membrane proteins, although the situation remains unclear for proteins that are loosely associated with membranes. It is thought, however, that cytoplasmic factors such as chaperones are essential for plasma membrane targeting of proteins (Rhee and Hunter, 1990; Ellis and van der Vies, 1991)

10.4 Possible DHlipDH plasma membrane functions

DHlipDH is found as an integral component in four main multienzyme complexes : pyruvate dehydrogenase complex (PDHC), 2-oxoglutarate dehydrogenase complex and branched-chain 2-oxoacid dehydrogenase multienzyme complexes (Mattevi et al, 1992; Perham, 1991) and the glycine cleavage system (Kikuchi and Hiraga, 1982). DHlipDH traditionally functions in the mitochondrion in association with a multienzyme complex. DHlipDH in bloodstream *T.brucei* has been shown to be extramitochondrial and operating independently of the mitochondrial 2-oxo-acid dehydrogenase components with which it is usually associated. Such an extra-mitochondrial plasma membrane DHlipDH in *T.brucei* and now possibly *Phytomonas sp.* (chapter 9), may be similarly present as a complexed enzyme, or else it may exist in a free, uncomplexed form, possibly with a novel function. There is some evidence to suggest that the bloodstream *T.brucei* DHlipDH is indeed complexed at the plasma membrane (Else et al, 1994) as in terms of its size, stability and kinetics, *T.brucei* bloodstream form DHlipDH appears to be similar to DHlipDHs associated with the 2-oxo acid dehydrogenase complexes of other species. Furthermore, although the level of DHlipDH and its substrate lipoic acid has been found to be higher in procyclic cells compared to bloodstream forms, they are both expressed in a ratio of 1:1 in both cell types. While procyclic DHlipDH would be expected to be a component of mitochondrial 2-oxo acid dehydrogenase complexes, this suggests that the bloodstream form DHlipDH may exist in a similar enzyme complex at the plasma membrane. However, the possibility of an uncomplexed DHlipDH in bloodstream *T.brucei* cannot be entirely ruled out and the nature of the membrane-associated DHlipDH in *Phytomonas* sp. remains to be determined.

10.4.1 Multienzyme complex functions

There are examples of plasma membrane DHlipDHs involved in multienzyme complexes in various cells. Plasma membrane-associated DHlipDH has been found in the glycine cleavage system in the purinolytic *Clostridium cylindrosporum* (Dietrichs et al, 1991) and the glycine-utilising *Eubacterium acidaminophilum* (Freudenberg et al, 1989). However, these are both prokaryotic cells, and the GCS has never been found outside the mitochondrion in a eukaryotic cell. DHlipDH involvement in a

plasma membrane GCS in either bloodstream *T.brucei* or *Phytomonas sp.* is therefore highly unlikely.

The DHlipDH-containing pyruvate dehydrogenase enzyme complex (PDHC) has been localised to plasma membranes of many species. PDHC enzyme activity has been detected in the mycoplasma *Acholeplasma laidlawi* (Manolukas et al, 1988; McGarrity et al, 1984) and has been shown to be at least partially associated with the membrane fraction (Wallbrandt et al, 1992). *A laidlawi* ferments glucose to lactate, pyruvate and acetate but lacks the tricarboxylic acid cycle and cytochromes (Manolukas et al, 1988, Pollack et al, 1983). In this respect, it exhibits metabolic characteristics similar to *Phytomonas sp.*, suggesting that a similar plasma membrane - associated PDH may possibly be present in *Phytomonas sp.*

Other DHlipDH-containing complexes include the lactate-degrading system of *Butyribacterium rettgeri* (Wittenberger and Haaf, 1964) and the acetoin dehydrogenase system of *Pelobacter carbinolicus* (Oppermann et al, 1991). Both of these are rather specialised, but they do demonstrate that DHlipDH is not confined to the four most common complexes, but could be part of a complex catalysing a completely different dehydrogenase reaction

10.4.2 Thiol:protein disulphide interchange

DHlipDH and lipoic acid form part of an active system of flavoenzymes that maintains redox balance and catalyses reductive processes in many cell types (Freedman, 1979). Lipoic acid has been used therapeutically in a number of diseases, including liver cirrhosis, heavy metal intoxication, and diabetic polyneuropathy, and in 1988 it was proposed that the antioxidant activity of lipoic acid might play an important role in its therapeutic efficacy (Bast and Haenen, 1988). Lipoic acid is reduced to dihydrolipoic acid (DHLA) *in vivo*, and it thought that it is the DHLA which is primarily responsible for antioxidant activity (Haenen and Bast, 1991; Scott et al, 1994; Kagan et al, 1995). In contrast to many other antioxidants, dihydrolipoate may function as a universal free radical quencher that scavenges peroxyl radicals both in the aqueous cytosol and the hydrophobic membrane environment (Kegan et al, 1992, Matsugo et al, 1995, Scholich et al, 1989). In this context, a plasma membrane location of DHlipDH in *T.brucei* and *Phytomonas sp.* is particularly interesting.

It has been suggested that dithiol-disulphide interchanges play a general role in membrane-related processes including receptor signal transduction and solute transport (Robillard and Konings, 1982; Malbon et al, 1987). Furthermore, it has been suggested that DHlipDH and lipoic acid may be directly or indirectly involved in these phenomena, giving rise to further possible functions of a plasma membrane DHlipDH

10.4.3 Solute transport

DHlipDH has been directly implicated in the transport of various sugars, mediated through its cofactor dihydrolipoamide. African trypanosomes are totally dependent on glycolysis for ATP production and it is conceivable that a plasma-membrane-associated DHlipDH may be involved in the very high glucose uptake of African trypanosomes which exceeds that of erythrocytes by a factor of approximately 300.

A lipoic acid-dependent import of sugars has been suggested for *E.coli* and adipocytes (Richarme and Heine,1986; Frost and Lane, 1986). *E.coli* has been found to express both a mitochondrial enzyme associated with the 2-oxoacid dehydrogenase complexes (reviewed by Carothers et al, 1989) and an extramitochondrial, possibly plasma membrane located DHlipDH thought to be involved in sugar transport (Richarme, 1989). A strong link between DHlipDH-containing multienzyme complex activity and binding protein-dependent transport of maltose, ribose and galactose has been clearly demonstrated in *E.coli* (Richarme 1985;1987; Richarme and Heine, 1986).

E.coli has two different genes encoding DHlipDH , specific for the intra and extramitochondrial enzyme (Richarme, 1989). However, this does not seem to be the case in *T.brucei* where it appears that both the plasma membrane and mitochondrial forms are encoded by the same gene and that there is little, if any, difference between the two proteins.(Else et al, 1993, 1994).

10.4.4 Other possible roles of DHlipDH

Evidence of additional roles for DHlipDH may be seen in archaebacteria, where 2-oxoacids are metabolised by a different mechanism, employing oxidoreductases that do not use lipoic acid (Plaga et al, 1992 ; Kersher and Oesterhelt,

1982), and where DHlipDH is still found but as a non-complexed enzyme (Danson , 1988). The role of such a DHlipDH remains undetermined.

DHlipDH from *H. volcanii* appears not to be involved in sugar transport (Jolly et al, 1996) nor to play a role in a glycine cleavage enzyme complex. It has been suggested that dihydrolipoic acid, which is also present in *H.volcanii* and is known to possess antioxidant properties (Suzuki et al, 1994), may play a role in the repair of membrane thiol groups in mitochondria (Zimmer et al, 1991). Hence the role of lipoic acid and DHlipDH in the halophilic bacteria may be in protection and repair.

10.5 Trypanosomal DHlipDH : role in oxidative stress and potential drug target

Lipoic acid is able to reduce the major intracellular thiol, glutathione (Bast and Haenan, 1988) and thereby contributes to the maintenance of redox balance and to reduction of oxidative stress (Meister and Anderson, 1983) in many eukaryotic cells. Kinetoplastida appear to possess an antioxidative thiol metabolism which differs qualitatively from that of other eukaryotes. In the trypanosome, glutathione is replaced by trypanothione, and the cell is much more vulnerable to reactive oxygen species (Boveris et al, 1980; Carnieri et al, 1993) Similar in structure to trypanothione reductase (TR), a key enzyme of the parasite's thiol metabolism, DHlipDH may also have a general function in creating and oxidising free intracellular thiol groups. Hence in the trypanosome environment, lipoic acid and DHlipDH may have considerable influence upon redox levels and may well be an effective drug target as many anti-trypanosomal drugs act as oxidative stressors and/or interfere with the defence mechanisms of the parasite (Fairlamb et al, 1992; Krauth-Siegel and Schoneck, 1995). In this context DHlipDH may be additionally attractive as a drug target because of its plasma membrane location in bloodstream *T.brucei* and now *Phytomonas sp.*

DHlipDH appears to be a target for a number of drugs which are already being used against trypanosomal infections. Melarsenoxide, an arsenical used in the treatment of sleeping sickness, covalently inhibits reduced DHlipDH (Fairlamb et al, 1992) while DHlipDH is implicated in the chemotherapeutic effect of Nifurtimox. DHlipDH triggers the production of O_2^- and H_2O_2 by reducing Nifurtimox, the most effective drug against *T.cruzi* (Docampo and Stoppani, 1979).

10.6 DHlipDH in *Crithidia fasciculata*

Although DHlipDH activity was found to be present in *Crithidia fasciculata* cell homogenates (chapter 7), it must be assumed that most of this DHlipDH is associated with 2-oxoacid dehydrogenase enzyme complexes in the active mitochondrion. To this effect, the enzyme has recently been characterised in the pyruvate dehydrogenase complex of *C. fasciculata* (Diaz and Komuniecki, 1995).

Subcellular fractionation studies were unable to determine conclusively a plasma membrane location for DHlipDH in *Crithidia fasciculata* in this study (chapter 9), paralleling findings by Jackman et al (1991) who were unable to show by subcellular fractionation whether a small proportion of the DHlipDH was present at the plasma membrane of *T.brucei* procyclic cells. A plasma membrane location in either of these cell types would be highly unusual as there are few enzymes located in both the mitochondrion and the plasma membrane of an organism. However, recent findings of normally mitochondrial dihydrolipoamide succinyltransferase (the core enzyme of the 2-oxoacid dehydrogenase complex) located both in the mitochondrion and plasma membrane of skeletal muscle cells (Matuda et al, 1997) confirm that, although unusual, the possibility of a plasma membrane location of DHlipDH in these cells is possible.

Interestingly, immunofluorescence studies performed by Tyler et al (1997) using antibodies specific for *T.cruzi* DHlipDH localised DHlipDH to the mitochondrion of procyclic forms of *T.brucei* but failed to detect the enzyme in the plasma membrane of procyclic cells. However, Tyler et al failed to detect the protein in the plasma membrane of *T.brucei* bloodstream cells using these antibodies, a finding which directly contradicts a number of reports (Danson et al, 1987, Jackman et al, 1991, Else et al, 1994). The negative result reported by Tyler et al is most likely due to insensitivity of the immunofluorescence assay used for the detection of very small amounts of enzyme expected to be present at the plasma membrane, as discussed earlier (section 10.1). This limited sensitivity could be overcome by using the more sensitive immunogold labelling method of localisation.

10.7 DHlipDH in *Phytomonas sp.*

Phytomonas sp. cells are highly specialised for glycolysis, with mainly glucose being consumed as energy and carbon source during logarithmic growth in culture (Chaumont et al, 1994). No evidence has been found for the presence of functional citric acid cycle enzymes in any species of *Phytomonas* studied to date (Fernandez-Beccerra et al, 1997; Chaumont et al, 1994; Sanchez-Moreno et al, 1992). When mitochondria were supplemented with α-oxoglutarate, pyruvate, malate or proline, none of these substrates increased endogenous respiration (Fernandez-Beccerra et al, 1997). The absence of detectable cytochromes and the fact that respiration is not inhibited by the classical inhibitors of the mitochondrial respiratory chain, such as cyanide and antimycin (Sanchez-Moreno et al, 1992) indicates that in *Phytomonas sp.* the mitochondrion is metabolically inactive and the glycolytic pathway serves as the major, if not the only, source of ATP within the cell.

The capacity for reoxidation of the glycolytically produced NADH by a putative glycerol-3-phosphate dihydroxyacetone cycle (involving a mitochondrial glycerol-3-phosphate oxidase and a glycosomal NAD-dependent glycerol-3-phosphate dehydrogenase) is not sufficient and some of the NADH is reoxidised by the reduction of acetate to ethanol following the decarboxylation of pyruvate (Fernandez-Becerra, 1997)

There have been no reports in the literature for the activity of any of the mitochondrial multienzyme complexes with which DHlipDH is normally associated. While preliminary work, presented here, suggests an extramitochondrial plasma membrane location for DHlipDH in *Phytomonas sp.*, the function of such an enzyme remains unknown at present, mirroring the situation in bloodstream *T.brucei.*

10.8 Future work

10.8.1 Further characterisation of *Phytomonas sp.* DHlipDH

Further studies should be performed to identify and characterise DHlipDH conclusively in *Phytomonas sp.* Size, stability and kinetics of *Phytomonas* DHlipDH should be thoroughly investigated and compared with DHlipDHs associated with the 2-oxo acid dehydrogenase complexes of other species to elucidate whether the enzyme is present as part of a multienzyme complex (as has been suggested for bloodstream *T.brucei*) or

uncomplexed (as in some species of prokaryotes including archaebacteria),. When DHlipDH functions in the 2-oxo acid dehydrogenase complexes, its substrate, dihydrolipoic acid, is attached to the transacetylase component of the multienzyme. The question hence arises as to whether lipoic acid is still the substrate of the apparently extra-mitochondrial DHlipDH in *Phytomonas sp.* Elucidation of the ratio of lipoic acid to DHlipDH would help to determine if DHlipDH is present in a complexed or uncomplexed form. Hence, future work should focus on a determination of this cofactor in *Phytomonas sp.*

Additionally, purification of the enzyme and N-terminal sequencing could provide information on the possible mechanism of plasma membrane targeting in this trypanosome.

10.8.2 Study of DHlipDH in other trypanosomatids

As well as *Phytomonas sp.* and bloodstream *T.brucei*, a complete suppression of mitochondrial activity and a total absence of cytochromes has been reported in another trypanosomatid *Herpetomonas muscaeum ingenoplastis.* (Coombs., 1989). Furthermore, a recent report that in the culture-adapted trypanosomatid *Leishmania tarentolae* most of the guide RNAs required for the editing of mitochondrial transcripts have been lost (Simpson et al, 1993), suggests also that this organism is able to survive in the absence of mitochondrial activity.

Hence utilisation of the glycolytic pathway as the sole carbon and energy source, together with a complete suppression of mitochondrial activity, appears to be found in at least three distantly-related representatives within the trypanosomatid family. Evidence for an unusual plasma-membrane associated DHlipDH in bloodstream *T.brucei* (Danson et al, 1987) and now *Phytomonas sp.* (chapter 9) suggests that such a DHlipDH may be a common feature of mitochondrially-inactive trypanosomatids. It would be therefore of interest to determine if such a DHlipDH may be found in the plasma membrane of *Herpetomonas muscarum ingenoplastis* and *Leishmania tarentolae.*

The separation of the African trypanosome from the main line of trypanosomatid evolution took place several hundreds of millions of years ago, well before the other representatives of the family Trypanosomatidae diverged (Fernandes

et al, 1993). The utilisation of glycolysis as the sole carbon and energy source in various diverse members of the trypanosomatid family indicates that such metabolic flexibility must represent an ancestral property, rather than a recent adaptation to life in a specific host. It may well be that this metabolic flexibility has allowed descendants of the ancestral trypanosomatid to adapt to the great variety of metazoan hosts, ranging from plants via insects, fishes, amphibians and reptiles to mammals, that are all parasitised today by trypanosomatids.

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