Dihydrolipoamide dehydrogenase in Trypanosoma brucei brucei

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DIHYDROLIPOAMIDE DEHYDROGENASE IN *TRYPANOSOMA BRUCEI BRUCEI*

submitted by Simon Andrew Jackman
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To my God, and my wife, Carol, with love and thanks

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ABBREVIATIONS

2OGDC	2-Oxoglutarate dehydrogenase complex
AAPDCA	*p*-Acetylaminophenyldichloroarsine
AMAs	Anti-mitochondrial antibodies
APDCA	*p*-Aminophenyldichloroarsine
ATPase	Adenosine triphosphatase
BCKDC	Branched-chain 2-oxo acid dehydrogenase complex
BSA	Bovine serum albumin
CNS	Central nervous system
DFMO	α-Difluoromethylornithine
DHAP	Dihydroxyacetone phosphate
DHlip	Dihydrolipoamide
DHlipDH	Dihydrolipoamide dehydrogenase
DMF	Dimethylformamide
DNP-lys	Dinitrophenyl-L-lysine
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene guanine tetraacetic acid
FAD	Flavin adenine dinucleotide
FPLC	Fast Protein Liquid Chromatography
$Fru(2,6)P_2$	Fructose 2,6-bisphosphate
GC/MS	Gas chromatography/mass spectrometry
GCS	Glycine cleavage system
GPI	Glycosylphosphatidyl inositol
GSH	Glutathione
KRB	Krebs Ringer buffer
KRBG	Krebs Ringer buffer containing glucose & sucrose
LDL	Low density lipoprotein
LS	Long slender
Mel O	Melarsen oxide
MICA	5-Methoxyindole-s-carboxylic acid
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NAD-IDH	NAD^+-linked isocitrate dehydrogenase
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP-IDH	$NADP^+$-linked isocitrate dehydrogenase
ODC	Ornithine decarboxylase
PAO	Phenylarsenoxide

PARP	Procyclic acidic repeat protein
PBC	Primary biliary cirrhosis
PDC	Pyruvate dehydrogenase complex
PEP	Phosphoenolpyruvate
PFK2	6,phosphofructo-2-kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHAM	Salicylhydroxamic acid
SIE	Sucrose-imidazole-EGTA
SS	Short stumpy
STE	Sucrose-Tris-EDTA
$Try(SH)_2$	Dihydrotrypanothione
VSG	Variable surface glycoprotein

ENZYMES

Acid phosphatase	EC 3.1.3.2
Adenylate cyclase	EC 4.6.1.1
Branched-chain 2-oxo acid dehydrogenase complex	EC 1.2.4.4
Catalase	EC 1.11.1.6
Citrate synthase	EC 4.1.3.7
Dihydrolipoamide dehydrogenase	EC 1.8.1.4
α-Glucosidase	EC 3.2.1.20
Hexokinase	EC 2.7.1.1
Isocitrate dehydrogenase ($NADP^+$-linked)	EC 1.1.1.42
Lactate dehydrogenase	EC 1.1.1.27
Malate dehydrogenase	EC 1.1.1.37
α-Mannosidase	EC 3.2.1.24
3'-Nucleotidase	EC 3.1.3.6
2-Oxolutarate dehydrogenase complex	EC 1.2.4.2
Pyruvate dehydrogenase complex	EC 1.2.4.1

ABSTRACT

Dihydrolipoamide dehydrogenase (DHlipDH) has been discovered in the mammalian bloodstream form of the protozoal parasite *Trypanosoma brucei brucei* (Danson, M.J., Conroy, K., McQuattie, A. & Stevenson, K.J. (1987) *Biochem. J. 243*, 661-665). This organism has a repressed mitochondrion which does not contain the 2-oxo acid dehydrogenase complexes, of which DHlipDH is normally a component part.

Within the cell, DHlipDH has been located to the whole surface of the plasma membrane, including the flagellar pocket region, by subcellular fractionation. Latency studies demonstrated that it is at the cytoplasmic face of this membrane. Using Triton X-114 phase partitioning, this DHlipDH was shown not to be attached to the membrane by any form of direct lipid or hydrophobic anchorage. This is the first report of a eukaryotic extra-mitochondrial DHlipDH.

Lipoic acid, the presumed substrate of DHlipDH, has been detected in acid hydrolysates of bloodstream form cells at a level of 1.7 (± 0.2) ng of the cofactor/mg protein. Its chemical identity has been confirmed by gas chromatography/mass spectrometry.

In the procyclic (Tsetse fly midgut) stage of the parasite, DHlipDH is 13-fold more active than in the bloodstream form, and there is also 10-fold more lipoic acid at this stage. These increases are coincident with the activation of the mitochondrion and expression of the 2-oxo acid dehydrogenase complexes. In agreement with this, the majority of DHlipDH in these cells was shown to be intra-mitochondrial by subcellular fractionation.

The DHlipDH of bloodstream form cells has been purified to homogeneity by Triton X-114 phase partitioning, heat treatment, chromatofocussing and gel filtration. It appears to be a dimer of similar molecular weight sub-units (M_r = 53 kDa). In terms of size and kinetics, this enzyme is similar to those isolated from the mitochondria of other species.

The active site vicinal dithiol of DHlipDH is susceptible to inactivation by arsenical reagents. As these are also the drugs of choice for late-stage African trypanosomiasis, DHlipDH has been assessed as a possible target for chemotherapy. The enzyme was not inactivated at arsenical concentrations required to immobilise cells, and was particularly insensitive to the drug melarsen oxide, indicating that it may not be a major target for these compounds.

The possible functions of the plasma membrane DHlipDH are discussed together with methods of functional analysis.

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CHAPTER 1 : INTRODUCTION

1.1 The trypanosomes

1.1.1 Classification of the trypanosomes

Trypanosomes are parasitic protozoan haemoflagellates (class Zoomastigophora, order kinetoplastida) which infect humans and domestic animals in tropical Africa and South America. The genus *Trypanosoma* is divided into two groups on the basis of the mode of transmission of the parasite by the insect vector: the stercoraria (faecal transmission) and the salivaria (salivary transmission) (Fairlamb, 1982).

1.1.2 The stercoraria

The most prominent stercorarian trypanosome is *Trypanosoma cruzi*, the causative agent of Chagas' disease in humans in South America. The parasite is transmitted between hosts by the Reduviid bug, which ingests the trypanosome during a blood meal, and excretes it in the faeces, from where it enters the host by being rubbed into skin abrasions or mucous membranes. Young children die of infection in the acute febrile stage, whilst adults who survive to the chronic phase suffer parasite-induced heart muscle and autonomic nervous tissue damage, leading to congestive heart failure and dilatation of the oesophagus, respectively (Boreham, 1979). The disease is very prevalent in South America, with 35 million people exposed to infection, and 13-14 million possibly carrying the parasite (Fairlamb, 1982). At present, there is no effective treatment available (Gutteridge, 1985).

1.1.3 The salivaria

The salivarian trypanosomes that infect humans are *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. These cause African sleeping sickness, 10,000 new cases of which are reported every year. In total, 45 million people are at risk from infection, and the disease constantly threatens to reach epidemic proportions (Goodwin, 1985). Acute infection is characterised by a swollen chancre at the site of inoculation, and by general oedema of the face. If the infection is not treated after several weeks of intermittent and irregular fever, invasion of the central nervous system (CNS) is inevitable and rapidly fatal (Fairlamb, 1982).

Treatment regimens have not altered significantly over many years, with suramin or pentamidine employed for early-stage sleeping sickness; combinations of these with aromatic arsenicals such as melarsoprol and tryparsamide are required when the CNS becomes involved, because the former compounds cannot cross the "blood-brain barrier" (Raseroka & Ormerod, 1985; Thomson, 1989). Such treatment is highly unsatisfactory as the arsenicals cause serious encephalopathy even at therapeutic doses.

The diseases of domestic animals caused by salivarian trypanosomes include nagana in cattle (*T.b.brucei*, *T.vivax* & *T.congolense*), surra in horses and camels (*T.evansi*) and dourine in horses (*T.equiperdum*). Over 3 million cattle die from animal trypanosomiasis each year, and the prevalence of the Tsetse fly, the major insect vector, means that 10 million square kilometres of land cannot be farmed in Africa.

The lack of effective cure for any of the trypanosomiases, together with the associated economic and nutritional problems and the possibilities of epidemics, led the World Health Organisation, in 1979, to rate trypanosomiasis among the top six tropical diseases for study with a view to developing more effective treatments (Trigg, 1979). In response to this, the last decade has seen a rapid increase in biochemical research into the trypanosomiases, with much work concentrating on the exploitation of biochemical differences between the parasite and its mammalian host.

T.b.brucei has become the main organism for the study of African trypanosomiasis, and its particularly interesting biochemistry has drawn many workers into this field of research.

1.2 *Trypansoma brucei brucei*

1.2.1 Life-cycle of *T.b.brucei*

T.b.brucei undergoes a cyclical transmission from mammal to mammal effected by the insect vector, the Tsetse fly (*Glossina* spp.) (Fig. 1.1). Different developmental stages of the life-cycle are accompanied by markedly different morphology and biochemical activities. Most notable among 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 & 1985).

In the mammalian bloodstream, a pleomorphic population of trypanosomes is observed, 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. This form is able to divide rapidly (doubling time = 6 h), and evades the host immune system by antigenic variation of its surface coat (Vickerman, 1985). 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

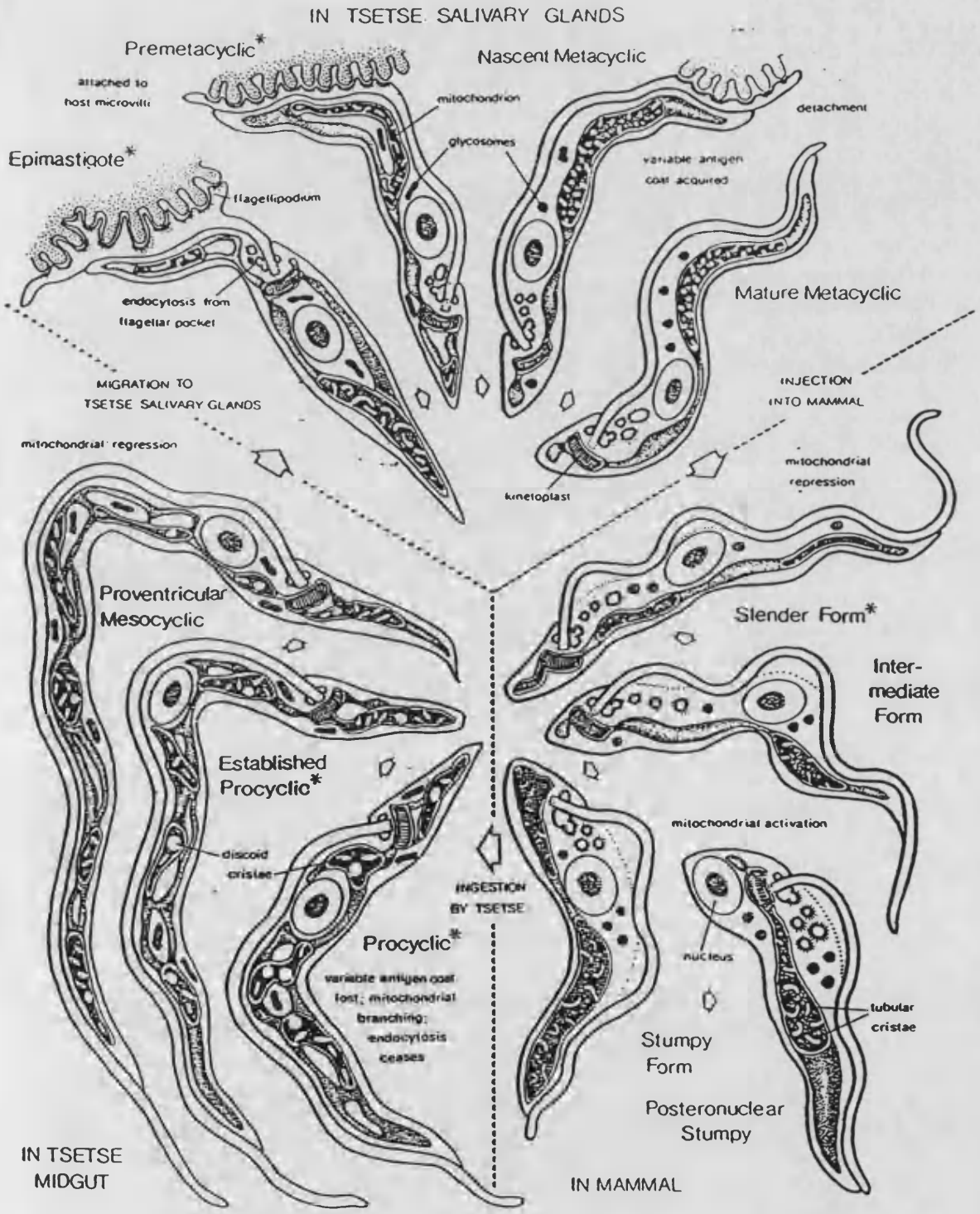


Fig. 1.1. Schematic diagram of *Trypanosoma brucei brucei* developmental cycle in mammal and tsetse fly, showing changes in cell surface, mitochondrion, glycosomes and receptor-mediated endocytosis, and relative sizes of different stages. Stages possessing the variable antigen coat lie to the right, and uncoated stages to the left. The mitochondrion is depicted in section to show changes in its cristae (from Vickerman, 1985).

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. For many years, this has been thought to be a pre-adaption 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. There is some doubt, however, about whether this form, or a closely related intermediate is the true form that is able to infect the Tsetse fly (Giffin & McCann, 1989; Bienen *et al.*, 1991).

Following a blood meal, ingested trypanosomes enter the Tsetse fly midgut lumen. Transformation to the procyclic form occurs within the endoperitrophic space in the posterior of the midgut. This insect form has lost the variable surface antigen coat, which has been replaced by a procyclic antigen (Roditi *et al.*, 1989). The mitochondrion has become a fully-developed, highly-branched network with many plate-like cristae (Fairlamb, 1982).

From 4 days onwards, procyclics invade the ectoperitrophic space by penetrating the peritrophic membrane. During the following week this space becomes packed with dividing trypanosomes. As they move to the proventriculus, they become longer (up to 60 μm) and cease to divide. Mitochondrial regression begins at this stage. Proventricular mesocyclics re-invade the endotrophic space and journey via the oesophagus, mouthparts and salivary ducts to the salivary glands (Vickerman, 1985).

In the salivary gland, the proliferative form is the epimastigote, which becomes attached by its flagellum to the microvilli of the epithelial cells. Here it undergoes metacyclogenesis, a process requiring the formation of junctional complexes with the epithelial cells. As cells become premetacyclics they lose their ability to divide, and during maturation, the metacyclic acquires an antigenic coat (Vickerman, 1985; Donelson, 1988).

When an infected Tsetse fly bites, metacyclic forms are injected into the mammalian host along with the salivary secretions. These enter the draining lymphatics, and from there infiltrate the bloodstream. Further mitochondrial regression accompanies these movements, producing the LS form to complete the life-cycle of *T.b.brucei* (Vickerman, 1985).

1.2.2 The structure and metabolism of *T.b.brucei*

The trypanosome is characterised by 5 main structural features: a single flagellum, a single mitochondrion containing a very large network of DNA (the kinetoplast), a complex network of subpellicular microtubules, glycosomes (microbody-like organelles) and a surface coat.

The ultrastructure of the trypanosome through all the various stages of its life-cycle has been described in detail by Steiger (1973), and the structure and role of the kinetoplast DNA has been reviewed by Simpson (1987). Of the remaining features, the glycosome and the surface coat have attracted a great deal of interest in the last few years, partly because

they are potential sites for chemotherapy. Furthermore, the pathways of purine, polyamine, thiol and carbohydrate metabolism and terminal oxidation, with their unique features, have been extensively investigated, as has the overall nutrition throughout the life-cycle of the trypanosome.

1.2.2.1 The glycosome

Glycosomes are found in all members of the family of Trypanosomatidae. They are ellipsoid or circular organelles of 0.3 μm diameter bounded by a single membrane. Each *T.b.brucei* cell has 200-300 such microbodies, which make up approximately 6 % of the cell volume (Hart *et al.*, 1984; Opperdoes, 1988). They contain the first nine glycolytic enzymes, enzymes of pyrimidine and ether-lipid biosynthesis, of glycerol metabolism and of carbon dioxide fixation, and adenylate kinase (Fairlamb & Opperdoes, 1986).

The high degree of compartmentalisation of the glycolytic enzymes is thought to be of considerable benefit to the trypanosome, which is totally dependent upon substrate-level phosphorylation for ATP synthesis. The glycosomal membrane probably acts as a selective permeability barrier, allowing the entry and exit of metabolites, whilst maintaining high concentrations of the glycolytic enzymes (up to 1 mM; Fairlamb, 1989). Despite the capacity for free diffusion through the glycosomal membrane, metabolites are concentrated within the organelle to five times their levels in the cytosol, and are postulated to be channelled through a complex of the enzymes. This channelling may be facilitated by the very high isoelectric points of the enzymes (the highest found in nature !), which could play a role in ionic binding of phosphorylated intermediates (Fairlamb, 1989).

The biogenesis of the glycosomes has come under particular scrutiny as a possible site for chemotherapeutic intervention because of the dependence of the trypanosome upon glycolysis (Michels, 1988). These organelles possess no DNA, with all their proteins being manufactured in the cytosol on free ribosomes. Proteins are imported into the glycosome within 1-3 min of completion (Opperdoes, 1987 & 1988).

Several of the glycolytic enzymes have 2 "hotspots" of positive charges separated by 40 Å, which are thought to be an internal import motif. This suggestion has been borne out by experiments in Wang's laboratory using chimaeras of glycosomal and cytosolic phosphoglycerate kinase to investigate the structural basis for import in *in vivo* assays (Wang, 1988). Suramin, one of the major trypanocidal drugs, has two negatively-charged domains separated by a distance of 40 Å. This compound has a high affinity for several glycolytic enzymes, and could be inhibiting glycolysis by interacting with the "hot spots" (Fairlamb, 1989). Due to the lack of hotspots in some glycosomal proteins (Kendall *et al.*, 1990), current theory has had to be modified to include other possible signal sequences, including the C-terminal peroxisomal import sequence (SKL) (Fung & Clayton, 1991).

1.2.2.2 The surface coat

The surface coat of the bloodstream form of *T.b.brucei* consists of a unique variable surface glycoprotein (VSG), which has a protective function in enabling the parasite to evade the host immune system. The surface of the trypanosome is covered by 1.2×10^7 tightly-packed molecules of VSG, forming a 15 nm thick coat. The glycoprotein comprises a 450-480 amino acid polypeptide with 2 or more attached carbohydrate chains, linked to the plasma membrane by a glycosylphosphatidyl inositol (GPI) anchor containing dimyristoyl fatty acid (Boothroyd, 1985; Low, 1989).

The trypanosomal genome has a repertoire of over 1000 possible antigenic variants of VSG, only one of which is expressed at any one time. It is the switching between different VSGs that enables the parasite to escape destruction by the host immune system (see section 1.2.1). Expression occurs at specific telomeric sites, to which new VSG genes are transferred by homologous recombination. Towards the start of an infection, switching proceeds according to an ordered program, but with later gene conversions and mutations, the order becomes less predictable (Donelson, 1988; Pays, 1988).

Trypanosomes are able to transfer their VSGs to host cells, which are then destroyed by their own immune system, resulting in the anaemia often observed with trypanosomal infection (Rifkin & Landsberger, 1990).

Upon entering the Tsetse fly, the VSG coat is shed (Seyfang *et al.*, 1990) and is replaced by one of a group of GPI-anchored procyclic acidic repeat proteins (PARPs; Clayton & Mowatt, 1989). The most well characterised of these is procyclin, which has an unusual 14-18 nm long rod-like structure (Richardson *et al.*, 1988; Roditi *et al.*, 1989).

Once the trypanosome reaches the metacyclic stage, it re-acquires the VSG coat, often displaying the last coat which was expressed in the mammalian bloodstream, though only a small fraction (14-17 in *T.b.brucei*) of the VSGs are available for expression at this stage (Pays, 1988).

Vaccine development to early bloodstream and metacyclic VSGs may eventually prove effective, but blocking the biosynthesis of the glycoprotein, through an understanding of the component enzymes in the pathway, should be a more successful strategy for surface coat-directed chemotherapy (Pays, 1988).

1.2.2.3 Purine salvage

All members of the Trypanosomatidae are unable to synthesize purines *de novo*, and depend upon active salvage mechanisms for purine incorporation. A diversity of enzyme pathways can be involved in salvage, so it is difficult to design specific therapeutic agents. Allopurinol, however, is active as a substrate for salvage enzymes, being accumulated in and lethal to trypanosomes (Opperdoes, 1985).

1.2.2.4 Polyamine metabolism

Polyamines have been implicated in many vital cellular processes, and are significantly elevated in rapidly growing cells (Fairlamb, 1989). In *T.b.brucei* the major polyamines are putrescine and spermidine, produced via the same biosynthetic routes as in mammals. Putrescine is produced from ornithine by ornithine decarboxylase (ODC), which is the controlling enzyme in the formation of polyamines (Wang, 1988). ODC is specifically inhibited by α-difluoromethylornithine (DFMO), causing depletion of polyamines. The trypanosome treated with this drug is transformed from the long slender to short stumpy bloodstream form (Giffin & McCann, 1989). This form is unable to change its surface coat, and is caught up by the host immune system. The particular effectiveness of DFMO against trypanosomes appears to be because ODC has a low turnover rate *in vivo*, and is structurally different from the mammalian enzyme (Wang, 1988). DFMO is currently undergoing clinical evaluation against African trypanosomiasis.

1.2.2.5 Thiol metabolism

Most eukaryotic and prokaryotic cells contain high concentrations of at least one low molecular weight thiol. In eukaryotes, the principal thiol is glutathione (GSH), which is involved in maintenance of correct intracellular thiol redox balance, enzymatic removal of hydrogen peroxide, scavenging of free radicals, detoxification of xenobiotics and control of protein synthesis. It also produces deoxyribonucleotides (via ribonucleotide reductase) for DNA synthesis (Meister & Anderson, 1983). GSH is maintained in its reduced form within the cell by glutathione reductase.

In trypanosomatids, glutathione has been detected in a unique conjugate with spermidine, termed trypanothione (N^1,N^8-bis(glutathionyl)spermidine; Fairlamb & Henderson, 1987). This performs an analogous role to that of glutathione in mammals, and is kept in its reduced state by trypanothione reductase, which is located in the cytosol of the cell (Smith *et al.*, 1991) and shows close homology to glutathione reductase. Trypanothione peroxidase is also active in these cells, and, together with the reductase, it ensures low intracellular concentrations of H_2O_2 (Fairlamb & Henderson, 1987).

The enzymes trypanothione reductase, peroxidase and synthetase are all unique to trypanosomatids, and, because of their role in reducing oxidative stress, may provide excellent targets for chemotherapeutic drug design. Indeed, one of the major drugs for late-stage sleeping sickness, melarsen oxide, has been shown to form a stable adduct with trypanothione (Fairlamb *et al.*, 1989). The selective toxicity of this arsenical may result from sequestration of intracellular trypanothione and/or inhibition of trypanothione reductase. Progress has also been made in the use of "subversive substrates" for trypanothione reductase (Henderson *et al.*, 1988). These substances, when reduced, undergo redox cycling processes to produce toxic metabolites of oxygen. Therefore, this enzyme, which normally reduces

oxidative stress, has had its function subverted to produce a cytotoxic effect. Preliminary studies with *T.cruzi* have demonstrated that a number of these compounds are trypanocidal (Henderson *et al.*, 1988).

1.2.2.6 Terminal respiratory systems

Bloodstream forms of *T.b.brucei* have very high rates of respiration, with whole cell suspensions using 70-140 nmol O_2/min/mg protein with glucose as the oxidisable substrate. This respiration rate is up to 50 times that observed in mammalian tissues. In the absence of any cytochromes or lactate dehydrogenase, the trypanosomes use a novel L-glycerol-3-phosphate oxidase to re-oxidise NADH generated from glycolysis (Grant & Sargent, 1960; Bowman & Flynn, 1976). This terminal oxidase is in the mitochondrion, and, together with the glycosomal NAD^+-dependent glycerophosphate dehydrogenase, it forms a shunt for reducing equivalents into the mitochondrion (Opperdoes *et al.*, 1977b). By the concerted action of these two components, dihydroxyacetone phosphate (DHAP) is first reduced to glycerol-3-phosphate, which is then re-oxidised to DHAP by the glycerophosphate oxidase. This re-oxidation allows the quantitative conversion of glucose to pyruvate and the maintenance of redox balance in the cell (Fig. 1.2; Bowman & Flynn, 1976).

The bloodstream form terminal oxidase resembles those of some plants and fungi, and is inhibited by metal-chelating compounds such as salicylhydroxamic acid (SHAM), but not cyanide (Opperdoes, 1985).

Transformation to the insect form of the parasite is a two-stage process for terminal oxidation. Firstly, new dehydrogenases appear, capable of transferring reducing equivalents from succinate, proline and NADH to O_2 via the alternative oxidase. This process occurs during the development of the short-stumpy bloodstream forms (Bienen *et al.*, 1991). The second stage is the emergence of a complete cytochrome chain, and the partial attenuation of the glycerophosphate oxidase, which may still account for up to 30 % of terminal oxidation in procyclics (Opperdoes, 1985; Fairlamb & Opperdoes, 1986). The cytochrome chain appears branched, with cytochromes aa_3 and o as oxygen acceptors. Cytochrome c has a particularly unusual structure in trypanosomes, resulting in a change in its absorbance maximum from 550 nm (mammalian) to 558 nm (Opperdoes, 1985). The cytochrome chain is inhibited by cyanide, but not SHAM.

In addition, Turrens (1989) has recently demonstrated that the NADH-Ubiquinone segment of the respiratory chain of procyclics is replaced by direct formation of succinate from fumarate reduction (by fumarate reductase). Succinate dehydrogenase also passes electrons to the respiratory chain, regenerating fumarate from succinate.

T.b.brucei represents the most extreme case of mitochondrial repression and transformation of all the trypanosomatids, as many others still possess active mitochondria in their bloodstream forms (Vickerman, 1965).

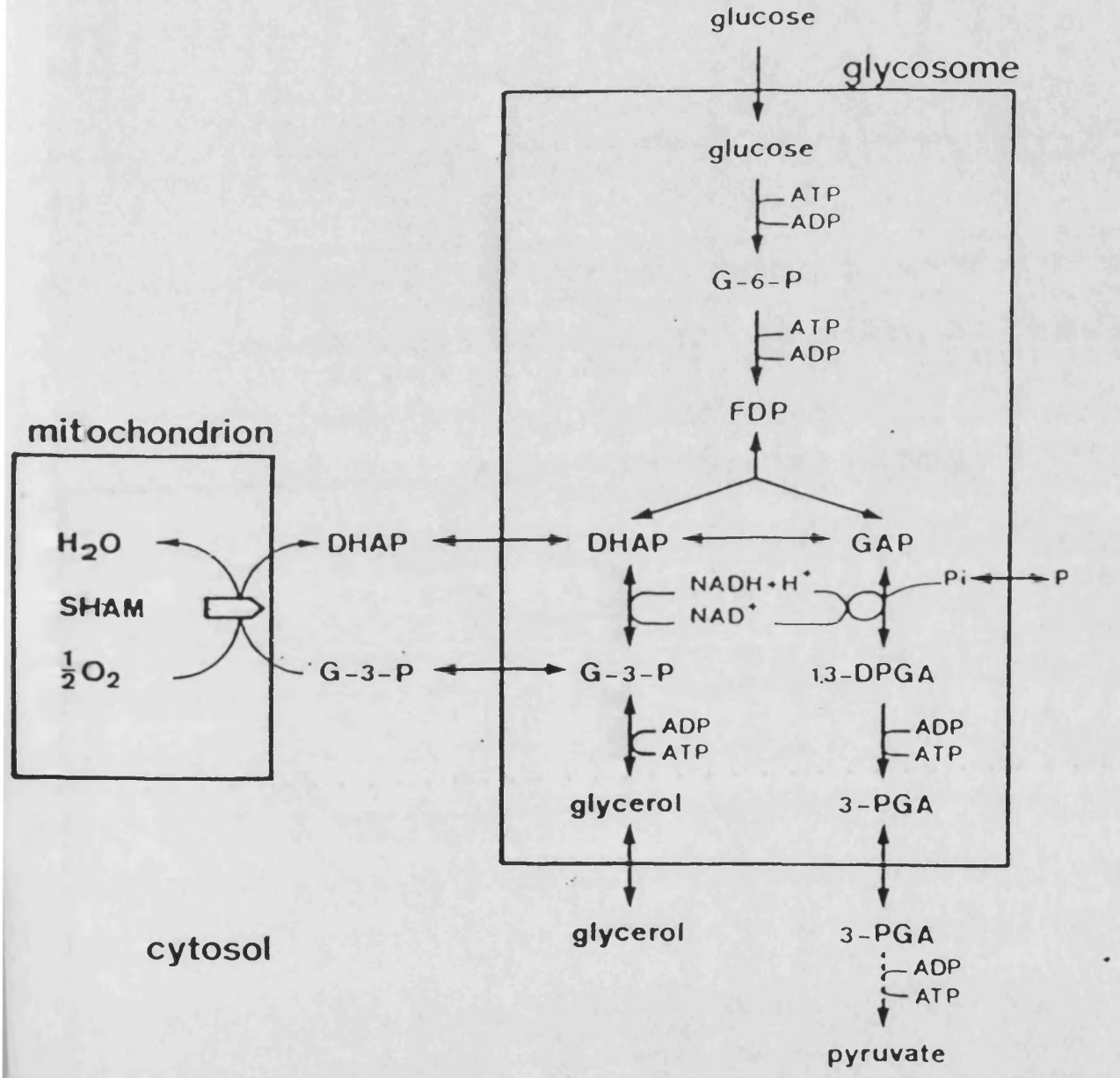


Fig. 1.2. Glycolysis in the long-slender bloodstream form of *Trypanosoma brucei brucei*. Abbreviations: DHAP, dihydroxyacetone phosphate; 1,3-DPGA, 1,3-diphosphoglyceric acid; FDP, fructose-1,6-diphosphate; G-6-P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; G-3-P, glycerol-3-phosphate; 3-PGA, 3-phosphoglyceric acid; SHAM, salicylhydroxamic acid.

1.2.2.7 Nutrition

In the mammalian bloodstream, *T.b.brucei* circulates in an extremely rich environment, capable of supplying all its metabolic needs. Glucose, its primary energy source, is present at high concentrations (5 mM in plasma), and is acquired by facilitated diffusion into the cell (Fairlamb & Opperdoes, 1986). A series of transporters exist for the amino acids (Voorheis, 1971), and fatty acids are imported into the cell because they cannot be synthesized *de novo* (Gilbert *et al.*, 1983). *T.b.brucei* is, however, capable of chain elongation of fatty acids, for which a ready supply of acetyl-CoA is available from the oxidation of threonine. In plentiful supply in the blood, threonine is transported into the trypanosome through the N_1-amino acid transporter, and broken down by threonine dehydrogenase and acetyl-CoA : glycine acetyltransferase to acetyl-CoA (solely available for lipid biosynthesis) and glycine (exported into the medium) in the mitochondrion (Linstead *et al.*, 1977; Gilbert *et al.*, 1983).

Macromolecular material is taken up by receptor-mediated endocytosis in the flagellar pocket (Opperdoes *et al.*, 1987). Receptors for transferrin and host low density lipoprotein (LDL) have been identified (Opperdoes *et al.*, 1987; Coppens *et al.*, 1988). LDL is of particular importance in providing cholesterol, the major membrane sterol, to bloodstream forms of *T.b.brucei.*

Endocytosis ceases in the more self-sufficient procyclic form (Vickerman, 1985). Energy metabolism switches from a carbohydrate to an amino acid base, with proline replacing glucose as the major energy source. Proline is present in high concentrations in the Tsetse fly haemolymph as a fuel for flight (Bursell, 1978). Within the trypanosome, it is oxidised with ring opening to glutamate followed by transamination to 2-oxoglutarate, which enters the citric acid cycle (Bowman & Flynn, 1976). Threonine metabolism is an order of magnitude more active than in the bloodstream form, but procyclics are also only capable of chain elongation of fatty acids (Klein, 1981).

1.2.3 Carbohydrate metabolism

1.2.3.1 Long-slender bloodstream forms

Glucose is the major respiratory substrate for the long-slender (LS) bloodstream form of *T.b.brucei.* It is quantitatively converted to pyruvate by a standard glycolytic pathway, the majority of which is located in the glycosomes of the parasite (Fig. 1.2; see section 1.2.2.1; Fairlamb & Opperdoes, 1986). The action of the glycerophosphate shunt allows the production of 2 moles of 3-phosphoglyceric acid from 1 mole of glucose under aerobic conditions. Net ATP and NADH production is zero within the glycosome, the glycolytic energy yield of 2 moles of ATP coming from the action of pyruvate kinase in the cytosol. Pyruvate is excreted into the surrounding medium because the pyruvate dehydrogenase

complex and the majority of the citric acid cycle enzymes are absent from LS forms (Fairlamb & Opperdoes, 1986). The rate-limiting step of glycolysis is thought to be the transport of glucose across the plasma membrane of the trypanosome (Eisenthal *et al.*, 1989). The oxidative branch of the pentose phosphate pathway is present in both this and the procyclic form of *T.b.brucei* (Hunt *et al.*, 1986).

Under anaerobic conditions, or in the presence of glycerophosphate oxidase inhibitors such as SHAM, glucose is converted to equimolar amounts of pyruvate and glycerol (Fairlamb & Opperdoes, 1986).

1.2.3.2 Short-stumpy bloodstream forms

The major end-product of glucose metabolism in short-stumpy (SS) forms is pyruvate, but CO_2, acetate and succinate are also formed (Flynn & Bowman, 1973). This is because, whilst the glycolytic metabolism of the LS form is still predominant, the mitochondrion of the cell has begun activation. Most CO_2 is produced from the decarboxylation of pyruvate by the pyruvate dehydrogenase complex (PDHC). Cells are also able to respire on 2-oxoglutarate (Vickerman, 1965), but the citric acid cycle is still thought to be inoperative due to the low levels of succinate oxidase and citrate synthase (Flynn & Bowman, 1973).

1.2.2.3 Procyclic forms

The state of carbohydrate metabolism in procyclic *T.b.brucei* is less well understood than in the bloodstream form. Therefore parts of the metabolic scheme shown in Fig 1.3 are constructed with data from *T.b.rhodesiense*. This trypanosome is closely related to *T.b.brucei*, but does have some subtle differences in the contribution of different pathways to its glucose catabolism (Bowman & Flynn, 1976; Fairlamb & Opperdoes, 1986).

With a reduction in the oxygen tension of the surrounding medium and a change from a glucose- to proline-based metabolism, the glycolytic pathway is less important to the procyclic cell (Fairlamb & Opperdoes, 1986). The specific activities of glycolytic enzymes are 30-70 % of their respective levels in the bloodstream form, except hexokinase, which is reduced 25-fold (Opperdoes *et al.*, 1981).

Despite the reduced activity of the glycerophosphate shunt, there remain no net changes in ATP/ADP or NAD^+/NADH ratios in the glycosome. To maintain this balance, 1,3-diphosphoglycerate leaving the glycosome must re-enter as phophoenolpyruvate (PEP). To this end, phosphoglycerate kinase is expressed in the cytosol (it is in the glycosome of the bloodstream form) and pyruvate kinase (which, in the bloodstream form, catalyses the conversion of PEP to pyruvate) is absent (Fig. 1.3; Fairlamb & Opperdoes, 1986). There are large increases in PEP carboxykinase and malate dehydrogenase in the glycosome. PEP carboxykinase enables CO_2 fixation, as well as maintaining the ATP balance. Malate

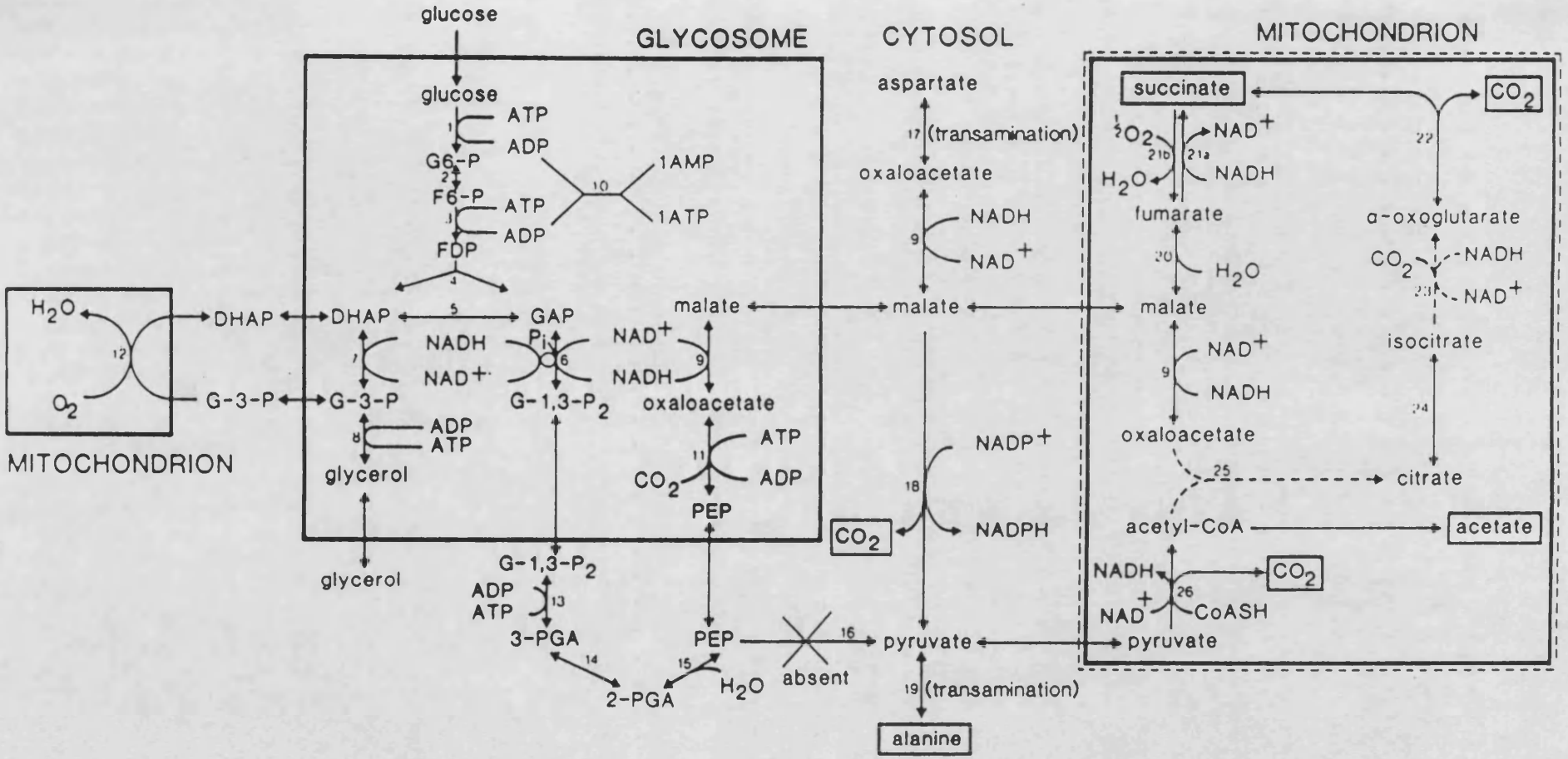


Fig. 1.3. Pathways of glucose metabolism in procyclic trypomastigotes of *Trypanosoma brucei brucei*. End products of aerobic or anaerobic metabolism are enclosed in boxes. The dashed lines indicate enzymes whose activity has not been proved *in vivo*. Enzymes: 1, Hexokinase; 2, phosphoglucose isomerase; 3, phosphofructokinase; 4, aldolase; 5, triose phosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, glycerol-3-phosphate dehydrogenase; 8, glycerol kinase; 9, malate dehydrogenase; 10, adenylate cyclase; 11, phosphoenolpyruvate carboxykinase; 12, glycerol-3-phosphate oxidase; 13, phosphoglycerate kinase; 14, phosphoglycerate mutase; 15, enolase; 16, pyruvate kinase; 17, aspartate aminotransferase; 18, malic enzyme; 19, alanine aminotransferase; 20, fumarate hydratase; 21a, fumarate reductase; 21b, succinate dehydrogenase; 22, 2-oxoglutarate dehydrogenase complex; 23, isocitrate dehydrogenase; 24, aconitase; 25, citrate synthase; 26, pyruvate dehydrogenase complex (from Fairlamb & Opperdoes, 1986).

dehydrogenase reduces oxaloacetate, making NADH available for glycerol-3-phosphate dehydrogenase, and producing malate, the final product of glycosomal metabolism (Opperdoes *et al.*, 1981; Fairlamb & Opperdoes, 1986).

Malate from the glycosome has a number of possible metabolic routes. In the cytosol, it can be converted by malate dehydrogenase to oxaloacetate, followed by transamination to aspartate, or, through the malic enzyme, it can be oxidised to pyruvate. A proportion of this pyruvate is transaminated to alanine, the major free amino acid in procyclics, but the majority is converted to acetyl-CoA by the PDHC (Opperdoes *et al.*, 1981; Fairlamb & Opperdoes, 1986). Malate can also enter the mitochondrion. In this way it acts as a shunt for reducing equivalents from the other two metabolic compartments. In the mitochondrion, malate can be oxidised to oxaloacetate or reduced to fumarate depending on the metabolic requirements of the organelle. With the proposed absence of the NADH-Ubiquinone segment of the respiratory chain, it has been suggested that the majority of the reducing equivalents from NADH are fed into the respiratory chain by the action of the fumarate reductase and succinate dehydrogenase (Turrens, 1989). Of the other enzymes of the citric acid cycle, aconitase and the 2-oxoglutarate dehydrogenase complex (2OGDHC) have been identified (Ryley, 1962), but an NAD^+-dependent isocitrate dehydrogenase (NAD-IDH) has never been detected (Ryley, 1962; Evans & Brown, 1972), and there remains only one report of citrate synthase activity, which was at a very low level (Jenkins *et al.*, 1988). Another indicator of an inoperative citric acid cycle has been the inability to detect citrate as a metabolic intermediate in both *T.b.brucei* and *T.b.rhodesiense* (Evans & Brown, 1972; Fairlamb & Opperdoes, 1986). With 3-10 % of glucose carbon being converted to acetate through an active deacylase (Fairlamb & Opperdoes, 1986), and glycosomal malate and proline-derived 2-oxoglutarate entering midway through the citric acid cycle to be converted to CO_2 and succinate (Ryley, 1962), it is perhaps not surprising that the cell does not require a full citric acid cycle.

This incomplete oxidation of glucose appears to be a feature of most parasitic protozoa (see Marr, 1980). In *Leishmania*, pyruvate is mostly converted to succinate, with the parasite containing only low levels of PDHC and pyruvate carboxylase. Citrate synthase activity is very low, NAD-IDH is absent, and 2OGDHC and succinate dehydrogenase levels are low (Marr, 1980). Therefore, this cell has many similarities to procyclic *T.b.brucei*. *T.cruzi* epimastigotes, which have an amino acid-based metabolism, also have low citrate synthase activity and no NAD-IDH, and, in addition, they lack the 2OGDHC (Adroher *et al.*, 1988).

1.3 Biochemical research into trypanosomiasis

From a biochemical point of view, it is clear that the trypanosome is a fascinating organism, with some features which are unique, and others which are very different from those of other eukaryotic cells. The parasite also represents a significant health hazard in Africa and South America. These two motivating factors have led to a considerable amount of research in recent years. From a chemotherapeutic point of view, much of this research has been aimed at identifying and characterising specific biochemical differences between trypanosomal cells and those of its mammalian host. Previous work from our laboratory has identified one such peculiarity, namely the enzyme dihydrolipoamide dehydrogenase (Danson *et al.*, 1987). The investigation of the cellular location(s) and characteristics of this enzyme is the subject of this thesis, and our current knowledge of dihydrolipoamide dehydrogenase will now be discussed.

1.4 Dihydrolipoamide dehydrogenase

1.4.1 Catalytic function

Dihydrolipoamide dehydrogenase (DHlipDH) catalyses the NAD^+-dependent oxidation of dihydrolipoamide :

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

Catalysis proceeds via alternate oxidation and reduction of the enzyme's active-site dithiol, as shown in Fig. 1.4. The first step is the oxidation of dihydrolipoamide to lipoamide. Two electrons are removed, and shared between the active-site dithiolate anion and a molecule of FAD in a charge-transfer complex. NAD^+ accepts the electrons from this complex, and, in being reduced to NADH, re-oxidises the enzyme in preparation for another catalytic cycle (for Reviews see Guest, 1978).

1.4.2 Role in multienzyme complexes

DHlipDH performs its catalytic role as part of the pyruvate dehydrogenase complex (PDHC), 2-oxoglutarate dehydrogenase complex (2OGDHC), branched chain 2-oxo acid 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. Each complex is situated at a key position in carbon and energy metabolism (see Fig. 1.5), and they are of particular importance because the PDHC, 2OGDHC and BCKDHC

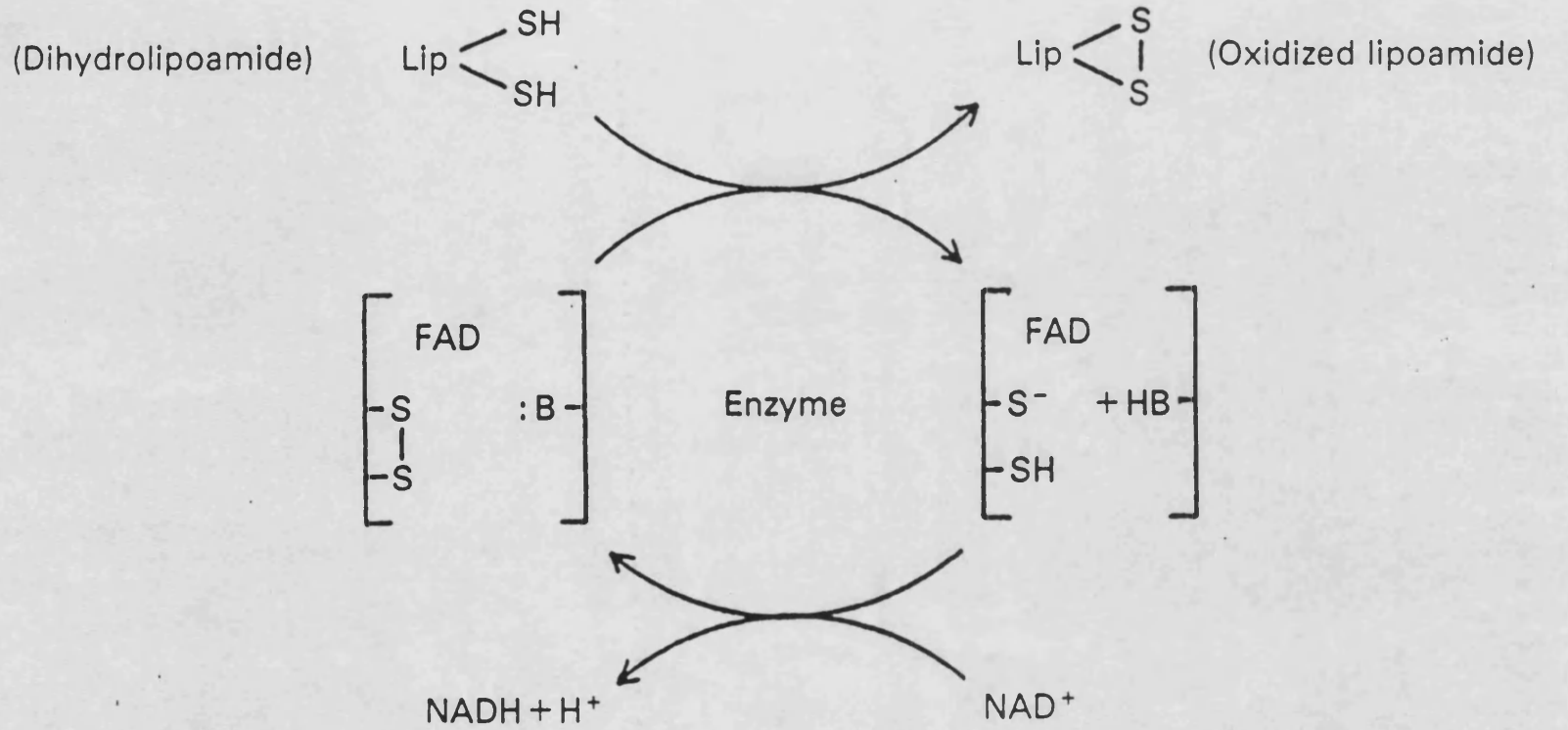


Fig. 1.4. NAD^+-dependent oxidation of dihydrolipoamide catalysed by dihydrolipoamide dehydrogenase. Catalysis proceeds via alternate oxidation and reduction of an intrachain disulphide bond and a base (B) on the enzyme. In the reduced enzyme, the electrons are shared between the active-site dithiolate anion and a molecule of FAD (from Danson, 1988).

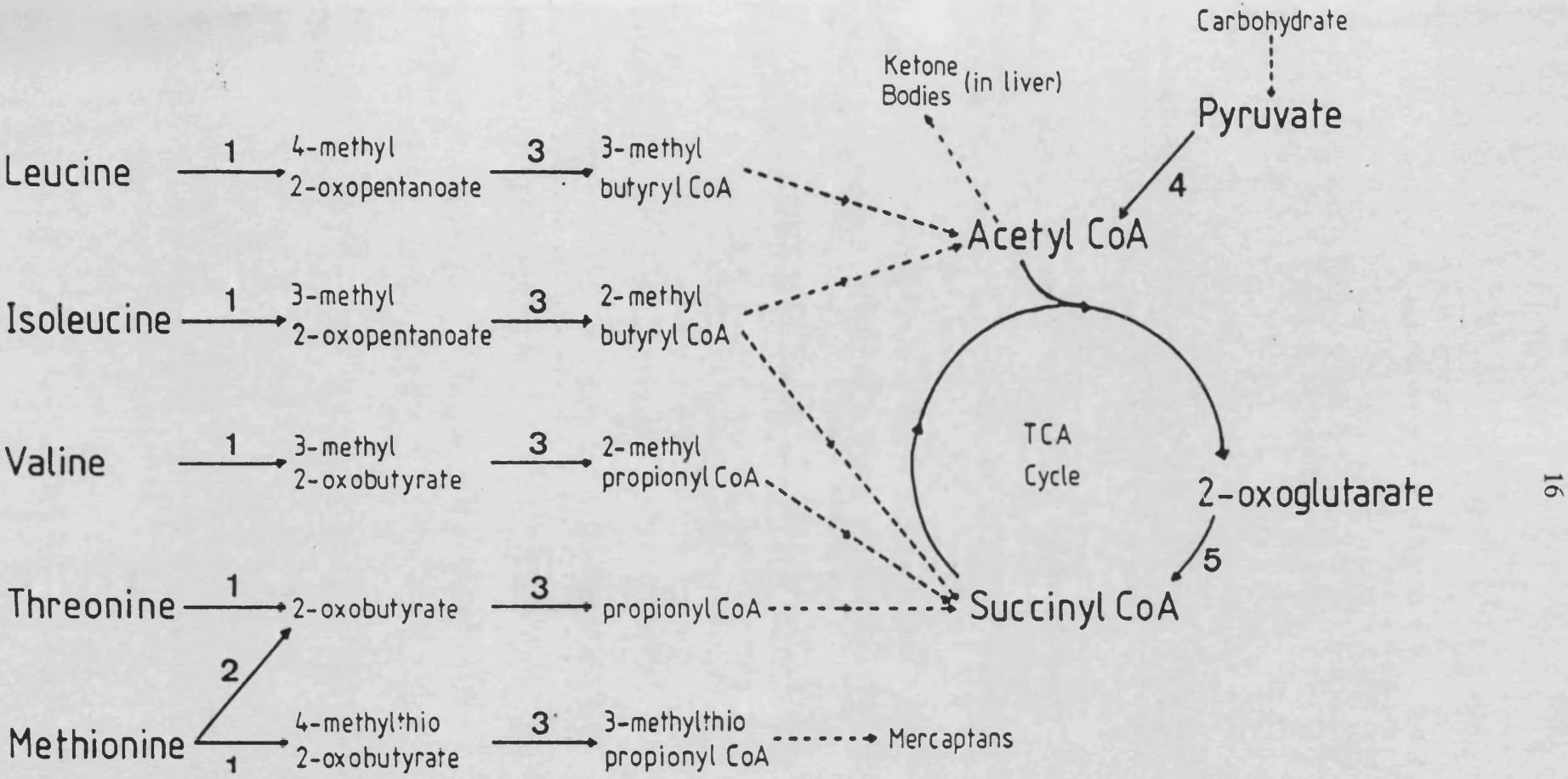


Fig. 1.5. Outline of the metabolic role of the 2-oxo acid dehydrogenase complexes. 1, transamination; 2, trans-sulphuration; 3, branched-chain 2-oxo acid dehydrogenase complex; 4, pyruvate dehydrogenase complex; 5, 2-oxoglutarate dehydrogenase complex. The broken lines indicate that several reaction steps are involved (from Yeaman, 1986).

catalyse irreversible catabolic steps. Molecular studies on these complexes have been recently reviewed in detail (Perham, 1991).

1.4.2.1 Pyruvate dehydrogenase complex

The pyruvate dehydrogenase complex (PDHC) is the most studied of the 2-oxo acid dehydrogenase complexes (see Patel & Roche, 1990 for Review). It catalyses the following enzyme reaction :

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

The PDHC is a multimer [M_r (3-10) x 10^6] the size of a ribosome, and has a pivotal role in the utilisation of pyruvate by most cells, producing acetyl-CoA for energy or biosynthesis. As can be seen from Fig. 1.6, it consists of 3 catalytic components, denoted E1, E2 and E3, which have similar properties in all species :

E1 (pyruvate decarboxylase) catalyses the thiamin pyrophosphate-dependent decarboxylation of pyruvate to a hydroxyethyl group followed by reductive acetylation of the lipoamide coenzyme of E2. In eukaryotes and Gram positive eubacteria, the enzyme consists of 2 subunits forming an $\alpha_2\beta_2$ oligomer, whereas in Gram negative bacteria there is a single E1 chain type forming a homodimer.

E2 (dihydrolipoyl transacetylase) possesses a lipoamide coenzyme bound to a lysyl ε-amino group at the end of a 1.4 nm swinging arm. This arm is thought to be able to rotate among the catalytic centres of the 3 component enzymes (Guest, 1978; Perham, 1991). As a transacetylase, E2 hands the acetyl group, derived from pyruvate, to CoA. In doing this, it maintains the energy-rich thioester bond, produced upon decarboxylation of the pyruvate, in the acetyl-CoA product.

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

E2 forms the structural core of the complex, with the mammalian PDHC possessing 60 copies arranged in icosahedral symmetry (Patel & Roche, 1990). To this core are bound 20-30 oligomers of E1 and 6 molecules of E3. E3 dimers are arranged in identical non-interacting sites, with each one extending across 2 of the 12 faces of the transacetylase core. In addition, the mammalian complex has 6 copies of a lipoyl-bearing component, Protein X, the role of which is not precisely known, although it appears to act as a high affinity binding site for E3 and may have a critical role in the transmission of reducing equivalents to the E3 component (Patel & Roche, 1990). This protein is unique to the mammalian PDHC.

The mammalian PDHC is regulated by phosphorylation, there being specific E1-kinases and E1-phosphatases which are bound to the complex, and phosphorylate and dephosphorylate, respectively, the E1α subunit (Yeaman, 1986 & 1989). The kinase is more

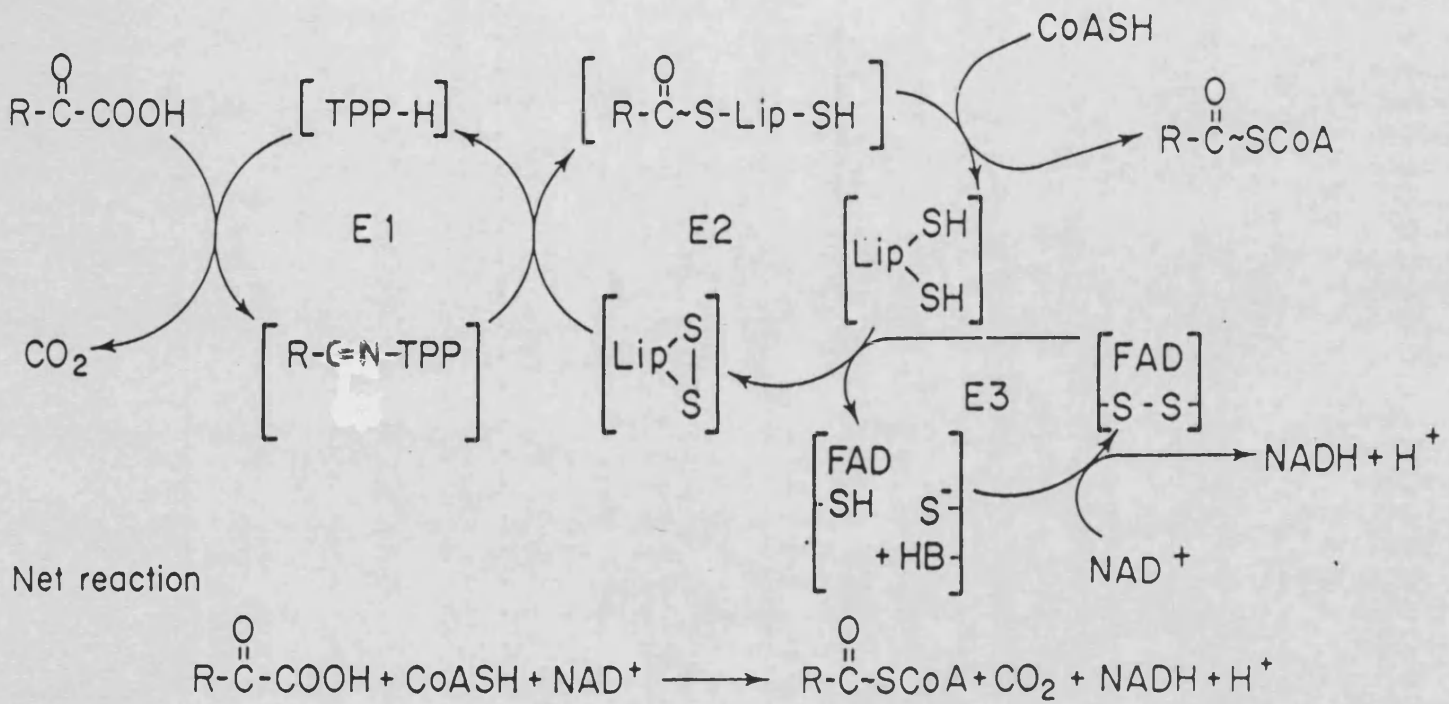


Fig. 1.6. Schematic representation of the reactions catalysed by the E1, decarboxylase, E2, dihydrolipoyl transacylase, and E3, dihydrolipoamide dehydrogenase components of the 2-oxo acid dehydrogenase complexes. R = CH_3 for the pyruvate dehydrogenase complex; R = CH_2CH_2COOH for the 2-oxoglutarate dehydrogenase complex; R = $(CH_3)_2CH$, $(CH_3)_2CHCH_2$ or $(CH_3)(C_2H_5)CH$ for the branched-chain 2-oxo acid dehydrogenase complex. Abbreviations: Lip, lipoic acid; TPP, thiamin pyrophosphate.

tightly bound than the phosphatase, which can be lost during purification (Guest, 1978). Three serine residues are available for phosphorylation, but 60-70 % inhibition occurs with phosphorylation of just one residue (Patel & Roche, 1990).

Neither protein X nor phosphorylation are features of the bacterial PDHC, and their presence in other eukaryotic systems such as yeast has not been proved. In Gram-negative bacteria the PDHC possesses octahedral symmetry, with 24 copies of E2 forming its core (Yeaman, 1989).

1.4.2.2 2-Oxoglutarate dehydrogenase complex

The 2-oxoglutarate dehydrogenase complex (2OGDHC) is of the same basic structure as the PDHC, but has an E2 octahedral core in all species, and is not regulated by phosphorylation. One further difference is that the E1 component is a homodimer as opposed to a hetero-oligomer. 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.

1.4.2.3 Branched-chain 2-oxo acid dehydrogenase complex

The BCKDHC catalyses the oxidative decarboxylation of branched-chain amino acids including ketoleucine, ketoisoleucine and ketovaline (Patston *et al.*, 1988). The process is thiamin pyrophosphate- and Mg^{2+}-dependent, and can be summarised by the following general reaction mechanism :

$$R\text{-}CO\text{-}COOH + CoA + NAD^+ = R\text{-}CO\text{-}CoA + CO_2 + NADH + H^+$$

The eukaryotic BCKDHC is similar in structure to the PDHC, having an E1 component that is $\alpha_2\beta_2$, and being regulated by phosphorylation (Patston *et al.*, 1988). 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 (Fig. 1.5; see Yeaman, 1986). In oxidising the branched chain amino acids, it is able to prevent their otherwise toxic effects on the cell (Harris *et al.*, 1986). The complex is found in eukaryotic systems and in those bacteria that utilise branched chain amino acids (e.g. *Pseudomonas putida*; Burns *et al.*, 1989a).

1.4.2.4 Glycine cleavage system

The glycine cleavage system (GCS) consists of four protein components: P-protein (pyridoxal-phosphate-containing glycine decarboxylase), H-protein (lipoyl-bearing aminomethyltransferase), T-protein (N^5, N^{10} methylene tetrahydrofolate synthetase) and L-

protein (DHlipDH) (Kikuchi & Hiraga, 1982; Olson *et al.*, 1986). It catalyses the pyridoxal phosphate-dependent decarboxylation of glycine :

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

This complex is also capable of glycine synthesis from methylene-tetrahydrofolate, ammonia and CO_2 in the reverse of the above reaction (see Kochi & Kikuchi, 1974). Therefore, in contrast to the 2-oxo acid dehydrogenase complexes, it catalyses a reversible reaction.

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

1.4.2.5 Other multienzyme complexes

In addition to the complexes mentioned above, DHlipDH has also been found as a component of the complex catalysing the NAD^+-dependent conversion of lactate to pyruvate in *Butyribacterium rettgeri* (Wittenberger & Haaf, 1964), and in the acetoin dehydrogenase enzyme system of *Pelobacter carbinolicus* (Oppermann *et al.*, 1991).

1.4.3 Diversity of dihydrolipoamide dehydrogenase

DHlipDHs have been isolated from a number of sources. They are all homodimers containing 1 mol of FAD and two redox-active cysteine residues per subunit. The sizes of individual subunits vary in the range 49,000 to 58,000 Daltons (see Dietrichs & Andreesen, 1990). DHlipDHs from different species are highly homologous, with human DHlipDH displaying 44 % identity to the *E.coli* enzyme (Pons *et al.*, 1988), and the human and pig enzymes being 96 % identical (Otulakowski & Robinson, 1987).

It is now accepted that eukaryotic PDHC, 2OGDHC and BCKDHC all contain the same E3 component (i.e. the product of one gene), but recent immunological observations have suggested that a different DHlipDH is involved in the GCS (Carothers *et al.*, 1987). On the other hand, some prokaryotes possess several different DHlipDHs that are thought to function in different multienzyme complexes. In this respect, three DHlipDHs have been characterised in *Pseudomonas putida* (Burns *et al.*, 1989a & b). One is part of the 2OGDHC and probably also the PDHC, the second is part of the BCKDHC, and the function of the third is unknown.

E.coli is thought to have one gene for DHlipDH, although a second protein has been identified, which is not involved in any of the complexes (Richarme, 1989). In this organism, DHlipDH has been implicated in both ubiquinone-mediated transport of proline (Owen *et al.*,

1980) and binding protein-dependent transport of maltose, ribose and galactose (Richarme, 1988). In other organisms, DHlipDH may be involved in the link between the mitochondrial ATPase and the respiratory chain (Partis *et al.*, 1977). These other possible roles for DHlipDH are discussed in more detail in chapter 8.

A crystal structure has now been solved for the DHlipDH from *Azotobacter vinelandii* (Schierbeek *et al.*, 1989), and this has now been refined to 2.2 Å (Mattevi *et al.*, 1991).

1.4.4 Other reactions of dihydrolipoamide dehydrogenase

In addition to performing its normal function as an NAD^+-dependent dehydrogenase, DHlipDH is also capable of mediating other NADH-linked reactions (see Tsai, 1980; Carothers *et al.*, 1989), including: (a) hydrogen transfer to nicotinamide nucleotides. This tranhydrogenase activity may effect hydride ion translocation across the mitochondrial inner membrane in certain organisms (Komuniecki & Saz, 1979) (b) electron transfer to inorganic acceptors, and (c) reduction of quinone dyes such as nitro-blue tetrazolium (the basis of diaphorase activity, a common histochemical test for mitochondria [Vickerman, 1965]).

1.4.5 The disulphide oxidoreductases

DHlipDH belongs to the family of enzymes, the flavin-containing pyridine nucleotide disulphide oxidoreductases (see Carothers *et al.*, 1989 for review), which includes thioredoxin reductase (Gleason & Holmgren, 1988), dihydroasparagusate dehydrogenase (Yanagawa & Egami, 1976), glutathione reductase (Pigiet & Conley, 1977), trypanothione reductase (Shames *et al.*, 1986), bis-γ-glutamylcystine reductase (Sundquist & Fahey, 1988), pantethine 4',4''-diphosphate reductase (Swerdlow & Setlow, 1983) and mercuric reductase (Fox & Walsh, 1982). These enzymes show considerable homology around their common active site dithiols, FAD binding sites, NAD(P) binding sites and subunit interfaces (Carothers *et al.*, 1989). In illustration of this fact, human and pig DHlipDHs show 30 and 28 % identity to glutathione reductase and mercuric reductase, respectively (Otulakowski & Robinson, 1987).

1.5 DHlipDH in *T.b.brucei*

With the bloodstream form of *T.b.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. They added further intrigue to their discovery by finding that this DHlipDH co-purified with *T.b.brucei* plasma membranes.

It has been the goal of this project to continue on from the work of Danson *et al.* (1987) by

a) providing further information about the subcellular location of DHlipDH in bloodstream forms

b) looking at the procyclic form, in which a more active mitochondrion might result in altered levels and locations of DHlipDH and the 2-oxo acid dehydrogenase complexes

c) searching in the bloodstream and procyclic forms for lipoic acid, the presumed substrate of DHlipDH, as a first step towards elucidating the function of the enzyme *in vivo*

d) analysing the properties of the bloodstream form DHlipDH to see if there are any differences from other DHlipDHs, and purifying and N-terminal sequencing the enzyme to enable a gene cloning approach to structural and functional studies

e) investigating the inactivation of the bloodstream form DHlipDH by arsenicals in order to elucidate the role of this enzyme in the toxicity of arsenicals towards trypanosomes, and possibly to develop these compounds for *in vivo* functional studies on DHlipDH

These five areas of work are described in chapters 3 to 7 (a to e respectively).

CHAPTER 2 : MATERIALS AND GENERAL METHODS

2.1 Materials

2.1.1 Chemicals and enzymes

Chemicals used were of analytical grade or the finest grade commercially available. NAD^+, 3-acetyl-NAD^+ and NADH were from Boehringer Mannheim, Lewes, E.Sussex. High purity Triton X-100 was from Pierce and Warriner (U.K.), Chester. D,L-Lipoamide, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and Triton X-114 were obtained from Sigma Chemical Co., Poole, Dorset. Of the components of the SDM-79 medium (see table 2.1), Dulbecco's modified Eagle medium, Medium 199, MEM amino acid solution, MEM non-essential amino acids, Penicillin/Streptomycin and Gentamycin were from Gibco, Uxbridge, Middlesex. The remainder were purchased from Sigma. Protein assay reagent was purchased from Bio-Rad Laboratories, Hemel Hempstead, Herts. The arsenical phenylarsenoxide was from Sigma or Aldrich Chemical Co., Poole, Dorset, and Melarsen oxide was provided by Specia, Paris, France. *p*-Aminophenyldichloroarsine and *p*-acetylaminophenyldichloroarsine were kind gifts from Prof. K. J. Stevenson (University of Calgary, Canada); these had been synthesized as described by Stevenson *et al.* (1978). DE-52 (DEAE-cellulose) was purchased from Whatman, Maidstone, Kent. Vitamin assay casamino acids and LB medium were from Difco Laboratories, West Moseley, Surrey. SDS-PAGE low molecular weight markers and 8-25 % gradient gels were obtained from Pharmacia LKB Biotechnology, Milton Keynes. Of the markers used for gel filtration, 2,4-dinitrophenyl-L-lysine, Dextran Blue, bovine liver catalase and bacteriorhodopsin were from Sigma, and pig heart malate dehydrogenase, citrate synthase and lactate dehydrogenase were obtained from Boehringer. Acetyl-CoA was produced by the method of Stadtman (1957) from CoA purchased from Boehringer. Unheparinised microhaematocrit tubes, for storing trypanosome stabilates, were purchased from Bilbate Ltd., Daventry, England.

2.1.2 Organisms

Monomorphic long-slender bloodstream forms of *Trypanosoma brucei brucei* strain EATRO 427 were obtained from Dr H. P. Voorheis (Trinity College, Dublin, Eire). These cells had been rendered monomorphic by syringe passage in laboratory animals (see Vickerman, 1965). No short-stumpy forms were ever seen when cells were counted prior to use in experiments. Procyclic forms of EATRO 427 were originally supplied by Dr W. Gibson (Tsetse Research Centre, Langford, Bristol, U.K.). They were grown by Marjan Kasraeian (Dept. of Biochemistry, University of Bath). *Escherichia coli* strain TG1 (an EcoK derivative of JM101) was a gift from Dr A. Bankier (MRC Laboratory of Molecular

Table 2.1 SDM-79 Medium for the growth of procyclic forms of *T.b.brucei*

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	8.0 g
MOPS	5.0 g
$NaHCO_3$	2.0 g
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 (5U/ml)/Streptomycin (5 mg/ml)	12 ml
Gentamycin	20 mg
Hemin solution (2.5 mg/ml)	2 ml
Fetal calf serum	10 % (v/v)

Biology, Cambridge, U.K.). The lipoic acid-requiring mutant *E.coli* K12 (W1485 *lip*2) was a kind gift from Prof J. R. Guest (University of Sheffield, U.K.).

2.2 General Methods

2.2.1 Growth and harvesting of bloodstream forms of *Trypanosoma brucei brucei*

Stocks of *Trypanosoma brucei brucei* (strain EATRO 427) were stored in the form of stabilates in microhaematocrit tubes under liquid nitrogen. These stabilates were produced from whole rat blood containing trypanosomes, to which 10-15% (w/v) glycerol was added. Haematocrit tubes were filled with blood by capillary action and sealed at both ends over a Bunsen flame. The process of glycerol addition to the blood was carried out slowly at 4°C due to the inhibition of glycolysis in bloodstream forms of *T. brucei* by high concentrations of glycerol (Fairlamb & Opperdoes, 1986). This was also the reason for using less glycerol than is common for the storage of other cells.

For the inoculation of rats, stabilate tubes were thawed, opened at each end, and the blood expelled by passing through Krebs Ringer Buffer (KRB: 98 mM NaCl, 22 mM KH_2PO_4, pH 8.0, 1 mM $MgSO_4$, 2 mM KCl) containing 10 mM glucose and 3 % (w/v) sucrose (denoted KRBG). The number of live trypanosomes present was measured using a Neubauer haemocytometer. Male Wistar or Sprague-Dawley rats were inoculated intraperitoneally with 1.7-1.9 x 10^7 cells per rat (see Eisenthal & Panes, 1985). Approximately seventy-two hours post-infection, rats were exsanguinated from the heart or common ileac artery, and the blood was rapidly mixed with 1 ml heparin solution (200 U/ml in KRB). Infected blood was centrifuged at 1000 x g (4°C) for 10 min in an IEC-CENTRA-3R bench centrifuge. The serum supernatant was discarded and the upper white layer containing trypanosomes and white blood cells was resuspended in a minimum volume of KRBG. Inevitably, a few red blood cells were also resuspended by this process, so a further one or two washes of the cells (by centrifugation and resuspension as before) was carried out prior to loading the cell suspension onto a DE-52 (DEAE-cellulose) column pre-equilibrated with KRBG. The column had a bed volume of 10 ml, and one column was used for every 3-4 rats bled. Uncharged trypanosomal cells passed straight through the column, while negatively charged blood cells were adsorbed (Lanham & Godfrey, 1970). The trypanosomes were washed through with 30-40 ml KRBG.

2.2.2 Growth of other cells

Escherichia coli strain TG1 was grown in LB (Luria-Bertani) medium according to Maniatis *et al.* (1982).

Trypanosoma brucei brucei procyclic forms were grown at 26°C in SDM-79 medium (Table 2.1; Brun & Schonenberger, 1979). In preparing this medium, hemin and fetal calf

serum were sterilised separately from the other components. Procyclic cells were harvested by centrifugation at 600 x g and washed in phosphate-buffered saline.

2.2.3 Preparation of sonic extracts

Sonic extracts of either form of *T.b.brucei* or *E.coli* were prepared by sonicating cells in Eppendorf microfuge tubes for 3 x 30 s at 40 W using a 3 mm probe. Sonication tubes were incubated at 4°C to minimise heating effects, and each 30 s sonication was separated by a 30 s time period to allow maintenance of the temperature at 4°C.

2.2.4 Preparation of dihydrolipoamide

Dihydrolipoamide was synthesized from D,L-lipoamide by reduction with sodium borohydride (Reed *et al.*, 1958). 800 mg D,L-lipoamide were dissolved in 20 ml methanol : water (4:1) over ice. Sodium borohydride (800 mg) was added in 4 ml cold distilled water, and the mixture stirred over ice for 1 h. The solution was acidified with 1 M HCl until effervescence stopped. The product was extracted three times with 50 ml chloroform (bottom layer), transferred to a round-bottom flask, and rotary evaporated to dryness. The resulting crystals were dissolved in toluene (40 ml) with gentle warming. Hexane (16 ml) was added, and the precipitate collected by filtration under vacuum and dried in a desiccator.

The purity of the product (dihydrolipoamide) was assessed by titration with DTNB. Reaction with DTNB is described by the following equation :

Dihydrolipoamide + DTNB = Lipoamide + 2 TNBS

The reaction was carried out in 50 mM sodium phosphate buffer (pH 7.0), containing 0.2 mM DTNB and 0.05 mM dihydrolipoamide (assuming 100 % purity). E_m TNBS at 412 nm is 13,600 M^{-1} cm^{-1}, so the expected absorbance at 412 nm produced by a solution of 0.1 mM SH groups (0.05 mM dihydrolipoamide) reacting with DTNB is 1.36.

2.2.5 Protein determination

Protein determination was carried out by the method of Markwell *et al.* (1981). Staining solution was either purchased from Bio-Rad or made according to Bradford (1976). The Bradford method involved dissolving 100 mg Coomassie G-250 in 50 ml 95 % ethanol, adding 100 ml 85 % (w/v) orthophosphoric acid, and making up to 1 litre with distilled water. The reagent was filtered prior to use. The assay was carried out in 1 ml cuvettes, to which were added 100 µl sample/standard BSA (0-25 µg when using Bio-Rad solution, or 0-10 µg when using reagent produced by the Bradford method) and 900 µl Bio-Rad reagent (1 in 5 dilution of stock solution in distilled water) or Bradford reagent. Protein content was assessed by measuring A_{595} at 10 min to 1 h after mixing by inversion.

2.2.6 Enzyme assays

(a) Dihydrolipoamide dehydrogenase was assayed at 30°C in 50 mM potassium phosphate (pH 7.0)/2 mM EDTA containing 0.4 mM dihydrolipoamide and 1 mM NAD^+. The reaction, in a final volume of 1 ml, was started with enzyme and its progress was monitored by the increase in A_{340}.

(b) Malate dehydrogenase was assayed at 30°C in 50 mM potassium phosphate (pH 7.0)/ 2 mM EDTA containing 0.1 mM NADH and 0.2 mM oxaloacetate. The reaction, in a final volume of 1 ml, was started with enzyme and its progress monitored by the decrease in A_{340}.

(c) Lactate dehydrogenase was assayed by adding samples from fractions to a reaction mixture containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 mM pyruvate and 0.2 mM NADH. The reaction was monitored by following the decrease in A_{340} due to the oxidation of NADH.

(d) Control assays for the above dehydrogenases were carried out in the absence of substrates to ensure that no NADH oxidase activity was present.

The molar extinction coefficient for NADH at 340 nm is $6.22 \times 10^3\ M^{-1}cm^{-1}$. Therefore, a 1 mM solution of NADH will have an A_{340} of 6.22.

(e) Citrate synthase activity was detected by measuring the increase in absorbance at 412 nm due to the reaction of DTNB with the product CoA to liberate the yellow-coloured thionitrobenzoate. Samples were added to an assay mixture containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 mM oxaloacetate, 0.2 mM acetyl-CoA and 0.1 mM DTNB.

(f) Catalase was detected by adding hydrogen peroxide to eluted fractions. The generation of bubbles of oxygen indicated the presence of the enzyme.

2.2.7 PhastSystem SDS-PAGE

Samples for SDS-PAGE were mixed with sample buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2.5 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 0.01 % (w/v) bromophenol blue) and boiled for 3 min. These were loaded onto a sample applicator (8 wells; 1 µl per well) that was automatically lowered onto the gel surface during electrophoresis. The molecular weight markers used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie blue R and silver.

2.2.8 Coomassie staining

Stain : 0.1 % (w/v) Coomassie Blue R in 30 % (v/v) methanol / 10 % (v/v) acetic acid
Destain : 30 % (v/v) methanol / 10 % (v/v) acetic acid

Procedure :

1. Stain for 8 min @ 50°C
2. Destain for 5 min @ 50°C
3. Destain for 8 min @ 50°C
4. Destain for 10 min @ 50°C

2.2.9 Silver staining (Pharmacia PhastSystem Owners Manual, 1987)

Solutions

1 50 % (v/v) ethanol, 10 % (v/v) acetic acid (90 ml)
2 10 % (v/v) ethanol, 5 % (v/v) acetic acid (400 ml)
3 distilled H_2O (400 ml)
4 8.2 % (w/v) glutaraldehyde (90 ml)
5 0.25 % (w/v) silver nitrate (90 ml)
6 0.04 % (v/v) formaldehyde in 2.5 % (w/v) Na_2CO_3 (180 ml)
7 5 % (v/v) acetic acid (90 ml)
8 10 % (v/v) acetic acid, 5 % (v/v) glycerol (90 ml)

Procedure

Step	Solution	Time (min)	Temp. (°C)
1	1	2	50
2	2	2	50
3	2	4	50
4	4	6	50
5	2	3	50
6	2	5	50
7	3	2	50
8	3	2	50
9	5	13	40
10	3	0.5	30
11	3	0.5	30
12	6	0.5	30
13	6	4	30
14	7	2	50
15	8	3	50

CHAPTER 3 : SUBCELLULAR LOCALISATION OF DIHYDROLIPOAMIDE DEHYDROGENASE

3.1 Introduction

The dihydrolipoamide dehydrogenase of the bloodstream form of *Trypanosoma brucei 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. In this method, live trypanosomes were swollen to turgidity and homogenised with a tight-fitting Dounce homogeniser. Following treatment with leupeptin and DNAase, the homogenate was subjected to sucrose-density-gradient centrifugation (Voorheis *et al.*, 1979). A prominent dense band was extracted from the gradient, which contained plasma membranes as evidenced by their characteristic subpellicular microtubular array, identified by electron microscopy. Enzymatic analysis of these membrane fragments 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). The recovery of DHlipDH in these membranes was much higher than those of the two membrane markers adenylate cyclase (47 %) and the ouabain ATPase (58 %), measured by Voorheis *et al.*(1979).

Whilst this membrane purification provided evidence of a plasma membrane location for the DHlipDH of bloodstream form *T.b.brucei*, we felt it necessary to prove conclusively whether or not the enzyme was present only in this location within the cell. This was important because the possibility of contamination of the plasma membrane preparation with other cellular membranes was not ruled out by Danson *et al.* (1987), who used malate dehydrogenase, which is 90 % cytosolic and 10 % mitochondrial in bloodstream trypanosomes (Opperdoes *et al.*, 1981) as an indicator of contamination from other organelles. With residual amounts of other citric acid cycle enzymes having been detected in the bloodstream form promitochondrion by Jenkins *et al.* (1988) and Durieux *et al.* (1991), DHlipDH could also be mitochondrial, and its recovery with plasma membranes could be a result of contamination or adventitious adherence. An additional source of concern about the Voorheis method (Voorheis *et al.*, 1979) is the low recovery of marker enzymes, all were less than 60 % of their initial levels, and glycolytic marker enzyme contamination (Conroy, 1988), in the purified plasma membranes. Either a considerable proportion of the membranes have been lost, or the enzymes may have become detached during purification. Alternatively, these enzymes may not be good markers for the plasma membrane.

A number of other methods have been used to purify plasma membranes from Trypansomatids. Rovis and Baekkeskov (1980) disrupted *T.b.brucei* cells by nitrogen cavitation, and separated their components by differential centrifugation. They obtained

plasma membrane vesicles free of microtubules and organelles, and were able to maintain the intact nature of nuclei and mitochondria during their separation. Whilst mitochondrial contamination was negligible, the yield of plasma membranes, as assessed by enzyme recoveries, was very low (9.3 % of the ATPase and 7.6 % of adenylate cyclase). Hunt and Ellar (1974), who purified plasma membranes by blending a cell suspension of *Leptomonas collosoma* (a trypanosomatid) with glass beads, differential centrifugation and further separation on a sucrose gradient, had problems with contamination from other cellular compartments.

The only method to deal adequately with problems of recovery and contamination would be one which did not require full separation of cellular organelles. Subcellular fractionation partially separates organelles on a linear sucrose gradient, and the location of a particular enzyme can be investigated by comparing its distribution across the gradient with those of organelle markers (Beaufay and Amar-Costesec, 1976). This method should unambiguously determine the cellular location(s) of DHlipDH in the bloodstream form of *T.b.brucei*. It is also able to distinguish between the plasma membrane and flagellar pocket membrane of the trypanosome.

In addition to determining the cellular location of DHlipDH within the trypanosome by subcellular fractionation, it is possible to ascertain whether it is located at the cytoplasmic or external face of the plasma membrane by measuring the latency of the enzyme, which is the amount of enzyme accessible to externally-applied substrates in whole cells. This has been carried out as described by Steiger *et al.* (1980).

The third method used in this chapter to investigate the DHlipDH of the bloodstream form *T.b.brucei* is Triton X-114 phase partitioning. This has been used extensively to determine the form of attachment of proteins to membranes. Triton X-114 is a nonionic detergent which forms clear micellar solutions in aqueous buffer below 20°C. Above this temperature (the cloud point), a microscopic phase separation occurs in solution, which proceeds with increasing temperature until two distinct phases are produced (Bordier, 1981).

This detergent phase partitioning was first applied to the analysis of proteins by Bordier (1981), who demonstrated the partitioning of human erythrocyte membrane acetylcholinesterase, bacteriorhodopsin and cytochrome c oxidase into the detergent phase upon raising the temperatures of their solutions from 0°C to 30°C. Serum albumin, catalase, ovalbumin, concanavalin A, myoglobin and cytochrome c all remained within the aqueous phase. Subsequently, Balber & Ho (1988) applied this technique to the Variable Surface Glycoprotein (VSG) of *T.b.brucei*, showing that its membrane form partitioned into the detergent phase, whilst the phospholipase C-cleaved soluble form remained in aqueous solution.

With the trypanosomal DHlipDH, three forms of cellular material were chosen for partitioning. The use of sonicated material would reveal any anchorage following cell

disruption. Plasma membranes (prepared according to Voorheis *et al.* [1979]) allow the analysis of only that DHlipDH which is attached to the plasma membrane, and whole cells give the nearest to *in vivo* conditions for the enzyme.

To confirm that DHlipDH is not participating in any of the 2-oxo acid dehydrogenase complexes in long-slender bloodstream forms, assays using pyruvate, 2-oxoglutarate and branched-chain 2-oxo acids as substrates have been carried out.

3.2 Methods

3.2.1 Subcellular fractionation

The subcellular fractionation of bloodstream form *T.b.brucei* was carried out by Mr J. Van Roy in the research group of Dr F. Opperdoes (International Institute for Cellular and Molecular Pathology, Brussels).

Trypanosomes were homogenized by grinding with silicon carbide grain until more than 90 % were broken (Opperdoes *et al.*, 1977a). Homogenization was carried out in two different buffering systems: (a) 0.25 M sucrose, 1 mM imidazole-HCl and 1 mM EGTA (pH 7.0) and (b) 0.25 M sucrose, 25 mM Tris-HCl and 1 mM EDTA (pH 7.2). Following homogenization, the silicon carbide was removed by centrifugation, and the supernatant was subjected to differential centrifugation. Unbroken cells, nuclei and debris were removed by centrifugation at 1000 x g for 10 min, and a large-granule fraction was separated at 5,000 x g for 10 min. The post-large-granule extract was applied to a linear sucrose gradient (density = 1.10-1.26 g/cm^3) using an E-40 automatic zonal rotor and accelerated to 40,000 rpm for 140 min according to the method of Beaufay and Amar-Costesec (1976; see also Steiger *et al.*, 1980). A series of fractions were removed from the gradient and assayed for marker enzymes, protein and DHlipDH.

The marker enzymes α-glucosidase (plasma membrane), acid phosphatase (flagellar pocket), 3'-nucleotidase (plasma membrane), isocitrate dehydrogenase (mitochondrion), α-mannosidase (lysosomes), hexokinase (glycosomes) and adenylate cyclase (flagellar pocket) were assayed by Mr J. Van Roy according to the methods of Gbenle *et al.* (1986) and Walter & Opperdoes (1982). Protein was determined as described by Stein *et al.* (1973). These assays were performed at the International Institute for Cellular and Molecular Pathology, Brussels. Individual fractions from the gradient were frozen in liquid nitrogen, stored frozen, and sent to Bath. The assay of DHlipDH was carried out in Bath as described in section 2.2.6.

3.2.2 Latency studies

Gbenle *et al.* (1986) measured the latencies of RNase, 3'-nucleotidase, aryl acylamidase and alanine aminotransferase by determining their activities in the presence and

absence of 0.1 % (w/v) Triton X-100. For this method, they supplemented the normal assay buffers with 0.25 M-sucrose, 5 mM-glucose and 0.05 % bovine serum albumin. For DHlipDH, the method of Gbenle *et al.* (1986) was adapted for live, respiring trypanosomes by using KRBG containing 1 mM NAD^+. For these studies, the known intracellular enzyme malate dehydrogenase (Voorheis *et al.*, 1979) was used as a positive control to determine whether the cells were intact. This enzyme was assayed according to the procedure detailed in section 2.2.6, replacing assay buffer with KRBG.

3.2.3 Triton X-114 phase partitioning

Triton X-114 phase partitioning was carried out according to the method of Bordier (1981). Bacteriorhodopsin and citrate synthase were chosen as membrane and soluble protein controls, respectively.

(a) **Preparation of samples.** To investigate the phase partitioning of DHlipDH, three different forms of *T.b.brucei* cellular material were examined: whole cells, sonicated cells (3 x 30 s @ 40 W), and plasma membranes prepared by the method of Voorheis *et al.* (1979) and stored frozen at -80°C.

Each sample was suspended, to a total volume of 800 µl, in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (partitioning buffer) at 0°C. Triton X-114 was added to a final concentration of 1 % (w/v), and the sample mixed by vortexing.

Bacteriorhodopsin was dissolved to a final concentration of 4 mg/ml in 25 mM potassium phosphate buffer, pH 6.88, containing 0.1 % (w/v) Triton X-100 according to the method of Dencher & Heyn (1978). A 10 µl sample was added to 190 µl partitioning buffer containing 1 % (w/v) Triton X-114.

Citrate synthase (2 mg/ml) was diluted 20-fold into partitioning buffer containing 1 % (w/v) Triton X-114.

(b) **Phase partitioning.** To carry out the phase separation, each sample was layered onto a 300 µl sucrose cushion (Tris buffer containing 6 % (w/v) sucrose and 0.06 % (w/v) Triton X-114) in a conical Eppendorf microfuge tube. Each tube was incubated at 30°C for 3 min, and centrifuged for 20 s in an MSE bench microfuge at 8,500 x g. The separation was then repeated by removing the aqueous phase, adding to it a further 0.5 % (w/v) Triton X-114, cooling, layering back onto the cushion, incubating and centrifuging as before. The aqueous phase was removed once more and 2 % (w/v) Triton X-114 added. Following incubation and centrifugation, the separated detergent was discarded.

Buffer and detergent were added to the detergent and aqueous phases, respectively, to give the same salt/surfactant contents. Protein and DHlipDH activities were determined as in sections 2.2.5 and 2.2.6, respectively. Bacteriorhodopsin and citrate synthase were detected by SDS-PAGE (section 2.2.7).

3.2.4 Determination of DHlipDH and 2-oxo acid dehydrogenase complex levels

The assay for DHlipDH is described in section 2.2.6. Determination of the 2-oxo acid dehydrogenase complexes was performed according to the methods described in sections 4.2.1 and 4.2.2.

3.3 Results

3.3.1 Subcellular fractionation

The distribution profiles for DHlipDH and the marker enzymes in post-large-granule extracts of *T.b.brucei* homogenates after isopycnic centrifugation in linear sucrose gradients are shown in Fig. 3.1.

DHlipDH shows three clear peaks of activity in the imidazole-containing gradient at 1.05 g/cm^3, 1.14 g/cm^3 and 1.20 g/cm^3. The two outer peaks coincide with those of the enzymes α-glucosidase and 3'-nucleotidase which have been shown by Steiger *et al.* (1980) and Gbenle *et al.* (1986) to be markers for the plasma membrane in *T.b.brucei*. Upon changing to the Tris-containing buffer, the peak at 1.20 g/cm^3 was replaced by one at 1.15 g/cm^3 both for DHlipDH and the two marker enzymes. This shift in density is due to the detachment of the subpellicular microtubular array from the plasma membrane, and provides an unambiguous confirmation of the location of the three enzymes at the plasma membrane (Opperdoes & Steiger, 1981).

The additional peak of DHlipDH activity at 1.14 g/cm^3 in sucrose/imidazole/EGTA buffer was not present in the profiles of either of the two plasma membrane markers. This peak is at the same density as those of acid phosphatase and adenylate cyclase, which are located in the flagellar pocket membrane (Walter & Opperdoes, 1982; Gbenle *et al.*, 1986).

3.3.2 Latency studies

The activity of malate dehydrogenase in 4.5 x 10^6 trypanosomes in the presence of 0.1 % (w/v) Triton X-100 was 6.2 (± 0.3) nmol NADH oxidised/min. In the absence of Triton, none of this activity could be detected. Malate dehydrogenase is a known intracellular enzyme in *T.b.brucei*, and the fact that no enzyme activity could be detected without the presence of detergent indicates that all the cells in the assay tube were intact.

DHlipDH activity in Triton-treated cells was 0.8 (± 0.2) nmol NADH produced/min. Without Triton, no enzyme activity could be detected, indicating that DHlipDH is probably located at the inner surface of the plasma membrane of bloodstream *T.b.brucei*, and hence is not accessible to externally supplied substrate in the whole organism.

3.3.3 Triton X-114 phase partitioning

The recoveries of DHlipDH and protein are displayed in table 3.1.

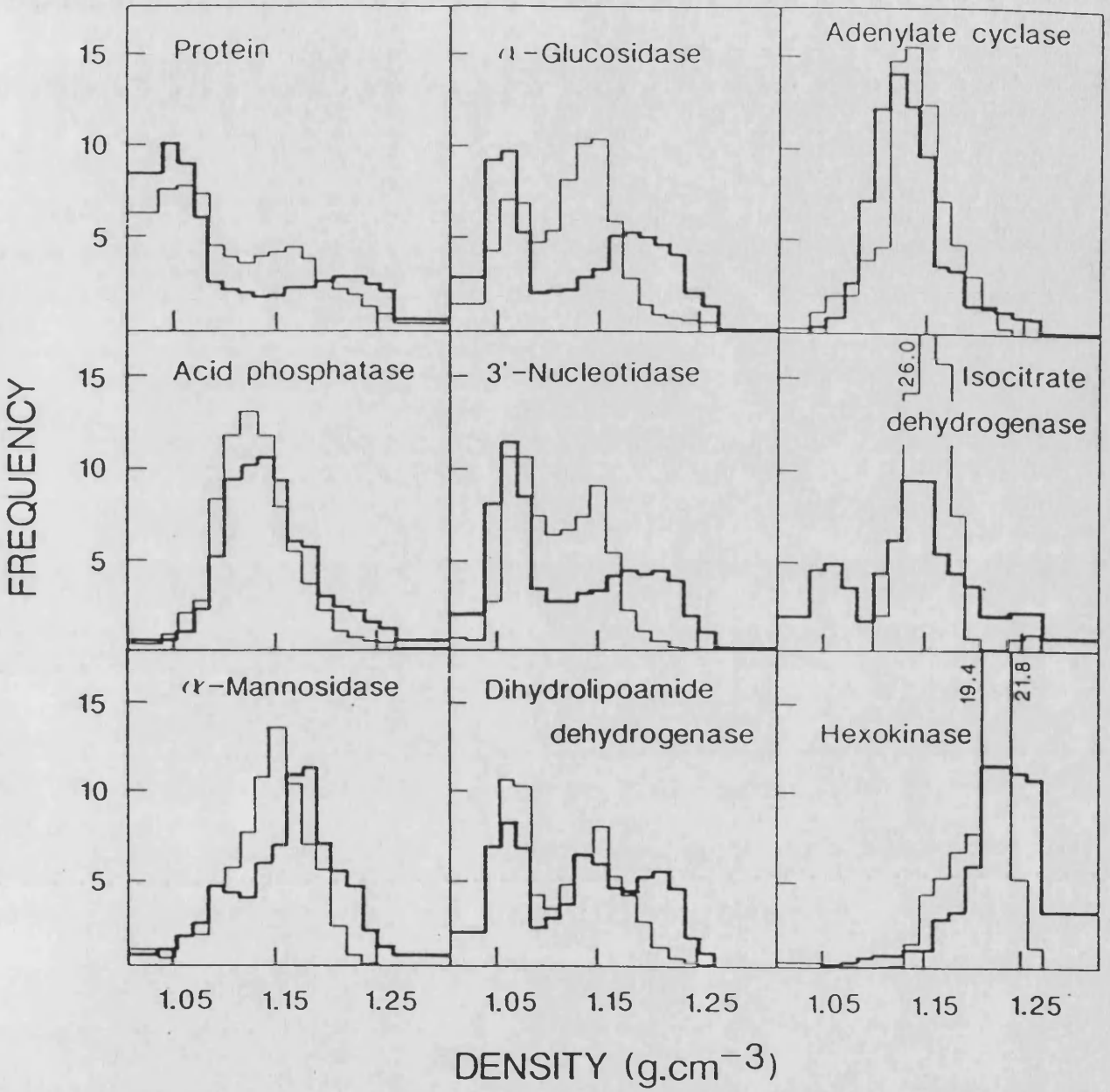


Fig. 3.1. Distribution profiles of marker enzymes and protein from post-large-granule extracts of bloodstream form *T.b.brucei* homogenates after isopycnic centrifugation in linear sucrose gradients. The marker enzymes present are : α-glucosidase and 3'-nucleotidase for plasma membrane; acid phosphatase and adenylate cyclase for flagellar pocket; α-mannosidase for lysosomes; isocitrate dehydrogenase for mitochondrion, and hexokinase for glycosomes. Thick lines represent the distribution in an imidazole-containing gradient, and thin lines that in a Tris-containing gradient. The recovery of marker enzymes and protein for both gradients was greater than 85 %; the recovery of dihydrolipoamide dehydrogenase was 95 %.

It was judged (from SDS-PAGE) that greater than 95 % of the bacteriorhodopsin partitioned solely into the detergent phase, and greater than 95 % of the citrate synthase was recovered in the aqueous phase following phase separation.

3.3.4 Determination of DHlipDH and 2-oxo acid dehydrogenase complex levels

The specific activity of DHlipDH in cell-free extracts of bloodstream form cells was 0.029 ± 0.003 U/mg protein. The pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched chain 2-oxo acid dehydrogenase complexes were undetectable in cell-free extracts. The validity of each assay was confirmed by the demonstration that each of the 2-oxo acid dehydrogenase complexes were active in the procyclic form of *T.b.brucei* (section 4.3.1).

3.4 Discussion

The location of dihydrolipoamide dehydrogenase to the plasma membrane of the bloodstream form of *T.b.brucei* was first reported by Danson *et al.* (1987) using the method of Voorheis *et al.* (1979) to purify plasma membranes. This method has a few limitations and a number of possible inaccuracies that have already been described in the introduction to this chapter (section 3.1).

Full subcellular fractionation of bloodstream forms of *T.b.brucei* has proved successful in confirming the intracellular location of a number of enzymes (Steiger *et al.*, 1980). The results presented in this chapter demonstrate that DHlipDH is located over the whole surface of the plasma membrane, including the flagellar pocket region. This is the first report of an enzyme located over the whole surface of the trypanosome, and also the only known example of an extra-mitochondrial eukaryotic DHlipDH.

Other enzymes located to the plasma membrane include α-glucosidase, which is on the external face of the membrane, and possibly galactosyl transferase, both of which may be involved in the turnover of variable surface glycoproteins (Cross *et al.*, 1975; Steiger *et al.*, 1980). 3'-Nucleotidase is buried within the plasma membrane of the bloodstream form of *T.b.brucei*, and is probably involved in the salvage of external purines (Gbenle *et al.*, 1986).

The flagellar pocket contains acid phosphatase, acid phosphodiesterase and acid pyrophosphatase (Steiger *et al.*, 1980; Opperdoes *et al.*, 1987). Adenylate cyclase has also been localised to this region (Walter & Opperdoes, 1982), as have receptors for host low density lipoproteins and transferrin (Opperdoes *et al.*, 1987; Coppens *et al.*, 1988). It is therefore thought that the flagellar pocket is the site for uptake of macromolecular material into the trypanosome (Opperdoes *et al.*, 1987).

Table 3.1. Phase partitioning of dihydrolipoamide dehydrogenase (DHlipDH) and protein with Triton X-114

	% Initial Material	
	Detergent Phase	Aqueous Phase
DHlipDH		
Whole Cells	0	69
Cell Sonicate	6	61
Plasma membrane sheets	0	82
Protein		
Cell sonicate	22	63
Controls		
Bacteriorhodopsin	> 95	< 5
Citrate synthase	< 5	> 95

For DHlipDH and protein, the data are mean results obtained from 3 separate experiments. Phase partitioning was carried out according to section 3.2.3.

The assay of the enzyme in intact trypanosomes in the presence and absence of detergent indicates that DHlipDH is present on the inner surface of the plasma membrane, so it is probably involved with cellular processes concerning the plasma membrane.

The DHlipDH of *Clostridium cylindrosporum* has been located to the plasma membrane of the cell by immunocytochemical techniques (Dietrichs *et al.*, 1991), and the enzyme has also been demonstrated to be an antigen in membrane vesicles of *E.coli* (Owen *et al.*, 1980). These are the only other examples of plasma membrane locations for DHlipDH. In our laboratory, we have also attempted an immunocytochemical localisation of the trypanosomal DHlipDH by producing rabbit polyclonal antibodies to the pig heart enzyme. Unfortunately, the serum obtained did not significantly cross-react with the trypanosomal enzyme on Western blots. Until sufficient quantities of the trypanosomal enzyme become available either by large-scale purification or the expression of a cloned gene, this confirmatory experiment is not possible.

Experiments carried out with Triton X-114 indicate that DHlipDH is probably not attached to the plasma membrane of *T.b.brucei* via a hydrophobic domain or a lipid anchor. Although 63 % of protein partitioned into the detergent phase, only 6 % of DHlipDH from sonicated cells was detected in this phase. As whole cells have not been disrupted prior to partitioning, and the plasma membranes also have attached DHlipDH, it is evident that the small DHlipDH content of the detergent phase from sonicates, in which cellular material has been more brutally treated, is probably the result of contamination. In the 2-oxo acid dehydrogenase complexes, DHlipDH is bound non-covalently to the E2 or protein X components by protein:protein interactions (Yeaman, 1989). Although the 2-oxo acid dehydrogenase complexes are absent from the bloodstream form of *T.b.brucei*, it is possible that the DHlipDH may exist as part of another multienzyme complex which could have a similar attachment to the plasma membrane as the pyruvate and 2-oxoglutarate dehydrogenase complexes may have to the inner mitochondrial membrane (see section 4.3.2 and Hunter & Lindsay, 1986). In this respect, Stanley and Perham (1980) found that the PDHC of ox heart was released from membranous material by treatment with Triton X-100, indicating some form of attachment of the complex, probably to the mitochondrial inner membrane. Another possibility may be that the enzyme possesses anchoring hydrophobic polypeptides which reside in the plasma membrane. This is the case for both fumarate reductase and succinate dehydrogenase in *E. coli*, each of which is a dimer linked by two hydrophobic polypeptides to the cytoplasmic membrane (Ingledew & Poole, 1984). In looking at membrane-bound hydrogenases, Knüttel *et al.* (1989) showed that the hydrogenase from *Paracoccus denitrificans* partitioned into the detergent phase upon treatment with Triton X-114. This was in contrast to earlier work from that laboratory (Podzuweit *et al.*, 1987) which demonstrated that the membrane-bound hydrogenase from *Alcaligenes eutrophus* remained in the soluble phase upon detergent treatment. The enzyme,

nevertheless, is thought to be an integral membrane protein. They therefore suggested the possibility of an intrinsic anchor protein, which could be detached upon purification. Another possibility raised by this work which might also be relevant to DHlipDH, is that an alteration in conformation due to detergent treatment may have exposed hydrophilic residues and domains, resulting in preferential partitioning into the aqueous phase. However, Podzuweit *et al.* (1987) found no evidence for this possibility from biochemical, immunological or catalytic experiments. Such work suggests that detergent partitioning may yield anomalous results, which cannot be explained without further detailed chemical characterization of the protein involved.

The level of DHlipDH in sonic extracts of bloodstream forms of *T.b.brucei* compares favourably with results obtained by Danson *et al.* (0.03 U/mg protein; 1987). The specific activity is half that found in *Halobacterium halobium* (Danson *et al.*, 1986), which also lacks the 2-oxo acid dehydrogenase complexes, and is 4-fold lower than in *S.cerevisiae* (Wren & Massey, 1965). *S.cerevisiae* does possess the enzymes of the citric acid cycle, but these are not always fully active, as the organism is capable of fermenting glucose when this substrate is supplied in high concentrations (Herbert & Guest, 1975), a process during which the citric acid cycle enzymes are less active. Therefore, this is perhaps not the best organism for a comparison of DHlipDH activity, but in working with other eukaryotes, it has been common to pre-purify mitochondria before purifying DHlipDH, and hence the specific activity of the enzyme in cell-free extracts has not been determined.

The inability to detect pyruvate dehydrogenase activity in *T.b.brucei* concurs with the work of Flynn & Bowman (1973), and the lack of 2-oxoglutarate dehydrogenase activity is consistent with the bloodstream form being unable to survive when 2-oxoglutarate is supplied as a growth substrate (Vickerman, 1965). The BCKDHC has not previously been measured in these cells, and it is not surprising that this complex appears absent, as it is intramitochondrial and feeds into the citric acid cycle in the mammalian cell (section 1.4.2.3).

In mammalian systems, the PDHC and BCKDHC are regulated by phosphorylation (Yeaman, 1989), with specific kinases and phosphatases existing to inactivate and re-activate the complexes, respectively. The activity states of the BCKDHC in rat liver, heart and kidney are 55 %, 5 % and 71 %, respectively (Patston *et al.*, 1988), indicating that phosphorylation is an important regulatory feature of this complex in mammals. Such phosphorylation has not been detected in other eukaryotic cells (Yeaman, 1989) and does not occur in prokaryotes. In the trypanosome, the majority of glucose metabolism is controlled by enzyme synthesis (Fairlamb & Opperdoes, 1986), with mitochondrial switching being under genetic regulation (Vickerman, 1965). Therefore, it is unlikely that phosphorylation would be used to inactivate the mitochondrial complexes in the bloodstream form cell. To be certain of this, however, future assays could be conducted under conditions that promote dephosphorylation (Patston *et al.*, 1988; Moreno-Sanchez & Hansford, 1988).

In summary, the results presented in this chapter demonstrate that DHlipDH is located over the whole of the cytoplasmic face of the plasma membrane. It is probably not attached to the membrane by direct anchorage, but rather through interaction with another membrane protein. It has been confirmed that this DHlipDH does not participate in the 2-oxo acid dehydrogenase complexes.

CHAPTER 4 : DIHYDROLIPOAMIDE DEHYDROGENASE AND THE 2-OXO ACID DEHYDROGENASE COMPLEXES IN PROCYCLIC FORMS OF *T.B.BRUCEI*

4.1 Introduction

An investigation into DHlipDH in *T.b.brucei* would not be complete without looking at the procyclic form of the parasite. This form possesses a highly-developed mitochondrion, and relies upon 2-oxo- and amino-acid oxidation linked to the phosphorylation of ADP at the level of the mitochondrial ATPase for production of energy (Opperdoes *et al.*, 1981). The 2-oxo acid dehydrogenase complexes, which contain DHlipDH, are intra-mitochondrial in other eukaryotes, and therefore their presence might be expected in a mitochondrially-active cell.

Whilst the pyruvate dehydrogenase complex (PDHC) and 2-oxoglutarate dehydrogenase complex (2OGDHC) have been detected in *T.b.rhodesiense* procyclics (Flynn & Bowman, 1973), neither they nor the branched chain 2-oxo acid dehydrogenase complex (BCKDHC) or DHlipDH have previously been measured in *T.b.brucei.* Therefore, the first part of this chapter describes the detection and measurement of these enzymes in *T.b.brucei* procyclics.

Secondly, with preliminary evidence of a plasma membrane location for DHlipDH in bloodstream form cells (Danson *et al.*, 1987), and the definitive evidence in chapter 3, it is necessary to investigate the location of the enzyme and its complexes in procyclics using a complete subcellular fractionation. This has been carried out for DHlipDH, the PDHC and NADH oxidase. The NADH oxidase activity has been measured because it could interfere with DHlipDH and PDHC assays, leading to inaccurate localisation.

4.2 Methods

DHlipDH was assayed as described in section 2.2.6.

4.2.1 Assay of the pyruvate and 2-oxoglutarate dehydrogenase complexes

The pyruvate and 2-oxoglutarate dehydrogenase complexes were assayed under identical assay conditions, with the exception of the substrates employed.

The assay mixture, in a total volume of 1 ml, consisted of 50 mM potassium phosphate, pH 7.0, 2.5 mM NAD^+, 0.2 mM thiamin pyrophosphate, 1.0 mM $MgCl_2$, 0.13 mM CoASH, 2.6 mM cysteine-HCl, 0.1 % (w/v) Triton X-100 and 2 mM sodium pyruvate or 2-oxoglutarate, to which was added an appropriate volume of cell extract. The assay was carried out at 30°C and the production of NADH followed by monitoring the increase in A_{340}.

A blank rate of reaction without substrate (pyruvate or 2-oxoglutarate) was determined for each sample. This was subtracted from the rate in the presence of substrate to yield the specific rate for complex activity.

CoASH and cysteine-HCl were stored together in a separate solution to the other assay components, the cysteine-HCl being present to ensure an acid pH for stabilisation of CoASH.

4.2.2 Assay of the branched-chain 2-oxo acid dehydrogenase complex

The branched-chain 2-oxo acid dehydrogenase complex (BCKDHC) has a broad specificity towards 2-oxo acid substrates (Patston *et al.*, 1988). Therefore, three different substrates were used for assays in trypanosomes: ketoleucine (α-ketoisocaproic acid), ketoisoleucine (DL-α-keto-β-methyl-n-valeric acid) and ketovaline (α-ketoisovaleric acid).

The assay mixture, in a total volume of 1 ml, contained 30 mM potassium phosphate, pH 7.5, 1 mM NAD^+, 0.4 mM thiamin pyrophosphate, 2 mM $MgSO_4$, 1.1 mM KCN, 2 mM dithiothreitol, 0.4 mM CoASH, 2.6 mM cysteine-HCl, 0.1 % (w/v) Triton X-100 and 0.2 mM 2-oxo acid. The assay was carried out at 30°C and monitored by increase in absorbance at 340 nm due to production of NADH. As with the PDHC & 2OGDHC assays, a blank rate was determined in the absence of substrate (2-oxo acid), and this was subtracted from the rate in the presence of substrate to give the true rate for the complex.

4.2.3 Subcellular fractionation

The same method was used for the subcellular fractionation of procyclic *T.b.brucei* as for the bloodstream forms (section 3.2.1). Cultured procyclic trypomastigotes were disrupted by grinding with silicon carbide, and subjected to differential centrifugation followed by isopycnic centrifugation in a linear sucrose gradient. The marker enzymes α-glucosidase (plasma membrane), acid phosphatase (microsomes), 3'-nucleotidase (plasma membrane), α-mannosidase (lysosomes), $NADP^+$-linked isocitrate-dehydrogenase (NADP-IDH, mitochondria) and hexokinase (glycosomes) were assayed as described previously (Opperdoes & Steiger, 1981; Walter & Opperdoes, 1982; Gbenle *et al.*, 1986). Subcellular fractionation and marker enzyme assays were carried out by Mr J. Van Roy, ICP, Brussels, and fractions from the gradient, stored in liquid nitrogen, were sent to Bath. DHlipDH, PDHC and NADH oxidase activities were measured in Bath. DHlipDH and the PDHC were assayed as described in sections 2.2.6 and 4.2.1. NADH oxidase was assayed in 50 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA, 0.1 mM NADH at 30°C. The reaction was monitored by decrease in absorbance at 340 nm.

4.3 Results

4.3.1 The 2-oxo acid dehydrogenase complexes

The levels of the 2-oxo acid dehydrogenase complexes and DHlipDH in the procyclic form of *T.b.brucei* are displayed in Table 4.1.

A blank rate of 1.84 x 10^{-3} µmol NADH produced/min/mg protein was obtained for extracts under the assay conditions for the PDHC, 2OGDHC and BCKDHC. This increase in A_{340} was proportional to the amount of extract added. The blank rate has been subtracted from each of the values obtained in the presence of substrate to give those shown in Table 4.1.

All three of the branched-chain 2-oxo acids (ketoleucine, ketoisoleucine & ketovaline) were equally active as substrates of the BCKDHC.

Of the 2-oxo acid dehydrogenase complexes, the highest level of activity was found for the PDHC, with the 2OGDHC having 2.7-fold less activity, and the BCKDHC activity being a further 6-fold lower. DHlipDH, a component of all of these complexes, has a 20-fold higher specific activity than the PDHC, and 14-fold more activity than all three complexes together.

4.3.2 Subcellular fractionation

The distribution profiles for DHlipDH, PDHC and NADH oxidase, together with marker enzymes for cellular compartments, in post-large-granule extracts of procyclic *T.b.brucei* homogenates after isopycnic centrifugation in linear sucrose gradients are shown in Fig. 4.1.

DHlipDH shows two clear peaks of activity at 1.09 g/cm^3 and 1.18 g/cm^3 in both the imidazole- and Tris-based buffering systems. This distribution is identical to that of the $NADP^+$-linked isocitrate dehydrogenase (NADP-IDH), which includes a soluble component at 1.09 g/cm^3 and a peak corresponding to the mitochondrial matrix at 1.18 g/cm^3.

There is no significant difference between the distributions of DHlipDH in each of the two buffers. This contrasts with the known plasma membrane markers α-glucosidase and 3'-nucleotidase, which display density shifts from 1.18 g/cm^3 in Sucrose-Imidazole-EGTA (SIE) buffer to 1.14 g/cm^3 in Sucrose-Tris-EDTA (STE) buffer. Such shifts are characteristic of the plasma membrane of both bloodstream and procyclic forms of *T.b.brucei*, the subpellicular microtubular arrays of the cells becoming detached from the plasma membrane upon changing from the imidazole- to the Tris-based buffer (Steiger *et al.*, 1980; Opperdoes & Steiger, 1981). This difference between results obtained for DHlipDH and the plasma membrane markers demonstrates that no DHlipDH is detectable at the plasma membrane of procyclic *T.b.brucei*.

Table 4.1 Specific activities of dihydrolipoamide dehydrogenase and the 2-oxo acid dehydrogenase complexes in the procyclic form of *T.b.brucei*

Enzyme	Specific activity (nmol/min/mg protein)
DHlipDH	400 ± 70
PDHC	20.0 ± 1.0
2OGDHC	7.5 ± 0.7
BCKDHC	1.3 ± 0.2

Enzyme activities have been measured according to sections 2.2.6, 4.2.1 and 4.2.2, and protein content has been determined according to section 2.2.5.

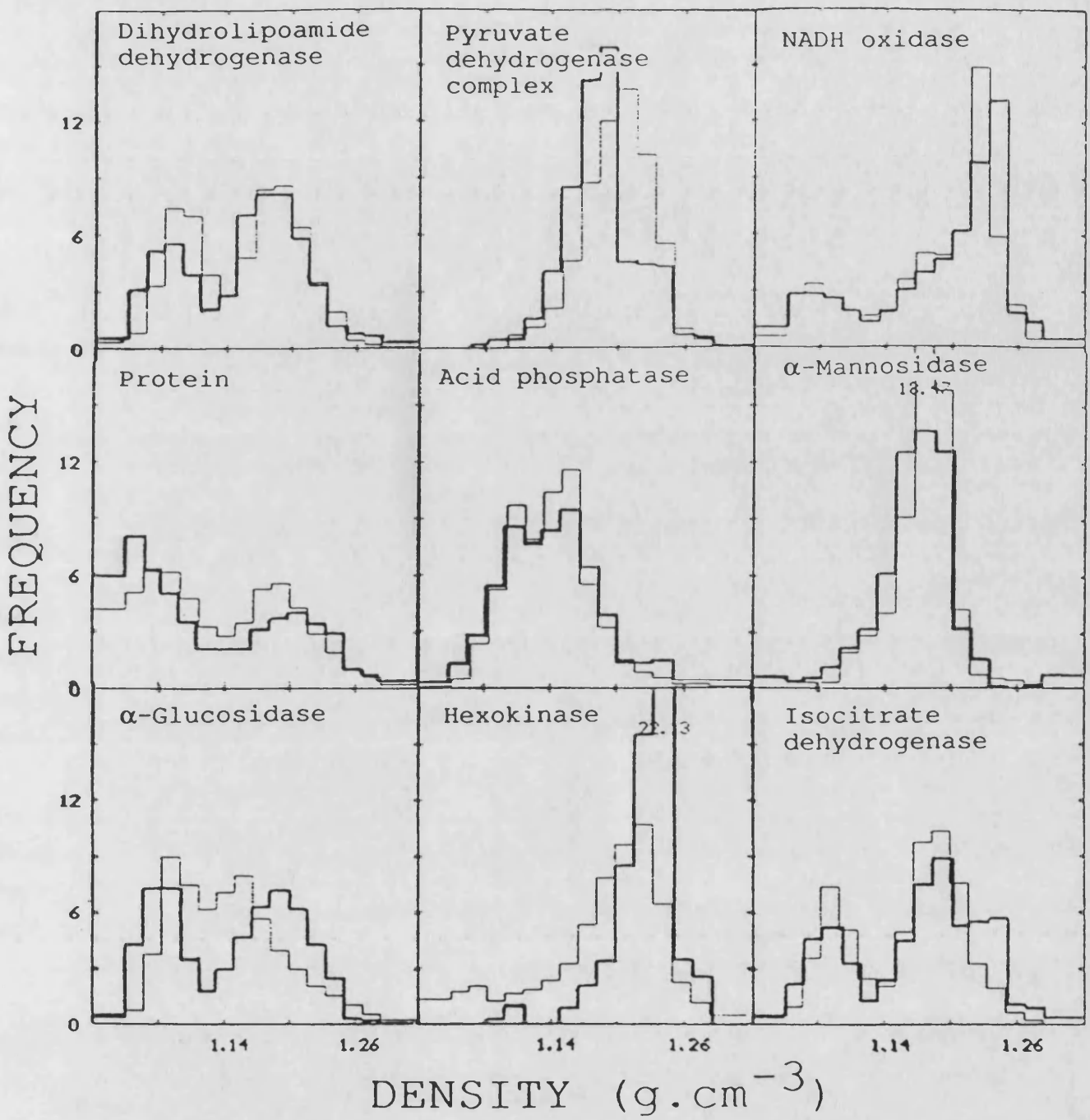


Fig. 4.1. Distribution profiles of marker enzymes and protein from post-large-granule extracts of procyclic *T.b.brucei* homogenates after isopycnic centrifugation in linear sucrose gradients. The marker enzymes present are : acid phosphatase for the endoplasmic reticulum; α-mannosidase for lysosomes; α-glucosidase for plasma membrane; hexokinase for glycosomes, and isocitrate dehydrogenase for mitochondrion. Thick lines represent the distribution in an imidazole-containing gradient, and thin lines that in a Tris-containing gradient. The recovery of marker enzymes, NADH oxidase and protein was greater than 85 %. Recoveries of pyruvate dehydrogenase complex and dihydrolipoamide dehydrogenase were 107 % and 121.2 %, respectively.

PDHC exhibited only one peak of activity in each sucrose gradient, at 1.18 g/cm^3 in the SIE gradient, and 1.20 g/cm^3 in the STE gradient. This peak coincides with the peaks of DHlipDH and NADP-IDH activity which correspond to the mitochondrial matrix. The lack of any soluble peak, together with the sharpness of the mitochondrial peak, may indicate that the PDHC is present at the mitochondrial membrane in procyclics.

A major peak of NADH oxidase activity was at 1.24 g/cm^3 in the imidazole buffer and 1.23 g/cm^3 in the Tris-based buffer, with an additional small peak in both buffers at 1.08 g/cm^3. The major peaks corresponded to those of hexokinase, a marker for the glycosomal compartment, and the minor peak at 1.08 g/cm^3 consisted of soluble enzyme activity.

4.4 Discussion

The work presented in this chapter demonstrates the presence of DHlipDH and the 2-oxo acid dehydrogenase complexes in the procyclic form of *T.b.brucei*.

The specific activity of DHlipDH is 4-fold higher than in epimastigotes of *T.cruzi* (Lohrer & Krauth-Siegel, 1990) and in *Saccharomyces cerevisiae* (Wren & Massey, 1965), which possess active citric acid cycles, and 7-fold higher than the activity in *Halobacterium halobium* (Danson *et al.*, 1986), which does not have the 2-oxo acid dehydrogenase complexes.

The PDHC activity is 4-fold lower than pyruvate oxidase (determined manometrically) in short-stumpy bloodstream forms of *T.b.rhodesiense* (Flynn & Bowman, 1973), but comparable to levels in other cells such as *E.coli* (35.6 nmol/min/mg protein; Bothe & Nolteernsting, 1975), rat liver (30 nmol/min/mg; Roche & Cate, 1977) and mouse and rat brain (4-24 nmol/min/mg; Hinman & Blass, 1981).

The 2.5-fold lower activity of 2OGDHC as compared to PDHC in procyclics contrasts with the higher levels of 2OGDHC found in other organisms including procyclic *T.b.rhodesiense* (1.25-fold more 2OGDHC; Flynn & Bowman, 1973) and rat liver (1.77-fold more 2OGDHC; Roche & Cate, 1977). This unusual difference may reflect the incomplete mitochondrial metabolism of procyclics (see section 1.2.3.3). The 2OGDHC receives 2-oxoglutarate from proline oxidation, and may also obtain it via the action of the NADP$^+$-linked isocitrate dehydrogenase (the NAD$^+$-linked enzyme, which normally forms the previous step in the citric acid cycle, is absent [see Fairlamb & Opperdoes, 1986]). The enzymes succinate thiokinase and succinate dehydrogenase, which follow 2OGDHC in the cycle, are also present (see section 1.2.3.3). Even with this metabolic context for the enzyme, there is surprisingly low activity of 2OGDHC in these cells.

The determination of the BCKDHC has not previously been reported in trypanosomes, and is evidence that branched-chain amino acids may be catabolised in procyclic *T.b.brucei*. The complex is less active in these cells than the PDHC and 2OGDHC,

which concurs with results from rat tissues, where Patston *et al.* (1988) found 3-10 nmol/min/mg BCKDHC and Roche & Cate (1977) measured PDHC activity at 30 nmol/min/mg (in liver).

At this point it is worth mentioning that both the PDHC and BCKDHC are regulated by phosphorylation in mammalian systems (Yeaman, 1989). It is possible, therefore, that their levels, as detected in the assays described above, may be underestimates of the true activities in the absence of phosphorylation (see section 3.4).

The 20-fold greater specific activity of DHlipDH in comparison to the PDHC in procyclics correlates well with results obtained by Komuniecki *et al.* (1979) working on *Ascaris* muscle (0.038 µmol/min/mg PDHC and 0.80 µmol/min/mg DHlipDH). The greater activity of DHlipDH in comparison to the total activity of all the complexes is understandable as the enzyme is produced in excess of the other components of the 2-oxo acid dehydrogenase complexes in mammalian mitochondria, where up to 25 % of the enzyme is uncomplexed (Lusty & Singer, 1964). Another reason for the higher activity is that the E1 component of the PDHC is the rate-limiting step of the reaction (Cate *et al.*, 1980), so, when E3 is assayed alone, it should show a higher activity than the whole complex.

The glycine cleavage system (GCS) has not been measured in either form of *T.b.brucei.* It is intramitochondrial in other eukaryotes (Motokawa & Kikuchi, 1971), and therefore might be expected to be absent from the bloodstream and present in the procyclic form of *T.b.brucei.* Glycine itself is a product of the active threonine catabolism of these cells, but appears to be expelled into the surrounding medium as opposed to being metabolised further (Linstead *et al.*, 1977). Nevertheless, growth on threonine does induce the synthesis of the glycine cleavage system in bacteria (Bell & Turner, 1976), so glycine cleavage commonly follows threonine catabolism in these cells. There is an amino acid transporter capable of importing/exporting glycine in trypanosomes (Voorheis, 1971).

In rat liver mitochondria, the specific activity of the GCS is 0.09 nmol/min/mg protein (Motokawa & Kikuchi, 1971). Therefore, if the enzyme were to perform an analogous role in *T.b.brucei*, it might be present at very low levels in procyclics.

Several anaerobic glycine-utilising bacteria contain high levels of the GCS (Dietrichs & Andreesen, 1990), with the DHlipDH of one, *Clostridium cylindrosporum*, having been located to the plasma membrane of the cell (Dietrichs *et al.*, 1991). These organisms, however, are anaerobic, and use glycine as their major respiratory substrate, whereas in *T.b.brucei* there is no evidence that the cells use glycine even as a minor nutrient. So, while the results from *C.cylindrosporum* are very interesting from the point of view of there being another plasma membrane DHlipDH, they have no bearing on the use of the GCS by *T.b.brucei.*

Further information concerning DHlipDH and the 2-oxo acid dehydrogenase complexes has been gained from a full subcellular fractionation of the procyclic cells. This

has demonstrated that at least the majority of the cellular DHlipDH is within the mitochondrion of the procyclic. It is not clear as to whether any DHlipDH exists in any other cellular location.

Both DHlipDH and the mitochondrial marker NADP-IDH have two peaks of activity in the sucrose gradient, one corresponding to mitochondrial, and the other to soluble, material. Opperdoes *et al.* (1981) detected similar bimodal distributions for threonine dehydrogenase and aminoacetone synthase in procyclics. They explained these results by suggesting that the highly developed mitochondria of these cells could be easily ruptured by grinding with silicon carbide, and enzymes such as those of the threonine pathway, which are present in the mitochondrial matrix, could leak out. Both DHlipDH and NADP-IDH may, at least in part, be free within the mitochondrial matrix, and therefore able to leak out.

There is much evidence for cytosolic and mitochondrial isoenzymes of NADP-IDH in several mammalian systems (Colman, 1983). For example, rat heart has 90 % mitochondrial/10 % cytosolic NADP-IDH (Plaut & Gabriel, 1983). The presence of the enzyme in both compartments may allow it to act as a flux for reducing equivalents between them (Plaut & Gabriel, 1983). In the kinetoplastida, *Crithidia fasciculata* also has mitochondrial and cytoplasmic isoenzymes (Morris & Weber, 1975). Therefore, there is clearly a precedent for a cytosolic NADP-IDH in procyclic cells, which may contribute to the soluble peak observed in the sucrose gradient.

Looking closer at the distribution profiles for DHlipDH and NADP-IDH (Fig. 4.1), 36 % of DHlipDH is present in the soluble peak, and the remaining 64 % in the mitochondrial peak. In the same gradients, NADP-IDH is 27 % soluble and 73 % mitochondrial. Therefore, 9 % more DHlipDH than NADP-IDH migrates in the soluble portion of the gradient. This difference may not be significant, and could be merely the result of differential leakage from ruptured mitochondria, but it does leave open the possibility that a proportion of the DHlipDH exists outside of the mitochondrion *in vivo*. The majority of the increase in DHlipDH upon differentiation to the procyclic form can, nevertheless, be put down to the switching on of the mitochondrial 2-oxo acid dehydrogenase complexes. This highlights a major drawback of the fractionation method, in that if 10 % of the DHlipDH in procyclic cells were at the plasma membrane, it would not be possible to observe the associated density shift against a high background of mitochondrial DHlipDH.

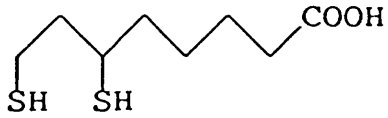
The distribution profile for the PDHC indicates its presence within the mitochondrion of the procyclic *T.b.brucei*. It has no soluble peak of activity, which is possibly indicative of attachment to the mitochondrial inner membrane. The enzymes succinate dehydrogenase, oligomycin-sensitive ATPase and glycerol-3-phosphate dehydrogenase, which are all components of the respiratory chains of these cells and are also attached to the inner membrane, possess distribution profiles identical to that of the PDHC (Opperdoes *et al.*, 1981).

Further experiments are required to elucidate whether or not a proportion of procyclic DHlipDH is located outside the mitochondrion, and possibly at the plasma membrane. Immunocytochemical localisation with antibodies to the bloodstream form DHlipDH would be one method. In the absence of suitable antibodies, the technique of digitonin-permeabilisation, whereby the plasma membrane can be selectively permeabilised, leaving the mitochondrial membrane intact, may allow detection of an extramitchondrial DHlipDH. The technique has recently been used successfully to investigate carbohydrate metabolism in both bloodstream (Kiaira & Njogu, 1989) and procyclic trypomastigotes (Turrens, 1989). Future work must also include the determination of GCS activity in procyclic cells, to complete the investigation of the major DHlipDH-containing multienzyme complexes.

CHAPTER 5 : DETECTION OF LIPOIC ACID

5.1 Introduction

In an investigation into the role of DHlipDH in the bloodstream form of *T.b.brucei*, it is important to look for its presumed substrate, lipoic acid, in these cells.

Lipoic acid 

The presence of lipoic acid 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, however, 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 latter feature is demonstrated by the electron-transferring flavoprotein of bacterium W6, which behaves more like thioredoxin reductase than DHlipDH, but can catalyse the oxidation of dihydrolipoamide (Dietrichs *et al.*, 1990; Meyer *et al.*, 1991). Certainly, the detection of lipoic acid would be suggestive of DHlipDH using it as a substrate *in vivo*. This still, however, gives no indication as to the source, or cellular location of the reduced lipoic acid. With the presence of both enzyme and presumed substrate within the cell, it becomes less likely that the DHlipDH is merely the result of aberrant expression.

Further analysis of the possible linkage of lipoic acid to a protein and any association of the cofactor with cell membranes will help to elucidate the role of enzyme and substrate in *T.b.brucei*.

A number of different methods have been developed for the determination of lipoic acid in biological systems. Following release of the cofactor from protein by acid or base hydrolysis, lipoic acid has been assayed as follows :

a) Manometric assay : *Streptococcus faecalis* (strain 10Cl), grown on lipoic acid-deficient medium contains the apoenzymes of the 2-oxo acid dehydrogenases (Gunsalus & Razzell, 1957). Addition of lipoic acid activates these enzymes in proportion to the amount of cofactor added. Activity is measured by following oxygen consumption manometrically. This assay is sensitive between 1 and 10 ng lipoic acid per assay tube.

b) Polarographic assay : The lipoic acid mutant of *Escherichia coli* K12, W1485*lip*2, contains the apoenzymes of the 2-oxo acid dehydrogenase complexes (Herbert & Guest, 1968). Therefore, when lipoic acid is supplied to these cells in the presence of pyruvate, oxygen consumption increases in proportion to the amount of cofactor added, and this can be measured with an oxygen electrode (Herbert & Guest, 1970 & 1975). The sensitivity range of this assay is 5 - 50 ng lipoic acid.

c) Turbidimetric assay : In this method, the growth of the lipoic acid-deficient *E.coli* K12, W1485*lip*2, is proportional to the amount of lipoic acid present. This growth is measured turbidimetrically. Growth is proportional to lipoic acid over the range 0.2 - 2.0 ng (Herbert & Guest, 1975).

d) Gas chromatography/mass spectrometry (GC/MS) : This method differs from the three detailed above in being qualitative, but giving an absolute chemical identification of lipoic acid (Pratt *et al.*, 1989).

For the purposes of this investigation, the turbidimetric assay was chosen to give a quantitative estimation of the lipoic acid content of *T.b.brucei* bloodstream form and procyclic cells. This method was favoured as the most sensitive and most straightforward of the quantitative techniques (Herbert & Guest, 1975). *Escherichia coli* TG1 was chosen as a control organism, as data already exist for the lipoic acid content of various strains of this organism.

GC/MS was used to confirm the chemical nature of the lipoic acid in bloodstream forms. This is of particular importance in proving that lipoic acid itself, rather than any chemically similar molecule, is present in *T.b.brucei*. It will not, however, definitively prove that lipoic acid is the chemical moiety *in vivo* because acid hydrolysis may have removed certain substituents prior to GC/MS.

At this point it is worth noting that another cellular thiol, glutathione, is replaced by trypanothione in all trypanosomatids. Although this has a different function to lipoic acid, there is nevertheless a precedent for differences in thiol metabolism in this organism.

5.2 Methods

5.2.1. Biological turbidimetric assay

The biological turbidimetric assay consisted of 3 main procedures: the preparation of lipoic acid, the growth of the mutant inoculum, and the assay itself.

5.2.1.1 Preparation of lipoic acid

(a) **Standard lipoic acid solution.** A standard solution of D,L-α-lipoic acid was prepared at 1 mg/ml in 0.2 M potassium phosphate buffer (pH 7.0). This stock solution could be stored at 4°C for several months.

(b) **Lipoic acid from cellular material.** Lipoic acid was liberated from protein by acid hydrolysis (Gunsalus & Razzell, 1957). Cells of *T.b.brucei* bloodstream and procyclic forms and *E.coli* TG1 were sonicated for 3 x 30 s at 40 W using a 3 mm probe at 4°C. Varying amounts of the sonicates were transferred to glass tubes and hydrolysed in 0.3 ml 6 M HCl at 120°C for 2 h *in vacuo*. As a control, 1 mg bovine serum albumin was also hydrolysed under the same conditions. The dried hydrolysates were resuspended in 1 ml

0.2 M potassium phosphate buffer (pH 7.0) and filtered through 0.22 μm pore filters to remove any insoluble material.

The protein contents of cell sonicates were determined prior to hydrolysis by the procedure described in section 2.2.5. Dry weight determinations were carried out by freeze-drying cell extracts and drying to constant weight over NaOH and conc. H_2SO_4 *in vacuo*.

5.2.1.2. Growth of the inoculum

The mutant inoculum was prepared by transferring a single colony of *E.coli* K12, W1485*lip*2, to a 5 ml culture of succinate-based minimal salts medium containing 35 mM K_2HPO_4, 16 mM $NaH_2PO_4.2H_2O$, 37 mM NH_4Cl, 1 mM Na_2SO_4, 5 μg thiamin/ml, 0.5 mM $MgCl_2.6H_2O$ and 50 mM sodium succinate supplemented with 1 ng/ml lipoic acid. Following overnight incubation at 37°C with vigorous shaking, cells were harvested by centrifugation at 600 x g, and washed twice with 5 ml portions of sterile 0.9 % saline; the cell pellet was resuspended in saline and diluted to an absorbance of approximately 0.2 at 660 nm. From this suspension, an inoculum of 0.1 ml was added to each assay tube.

5.2.1.3. Assay of lipoic acid

For the assay of lipoic acid, the following reagents were transferred to a series of capped 750 x 10 mm test tubes: 1.0 ml of Basal Growth Medium (Table 5.1), an appropriate volume of standard lipoic acid solution or hydrolysed sample, and the required volume of water to bring the total volume to 1.8 ml. The tubes were sterilised by autoclaving at 120°C for 10 min. Sterile 1 M sodium succinate solution (0.1 ml) and 0.1 ml mutant inoculum were added aseptically, and the tubes incubated for approximately 24 h at 37°C with shaking. The growth in each tube was determined by measuring the absorbance at 660 nm. From these results, a standard curve for lipoic acid was constructed and used to determine the lipoic acid contents of individual samples.

5.2.2 Gas chromatography/mass spectrometry

Gas chromatography/mass spectrometry (GC/MS) of bloodstream forms of *T.b.brucei* was carried out according to the method of Pratt *et al.* (1989) by Professor K. J. Stevenson at The University of Calgary, Calgary, Canada. Cells of *T.b.brucei* were grown and purified in Bath, and sent freeze-dried to Professor Stevenson.

The method can be summarised as follows. Cells were hydrolysed as described in the biological method, and any lipoic acid present reduced by slow addition of $NaBH_4$. The pH was lowered to pH 3 with HCl to remove excess $NaBH_4$, and the solution then made up to 30 % (v/v) with respect to ethanol, after which Na_2HPO_4 buffer was added and the pH re-adjusted to pH 9 with NaOH. This solution was added to *p*-aminophenylarsenoxide-agarose and gently agitated overnight. The matrix was then washed with 50 mM-sodium phosphate,

Table 5.1 Basal growth medium for the growth of the lipoic acid-requiring mutant of *Escherichia coli* K12, W1485*lip*2

Medium	Grams/litre
Casamino acids	4
K_2HPO_4	14
KH_2PO_4	6
Na_3 citrate.$3H_2O$	1
$MgSO_4.7H_2O$	0.20
$(NH_4)_2SO_4$	2
L-Asparagine	8
L-Arginine	0.20
L-Glutamate	0.20
Glycine	0.20
L-Histidine	0.20
L-Proline	0.20
L-Tryptophan	0.36
L-Cysteine	0.16
Na thioglycollate	0.20

pH 7.5, in 30 % (v/v) ethanol, resuspended in the same buffer containing 2,3-dimercaptopropane-1-sulphonic acid, and shaken for 3 h. The eluent was collected by filtration and the pH adjusted to pH 3 with HCl. The solution was extracted with benzene to recover the lipoic acid and the extract was washed with dilute acid to remove any extracted 2,3-dimercaptopropane-1-sulphonic acid. Extracted lipoic acid was allowed to oxidize overnight, yielding a dried extract, and methylated by dissolving in diethyl ether and reacting with cold diazomethane. The oxidised lipoic acid methyl ester was applied to a Kratos-MS 80 GC/MS.

5.3 Results

5.3.1. Biological turbidimetric assay

Using commercially obtained lipoic acid, the growth of the lipoic acid-requiring mutant of *E.coli* was proportional to the amount of cofactor present up to a level of 1.0 ng (Fig. 5.1). The amount of lipoic acid in each hydrolysed sample was calculated from the growth of the *E.coli* mutants, using a separate standard curve for each experiment. The lipoic acid contents of bloodstream and procyclic *T.b.brucei*, *E.coli* TG1 and the BSA control are displayed in Table 5.2. Those of procyclic *T.b.brucei* and *E.coli* TG1 are similar, and 7-fold greater than the lipoic acid content of bloodstream forms of *T.b.brucei*.

Each lipoic acid content (± SEM) has been calculated from 7 or more data points. Results were independent of protein hydrolysed and quantity of hydrolysate assayed for all cell types. At 25 mg of protein, *T.b.brucei* bloodstream form sonicates showed pronounced charring with hydrolysis and reduced lipoic acid recovery after filtration. Therefore, less than 20 mg protein were hydrolysed in each case.

5.3.2. Gas chromatography/mass spectrometry

The presence of lipoic acid in the bloodstream form of *T.b.brucei* was confirmed by GC/MS, a procedure which at the same time provided an unequivocal chemical identification of the cofactor. Following acid hydrolysis of bloodstream form *T.b.brucei* cells, any compounds containing vicinal thiol groups were separated from other cellular material by covalent chromatography on *p*-aminophenyldichloroarsine-agarose. Compounds such as lipoic acid were eluted from the column with 2,3-dimercaptopropane-1-sulphonic acid, extracted into benzene, and air-oxidised to the disulphide from. Treatment with diazomethane yielded their methyl esters which were subjected to GC/MS as described by Pratt *et al.* (1989).

A peak of oxidised lipoic acid methyl ester was identified in the gas chromatogram (Fig. 5.2a) by monitoring the presence of the fragmentation ion (m/z=123) and the molecular ion (m/z=220). Their coincidence at an elution time of 18 min 29 s, and the full mass

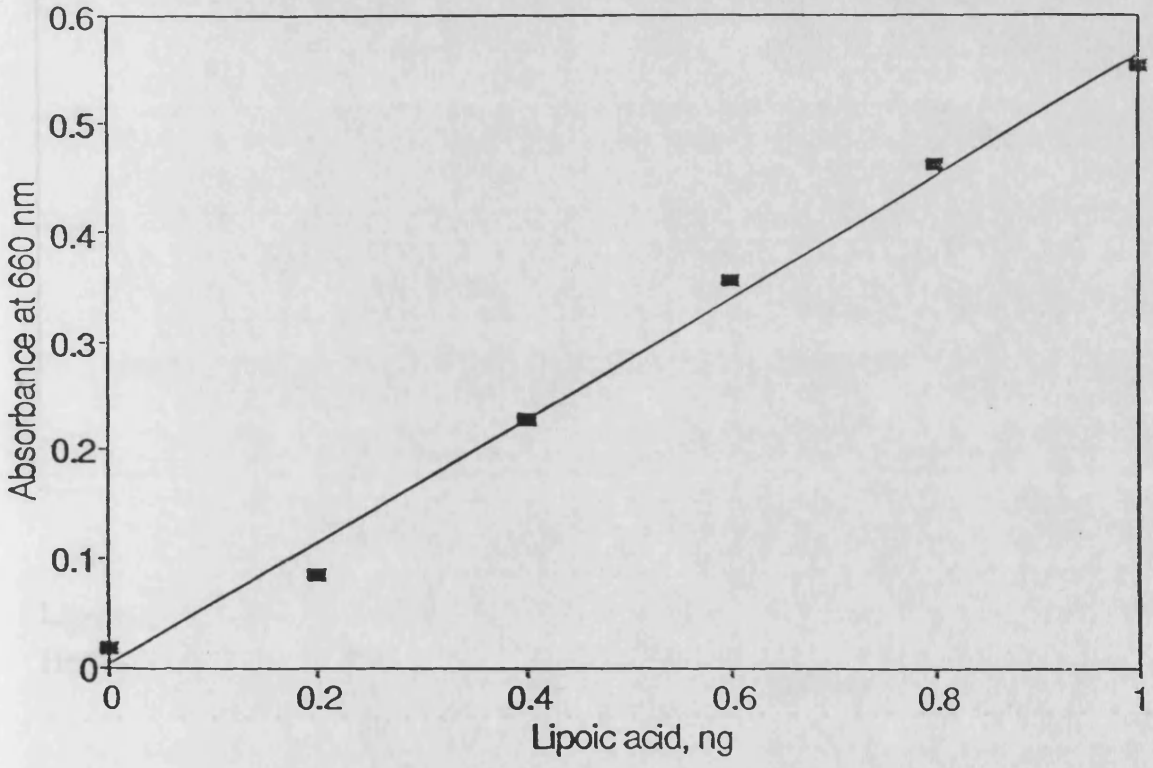


Fig. 5.1. Standard curve for the growth of the lipoic acid-requiring mutant *Escherichia coli* K12, W1485*lip*2, in the presence of increasing concentrations of commercial lipoic acid. The mutant *E.coli* were grown at 37°C for 24 h, with shaking, in a basal growth medium containing succinate. The appropriate amount of lipoic acid (ng) was added to each assay tube. Growth of cells was assessed turbidimetrically by determining the increase in absorbance at 660 nm. A standard curve has been fitted to these points by linear regression analysis.

Table 5.2 Lipoic acid contents of *T.b.brucei* and *E.coli* TG1

Organism	Lipoic acid content (± SEM)	
	ng/mg protein	ng/mg dry wt.
T.b.brucei (bloodstream)	1.7 ± 0.2	1.2 ± 0.1
T.b.brucei (procyclic)	17 ± 0.5	8.0 ± 0.2
E.coli TG1	19 ± 1.8	6.5 ± 0.6
bovine serum albumin	0	0

Lipoic acid contents have been determined using the biological turbidimetric assay of Herbert & Guest (1975), as described in section 5.2.1.

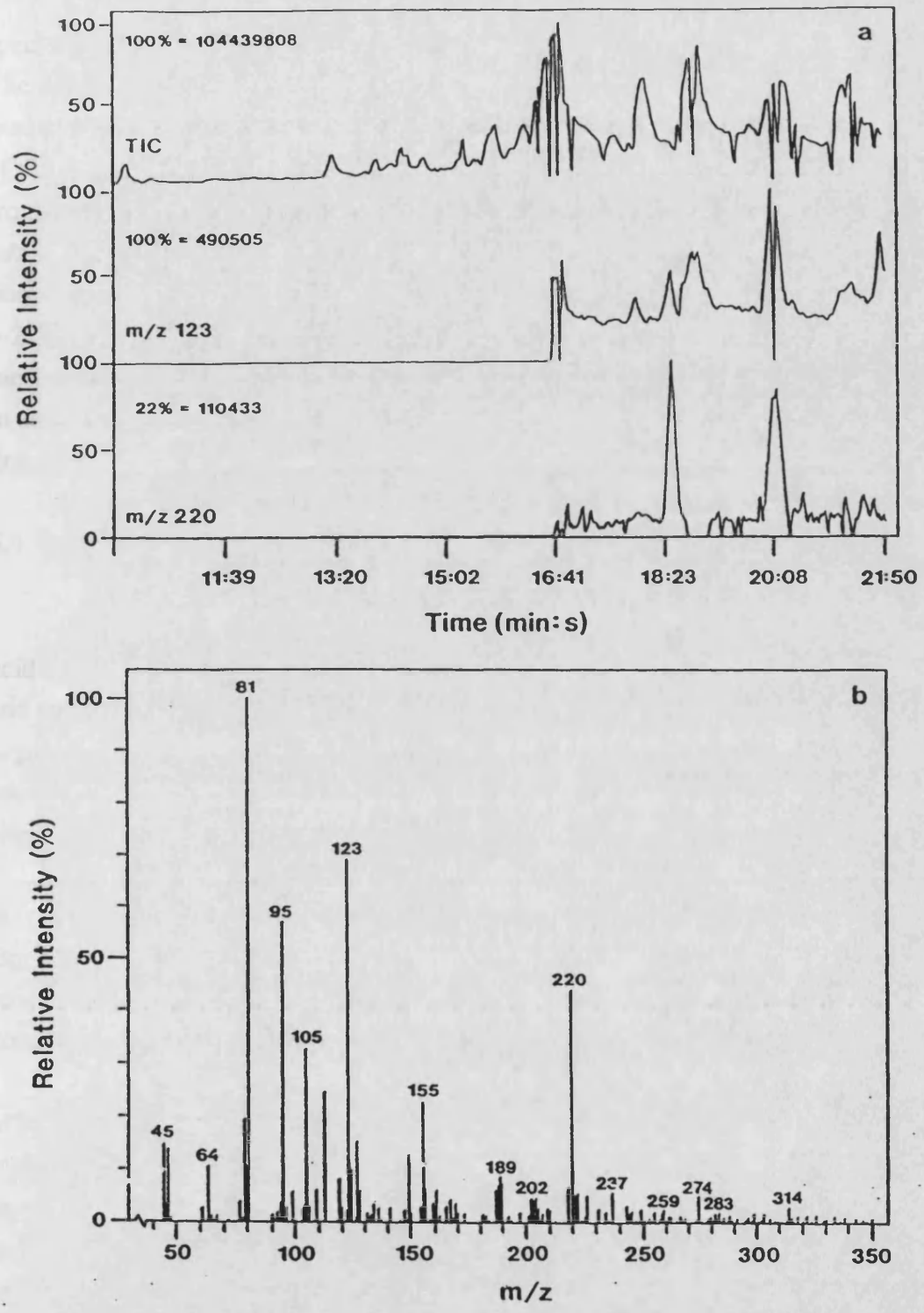


Fig. 5.2. Gas chromatography/mass spectrometry of oxidised lipoic acid methyl ester enriched from *T.b.brucei*. (a) Gas chromatogram monitored by mass spectroscopy of fraction enriched by covalent chromatography on *p*-aminophenylarsenoxide-agarose. TIC represents the total ion chromatogram. The profiles obtained by plotting the mass ions of *m/z* = 220 and 123 as a function of time are indicated by 220 and 123, respectively. (b) Mass spectrum of the peak eluted at 18.5 min (indicated by arrow) containing oxidised lipoic acid methyl ester. The spectrum was generated after subtraction of the 18-min baseline. The interpretation of the mass spectrum is described in the text, based on the assignment of molecular and fragmentation ions given in Pratt *et al.* (1989).

spectrum (Fig. 5.2b), provide the definitive identification of oxidised lipoic acid methyl ester. The expected mass spectral fragmentation pattern, the demonstration of the validity of the methodology using commercially available lipoic acid, and the rationale for the interpretation of the spectra have been described by Pratt *et al.* (1989). Thus, the mass spectrum (Fig. 5.2b) from the GC/MS analysis of hydrolysed cells of *T.b.brucei* shows clearly defined peaks at m/z = 220, 189, 155, 123, 105 and 95, the relative intensities of which are similar to those observed with pure lipoic acid. Moreover, the high $(m + 2)/m$ ratio of the molecular ion (m/z = 220) is consistent with this species possessing two sulphur atoms. Clearly the presence of ions above m/z = 220 indicates that the peak analysed from the GC has a minor contaminant in addition to the lipoic acid methyl ester, but this does not detract from the conclusions drawn.

5.4 Discussion

The work presented in this chapter represents the first report of the presence of lipoic acid in *Trypanosoma brucei brucei.* Levels of 1.2 & 8.0 ng/mg dry weight for bloodstream and procyclic *T.b.brucei*, respectively, are within the range of values obtained in various organisms by Herbert & Guest (1975). The negative result for the bovine serum albumin control indicates that individual amino acids, produced by hydrolysis, were not able to stimulate the growth of the mutant *E.coli.*

The lipoic acid content of *E.coli* TG1 (6.5 ± 0.6 ng/mg dry wt.) measured in this assay is lower than the range obtained for *E.coli* W1485 (Table 5.3) by Herbert & Guest (1975), but Bothe & Nolteernsting (1975), using the manometric method, detected only 4.1 ng/mg lipoic acid in *E.coli.* Such variation might be expected between different strains and growth conditions, but could also result from slightly lower yields after hydrolysis.

The recovery of lipoic acid and its release from protein by hydrolysis are key factors affecting the accuracy of lipoic acid determinations, and have been extensively investigated by other workers. Gunsalus & Razzell (1957) first showed that autoclaving at 120°C for 2 h in 6 M HCl gave least destruction of lipoic acid, and White (1980) demonstrated that this procedure released 95 % of the cofactor from *E.coli* proteins. A key determinant of lipoic acid recovery is the amount of protein hydrolysed with the cofactor. If lipoyllysine alone is the starting material, less than 70 % of the lipoic acid is recovered (Hayakawa & Oizumi, 1989), but the addition of bovine serum albumin to a lipoic acid solution was able to protect it against degradation during hydrolysis (Wagner *et al.*, 1956). Herbert & Guest (1975) looked at recovery in the presence of *E.coli* cell extracts. Addition of commercial lipoic acid to extracts yielded 90-110 % of the cofactor after hydrolysis. This particular study was carried out using the same experimental protocol as detailed in section 5.2.1, and therefore allows reasonable confidence in the recovery of lipoic acid from the cells under investigation.

Table 5.3 Lipoic acid contents of several micro-organisms

Organism	Lipoic Acid Content ng/mg dry wt.	Presence/absence of PDHC
Escherichia coli W1485[a]	13-41	+
Bacillus megaterium[a]	25	+
Pseudomonas aeruginosa[a]	8.5-9.0	+
Gloecapsa alpicola[a]	1.1	-
Clostridium pasteurinum[b]	0.05	-
Clostridium kluyveri[b]	0.15	-
Saccharomyces cerevisiae[a]	1.0	+ (low)
	7.5	+
Ankistrodesmus braunii[b]	0.8	-
Halobacterium volcanii[c]	0.8	-

[a] Herbert & Guest (1975); [b] Bothe & Nolteernsting (1975); [c] Noll & Barber (1988).

Other methods for releasing lipoic acid from protein include enzymatic cleavage by the enzyme lipoamidase, which is present in many species (Koike & Suzuki, 1970), and hydrolysis with organic acids (Matarese *et al.*, 1981) or sodium hydroxide (Noll & Barber, 1988). None of these techniques have been as well characterised in terms of yields and optimal cleavage conditions as acid hydrolysis with HCl or H_2SO_4.

Initial work on determining the lipoic acid contents of hydrolysates was performed according to Noll & Barber (1988). This procedure often produced revertants during overnight incubation at 37°C in the absence of lipoic acid (an addition to the method of Herbert & Guest [1970]). Another problem was the low reproducibility and inaccuracy of data. Noll & Barber (1988) used a simplified minimal salts medium for the assay, instead of the more complex medium of Herbert & Guest (1970). The latter medium is known to give an improved response of the cells to lipoic acid by reducing errors arising from small amounts of hydrolysed amino acids affecting growth (Herbert & Guest, 1975). On account of these problems, the method of Noll & Barber (1988) was soon abandoned in favour of that of Herbert & Guest (1970 & 1975).

Both *E.coli* TG1 and procyclic *T.b.brucei* possess the enzymes of the citric acid cycle, and therefore it is not surprising that they have similar levels of lipoic acid, presumably bound to the E2 components of the 2-oxo acid dehydrogenase complexes. The values for these two organisms also correlate with those of other organisms using the citric acid cycle, including *E.coli* W1485, *Bacillus megaterium* and *Pseudomonas aeruginosa* (table 5.3; Herbert & Guest, 1975). In *E.coli* W1485, a variation in lipoic acid content was observed with growth on different substrates, with glycerol resulting in only 13 ng lipoic acid/ mg dry wt., whereas pyruvate led to levels as high as 41 ng/ mg dry wt. (Herbert & Guest, 1975). The levels of the PDHC and 2OGDHC closely correlated with lipoic acid contents under all growth conditions.

In contrast to the procyclic form, the bloodstream form of *T.b.brucei* has 7-fold less lipoic acid, probably due to the absence of an active citric acid cycle and the 2-oxo acid dehydrogenase complexes. This is the first time lipoic acid has been found in a eukaryotic organism lacking the 2-oxo acid dehydrogenase complexes. The low level of lipoic acid is comparable to those in *Gloecapsa alpicola*, a cyanobacterium utilising a ferredoxin-linked pyruvate oxidoreductase, and *Ankistrodesmus braunii*, which contains low levels of the PDHC (table 5.3; Herbert & Guest, 1975).

Saccharomyces cerevisiae, when grown in the presence of high concentrations of glucose (2 %) ferments this substrate (see Herbert & Guest, 1975). This process does not employ the citric acid cycle, and therefore the level of lipoic acid is low, and is similar to that in the bloodstream form trypomastigote (table 5.3). When limiting amounts of glucose (0.1 %) are supplied to *S.cerevisiae*, the citric acid cycle plays a prominent role in energy production, and a correspondingly high level of lipoic acid is observed (table 5.3). This

illustrates the particular regulation of lipoic acid biosynthesis according to the type of metabolism in operation in yeast. Such regulation may be similar to that occurring in *T.b.brucei.*

Halobacterium volcanii utilizes the pyruvate:ferredoxin oxidoreductase system and lacks the 2-oxo acid dehydrogenase complexes, and yet has DHlipDH activity (Danson *et al.*, 1984) and low amounts of lipoic acid (table 5.3; Noll & Barber, 1988). In this respect, it is similar to the bloodstream form of *T.b.brucei.*

Two species of *Clostridia* have a further 10-fold less lipoic acid than in bloodstream forms of *T.b.brucei* (table 5.3), with similar trace amounts having been found in sulphur-metabolising and methanogen archaebacteria (Noll & Barber, 1988). The *Clostridia* use only the pyruvate: ferredoxin oxidoreducatse system to metabolise pyruvate.

GC/MS has provided positive identification of lipoic acid, but it cannot be ruled out that the cofactor exists in a modified form *in vivo*, which is converted to the identified form upon hydrolysis.

Following on from the detection and quantitation of lipoic acid in bloodstream form *T.b.brucei*, numerous other experiments can be envisaged. These include the investigation of the nature of possible protein attachment and the cellular location of lipoic acid. Preliminary acetone-precipitation experiments have demonstrated that the lipoic acid is probably attached to protein *in vivo* in the bloodstream form cell. In procyclics, the cofactor is presumably bound to the E2 components of the 2-oxo acid dehydrogenase complexes. Bothe & Nolteernsting (1975) were able to release free lipoic acid from cell extracts by boiling, and Griffiths *et al.* (1977) were able to detect the free form in purple membranes from *H.halobium.* Also, gel filtration and partial purification of extracts will show whether the lipoic acid is protein-bound. Purification of such a protein, followed by N-terminal sequencing would allow cloning of its gene, which may lead to an understanding of its function.

A recent technique to produce antibodies to lipoic acid by conjugating it to bovine serum albumin (Maclean & Bucher, 1991) may allow immunoprecipitation and immunocytochemical localisation of the cofactor. Also, the determination of the lipoic acid contents of fractions from a full subcellular fractionation (see Chapter 3) should reveal its cellular location.

In summary, lipoic acid is present in both bloodstream and procyclic forms of *T.b.brucei.* In each cell type, it is present in proportional amounts to the enzyme DHlipDH, for which it is presumed to be a substrate. In the procyclic cell, the majority of the lipoic acid is probably bound to the E2 components of, and functions as a cofactor within, the 2-oxo acid dehydrogenase complexes. In the bloodstream form cell, lipoic acid probably acts as an enzyme cofactor, but the metabolic process in which it is involved remains unknown.

CHAPTER 6 PURIFICATION AND PROPERTIES OF DIHYDROLIPOAMIDE DEHYDROGENASE FROM BLOODSTREAM FORMS OF *T.B.BRUCEI*

6.1 Introduction

The purification of proteins is necessary before detailed structural studies can be carried out, and is often a prelude to the cloning of the relevant gene via N-terminal sequencing or the production of antibodies.

The first half of this chapter is concerned with the purification of DHlipDH from the bloodstream form of *T.b.brucei*. With the enzyme being in low abundance within the cell (0.03 U/mg; Danson *et al.*, 1987), and the labour-intensive method of producing trypanosomes in rats, it was important to develop a procedure in which each individual step was maximised in terms of its yield.

The particular importance of purifying this enzyme is that, through N-terminal sequencing of the pure protein, and deduction of the corresponding DNA sequence, oligonucleotide probes can be constructed that will facilitate the identification and sequence determination of the DHlipDH gene from trypanosomal DNA. Once the gene for DHlipDH has been cloned, more detailed structural and functional studies can be carried out, as described in Chapter 8.

A second use for purified DHlipDH may be for the production of antisera for use in immunocytochemical localisation studies. Although such work has not been performed in this thesis, the purification of the enzyme enables it to be carried out in the future. Antibodies could also be used to identify the DHlipDH from a cDNA expression library.

The second half of this chapter is concerned with the investigation of several properties of the trypanosomal DHlipDH, with the aim of identifying any similarities to, and differences from, other known DHlipDHs. Studies of pH-dependence, temperature stability and kinetic parameters have been carried out.

6.2 Methods and Results

6.2.1 The development of a purification procedure for DHlipDH

Following initial disruption of cells by sonication (see section 2.2.3), sonic extracts were turbid, especially when high concentrations of cells were used. Some form of clarification was necessary before such extracts were amenable to column chromatography.

Centrifugation (8,500 x g for 10 min) brought about some clarification, but up to 30 % of the enzyme was pelleted with cell debris. Therefore another method was sought that would clarify the extract without significant loss of activity.

Extraction of lipid was attempted by adding chloroform/methanol (2:1) up to a concentration of 80 % (v/v). This produced significant clarification, but only 47 % of enzyme activity was recovered.

Ammonium sulphate fractionation, used effectively by Komuniecki & Saz (1979) and Dietrichs & Andreesen (1990), precipitated the majority of the *T.b.brucei* enzyme in a 50-75 % ammonium sulphate cut, but the yield of DHlipDH, after resuspension in buffer, was only 45 %.

Phase partitioning with Triton X-114 (see section 3.2.3 for detailed method), separates hydrophilic proteins from hydrophobic proteins and lipid, which are probably major sources of turbidity. When applied to cell extracts, a clear aqueous phase was obtained, with up to 100 % recovery of DHlipDH (given probable under-estimation in cell-free extracts). This was accepted as a first step in the purification procedure.

Following clarification, a good method for obtaining significant purification with good yields is affinity chromatography, where the enzyme is selectively bound to a column on the basis of its affinity for a particular ligand, which is usually related to one of its substrates. In investigating affinity steps for DHlipDH, an ideal situation could be envisaged where the enzyme is separated from other proteins on the basis of its affinity for each of the physiological substrates NAD^+ and dihydrolipoamide (DHlip).

With this in mind, a series of triazene dye columns was surveyed for DHlipDH binding. These dyes are related to Cibachron Blue, which is known to bind NAD^+-dependent dehydrogenases (Thompson & Stellman, 1986). Blue B PGR-10 (from ICI) linked to Sepharose 4B was able to bind DHlipDH, and the enzyme could be specifically eluted with 3 mM NAD^+. This specific elution was very dependent upon pH, and only occurred over a narrow range of approximately 0.2 pH units. It therefore proved difficult to reproduce consistently. Elution with a 0-1 M NaCl gradient gave a 10-fold purification, which was not considered adequate for a first affinity chromatography step.

Separation based upon the affinity of DHlipDH for its lipoyl substrate has been used by Schmincke-Ott & Bisswanger (1981) and Richarme (1989), with the latter author obtaining a 50-fold purification. Employing the method of Richarme (1989), lipoic acid was coupled to EAB Sepharose (Pharmacia) by carbodiimide coupling. *T.b.brucei* DHlipDH was adsorbed to the column at pH 7.8, and eluted with a 0-0.5 M NaCl gradient (elution was unsuccessful with any combination of substrates and products). A 7.5-fold purification of enzyme was obtained, with a 70 % yield. Once again this level of purification, together with the yield of enzyme, made this unacceptable as an initial affinity step.

One further method used by many workers to purify DHlipDH has been calcium phosphate-based column chromatography with calcium phosphate-cellulose (Schmincke-Ott & Bisswanger, 1981; Koike & Hayakawa, 1970; Komuniecki & Saz, 1979) or hydroxyl apatite (Dietrichs & Andreesen, 1990). Hydroxyl apatite chromatography of partially

purified *T.b.brucei* DHlipDH gave a broad elution peak with a 50 mM to 0.5 M potassium phosphate (pH 7.0) gradient. The enzyme was purified 1.5-fold, with a 49 % recovery.

With both affinity steps and hydroxyl apatite giving poor results, a heat treatment similar to those of Komuniecki & Saz (1979) and Oppermann *et al.* (1991) was attempted. This had not been initially favoured because of the possibility that DHlipDH may be structurally altered by incubation at high temperatures.

An investigation into the heat stability of DHlipDH from *T.b.brucei* is detailed later in section 6.2.3. When heating at 75°C for 10 min (followed by centrifugation at 8,500 x g for 10 min) after initial Triton X-114 phase partitioning, a 36.5-fold average purification was obtained for the heat treatment alone. With yield being a major consideration, the observed 100 % yield meant that this step was extremely useful.

To give further separation, a Mono P chromatofocussing step, using Fast Protein Liquid Chromatography apparatus (FPLC; Pharmacia-LKB Biotechnology, Milton Keynes, U.K.), was added after heat treatment. This enabled separation of proteins on the basis of their isoelectric points. A final gel filtration step was added for further purification and removal of the polybuffer, which was used to form the pH gradient in the Mono P column. Superdex 200 gel filtration on the FPLC was chosen both for rapidity and resolution.

6.2.2 Final purification procedure for DHlipDH

Frozen cell pellets of bloodstream form *T.b.brucei* were thawed and resuspended in 25 mM bis-Tris-HCl, pH 7.1. Suspensions were sonicated at 40 W for 3 x 30 s with a 3 mm probe, and the cell-free extracts subjected to Triton X-114 phase partition as described elsewhere (section 3.2.3), with the exception that the Tris buffer used previously was replaced by bis-Tris in this procedure.

The aqueous phase from Triton X-114 phase partitioning was heated at 75°C for 10 min, and aggregated material was removed by centrifugation (8,500 x g for 10 min). The sample was filtered (0.22 µm pore Durapore filter) and applied to a Mono P chromatofocussing column (linked to an FPLC apparatus) pre-equilibrated with 25 mM bis-Tris-HCl, pH 7.1. The extract was applied at a flow rate of 0.4 ml/min, and was followed by 3 ml of the bis-Tris buffer. Polybuffer 74, pH 5 (adjusted with HCl) was then introduced into the column, and this generated a pH gradient from pH 7.1 to pH 5 over a total volume of 26 ml. Peak fractions containing DHlipDH were pooled and passed through a prep grade Hi Load 16/60 Superdex 200 gel filtration column (attached to the FPLC) at a flow rate of 1 ml/min. The column had been pre-equilibrated with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA or 5 mM sodium phosphate, pH 8.0, the latter being used at a low concentration in preparation for N-terminal sequencing.

A calibration curve for gel filtration was constructed from two separate experiments. The first contained Blue Dextran & 2,4-DNP-L-lysine, to determine the excluded and

included volumes of the column, respectively. The second run included the enzymes catalase, lactate dehydrogenase, citrate synthase and malate dehydrogenase as molecular weight markers. Certain of the enzymes appeared to bind to Blue Dextran, yielding anomalous results, and therefore the enzymes were run separately from the volume markers.

The presence of catalase, lactate dehydrogenase, citrate synthase and malate dehydrogenase were confirmed by their relevant assays (section 2.2.6).

The purity of the DHlipDH was assessed following gel filtration by SDS-PAGE using an 8-25 % gradient gel with a Pharmacia PhastSystem (section 2.2.7).

The results of a typical purification procedure are displayed in Table 6.1.

Due to the high turbidity of the sonic extract, protein content and DHlipDH activity were probably underestimated. Therefore, the activity in the aqueous phase of the Triton X-114 extract was taken to be 100 % for the purpose of yield.

A typical elution profile for the Mono P column is displayed in Fig. 6.1. The protein content of the eluent from the column could not be measured accurately due to the presence of polybuffer, which absorbs at 280 nm. DHlipDH eluted from the Mono P column at pH 6.3. Because enzymes are eluted from the column when the pH of the buffer reaches their isoelectric points, pH 6.3 is the isoelectric point of the *T.b.brucei* DHlipDH.

An example of an elution profile from the Superdex 200 column is shown in Fig. 6.2. In this experiment, DHlipDH eluted in a total volume of approximately 12 ml, at a peak of 67.5 ± 1.0 ml. The column was calibrated with protein and chemical markers in order to calculate the native molecular weight for DHlipDH. Kd values for each protein were calculated using the following equation:

$$Kd = (V_e - V_0) / (V_{re} - V_0)$$

where Kd is the distribution coefficient, V_e is the elution volume of the protein, V_0 is the excluded volume, as determined by the elution volume for Blue Dextran (44.3 ml), and V_{re} is the included volume, as determined by the elution volume for DNP-lys (131.5 ml). Fig. 6.3 is a plot of log M_r vs Kd for the four enzymes. A standard curve has been fitted to these points by linear regression. From this graph, the experimental Kd value for DHlipDH corresponded to a native molecular weight of approximately 107 ± 25 kDa (the error from the DHlipDH experiments having been combined with that from the standard curve).

Assessment of protein purity by SDS-PAGE gave a single band after silver staining. Using molecular weight markers, a graph of log M_r vs distance migrated was plotted (Fig. 6.4), and from this, the sub-unit molecular weight of DHlipDH was calculated to be 53 ± 1.7 kDa. This result, together with that of the gel filtration, indicates that the enzyme has 2 (± 0.5) sub-units, and therefore appears to be a dimer of similar M_r sub-units of approx. 53 kDa.

Table 6.1. Purification of dihydrolipoamide dehydrogenase (DHlipDH) from the bloodstream form of *T.b.brucei*

Fraction	Protein (mg)	Activity (Units)	Spec.Act. (Units/mg)	Yield (%)
Sonic extract*	26.9	0.756	0.028	-
Phase partitioning	23.2	0.943	0.041	100
Heat-treatment	0.58	0.807	1.39	85.6
Chromatofocussing	-	0.731	-	77.5
Gel filtration	-	0.326	-	34.6

*Both protein content and DHlipDH activity were probably underestimated in the sonic extract due to its extreme turbidity.

The full purification procedure is described in section 6.2.2. Phase partitioning was carried out with Triton X-114, with DHlipDH partitioning solely into the aqueous phase. A Mono P column (attached to an FPLC apparatus) was used for chromatofocussing. The polybuffer used to create a pH gradient absorbs at 280 nm, and therefore an accurate determination of protein content was not possible at this stage. Gel filtration was carried out with a HiLoad 16/60 Superdex 200 column (attached to an FPLC apparatus). The protein content of the DHlipDH-containing eluent was too low to be measured accurately.

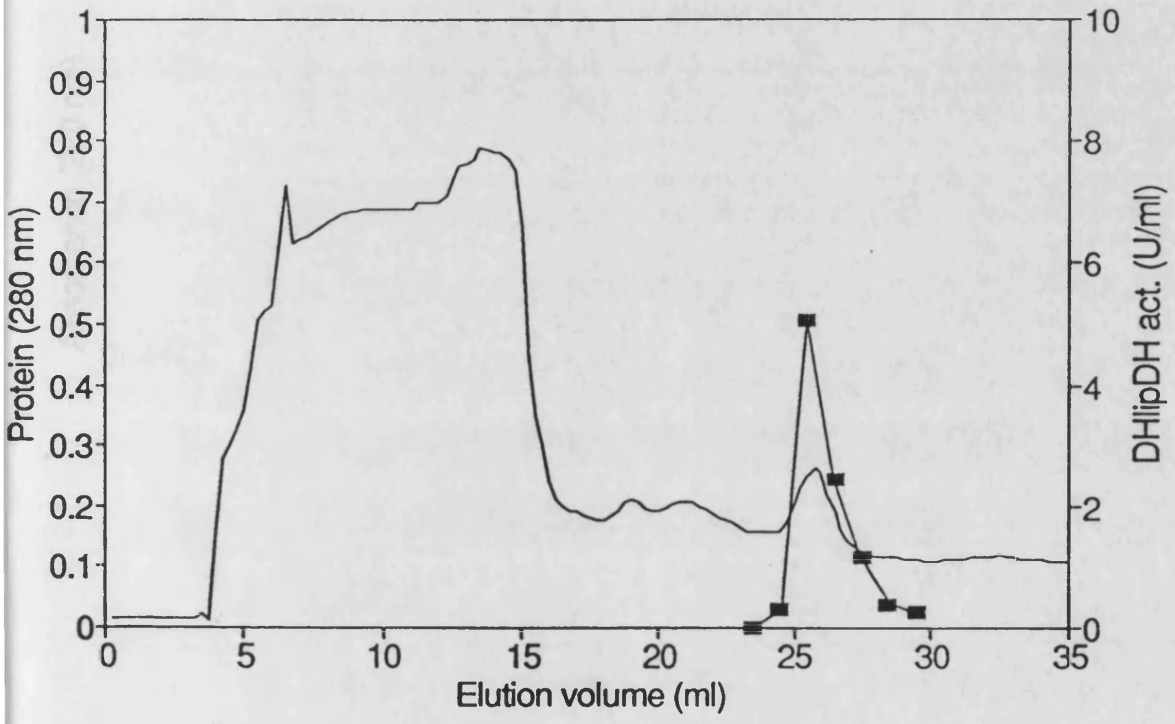


Fig. 6.1. Typical elution profile for protein and DHlipDH of bloodstream form *T.b.brucei* from a Mono P chromatofocussing column (attached to FPLC apparatus). Triton-partitioned and heat-treated cell-free extract of the bloodstream form of *T.b.brucei* was applied to the column in 25 mM bis-Tris-HCl, pH 7.1 at a flow rate of 0.4 ml/min. Polybuffer 74, pH 5, was introduced to the column, generating a pH gradient from pH 7.1 to pH 5 over a total volume of 26 ml. The protein content of the eluent (–) could not be accurately assessed because of the presence of polybuffer, which absorbs at 280 nm. The peak of DHlipDH activity (■) was at pH 6.3.

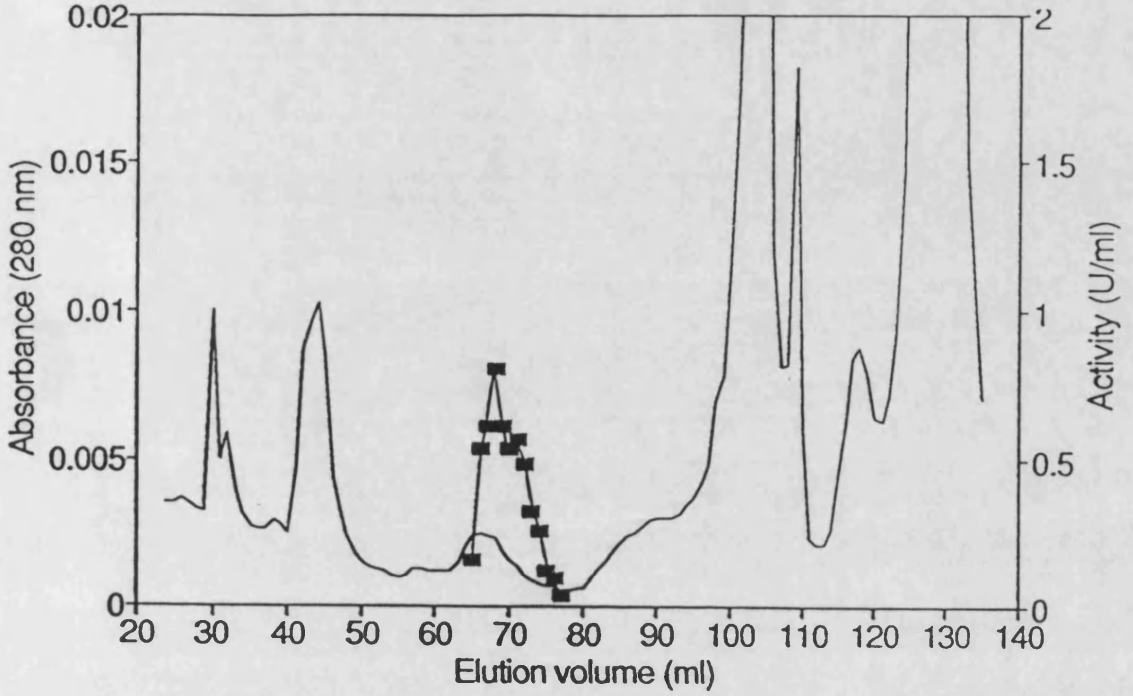


Fig. 6.2. Typical elution profile for protein and DHlipDH of bloodstream form *T.b.brucei* from a Superdex 200 gel filtration column (attached to FPLC apparatus). Cell-free extracts were subjected to Triton X-114 phase partitioning, heat treatment and Mono P chromatofocussing prior to gel filtration. The column was pre-equilibrated with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and the sample was loaded in polybuffer at a flow rate of 1 ml/min. Protein (–) content of the eluent was assessed by measuring A_{280}. DHlipDH activity (■) was measured using the standard assay.

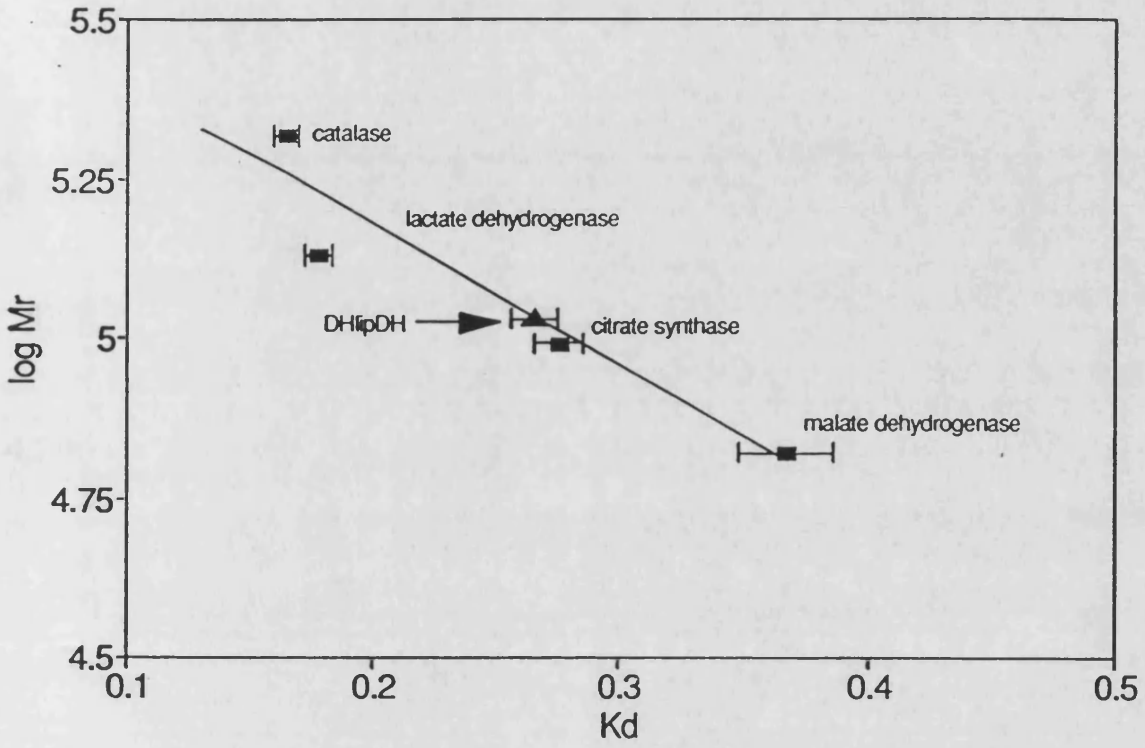


Fig. 6.3. Calibration curve for gel filtration on a Superdex 200 column (attached to an FPLC apparatus). The distribution coefficients, Kd, for each enzyme have been calculated from elution volumes according to section 6.2.2, and a standard curve has been fitted to the data points by linear regression. The data point for the *T.b.brucei* bloodstream form DHlipDH is the average of 6 separate experiments, and yields a native molecular weight of 107 (± 25) kDa.

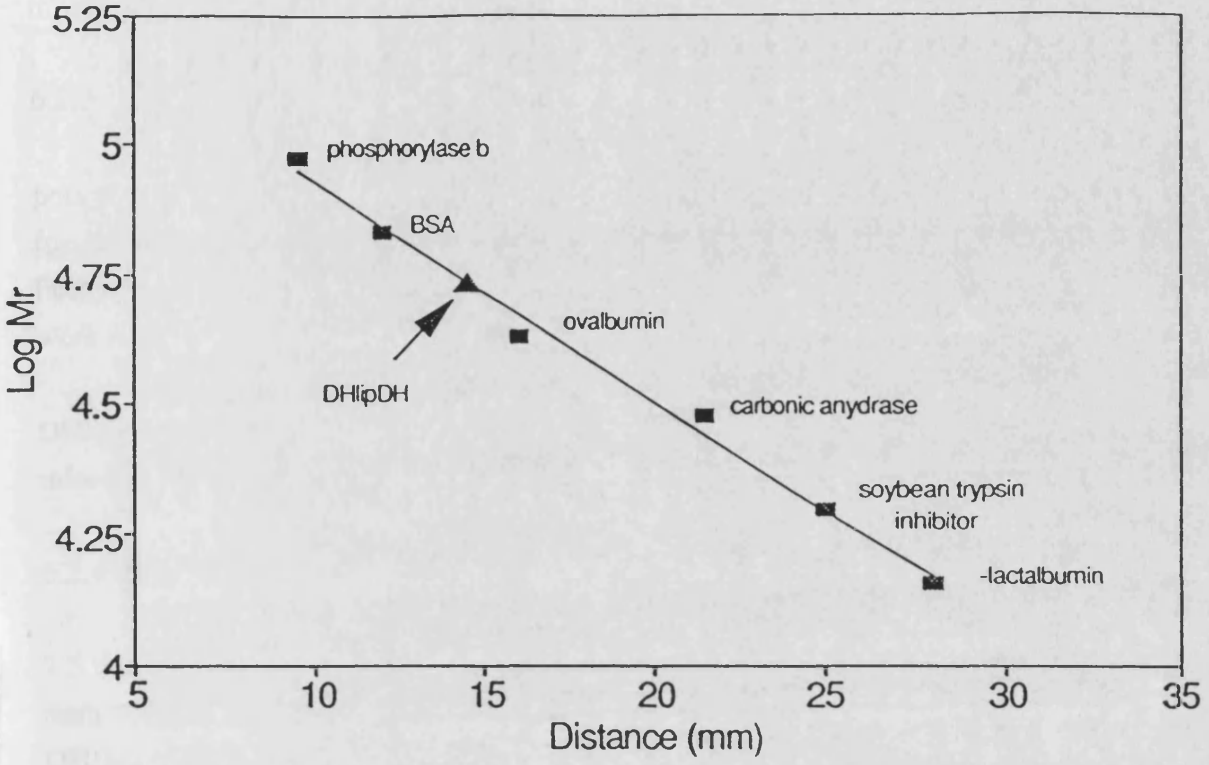


Fig. 6.4. Calibration curve for SDS-PAGE on an 8-25 % gradient gel using the Pharmacia PhastSystem. A standard curve has been fitted to the data points by linear regression, and this graph yields a sub-unit molecular weight of 53 (± 1.3) kDa for the *T.b.brucei* bloodstream form DHlipDH.

A total of 5.1 units of enzyme were produced for N-terminal sequencing. The eluent from the Superdex column (in sodium phosphate buffer) was concentrated to a volume of 1 ml, which was freeze-dried, and sent for N-terminal sequencing. Unfortunately no sequence information was obtained.

6.2.3 Heat stability

Bloodstream forms of *T.b.brucei* were sonicated at 40 W for 3 x 30 s in 50 mM potassium phosphate, 1 mM EDTA, pH 8.0. Samples of 200 µl of sonic extracts were heated for 10 min time periods at temperatures up to 100°C, using a Perkin-Elmer thermal cycler. Following heating, samples were centrifuged (8,500 x g for 10 min), and the supernatants were assayed for protein content and DHlipDH activity.

The results of the heat inactivation experiment are displayed in table 6.2. The DHlipDH is stable up to 70°C, with a small decrease in activity when the temperature is raised to 80°C, followed by a sharp decline to no activity at 90°C.

6.2.4 pH Profile

The activity of trypanosomal DHlipDH over a range of pH values from pH 6.5 to pH 8.5 was measured using 50 mM potassium phosphate for pH values up to pH 8.0, and 50 mM tetra-sodium pyrophosphate for pH 8.5. The concentrations of substrates used were 1.8 mM DHlip and 3.3 mM NAD^+.

The activity of trypanosomal DHlipDH over this pH range is displayed in Fig. 6.5. Activities were measured in duplicate, with maximal activity being observed at pH 8.0. The standard assay for DHlipDH is conducted at pH 7.0, at which the enzyme activity is only 66 % of that at pH 8.0. The calculated pH optimum can only be considered an operational one as two different buffers have been used, which possess different ionic strengths.

6.2.5 Kinetic analysis

Kinetic analysis of DHlipDH was carried out at 37°C, which is the ambient temperature of the trypanosome in the host bloodstream. The buffer used was 50 mM potassium phosphate, 2 mM EDTA, pH 8.0. A grid was constructed of 8 different concentrations of DHlip and 9 concentrations of NAD^+. Initial reaction rates were determined at each combination of substrate concentration, and kinetic plots were used to determine the kinetic parameters. All assays were performed in duplicate.

The kinetic data for DHlipDH in cell-free extracts are displayed as half-reciprocal plots, with the concentration of NAD^+ being varied at 8 different concentrations of DHlip (Fig. 6.6a & b). The data are displayed on two graphs for ease of presentation, and show that the enzyme follows Michaelis-Menten kinetics. All kinetic parameters have been determined by the direct linear plot of Eisenthal & Cornish-Bowden (1974). When data were plotted for

Table 6.2. Heat-treatment of dihydrolipoamide dehydrogenase (DHlipDH) in cell-free-free extracts of bloodstream form *T.b.brucei*.

Temp. °C	DHlipDH Activity U/ml	Protein µg/ml	Spec.Act.of DHlipDH U/mg
4	0.181	1760	0.103
50	0.196	1110	0.176
60	0.202	340	0.593
70	0.198	150	1.32
80	0.166	145	1.14
90	0	145	0
100	0	160	0

Protein content and DHlipDH activity have been measured following centrifugation of heated extracts at 8,500 x g for 10 min. Further details of the method and results are presented in section 6.2.3.

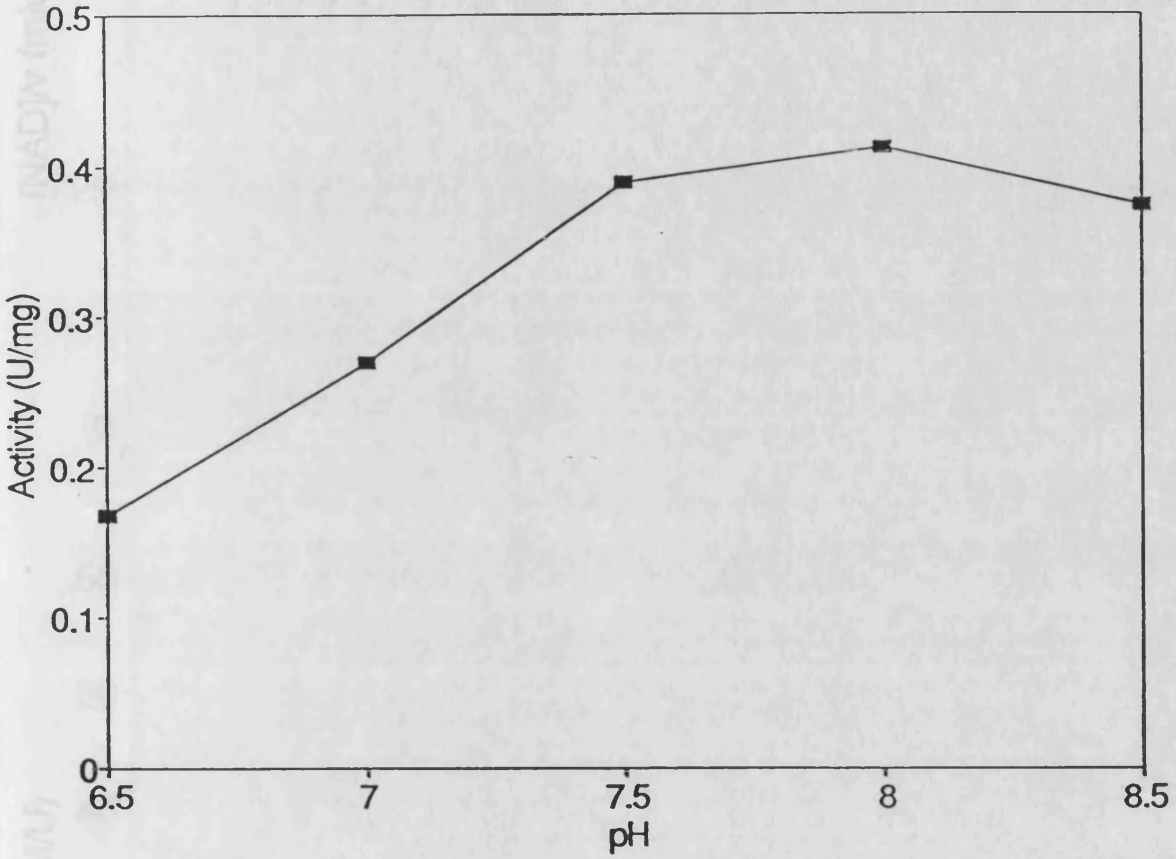


Fig. 6.5. pH Profile for DHlipDH from the bloodstream form of T.b.brucei. Each assay was conducted at 30°C in 50 mM potassium phosphate (for pH 6.5 to pH 8.0), or 50 mM tetra-sodium pyrophosphate (for pH 8.5), containing 1.8 mM dihydrolipoamide and 3.3 mM NAD^+. The data points are the mean of two experiments.

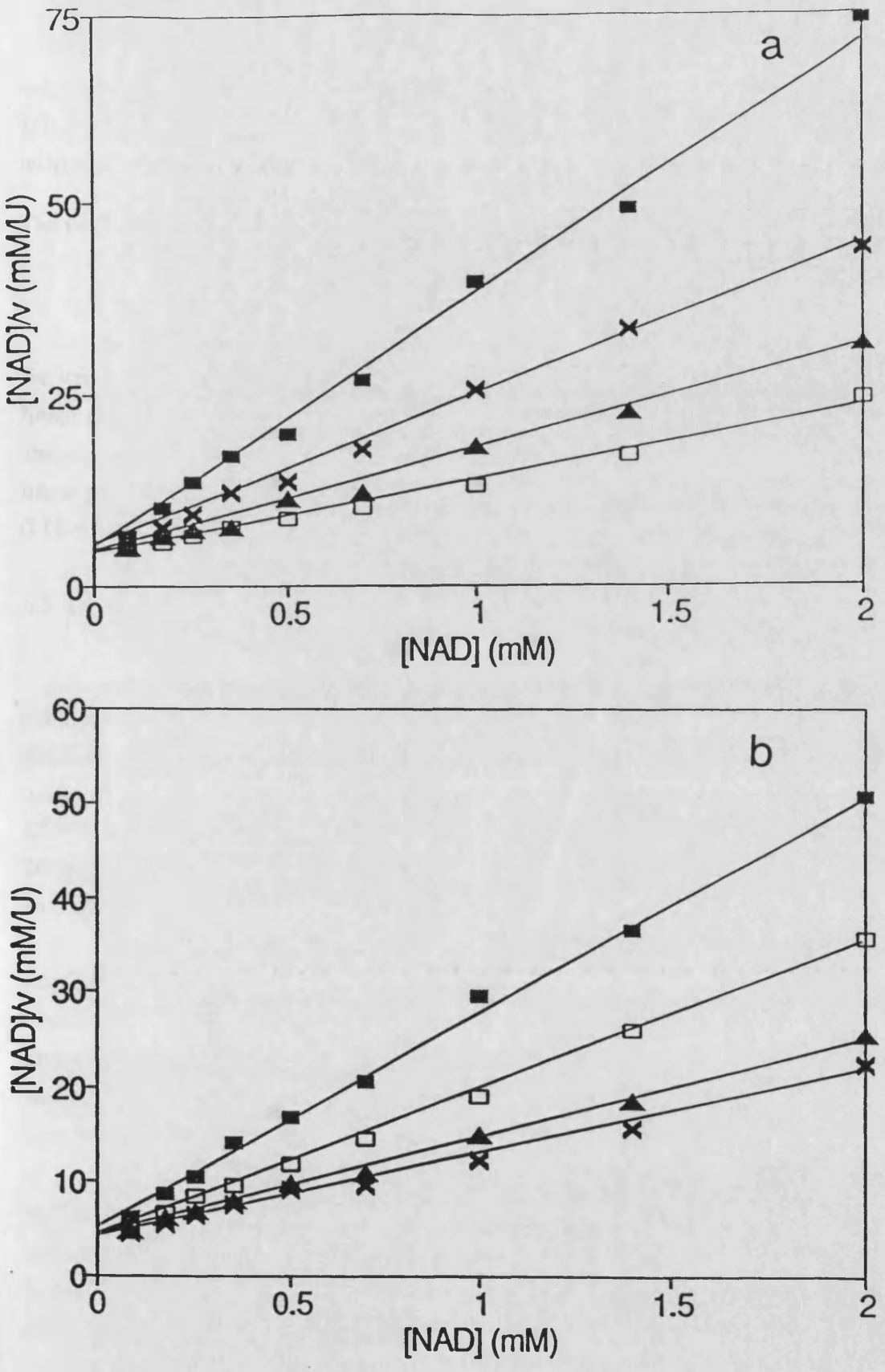


Fig. 6.6. Half-reciprocal (Hanes-Woolf) plots for the activity of DHlipDH from the bloodstream form of *T.b.brucei*, in the presence of varying concentrations of NAD^+, at 8 different concentrations of dihydrolipoamide. DHlipDH was assayed at 37°C in 50 mM potassium phosphate, pH 7.0, 2 mM EDTA, with the reaction being monitored by the increase in A_{340}. The data are displayed on two graphs (a and b) for ease of presentation. The concentrations of dihydrolipoamide are : (a) 0.09 mM (■), 0.18 mM (×), 0.36 mM (▲), and 0.72 mM (□); (b) 0.14 mM (■), 0.27 mM (□), 0.54 mM (▲), and 1.0 mM (×). Data are from duplicate experiments.

varying [NAD^+] or [DHlip], both graphs gave patterns of lines intersecting on the vertical axis, which is indicative of a ping-pong mechanism where Ks^A is zero and V^{app}/Km^{app} is independent of the concentration of fixed substrate (Wharton & Eisenthal, 1981).

The rate equation for this mechanism is : $$v_0 = \frac{V}{1 + \frac{Km^A}{(A)} + \frac{Km^B}{(B)}}$$

The slopes at each concentration of DHlip are equal to $1/V^{app}$, and the intercept on the vertical axis is equal to Km^B/V. A secondary plot of $[DHlip]/V^{app}$ against [DHlip] is linear (Fig. 6.7) with a gradient of 6.33 min, which is equal to 1/V. The intercept on the abscissa of -0.39 mM is equal to $-Km^A$. The calculated kinetic constants from the direct linear plot are :- Km^A (DHlip) = 0.39 ± 0.03 mM; Km^B (NAD^+) = 0.63 ± 0.01 mM; V = 0.16 ± 0.09 $\mu mol.min^{-1}$.

6.3 Discussion

The purification of DHlipDH from bloodstream forms of *T.b.brucei* has been achieved with the high yield of enzyme (34.6 %) that was needed to provide enough protein for N-terminal sequencing. Similarly high yields have been obtained by other groups working with DHlipDH, including Schmincke-Ott and Bisswanger (1981), who recovered 35.3 % of enzyme activity from *E.coli*, and Lohrer and Krauth-Siegel (1990) who recovered 26 % from *T.cruzi*. Presumably, this high level of recovery of enzyme from several species is an indication of the stability of DHlipDH throughout various purification procedures.

The procedure which has been developed in this work is extremely rapid, being capable of completion in 8-10 hours. The major reason for this short timescale is the implementation of the FPLC apparatus, through the use of which both the Mono P and Superdex 200 separation steps can be completed in less than 3 h. The Superdex gel filtration step is also very useful because it enables the final protein to be produced in whichever buffer may be required for subsequent experiments.

The failure of the N-terminal sequencing of DHlipDH has a number of possible explanations. Insufficient amounts of protein or too high a concentration of buffer salts could give unclear results. Alternatively, the N-terminus of the protein could be blocked. Up to 50 % of all proteins may be N-terminally blocked by formyl, acetyl or other acyl groupings or conversion to a pyroglutamyl residue (Aitken *et al.*, 1989). Chemical unblocking can occasionally be successful, but it is more usual to form large peptide fragments by cleaving with cyanogen bromide, for example, and then separating these fragments for sequencing (Aitken *et al.*, 1989). Electroblotting onto Polyvinylidene difluoride membranes enables accurate and effecient sequencing, and can be used in future for the trypanosomal DHlipDH (Wilson & Yuan, 1989).

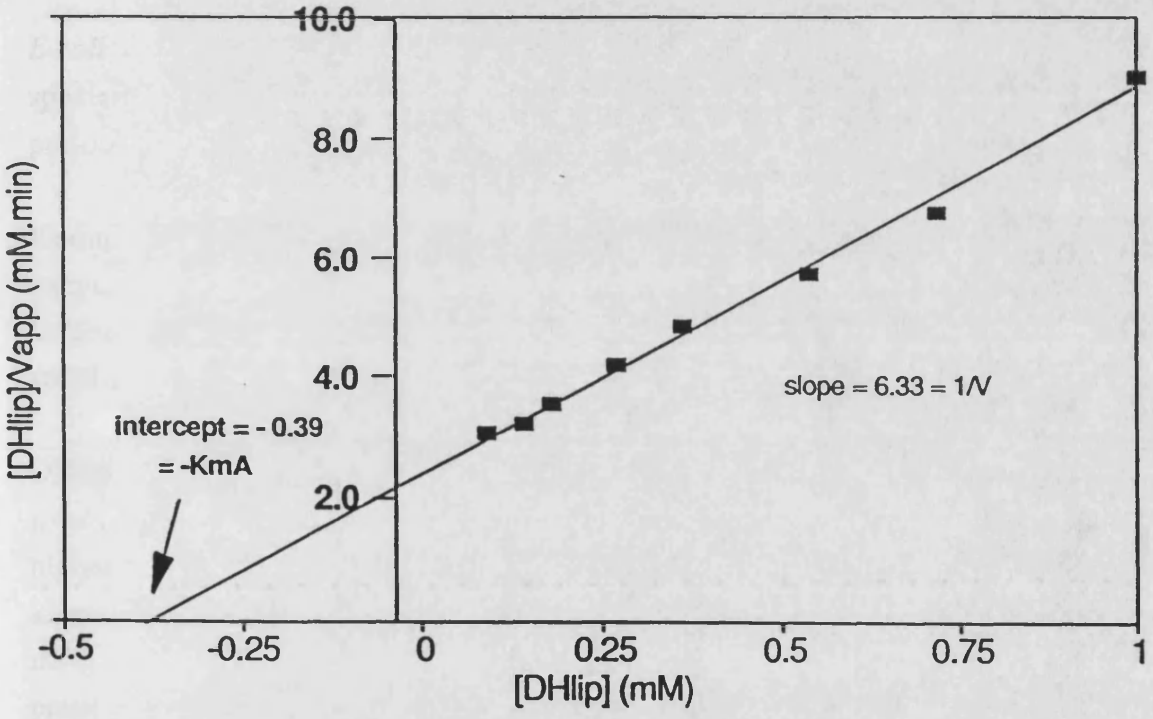


Fig. 6.7. Secondary plot to determine the kinetic parameters of DHlipDH from the bloodstream form of T.b.brucei. Vapp for each concentration of dihydrolipoamide has been calculated from a direct linear plot of the relevant data in Fig. 6.6. Km^A (dihydrolipoamide) = 0.39 (± 0.03) mM; Km^B (NAD+) = 0.63 (± 0.01) mM; V = 0.16 (± 0.09) mM.

To enable the comparison of the properties of *T.b.brucei* DHlipDH with those of a number of different species, table 6.3 displays several important properties of these enzymes taken from other published data.

The sub-unit and native molecular weights of the *T.b.brucei* enzyme are almost identical to those of the other DHlipDHs. The only known exception to this 50 kDa homodimer structure is the abnormally small DHlipDH of *E.acidaminophilum* (35.5 kDa per S.U.), which behaves more like thioredoxin reductase, with a subsidiary low DHlipDH activity (Freudenberg & Andreesen, 1989; Dietrichs *et al.*, 1990).

An isoelectric point of 6.3 for *T.b.brucei* DHlipDH is similar to those of other cytosolic enzymes of *T.b.brucei* and other unicellular organisms, and lower than the 8.8-10.2 range of the glycosomal glycolytic enzymes (Opperdoes, 1987).

A pH optimum of 8.0 for the *T.b.brucei* DHlipDH is identical to that obtained for the *E.coli* K12 enzyme by Schmincke-Ott & Bisswanger (1981), and similar to values from other species. The pH optimum is 0.5 to 1.0 units lower than those of the DHlipDHs from the purinolytic anaerobic bacteria (Dietrichs & Andreesen, 1990).

The stability of DHlipDH at high temperatures has been used very effectively by Komuniecki & Saz (1979) to purify the *Ascaris* muscle enzyme. They recovered 100 % of enzyme activity after heating at 70°C for 15 min. Similarly, Oppermann *et al.* (1991) retained 91 % of activity after heating at 75°C for 10 min. Therefore, the very high heat stability of the *T.b.brucei* enzyme is in keeping with its stability in other species.

Kinetic analysis of the trypanosomal DHlipDH has yielded kinetic constants for DHlip and NAD^+ which are similar to those of *E.coli* K12, *S.cerevisiae*, pig heart and rat liver (see table 6.3), with these being in the millimolar range, and the Km for NAD^+ being higher than that for DHlip (the only exception to this being the pig heart enzyme). Initial kinetic data, with half-reciprocal plots intersecting at the y-axis, are consistent with a ping-pong mechanism for the action of the enzyme. A bi-bi ping-pong mechanism was initially proposed for DHlipDH by Massey *et al.* (1960), and has been confirmed for the pig heart enzyme by Koike & Koike (1976). Therefore, in this respect, the *T.b.brucei* DHlipDH is similar to those from other species.

In summary, all of the data concerning the size, pH profile, heat stability and kinetics of bloodstream form *T.b.brucei* DHlipDH indicate that it is a normal DHlipDH, despite the fact that it may be performing a novel role in metabolic processes in the bloodstream trypomastigote.

Table 6.3. Comparison of some properties of dihydrolipoamide dehydrogenase from different organisms

Organism	M_r(S.U.)	pH_{opt}	Km(DHlip) (mM)	Km(NAD^+) (mM)
E.coli K12[a]	56	8.0	0.28	1.83
S.cerevisiae[bc]	51.5	n.d.	0.4	0.7
Pig Heart[de]	50	7.9	0.3	0.2
Rat liver[f]	56	8.0	0.49	0.52
T.cruzi[g]	55	n.d.	n.d.	n.d.
T.b.brucei	53	8.0	0.39	0.63

[a]Schmincke-Ott & Bisswanger (1981); [b]Browning *et al.* (1988); [c]Wren & Massey (1966); [d]Williams (1976); [e]Otulakowski & Robinson (1987); [f]Reed (1973); [g]Lohrer & Krauth-Siegel (1990).

CHAPTER 7 : INACTIVATION STUDIES USING ARSENICAL REAGENTS

7.1 Introduction

Compounds containing arsenic have been used in Oriental medicine for two to three thousand years, and more recently to treat almost every kind of disease (Webb, 1966).

Arsenite has long been known to have some effect on trypanosomiasis, but for many years its high toxicity precluded its use. It was the work of Thomas and Breinl in 1905 which renewed interest in the arsenical treatment of trypanosomiasis. They discovered that atoxyl (sodium arsanilate) was an effective treatment for murine trypanosomiasis. Ehrlich went on to show that pentavalent arsenicals such as atoxyl, must be broken down to their trivalent forms in order to be active, and he postulated the presence of arsenoreceptors to explain the actions of these compounds. This began the search for the mode of action of arsenicals (see Webb, 1966).

Voegtlin *et al.* (1923) demonstrated that the effect of oxophenarsine on trypanosomal motility could be antagonized by various thiols. Trivalent arsenical derivatives were shown to form stable complexes with enzymes, coenzymes or other compounds containing two or more proximal thiol groups (Johnstone, 1963), and this led to the theory that arsenicals might interact with cellular thiols and thereby interrupt oxidative processes. Cellular thiols were soon to be considered the major site of arsenical attack (Webb, 1966).

Further metabolic studies by Krebs (see Johnstone, 1963) showed that arsenite specifically blocks the oxidation of 2-oxo acids. Peters then demonstrated the extreme sensitivity of pyruvate oxidase to arsenicals and suggested this to be due to the presence of the dithiol, lipoic acid (Peters *et al.*, 1946).

Two components of the pyruvate dehydrogenase complex possess vicinal thiols: the transacetylase component, E2, through its attached lipoic acid residue, and dihydrolipoamide dehydrogenase (DHlipDH), E3, which has an active site dithiol (see section 1.4.1). Both the dithiol of DHlipDH (Massey & Veeger, 1960) and the lipoyl dithiol (Stevenson *et al.*, 1978) can be modified by arsenicals. Stevenson *et al.* (1978) showed that in the presence of pyruvate and Coenzyme A, a multifunctional arsenoxide formed a stable 6-membered cyclic dithiolarsinite with the substrate-generated dihydrolipoyl domain of E2. The modified lipoyl residue can also pass the arsenical to the active site dithiol of E3 (Adamson & Stevenson, 1981).

For many years, the main arsenical treatment for trypanosomiasis was tryparsamide (Thomson, 1989), but resistance to this drug, especially in populations of *T.b.gambiense*, prompted the introduction of melarsen, melaminyl (2,4,6-triamino-S-triazinyl)phenylarsonate, which was first developed by Friedheim (1939). Melarsen itself was more toxic than tryparsamide, but its conjugate with dimercaprol, melarsoprol, proved to have a much lower toxicity, and was of particular value for the treatment of late-stage

sleeping sickness (Newton, 1963). The active component of melarsoprol, melarsen oxide (Mel O), is extremely toxic to the mammalian bloodstream form of *T.b.brucei*, causing rapid loss of motility and subsequent cell lysis. For many years, pyruvate kinase was considered a major site of arsenical action in trypanosomes, with the evidence for this largely based upon the accumulation of its substrate, phosphoenolpyruvate, in extracts treated with arsenical, along with simple kinetic studies which demonstrated enzyme inactivation (Flynn & Bowman, 1974). Closer investigation by Van Schaftingen *et al.* (1987) revealed that the inhibition of pyruvate kinase by melarsen oxide was rather poor (Ki > 100 μM), and that fructose 2,6-bisphosphate ($Fru(2,6)P_2$), a potent stimulator of pyruvate kinase, was depleted in arsenical-treated cells. Investigation of 6-phosphofructo-2-kinase (PFK2), the enzyme responsible for production of $Fru(2,6)P_2$, showed that it was potently inhibited by Mel O (Ki < 1 μM). Fructose-2,6-bisphosphatase (Ki = 2 μM) was also much more potently inhibited than pyruvate kinase (Van Schaftingen *et al.*, 1987). Although these workers have used the word inhibition, the effect of Mel O on these enzymes may, more accurately, be termed inactivation.

This work established the point at which Mel O interrupted glycolytic flux, namely at PFK2, but there were several arguments against this being the mechanism for the trypanocidal action of Mel O. Firstly, the trypanocidal action of Mel O was almost fully expressed after 5 min, after which time the level of $Fru(2,6)P_2$ had not changed significantly. Furthermore, susceptibility to Mel O was not changed when cells were grown on glycerol, a substrate which yielded no $Fru(2,6)P_2$. Lastly, trypanosomes can adapt to a 70 % decrease in ATP without any loss of viability when salicylhydroxamic acid is used to inhibit respiration. Therefore, to kill cells by depletion of ATP, melarsen oxide would have to inhibit glycolytic flux by more than 70 %. What is seen *in vivo* is that changes in glycolytic flux parallel cell lysis, indicating that pyruvate production is normal in those cells that remain intact (Van Schaftingen *et al.*, 1987). All of these results demonstrate that inhibition of glycolysis does not appear to be the cause, but rather the consequence, of cell lysis.

In the light of these results, the discovery of the glutathione analogue, trypanothione (see section 1.2.2.5), in trypanosomes by Fairlamb *et al.* (1989) brought cellular thiols back to the centre stage of investigations into the mode of action of arsenicals. Mel O forms a stable adduct, termed Mel T, with the dithiol form of trypanothione [dihydrotrypanothione, $Try(SH)_2$]. This adduct accumulates to levels approaching 17 μM (approximately 10 % of the intracellular $Try(SH)_2$) before cell lysis commences. It has been suggested that Mel T exerts a cytotoxic effect on the cell, which results either from sequestration of intracellular trypanothione or from the inhibition of trypanothione reductase (Ki = 9 μM). Studies using a combination of the ornithine decarboxylase inhibitor α-difluoromethylornithine (DFMO) and Mel O have demonstrated a pronounced synergism between the trypanocidal activities of the two drugs (Jennings, 1988). DFMO selectively blocks the biosynthesis of spermidine, a

precursor of trypanothione, in trypanosomes, and therefore a synergism between its action and that of Mel O, which sequesters trypanothione, lends further weight to the proposal that trypanothione is the primary target for arsenical drugs against African trypanosomes.

The absence of the 2-oxo acid dehydrogenase complexes from the bloodstream form of *T.b.brucei* precludes these most studied targets of arsenical inactivation from investigation in this organism, but the recent discovery of DHlipDH (Danson *et al.*, 1987) and lipoic acid (Jackman *et al.*, 1990) re-introduces these species as possible targets for arsenical action.

One aim of the work detailed in this chapter is to investigate DHlipDH as a target for trivalent arsenicals in the bloodstream form of *T.b.brucei* by looking at the inactivation of the enzyme with a range of arsenicals including melarsen oxide. The correlation of results on DHlipDH inactivation in cell-free extracts and whole cells with those for the immobilisation of cells will give some indication of the relevance of DHlipDH as a target for arsenicals in these organisms.

Another aim of these studies is to assess the feasibility of using trivalent arsenicals as tools for the elucidation of the function of DHlipDH *in vivo*. In this respect, phenylarsenoxide has been used extensively to probe the role of vicinal dithiols in mammalian glucose transport (Douen & Jones, 1986) and signal transmission from the insulin receptor (Bernier *et al.*, 1987). Were DHlipDH to be involved in a membrane transport or signal transduction mechanism, similar inactivation studies with arsenicals may reveal the link between the enzyme and such processes.

Inactivation studies have been used successfully by Richarme (1988) to demonstrate a relationship between lipoic acid, DHlipDH and the binding-protein-dependent transport of maltose and galactose in *Escherichia coli*. He used arsenite (Richarme, 1988) and 5-methoxyindole-2-carboxylic acid (a known inhibitor of 2-oxo acid dehydrogenases; Richarme, 1985) as inhibitory probes to prove the link between the enzyme and transport mechanisms. Because of the effectiveness of Richarme's studies, 5-methoxyindole-2-carboxylic acid has also been investigated as an inhibitor of DHlipDH in bloodstream form *T.b.brucei* as a prelude to functional studies.

7.2 Methods

All of the experiments described in this chapter were performed on the bloodstream form of *T.b.brucei*.

7.2.1 Preparation of stock solutions of arsenicals

Phenylarsenoxide (PAO) and Melarsen oxide (Mel O) were dissolved in dimethylformamide (DMF) to concentrations of up to 100 mM. *p*-Aminophenyldichloroarsine (APDCA) and *p*-acetylaminophenyldichloroarsine (AAPDCA) were dissolved to

similar concentrations in 95 % (v/v) ethanol. The structures of these arsenicals are displayed in Fig. 7.1.

The concentration of arsenical in each stock solution was determined by titration with dihydrolipoamide (DHlip). The arsenical and DHlip form a stable adduct in solution. Therefore, if arsenical is mixed with an excess of DHlip, the free DHlip can be titrated against DTNB as described in section 2.2.4. From the known concentration of DHlip in the stock solution, the concentration of arsenical in the mixture can be calculated.

7.2.2 Inactivation of DHlipDH in cell-free extracts

Cell-free extracts for inactivation studies with arsenicals were prepared by sonication of cells suspended in 50 mM KPO_4, pH 7.0, 2 mM EDTA. Sonication was carried out at 40 W for 3 x 30 s using a 3 mm probe. 500 µl diluted sonicate (1.5 mg protein/ ml) was incubated with 0.1 % (w/v) Triton X-100, 0.5 mM NADH and the appropriate concentration of arsenical at 30°C in a microfuge tube. The reaction was started by addition of arsenical, and 10 or 20 µl samples were taken at time points and assayed for DHlipDH activity (see section 2.2.6 for assay details).

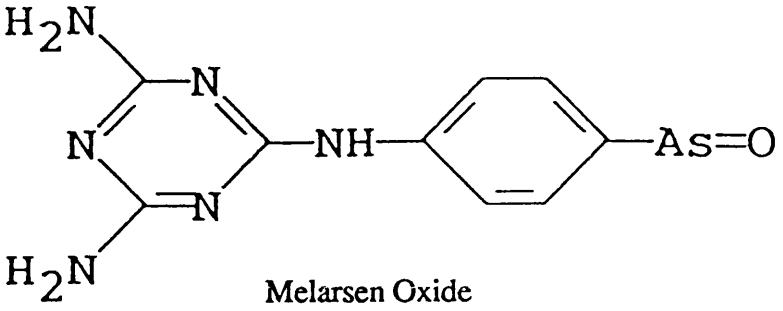
7.2.3 Inactivation of DHlipDH in whole, respiring cells

Live cells were pre-incubated at a concentration of 1 x 10^8 cells per ml in Krebs Ringer buffer containing glucose and sucrose (KRBG; see section 2.2.1) at 30°C for 5 min. This pre-incubation period was used to equilibrate the trypanosomal cells to the assay temperature of 30°C. The reaction was started by addition of arsenical, and 15 s before each time point, the reaction tube was centrifuged at 8,500 x g for 30 s in a bench microfuge. The buffer containing arsenical was removed with a Pasteur pipette, and 500 µl ice-cold KRBG added. The cell pellet was resuspended and sonicated for 30 s at 40 W using a 3 mm probe. A sample of 100 µl was removed for assay of DHlipDH. DHlipDH activity was calculated from the rate of reaction after 2 min incubation at 30°C.

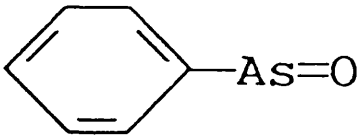
7.2.4 Immobilisation of whole respiring cells

Live cells were pre-equilibrated for 5 min at 30°C in KRBG at a concentration of 1 x 10^7 cells per ml. The appropriate volume of arsenical was added to start the reaction, and the suspension was mixed by vortexing. A sample of the reaction mixture was transferred to a haemocytometer, and the number of motile cells in a 4 x 4 grid was counted at each time point using a microscope. At this concentration of cells, approximately 40 were present in one grid, the counting of which took about 30 s. Counting was therefore begun 15 s before the time recorded. All cells that showed any sign of motility were counted as motile for the purpose of these experiments. From the total number of cells in the grid, the percentage of total cells that were motile was calculated.

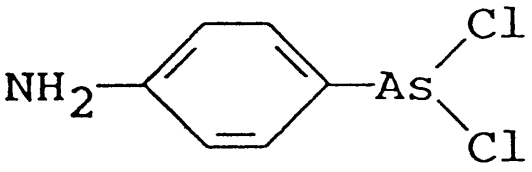
Fig. 7.1. Trivalent arsenical reagents used to inactivate DHlipDH



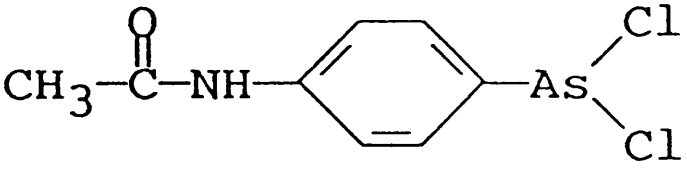
Melarsen Oxide



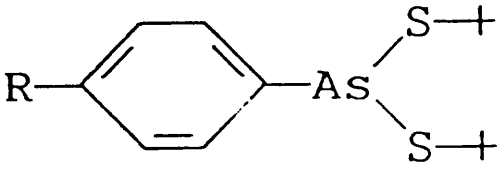
Phenylarsenoxide



p-Aminophenyldichloroarsine



p-Acetylaminophenyldichloroarsine



Arsenical reagent bound to protein sulphydryl residues

7.2.5 Inhibition of DHlipDH in cell-free extracts by 5-methoxyindole-2-carboxylic acid

The assay of DHlipDH in the presence of 5-methoxyindole-2-carboxylic acid (MICA) was carried out under standard assay conditions, except that, due to the high absorbance of MICA at 340 nm, NAD was replaced by its analogue 3-acetyl-NAD. The reaction was monitored at 366 nm instead of 340 nm.

7.3 Results

7.3.1 Inactivation of DHlipDH in cell-free extracts

In order to calculate the rate constants for inactivation of DHlipDH in cell-free extracts, the first-order rate equation was employed :

$$\ln C/C_0 = -kt$$

where C_0 is the DHlipDH activity at time 0, C is the activity at time t, and k is the pseudo-first-order rate constant for inactivation. C/C_0 is the percentage of initial activity at time t, so if log C/C_0 is plotted against t, the slope of the line is equal to -k/2.303, for each concentration of arsenical (or NADH). Assuming that the inactivation proceeds according to pseudo-first-order kinetics, k = k'(R), where k' is the true second-order rate constant and (R) is the concentration of arsenical. Therefore, k' is equal to the slope of a secondary plot of k vs (R).

DHlipDH was inactivated in cell-free extracts of *T.b.brucei* by all the arsenicals tested under the reaction conditions described. Control experiments with DMF and 95 % (v/v) ethanol indicated that these did not interfere with enzyme assays. Arsenical inactivation was dependent upon the presence of NADH in the reaction mixture, as demonstrated in Fig. 7.2. The inactivation rate constant in the presence of 1 mM APDCA alone was only 0.11 min^{-1}, but when 0.5 mM NADH was added, the inactivation rate constant increased to 1.5 min^{-1}. 0.5 mM NADH alone inactivated the enzyme with a rate constant of 0.074 min^{-1}.

When measuring the activity of DHlipDH in the presence of arsenical, a short lag phase of 2 min was observed in the assay prior to the constant increase in absorbance due to production of NADH. This lag period was not seen when no arsenical was present. It may reflect a partial re-activation of the enzyme by the substrate, dihydrolipoamide, removing the arsenical.

Another difference between the assay reaction profiles of DHlipDH in the presence and absence of arsenicals, was a gradual increase in the rate of reaction after approximately 5 min when arsenicals were added to extracts. The most probable explanation for this is that the trivalent arsenical forms a more stable adduct with DHlip than with DHlipDH. Therefore DHlip slowly removes the arsenical from the active site of DHlipDH during the assay.

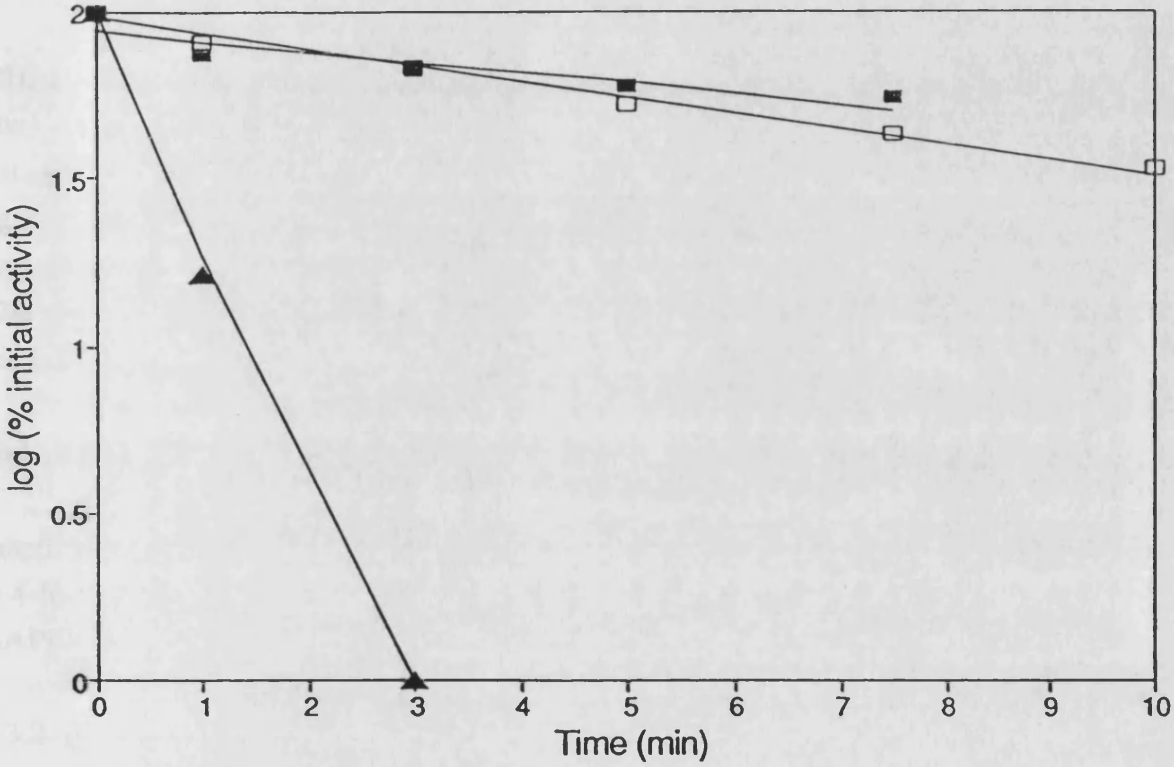


Fig. 7.2. Inactivation of DHlipDH in cell-free extracts of *T.b.brucei* by 0.5 mM NADH (■), 1 mM *p*-aminophenyldichloroarsine (APDCA;□), and 0.5 mM NADH and 1 mM APDCA (▲). Each experiment, carried out at 30°C, was started by adding NADH/APDCA to cell-free extract (1.5 mg protein/ml in 50 mM potassium phosphate, pH 7.0, 2 mM EDTA) and samples were taken at time points and assayed for DHlipDH activity.

Having commented on the two major sources of inaccuracy within measurements of DHlipDH activity, it is clear that the inactivation rate constants for the arsenicals are only an approximation to the true inactivation rate constants in the presence of each arsenical.

For the purpose of brevity, the full details of the inactivation results in this and both of the subsequent sections are only shown for PAO. Unless otherwise stated, the three other arsenicals behaved in exactly the same fashion as PAO, but obviously possessed different inactivation parameters.

Fig. 7.3 shows the effect of varying concentrations of PAO on the activity of DHlipDH in cell-free extracts. In each case, the rate of inactivation was approximately constant over the first 7.5 min of the reaction, so lines were fitted by linear regression to the data points up to this time. When the pseudo-first-order rate constants (k) were plotted against the concentration of PAO (Fig. 7.4), a linear relationship between the two parameters was obtained, indicating that inactivation was proportional to the amount of arsenical added. The slope of the line through these points is equal to the second-order rate constant (k') for the inactivation of DHlipDH. For PAO, this rate constant is 0.027 $\mu M^{-1}min^{-1}$.

The rate constants for each of the other arsenicals were calculated in the same way, and all four are displayed in Table 7.1.

The arsenical possessing the highest rate constant, and therefore the most potent inactivator of DHlipDH in cell-free extracts, was PAO. Its rate constant of 0.027 $\mu M^{-1}min^{-1}$ is 4-fold greater than that of APDCA, and 5-fold greater than that which was obtained for AAPDCA. Mel O was also approximately 5-fold less potent than PAO.

7.3.2 Inactivation of DHlipDH in whole cells

All four trivalent arsenicals were able to inactivate DHlipDH *in vivo* in whole respiring cells of *T.b.brucei*. Control experiments with DMF and 95 % (v/v) ethanol indicated that these solvents did not affect cell motility and viability. When log % initial activity of DHlipDH in whole cells was plotted against time, the rate of inactivation was not constant over the first 7.5 min. Therefore, in order to calculate the inactivation constants, % initial activity was plotted against time (Fig 7.5), and the time taken for 50 % inactivation of enzyme activity (t_{50}) was determined for each concentration of arsenical.

Although not as accurate as an initial inactivation rate, the t_{50} value does give some indication of how rapidly inactivation is occurring. Therefore, employing the first-order rate equation, ln $C/C_0 = -kt$; when $t = t_{50}$, $C/C_0 = 0.5$. Therefore ln $C/C_0 = -0.693$ and $0.693 = kt_{50}$. The second-order rate constant can be calculated from a secondary plot of $1/t_{50}$ vs arsenical concentration using the equation $0.693/t_{50} = k'(R)$. The value for PAO, calculated from Fig 7.6, is 3.6×10^{-3} $\mu M^{-1}.min^{-1}$.

When the inactivation of DHlipDH by Mel O was attempted over the same concentration range as for PAO, there was no linear relationship between $1/t_{50}$ and

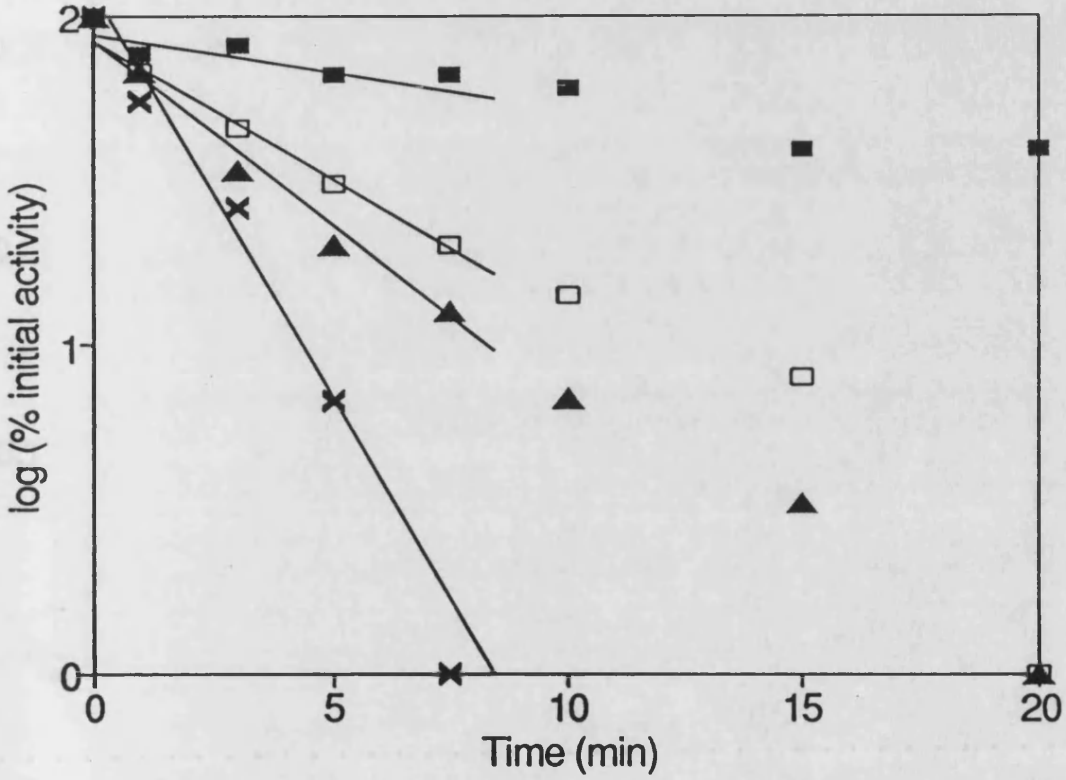


Fig. 7.3. Inactivation of DHlipDH in cell-free extracts of *T.b.brucei* by phenylarsenoxide (PAO). Cell-free extracts (1.5 mg protein/ml in 50 mM potassium phosphate, pH 7.0, 2 mM EDTA) were incubated at 30°C in the presence of 0.5 mM NADH alone (■), and 0.5 mM NADH together with 5 mM PAO (□), 10 mM PAO (▲) and 20 mM PAO (✕). Samples were taken at time points and assayed for DHlipDH activity. To determine the initial rates of inactivation, regression lines have been fitted to points up to 7.5 min of incubation.

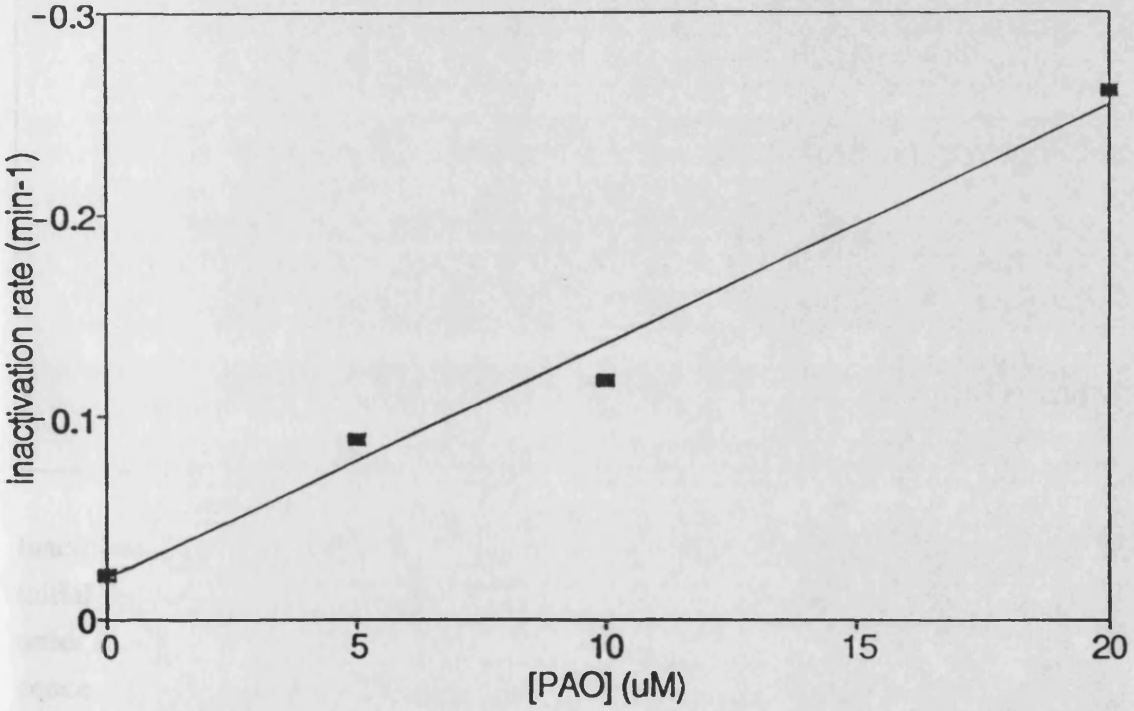


Fig. 7.4. Secondary plot for the inactivation of DHlipDH in cell-free extracts of T.b.brucei by phenylarsenoxide (PAO). Initial reaction rates have been determined from Fig. 7.3. A regression line has been fitted to the data points, yielding a second order rate constant of 0.027 μM-1min-1 for inactivation by PAO.

Table 7.1. Second-order rate constants for the inactivation of DHlipDH in cell-free extracts of bloodstream forms of *T.b.brucei* by a range of arsenical reagents

ARSENICAL	Rate Constant ($M^{-1}.min^{-1}$)
PAO	27×10^{-3}
APDCA	6.8×10^{-3}
AAPDCA	5.0×10^{-3}
Mel O	5.0×10^{-3}

Inactivation experiments were carried out according to section 7.2.2. For each arsenical, the initial rate of inactivation was measured over a range of concentrations, and the second-order rate constant was determined from a secondary plot of inactivation rate vs. concentration of arsenical (see Figs. 7.3 & 7.4 for PAO). Abbreviations: PAO, phenylarsenoxide; APDCA, *p*-aminophenyldichloroarsine; AAPDCA, *p*-acetylaminophenyl-dichloroarsine; Mel O, melarsen oxide.

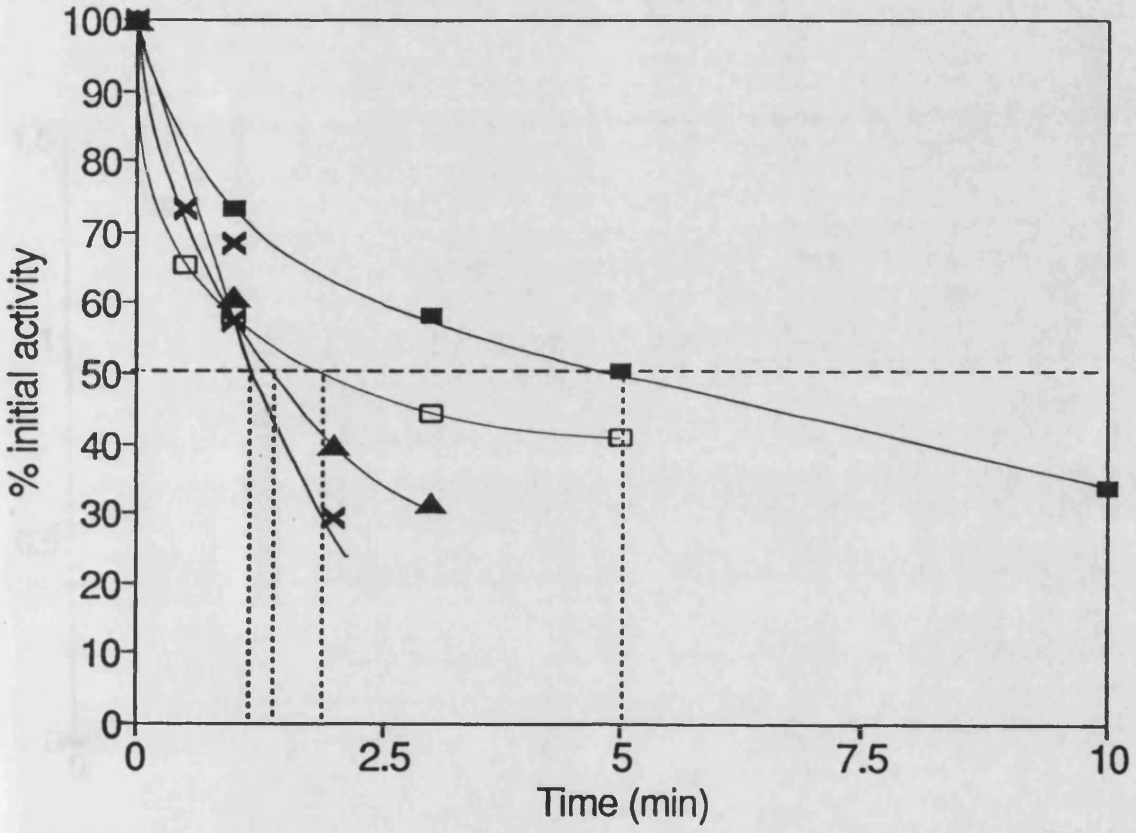


Fig. 7.5. Inactivation of DHlipDH in whole cells of *T.b.brucei* by phenylarsenoxide (PAO). Live cells (10^8 cells/ml in Krebs Ringer buffer containing glucose and sucrose) were incubated at 30°C in the presence of PAO at the following concentrations: 100 μM (■), 200 μM (□), 250 μM (▲) and 500 μM (✕). The reaction was started by addition of arsenical, and 15 s before each time point, the reaction tube was centrifuged at 8,500 x g for 30 s in a bench microfuge. The buffer containing arsenical was removed, and the cell pellet was resuspended in 500 μl ice-cold buffer and sonicated. A sample of 100 μl was removed for assay of DHlipDH. From these data, the times taken for 50 % of DHlipDH activity to be present (t_{50}) were calculated.

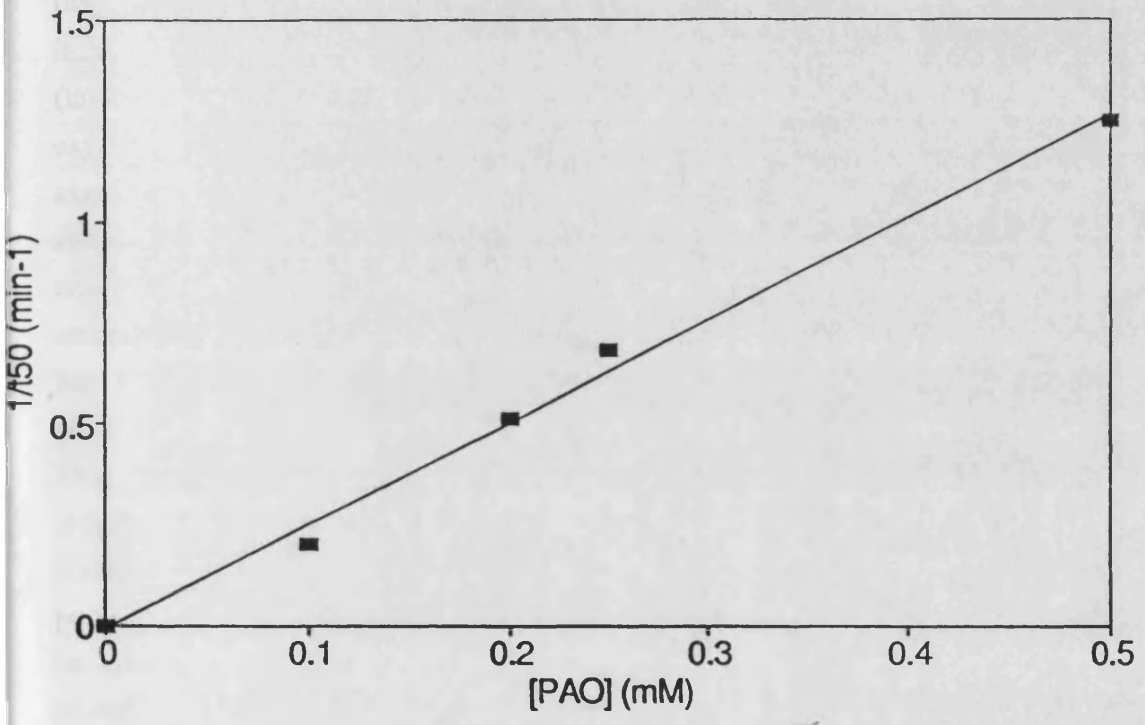


Fig. 7.6. Secondary plot for the inactivation of DHlipDH in whole cells of *T.b.brucei* by phenylarsenoxide (PAO). For each concentration of PAO, the time taken for 50 % inactivation of DHlipDH has been calculated from Fig. 7.5. A regression line has been fitted to the data points, yielding a second order rate constant of 3.6×10^{-3} $mM^{-1}min^{-1}$ for PAO.

concentration. To investigate this phenomenon further, the graph was extended up to 2 mM Mel O (Fig 7.7), a concentration at which results could not be obtained with PAO because of the very high rate of inactivation with this arsenical. The data in Fig. 7.7 demonstrate that the rate of inactivation of DHlipDH continued to increase with increasing arsenical concentration, even up to 2 mM Mel O. If it is assumed that the gradual increase in the rate of inactivation between 0.5 and 2 mM Mel O is due to a linear component similar to that displayed in Fig. 7.6, then by fitting a regression line to the points between 0.5 and 2 mM Mel O, redrawing this line to start from the origin, and subtracting the values from Fig. 7.7, two components are resolved in Fig. 7.8. The linear component gives a second-order inactivation constant of 6.7×10^{-5} $\mu M^{-1}min^{-1}$, which is 5-fold lower than that for AAPDCA (table 7.2). With this component, the concentration of arsenical to which the enzyme is exposed appears to be proportional to the external concentration of arsenical. So it can be assumed that the Mel O may be diffusing into the cell, but at a much lower rate than the other arsenicals. The second component approximates to a rectangular hyperbola, and can be analysed according to the equation: $1/t_{50} = n.M.(Mel\ O)/[Kd + (Mel\ O)]$, where $1/t_{50}$ gives an approximation to the rate of inactivation of DHlipDH, n is the number of binding sites for Mel O per molecule, M is the macromolecular concentration of the molecule to which Mel O binds, Kd is the distribution coefficient, and (Mel O) is the external concentration of Mel O. This has the form of a Michaelis-Menten equation. Therefore, from a half-reciprocal plot (Fig. 7.9), n.M = 0.18 mM and Kd = 0.21 mM. At low concentrations of Mel O, there is some non-linearity, but it is difficult to determine t_{50} values at such low concentrations. The parameters n.M and Kd may describe a binding site for Mel O which is saturable. This could be some form of receptor or transporter for the arsenical, and it appears to be the major route of entry for Mel O into the trypanosomal cell. The overall equation describing Fig. 7.7 is the sum of the diffusion and Michaelis-Menten processes :

$$\frac{1}{t_{50}} = \frac{k}{0.693} . [Mel\ O] + \frac{n.M.[Mel\ O]}{Kd + [Mel\ O]}$$

To investigate further the presence of a receptor/transporter, a mixture of 2 mM melarsen oxide (a concentration at which melarsen oxide inactivation has started to become limited) and 0.5 mM PAO was applied to live cells in the normal way, and the inactivation of DHlipDH monitored over time (Fig. 7.10). Inactivation of DHlipDH was extremely rapid, yielding a pseudo-first-order rate constant, k, of 1.4 min^{-1}. This is greater than would be expected if PAO and Mel O were competing for entry into the cell. Indeed, it is even greater than the combined rate constants of the two arsenicals (k[PAO] = 0.57, k[Mel O] = 0.17). Detailed discussion of this will be left to the section 7.4, but it does mean that the inactivation kinetics for Mel O cannot be compared to those of the other arsenicals.

The second-order rate constants for PAO, APDCA and AAPDCA, and the rate constant and Michaelis constants for Mel O are displayed in Table 7.2. The rate constant for

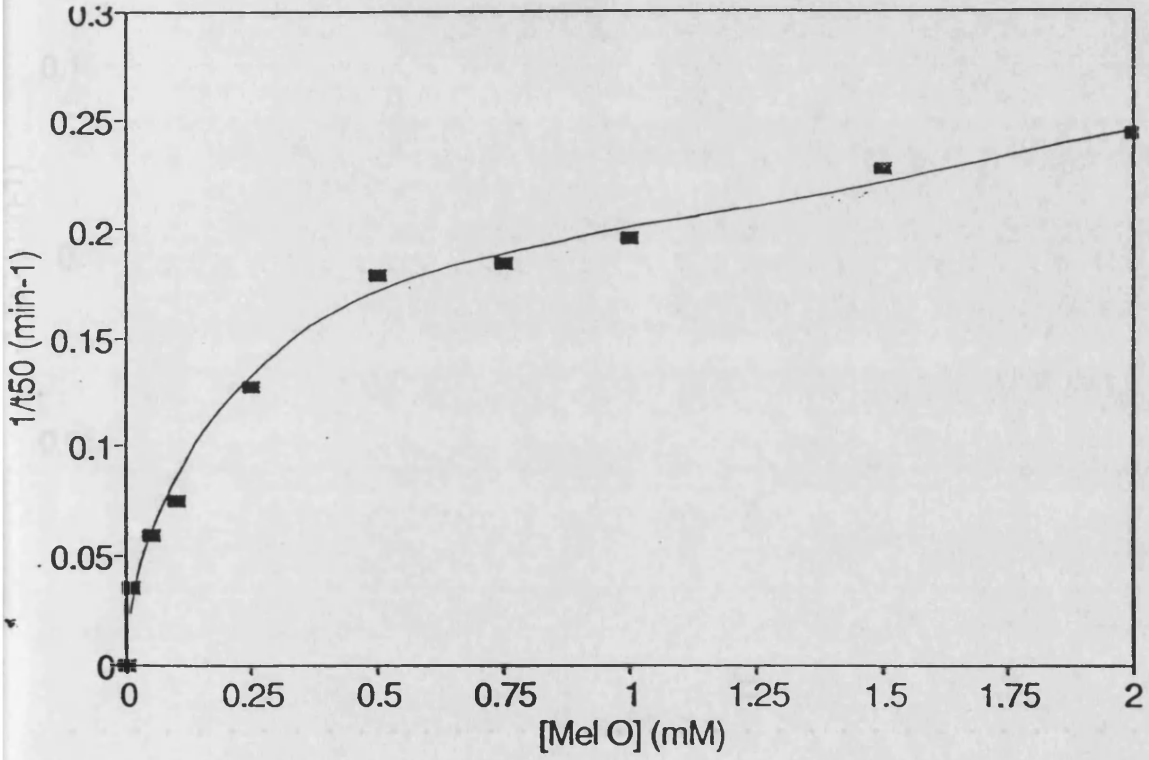


Fig. 7.7. Secondary plot for the inactivation of DHlipDH in whole cells of T.b.brucei by melarsen oxide (Mel O). Times for 50 % inactivation at each concentration of Mel O have been calculated from a plot similar to Fig. 7.5.

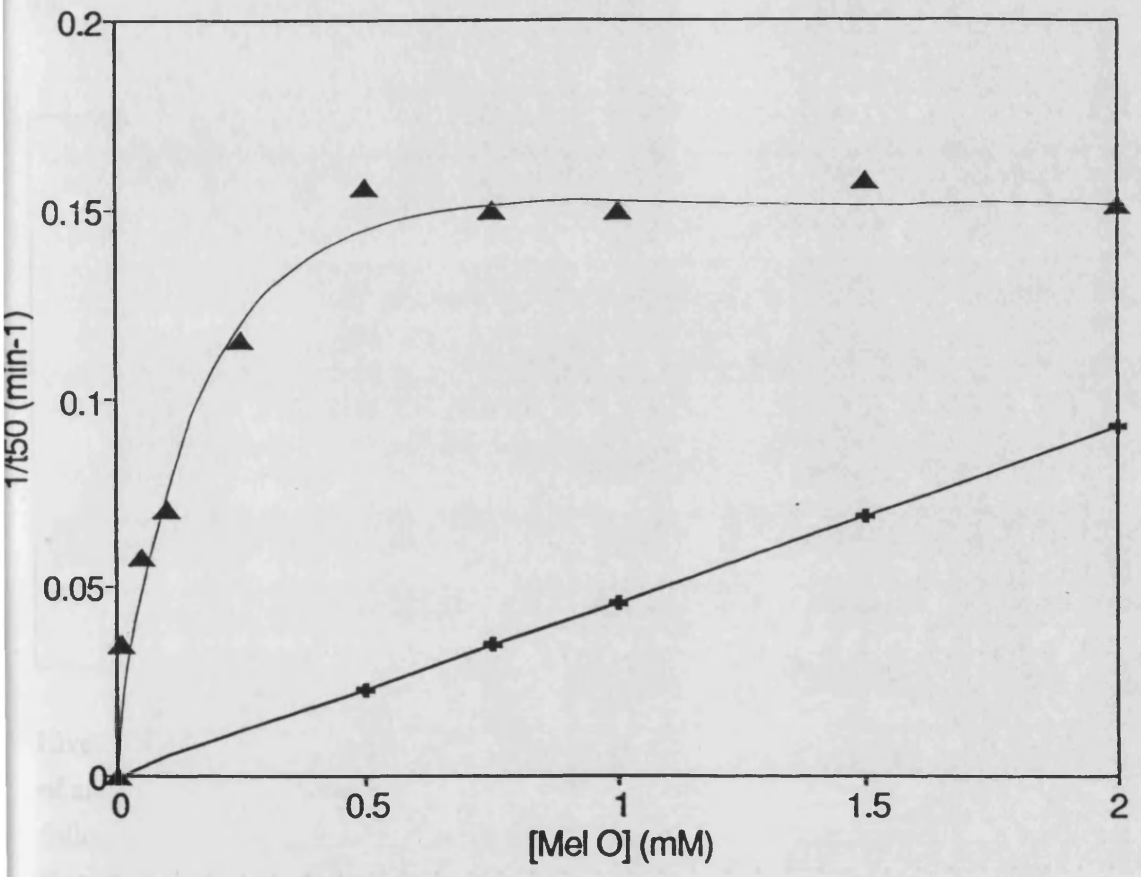


Fig. 7.8. Secondary plot for the inactivation of DHlipDH in whole cells of *T.b.brucei* by melarsen oxide (Mel O) resolved into two components. A regression line has been fitted to the points between 0.5 and 2.0 mM Mel O in Fig. 7.7, yielding a second order rate constant of 4.6×10^{-5} $mM^{-1}min^{-1}$ for the linear component. This component of the inactivation profile has been subtracted from the data in Fig. 7.7, to yield a second component which is saturable, and approximates to a rectangular hyperbola.

Table 7.2. Second-order rate constants for the inactivation of DHlipDH in whole cells of the bloodstream form of *T.b.brucei* by a range of arsenical reagents.

ARSENICAL	Rate Constant ($M^{-1}.min^{-1}$)
PAO	3.6×10^{-3}
APDCA	2.0×10^{-3}
AAPDCA	3.3×10^{-4}
Mel O	6.7×10^{-5}
	n.M = 0.18 mM, Kd = 0.21 mM

Live cells of the bloodstream form of *T.b.brucei* were incubated in the presence of a range of arsenical concentrations as described in section 7.2.3. Primary plots (to give t_{50} values), followed by secondary plots, allowed determination of the second order rate constants. Examples of these for PAO are displayed in Figs. 7.5 and 7.6. The secondary plot for Mel O was not linear, and has therefore been resolved into two components, one of which is linear, and comparable to those of the other arsenicals, and the other approximates to a rectangular hyperbola, the binding kinetics of which are displayed in the table (see section 7.3.2). Abbreviations: PAO, phenylarsenoxide; APDCA, *p*-aminophenyldichloroarsine; AAPDCA, *p*-acetylaminophenyldichloroarsine; Mel O, melarsen oxide.

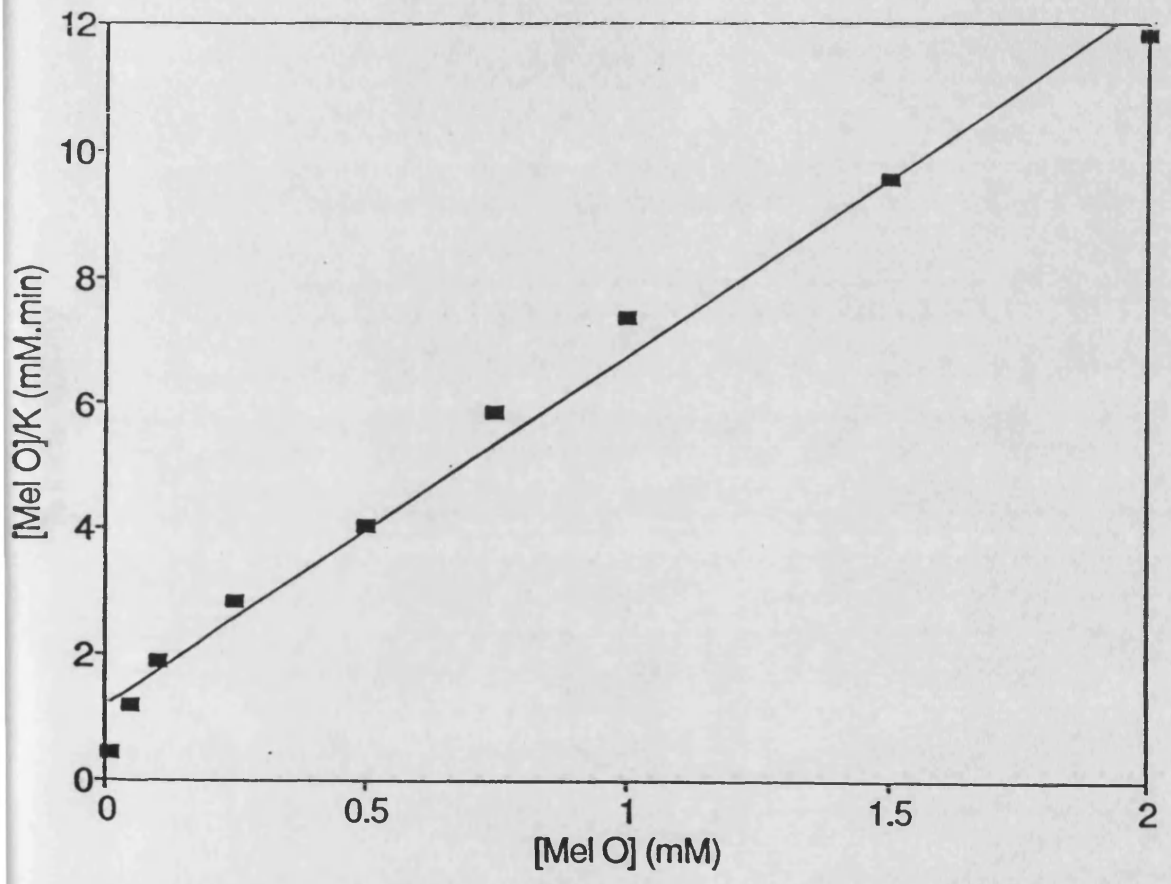


Fig. 7.9. A half-reciprocal (Hanes-Woolf) plot for the determination of the kinetic parameters of the saturable component (see Fig. 7.8) of inactivation of DHlipDH in whole cells of T.b.brucei by melarsen oxide (Mel O). The pseudo-first order rate constant, k, has been calculated from Fig. 7.8 using the equation k = 0.693/t50. Regression analysis of this data yields parameters : n.M = 0.18 and Kd = 0.21 mM, from the equation $1/t_{50}$ = n.M.(Mel O) / [Kd + (Mel O)].

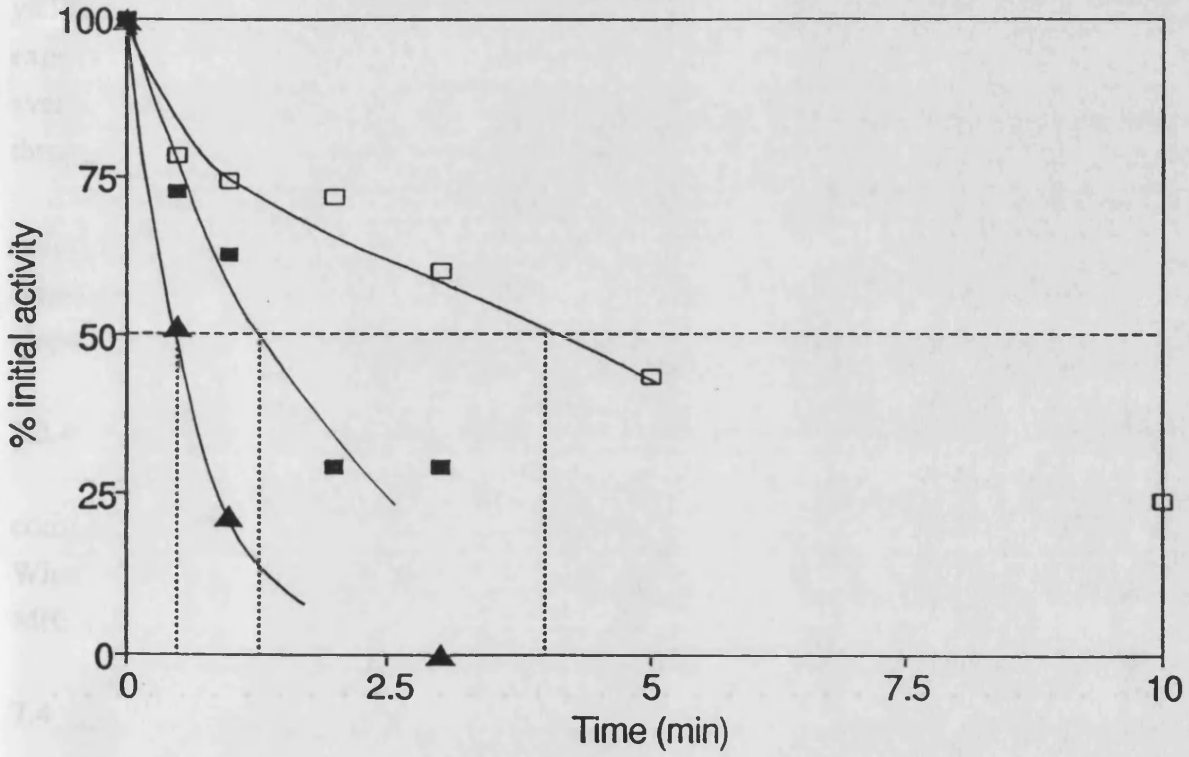


Fig. 7.10. Inactivation of DHlipDH in whole cells of *T.b.brucei* by melarsen oxide (Mel O) and phenylarsenoxide (PAO). Live cells (10^8 cells/ml in Krebs Ringer buffer containing glucose and sucrose) were incubated at 30°C in the presence of 2 mM Mel O (□), 0.5 mM PAO (■), and 2 mM Mel O and 0.5 mM PAO (▲). The reaction was started by addition of arsenical, and 15 s before each time point, the reaction tube was centrifuged at 8,500 x g for 30 s in a bench microfuge. The buffer containing arsenical was removed, and the cell pellet was resuspended in 500 ml ice-cold buffer and sonicated. A sample of 100 ml was removed for assay of DHlipDH.

PAO (3.6×10^{-3} $\mu M^{-1}min^{-1}$) is twice that of APDCA, and 10-fold greater than that of AAPDCA.

7.3.3 Immobilisation of whole cells

All of the arsenicals under investigation were able to immobilise whole cells of *T.b.brucei* in the micromolar concentration range. As with the inactivation of DHlipDH in whole cells, graphs of % initial motility vs time were plotted with different arsenical concentrations (see Fig 7.11 for PAO) and t_{50} values for each concentration were determined. Fig 7.12 shows that $1/t_{50}$ was proportional to the concentration of PAO used, yielding a second-order rate constant of 0.52 $\mu M^{-1}.min^{-1}$ (slope/0.693). A further experiment was carried out with PAO, giving another estimate of the rate constant, and the average value of 0.66 $\mu M^{-1}min^{-1}$ is shown in table 7.3. The secondary plots for the other three arsenicals were also linear, and all the rate constants are also displayed in Table 7.3.

Mel O was the most potent arsenical for immobilising cells, being approximately twice as potent as PAO (rate constants of 1.18 and 0.66 $\mu M^{-1}min^{-1}$ for Mel O & PAO, respectively). APDCA and AAPDCA were 20- and 40-fold less potent than Mel O, respectively.

7.3.4 Inhibition of DHlipDH in cell-free extracts by 5-methoxyindole-2-carboxylic acid

DHlipDH was inhibited in cell-free extracts of *T.b.brucei* by MICA. Inhibition was competitive as demonstrated by the parallel lines of the Cornish-Bowden plot (Fig. 7.13b; Wharton & Eisenthal, 1981) and the Dixon plot (Fig. 7.13a) yielded a Ki of 5.9 mM for MICA.

7.4 Discussion

The inactivation of DHlipDH by trivalent arsenicals is thought to occur by modification of the active site dithiol (see Fig.7.1; Hill, 1966). The results shown in Fig. 7.2 demonstrate that NADH is required for enzyme inactivation in cell-free extracts of *T.b.brucei*. This is consistent with DHlipDH operating by a catalytic mechanism of alternate oxidation and reduction of an interchain disulphide bond, with reduction by dihydrolipoamide or NADH rendering the enzyme susceptible to arsenical inactivation (Adamson & Stevenson, 1981). The low rate of inactivation in the absence of NADH shows that the vast majority of the enzyme in cell-free extracts is in the oxidised state.

The relative rate constants for inactivation with each arsenical (Table 7.1) are probably a function of their chemical structure. PAO, the smallest and most hydrophobic arsenical, was the most potent inactivator of DHlipDH. APDCA was 4-fold less potent, probably because of its amino group conferring polarity upon the molecule. The further

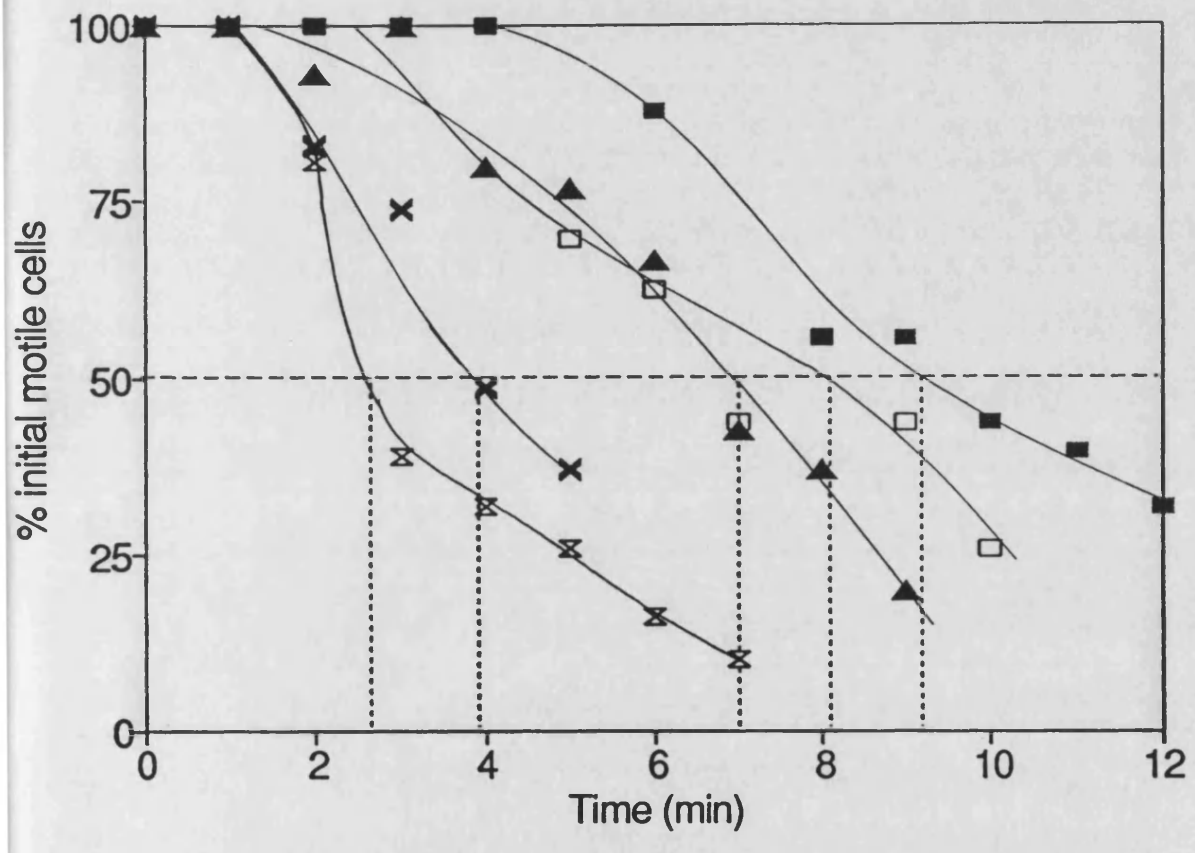


Fig. 7.11. Immobilisation of live cells of *T.b.brucei* by phenylarsenoxide (PAO). Live cells, at a concentration of 1 x 10^6 cells/ml, were pre-incubated at 30°C for 5 min. Following the addition of PAO, the number of motile cells present in a 4 x 4 grid was counted at different time points using a haemocytometer. The concentrations of PAO applied to the cells were: 0.3 mM (■), 0.4 mM (□), 0.5 mM (▲), 0.7 mM (✕), and 1.0 mM (⧖). From these data, the t_{50} values, that is, the times at which 50 % of cells are immotile, are calculated.

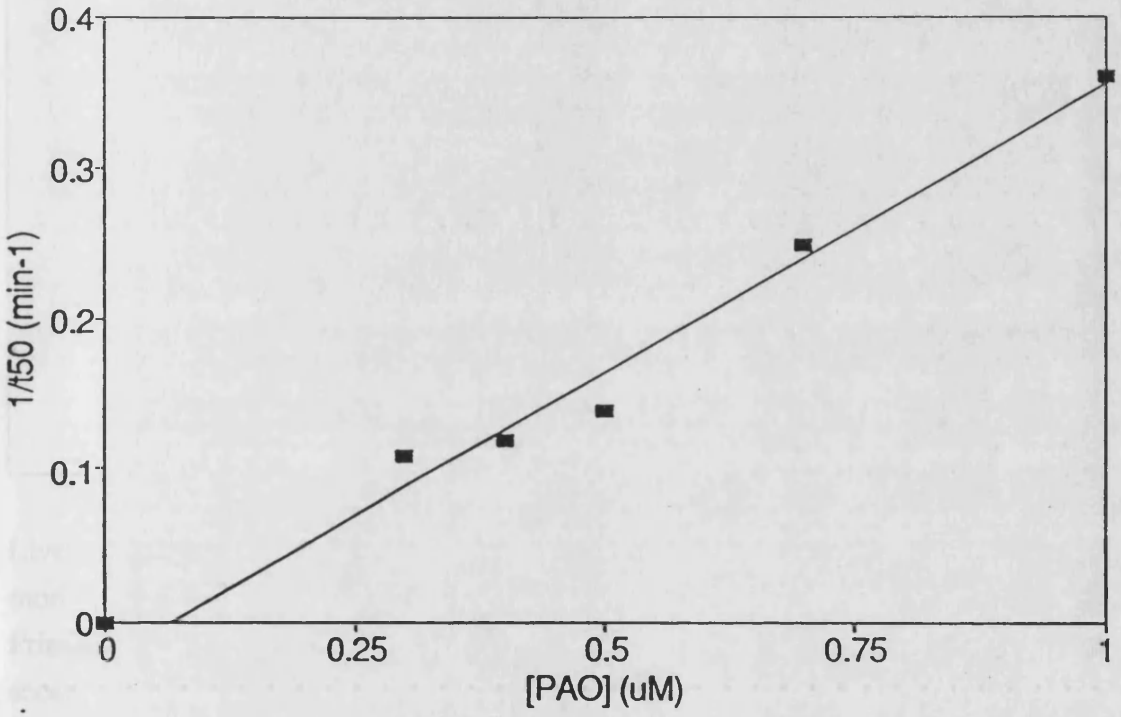
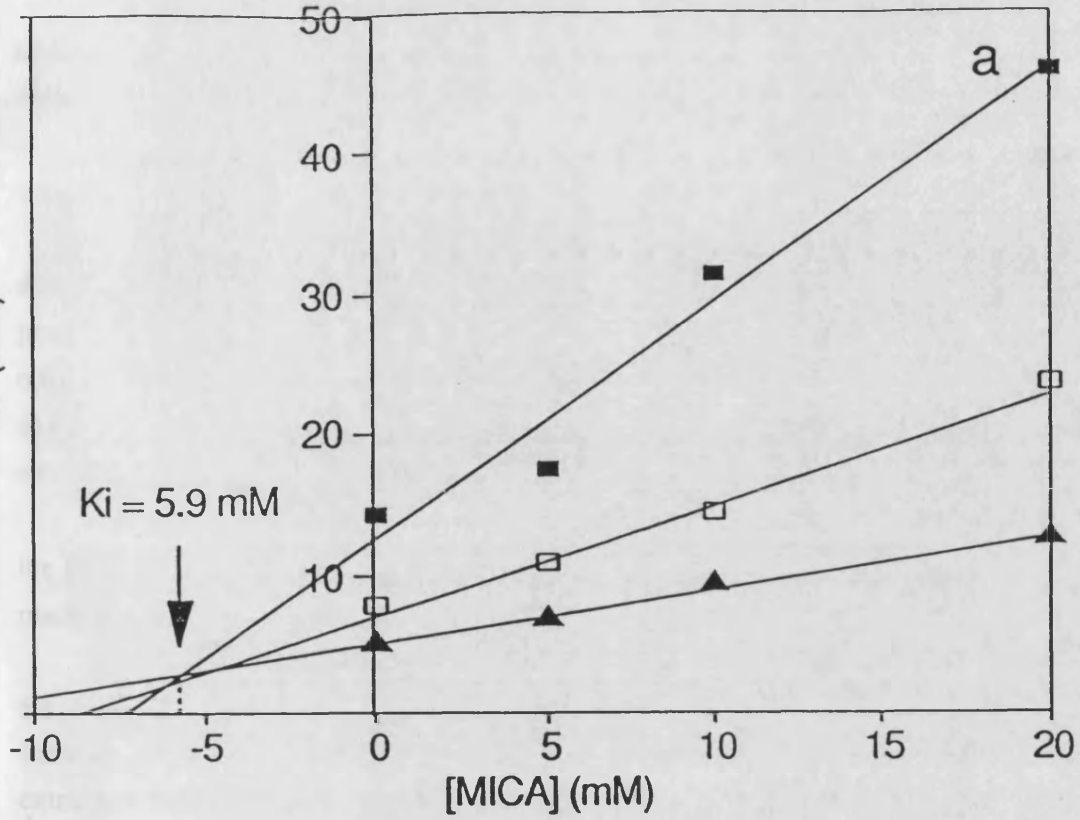


Fig. 7.12. Secondary plot for the immobilisation of cells of *T.b.brucei* by phenylarsenoxide (PAO). The t_{50} values have been calculated from Fig. 7.11, and a straight line fitted to these data points by regression analysis, yielding a second order rate constant of 0.521 $mM^{-1}min^{-1}$ for PAO.

Table 7.3. Second-order rate constants for the immobilisation of cells of the bloodstream form of *T.b.brucei* by a range of arsenicals

ARSENICAL	Second-order rate Constant ($M^{-1}.min^{-1}$)
PAO	0.66
APDCA	0.063
AAPDCA	0.039
Mel O	1.18

Live cells of the bloodstream form of *T.b.brucei* were incubated with arsenicals, and the motility of the cells monitored using a haemocytometer, as described in section 7.2.4. Primary plots were derived from t_{50} values for immobilisation. Examples of primary and secondary plots for PAO are displayed in Figs. 7.11 and 7.12. The second-order rate constants have been calculated from the slopes of the secondary plots. Abbreviations: PAO, phenylarsenoxide; APDCA, *p*-aminophenyldichloroarsine; AAPDCA, *p*-acetylamino-phenyldichloroarsine; Mel O, melarsen oxide.



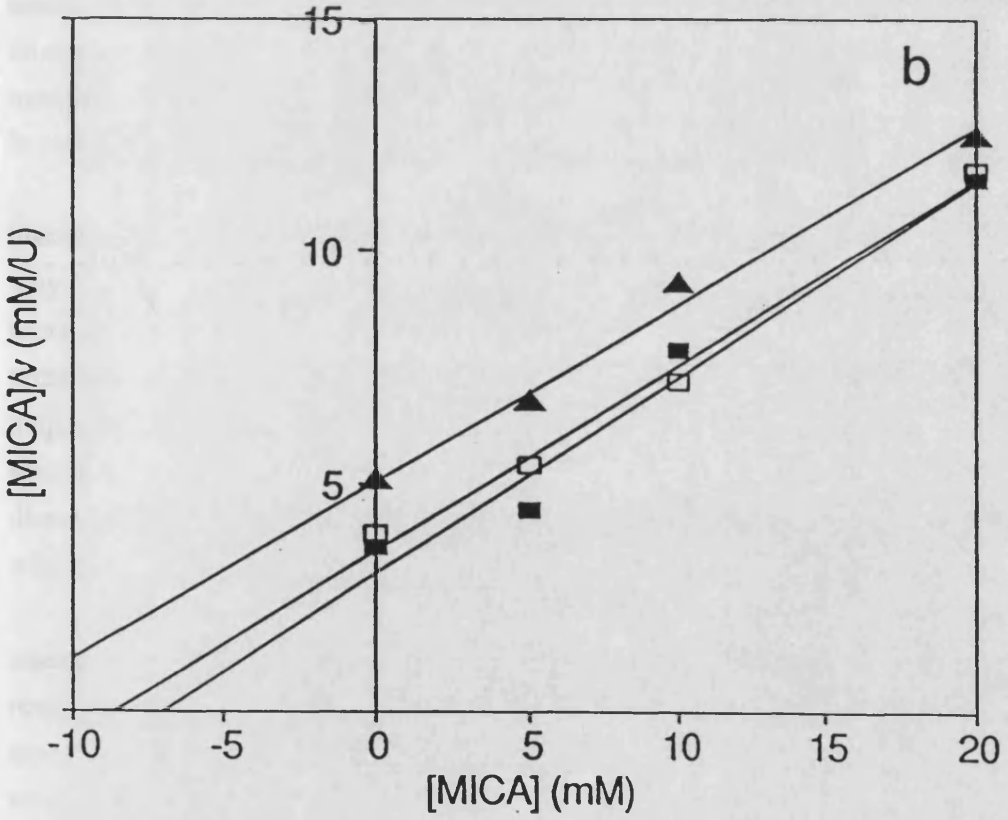


Fig. 7.13. Inhibition of DHlipDH in cell-free extracts of T.b.brucei by 5-methoxyindole-2-carboxylic acid (MICA). The assay of DHlipDH in the presence of MICA was carried out at 30°C in 50 mM potassium phosphate, pH 7.0, 2 mM EDTA, 0.4 mM DHlip in the presence of 0.25 mM (■), 0.5 mM (▭), and 1.0 mM (▲) acetyl-NAD+. The absorbance change due to the production of acetyl-NADH was monitored at 366 nm. The data have been plotted on (a) a Dixon plot, yielding a Ki of 5.9 mM for MICA, and (b) a Cornish-Bowden plot

addition of an acetyl group, despite reducing the polarity, reduced the inactivation rate constant to 5-fold lower than that for PAO, presumably because it increased the bulk of the molecule. Mel O had an equally low potency, even though it is a very much larger molecule than AAPDCA.

DHlipDH was inactivated in whole cells by all four arsenicals. It has already been shown that the enzyme must be in the reduced state for inactivation to occur. This state was produced in cell-free extracts by supplying NADH, but in whole cells DHlipDH is thought only to enter the reduced state during a catalytic cycle *in vivo*. This may indicate that the enzyme is turning over in whole cells. It is also possible, however, that the reducing environment of the cell may maintain the DHlipDH in its reduced state.

A comparison of the second-order rate constants for inactivation/immobilisation in the three different systems with each arsenical (Table 7.4) enables certain conclusions to be made about the nature of the inactivation process *in vivo*.

Excluding the data for Mel O, the lowest second-order rate constants were obtained for the inactivation of DHlipDH in live cells. The rate constants for inactivation in live cells were 2- to 10-fold lower than the rate constants for inactivation of DHlipDH in cell-free extracts, which were a further 10- to 40-fold lower than those for the immobilisation of cells. Mel O was different from the other arsenicals because, as has already been mentioned, the inactivation of DHlipDH in live cells proceeded, in part, according to Michaelis-Menten kinetics, and therefore it cannot be compared with the constants for cell-free extracts and motility. Mel O was 240-fold more potent at immobilising cells than inactivating DHlipDH in cell-free extracts. This is a 10-fold higher potency ratio than with the other arsenicals.

A combination of possible factors may contribute to the lower rate constants for inactivation of DHlipDH in whole cells as compared to cell-free extracts. Firstly, the enzyme may be turning over in whole cells, whereas it exists in a constantly reduced, and therefore more susceptible, condition in cell-free extracts. A second factor in whole cells is the presence of the plasma membrane, which acts as a permeability barrier. This may not be a major factor with PAO and APDCA, which are thought to diffuse freely into cells, and for which binding reactions within cells are more important than membrane penetration in determining their uptake kinetics (Hill, 1966). AAPDCA has a 10-fold reduced potency in whole cells, which may be caused, at least in part, by penetration effects.

Mel O possessed completely different kinetics, when compared to the other arsenicals, for inactivation of DHlipDH in whole cells. The inactivation process could be resolved into two components: a linear component, presumably similar to those for the other arsenicals, and a component described by a rectangular hyperbola, which may indicate a receptor/transporter mechanism of entry into the cell. The presence of the bulky melaminyl residue would dramatically reduce its permeability through membranes, but other workers have also shown that it is concentrated by the cell against a concentration gradient (Albert,

Table 7.4. Comparison of second-order rate constants for the inactivation of DHlipDH in cell-free extracts and live cells and the immobilisation of bloodstream form cells of *T.b.brucei*

ARSENICAL	RATIO OF INACTIVATION CONSTANTS		
	Live Cells	Cell-free extracts	Motility
PAO	1	7.5	180
APDCA	1	3.4	31
AAPDCA	1	16	120
Mel O	-	(1)	(240)

Treating PAO, APDCA & AAPDCA separately, each constant has been divided by that for inactivation of DHlipDH in live cells. For Mel O, the values are displayed in brackets because they are divided by the inactivation constant for DHlipDH in cell-free extracts. Abbreviations: PAO, phenylarsenoxide; APDCA, *p*-aminophenyldichloroarsine; AAPDCA, *p*-acetylaminophenyldichloroarsine; Mel O, melarsen oxide.

1973; Flynn & Bowman, 1974) and that uptake is via a carrier-mediated mechanism (Ojeda & Flynn, 1982). Furthermore, Ojeda & Flynn (1982) showed that the melaminyl residue of melarsen oxide was required for this uptake process. This presence of a carrier may explain the very different behaviour of Mel O in inactivating DHlipDH in live cells. The Kd of 0.21 mM and n.M of 0.18 mM, determined from kinetic plots, may therefore describe a transporter for Mel O.

The immobilisation of cells was achieved at lower arsenical concentrations than the inactivation of DHlipDH *in vivo*. PAO, APDCA & AAPDCA were 12- to 32-fold more potent at immobilising as compared to inactivating DHlipDH *in vivo*, and the order of potency was PAO > APDCA > AAPDCA. This is the same order of potency as for inactivation of DHlipDH in cell-free extracts. Presumably this is because the chemical structural differences mentioned earlier for inhibition of DHlipDH also apply for the interactions of the arsenicals with the cellular targets involved in immobilisation.

It is interesting that the concentrations of arsenicals used to inactivate DHlipDH are considerably higher than those which immobilise cells. This indicates that the DHlipDH may still be turning over, or may be reduced, when the cells have lost their motility.

When Mel O is administered therapeutically, concentrations between 0.6 and 6 μM can be achieved in the blood of host animals (Hill, 1966). This would be sufficient to immobilise trypanosomes, but would not inactivate DHlipDH over the timescales used in these experiments.

One major limitation of the experimental data for live cells presented in this chapter is that the timescales of exposure to arsenicals are very short, ranging up to 20 min. The concentration of arsenical needed to kill trypanosomes over a short period of time is known to be much greater than that required for longer term killing (Hill, 1966). For example, the results in this chapter show that 1 μM PAO immobilised trypanosomes in 5-10 min, whereas 18 nM PAO is able to kill trypanosomes in 6 h (King & Strangeways, 1942). Trypanosomes in the bloodstream of their host organism will be exposed to arsenicals for long periods of time, and therefore lower doses of arsenical will be effective. Over longer timescales, and at lower concentrations, the arsenicals may have different potencies at different sites, and the inactivation of DHlipDH, because it could be a cumulative process, may become more relevant. However, this is only speculation, and the likelihood is that the results obtained give a fair indication of the relative potencies of the arsenicals for the different cellular targets.

According to Hill (1966), the trypanocidal activity of arsenicals occurs in three phases:

1) fixation of arsenical - rapid & reversible

2) secondary chemical reactions

3) death process within cells - cells begin to swell, become distorted, and eventually lyse.

Various indicators of cell viability have been used by workers testing trypanocidal drugs. Motility is not the most direct method, as cells remain intact after immobilisation, and indeed can still be resuscitated by cysteine or dimercaprol after immobilisation with PAO (Eagle *et al.*, 1946). Nevertheless, Ercoli *et al.* (1980) found that determinations of immobilisation and parasite number led to similar comparative results using a range of trivalent antimonials against *T.venezuelense*. More direct measurements of cell lysis were carried out by Yarlett *et al.* (1991), recording lysis spectrophotometrically by reduction in A_{500}, Van Schaftingen *et al.* (1987), who used release of pyruvate kinase as a measure of lysis, and Betbeder *et al.* (1990), who measured reduction in cell number microscopically. A common viability assay for cultured animal cells involves the uptake of fluorescein diacetate and the exclusion of propidium iodide by live cells (see Mishell & Shiigi, 1980). With this technique live cells fluoresce green, and non-viable cells are stained red. This assay was attempted, but the trypanosomes appeared to lyse under assay conditions, and all fluoresced red.

As has already been mentioned, it is important with arsenical studies to assess the long-term effects of low concentrations upon cells. The motility determinations employed in this chapter could not be extended over longer time periods because of the depletion of glucose from the medium, which is known to affect arsenical reactions (Hill, 1966). Instead, other groups have assessed the longer-term effects by measuring the infectivity of treated trypanosomes (Loiseau *et al.*, 1988; Betbeder *et al.*, 1990), and the production of pyruvate by cells in continuous culture, followed with a pH indicator (Zinsstag *et al.*, 1991).

In conclusion, both the short- and long-term effects of the arsenicals on DHlipDH activity and cell viability should be assessed to give a complete picture of the relationship between these two phenomena. However, such studies were not practicable within the timescale of this project, and short-term work is sufficient to give an indication of the relative arsenical potencies.

As mentioned in the introduction to this chapter, several other trypanosomal enzymes are inhibited by melarsen oxide, including phosphofructo-2-kinase, for which Mel O has a Ki of < 1 μM (Van Schaftingen *et al.*, 1987), and fructose -2,6-bisphosphatase (Ki = 2 μM).

These enzyme studies were superseded by the work of Fairlamb *et al.* (1989) on trypanothione, who showed that this thiol forms a stable adduct with melarsen oxide (termed Mel T), which is the only acid-soluble form of Mel O in *T.b.brucei* (Fairlamb & Henderson, 1987). The conjugate has a Ka of 1.21×10^7 M^{-1}, and its concentration reaches 17 μM (equivalent to approximately 10 % of cellular trypanothione) when cell lysis occurs. It is a potent inhibitor of trypanothione reductase (Ki = 9 μM), so at its maximum level of 17 μM within the cell, Mel T is significantly inhibiting trypanothione reductase.

It is not possible directly to compare the second-order rate constants for inactivation of DHlipDH by arsenicals, with the Ki values for the inhibition of the other enzymes or the Ka for the affinity of Mel O with trypanothione, but the interaction with trypanothione appears to be much more relevant to cell lysis. Nevertheless, any role for DHlipDH in the toxicity of arsenicals to trypanosomes should not be ruled out too quickly, as it represents a significant difference between the enzymology of the parasite and its host. It is also worth bearing in mind that because the DHlipDH is not inactivated at concentrations of arsenicals sufficient to immobilise the cells, the enzyme could still be essential to the cell. In this case, a specific inhibitor of DHlipDH could be developed which would cause cell lysis.

Because cell lysis follows immobilisation even with the high concentrations of arsenicals used to inhibit DHlipDH *in vivo*, it may be possible to carry out functional studies on transport processes and signal transduction pathways in *T.b.brucei* using trivalent arsenicals as inhibitory probes.

The other possible inhibitor of DHlipDH which could be used for functional studies is MICA. Its Ki of 5.9 mM for *T.b.brucei* DHlipDH compares favourably with those for the rat liver (Ki = 3 mM) (Reed & Lardy, 1970) and *E.coli* (Ki = 6 mM) (Richarme, 1985) enzymes. The mechanism of MICA inhibition is very different from that of arsenicals, in that it probably forms an inhibitory complex with the FAD cofactor of DHlipDH (Reed & Lardy, 1970). MICA may therefore prove useful in functional studies, but trypanothione reductase is also a flavoprotein, and may be inhibited by this compound.

CHAPTER 8 GENERAL DISCUSSION AND CONCLUSIONS

Dihydrolipoamide dehydrogenase (DHlipDH) was discovered in the bloodstream form of *T.b.brucei* in the absence of the 2-oxo acid dehydrogenase complexes by Danson *et al.* (1987). In that report, it was shown to co-purify with plasma membranes and to be inactivated by a trivalent arsenical in a substrate-dependent process. This was a particularly interesting discovery in that it was the first time that DHlipDH had been found in a eukaryotic cell in the absence of the 2-oxo acid dehydrogenase complexes, and the first time that this enzyme had been located to the plasma membrane of a eukaryote. The aim of this project has been to proceed from these initial discoveries into gaining a greater understanding of DHlipDH in *T.b.brucei.*

8.1 Summary of Chapters 3 to 7

From the work of chapter 3, it is clear that the bloodstream form of *T.b.brucei* possesses a DHlipDH solely located over the inner surface of its plasma membrane, and that this enzyme is not attached to the membrane by any form of lipid or hydrophobic anchor.

The situation is very different in the procyclic form of the parasite (chapter 4), where the enzyme is definitely in the mitochondrion, but it was not possible to demonstrate whether any remained at the plasma membrane. The procyclic mitochondrion contains active 2-oxo acid dehydrogenase complexes, the presence of which accounts for at least the majority of the DHlipDH in these cells. The level of DHlipDH is 13-fold higher in the procyclic cell than in the bloodstream form trypomastigote.

Lipoic acid, the presumed substrate of DHlipDH, is present in both forms of the parasite (chapter 5), with the procyclic cell possessing 10-fold more of this cofactor than the bloodstream form cell. The increase in lipoic acid parallels that of DHlipDH, the ratio of enzyme to cofactor remaining approximately constant through these two stages of the lifecycle (0.018 & 0.024 U DHlipDH / ng lipoic acid in bloodstream form and procyclic cells, respectively). In the procyclic cell, it is thought that the majority of the lipoic acid and DHlipDH are components of the mitochondrial 2-oxo acid dehydrogenase complexes. Their respective levels are therefore optimised for the complexed situation. In the bloodstream form, a comparable ratio of enzyme to cofactor might suggest that a structurally similar complex to the 2-oxo acid dehydrogenase complexes contains the DHlipDH and lipoic acid in these cells, because, if the cofactor were free, much higher quantities of it would be required for efficient catalysis by the same amount of enzyme.

Production of lipoyl-bearing proteins and DHlipDH is probably co-ordinately regulated in both prokaryotic and eukaryotic systems, but the situation has only been clearly worked out in *E.coli.* Here, the *lpd* gene, which encodes E3, is regulated both by the PDHC (*ace* E and *ace* F genes) operon and a secondary promoter of the *lpd* gene (Patel & Roche,

1990). It is therefore likely that DHlipDH and lipoic acid are produced in a coordinated fashion in the mitochondrion of the procylic cell. No such suppositions can be made concerning the bloodstream form cell.

Focussing in further upon the bloodstream form DHlipDH, it appears, in terms of its size, stability and kinetics, to be very similar to the DHlipDHs associated with the 2-oxo acid dehydrogenase complexes of other species. The bloodstream form DHlipDH has also been purified to homogeneity in preparation for N-terminal sequencing and further analysis (chapter 6).

Experiments in chapter 7 demonstrated that the DHlipDH could be inactivated *in vivo* by a series of arsenical reagents. This might indicate that the enzyme is catalytically turning over in the living cell, but it may be possible for the DHlipDH to be maintained in the reduced state by a reducing environment within the cell. This result, together with the demonstration of the presence of lipoic acid in proportional amounts to DHlipDH, and the fact that this DHlipDH appears to be similar to those of other species, strongly suggests that the enzyme is not a metabolic aberration, sent to the plasma membrane by faulty processing or targetting. Instead, it may have a membrane function, possibly involving another protein/enzyme complex containing lipoic acid.

Further studies with the arsenical reagents demonstrated that DHlipDH is probably not a major target for arsenical drugs such as melarsen oxide, and is inactivated only after cells have been immobilised by these compounds. These studies do not, however, rule out the involvement of DHlipDH in arsenical toxicity, as the enzyme is located at the plasma membrane of the cell, whereas trypanothione reductase, thought to be the major arsenical target, is cytoplasmic (Smith *et al.*, 1991). DHlipDH may therefore be one of the first vicinal dithiol-containing molecules to be seen by arsenicals entering the cell. In addition, lipoic acid is able to form stable adducts with trivalent arsenicals. The reduction potential of lipoic acid (E_o = - 0.288 v) is such that it could receive an arsenical like melarsen oxide from its dimercaprol conjugate near the cell surface, and then pass it on to trypanothione, which has a lower reduction potential (E_o = - 0.242 v; Fairlamb & Henderson, 1987).

Until the functions of DHlipDH and lipoic acid have been elucidated, it remains to be seen how they may relate to arsenical toxicity *in vivo*.

With the numerous other cellular targets for arsenical reagents, it is unlikely that these reagents will prove useful as inhibitory probes for the function of DHlipDH, as it will be difficult to differentiate the DHlipDH-related effects from those involving other molecules.

8.2 Previous reports of DHlipDH activity in trypanosomes

The de-repression of mitochondrial enzymes during transformation from the bloodstream form of *T.b.brucei* has been investigated by Vickerman (1965) using the NADH-diaphorase test. Diaphorase is a secondary activity of DHlipDH (Massey, 1960), in

which the enzyme uses NADH as substrate and nitro-blue tetrazolium as electron acceptor. Activity is indicated by blue-black deposits of formazan dye. Small amounts of activity were observed in microbodies in long-slender bloodstream forms, but these were put down to the activity of another enzyme (Vickerman, 1965). Significant deposits were seen in the mitochondria of short-stumpy forms, which coincided with the ability to survive on 2-oxoglutarate.

The reverse reaction of DHlipDH (NADH-dependent lipoate reductase) has been measured in *T.b.rhodesiense* by Ryley (1962). He observed activity in bloodstream forms and procyclics, with slightly higher levels in the latter. These results cannot, however, be considered definitive because a pleomorphic population of bloodstream form cells was used, a significant proportion of which would be short-stumpy cells with active mitochondria. This is in contrast to the purely monomorphic long-slender bloodstream forms of *T.b.brucei* EATRO 427 used in this project (see section 2.1.2). In addition, trypanosomal cells were probably not adequately separated from blood cell material by the technique of blood cell lysis; column chromatography, as employed in this thesis, does give complete separation.

8.3 Possible functions of the plasma membrane DHlipDH

8.3.1 Multienzyme complex functions

In considering the possible function(s) of DHlipDH and lipoic acid *in vivo*, the first possibility is that they are part of a dehydrogenase multienzyme complex. The most prominent of these (apart from the 2-oxo acid dehydrogenase complexes) is the glycine cleavage system (GCS), which has been discussed in section 1.4.2.4. The GCS is particularly important in the metabolism of glycine-utilising and purinolytic anaerobes (Dietrichs & Andreesen, 1990). The latter produce glycine (which is further catabolised by the GCS) and formate from purines. As *T.b.brucei* produces significant quantities of glycine from threonine catabolism, and has an active purine salvage pathway, it would be possible for the GCS to be involved in the metabolism of these cells. There are also examples of plasma membrane DHlipDHs involved in the GCS in the purinolytic *Clostridium cylindrosporum* (Dietrichs *et al.*, 1991), and the glycine-utilising *Eubacterium acidaminophilum* (Freudenberg *et al.*, 1989), but these are prokaryotic cells, and the GCS has never been found outside the mitochondrion in eukaryotes.

Other DHlipDH-containing complexes include the lactate-degrading system of *Butyribacterium rettgeri* (Wittenberger & 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.

8.3.2 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 (see Freedman, 1979). Lipoic acid is able to reduce the major intracellular thiol, glutathione (Bast & Haenan, 1988), and thereby contributes to the maintenance of redox balance and to reduction of oxidative stress (see Meister & Anderson, 1983). DHlipDH may also affect oxidative stress, as it has a significant oxidase activity capable of producing reactive superoxide radicals (Bando & Aik, 1991; Grinblat *et al.*, 1991). In the trypanosome, glutathione is replaced by trypanothione, and the cell is much more vulnerable to reactive oxygen species. In this environment, lipoic acid and DHlipDH may have considerable influence upon redox levels.

Lipoic acid and DHlipDH can also catalyse the NADH-dependent reduction of thioredoxin (Holmgren, 1979; Spector *et al.*, 1988). This enzyme and its homologue, glutaredoxin, are capable of reducing a wide variety of cellular substrates, including insulin, glutathione, oxidised lens protein and ribonucleotides (Holmgren, 1988). Thioredoxin is also an important regulator of enzyme activity. It reduces photosynthetic fructose-1,6-bisphosphatase, and protein disulphide isomerase (Holmgren, 1979; Lundstrom & Holmgren, 1990), which catalyses thiol : protein disulphide interchange associated with the biosynthesis of secretory proteins such as insulin (Freedman *et al.*, 1988; Tang *et al.*, 1988). With thioredoxin being membrane-bound in both mammals (Lundstrom & Holmgren, 1990) and *E.coli* (Bayer *et al.*, 1987), it can be speculated that a plasma membrane DHlipDH may interact with this protein in trypanosomes, with significant effect upon cellular reductive processes.

The reaction mechanism for each of the interactions described above is thiol : protein disulphide exchange. This mechanism has also been implicated in several other cellular processes, including receptor signal transduction and transport phenomena (Robillard & Konings, 1982; Malbon *et al.*, 1987). Furthermore, it has been suggested that DHlipDH and lipoic acid may be connected with several of these phenomena. This is of particular relevance to the trypanosomal situation, where a plasma membrane-associated function for the enzyme and cofactor is being sought.

The insulin receptor represents the most studied example of sulphydryl involvement in receptors and transmission. When insulin binds to its receptor, the two undergo disulphide exchange, which activates the post-receptor transmission pathway (Clark & Harrison, 1983). A further site of sulphydryl involvement has been detected downstream of a tyrosine phosphorylation step by inactivation studies with phenylarsenoxide (Bernier *et al.*, 1987). These studies demonstrated that a vicinal dithiol is required for internalisation of the insulin-receptor complex, which is essential for subsequent translocation of glucose transporters to the plasma membrane (Frost & Lane, 1985; Douen & Jones, 1986).

The precise molecular details of the transduction pathway have not been elucidated, but a number of steps must require protein disulphide interchange (Maturo *et al.*, 1983). Although no role is suggested for DHlipDH, this system illustrates how such an enzyme catalysing thiol : disulphide exchange might have a role in transmission/transduction.

Robillard and Konings (1982) have proposed that in solute transport, the affinities of substrate binding sites are regulated by interchange between a dithiol and a disulphide located at different depths in the membrane. This is evidenced by the ability of phenylarsenoxide and other sulphydryl reagents to inhibit the phosphoenolpyruvate-dependent transport of hexoses in *E.coli* (Robillard & Konings, 1982) and glucose transport in 3T3-L1 adipocytes (Douen & Jones, 1986). A similar situation is seen with the mitochondrial proton-pumping NAD^+ transhydrogenase, which also operates by thiol : disulphide interchange (Persson & Rydstrom, 1987).

8.3.3 Solute transport

Besides the possible role of DHlipDH in thiol : protein disulphide interchange-related transport processes (section 8.3.2), the enzyme itself has been implicated in several solute transport mechanisms:

a) Ubiquinone-mediated transport of proline in *E.coli* is NADH-dependent, and it is thought that reduction of endogenous membrane-bound quinones by DHlipDH stimulates solute uptake *in vivo* (Owen *et al.*, 1980).

b) Binding-protein-dependent transport of maltose, ribose and galactose in *E.coli* is energised by dihydrolipoamide and NAD^+ (Richarme, 1988). Furthermore, lipoic acid has been shown to stimulate aggregation of maltose-binding proteins, resulting in inhibition of binding (Richarme, 1986). Studies with strains deficient in the 2-oxo acid dehydrogenase complexes (Richarme, 1987) and with inhibitors of these complexes (Richarme, 1985; Richarme & Heine, 1986) have demonstrated a clear link between complex activity and transport. However, according to recent work, the DHlipDH involved in transport may be distinct from the *lpd* gene product (the DHlipDH of the complexes), and may be associated with the *mgl* gene product, which forms part of the binding-protein-dependent transport system (Richarme, 1989).

8.3.4 Electron transport

The link between the mitochondrial ATP synthase and the respiratory chain may involve, amongst other components, lipoic acid (Partis *et al.*, 1977). This is present in stoichiometric amounts in ATP synthase preparations (Griffiths, 1976), and is required for the oxidative phosphorylation of NADH, succinate or D-lactate by the mitochondria of several cells. Indeed, in isolated mitochondria, dihydrolipoic acid stimulates ATP synthase by up to 45 %, and decreases ATPase activity by 36 % (Zimmer *et al.*, 1991). In parallel to

the increase in ATP synthesis, oligomycin-sensitive mitochondrial -SH groups are activated at 2-4 nmol lipoic acid/ mg protein. An analogous role for the cofactor has been postulated in the purple membranes of *Halobacterium halobium*, where photoreduction of lipoate might form the link between bacteriorhodopsin and the ATP synthase (Griffiths *et al.*, 1977).

In summary, DHlipDH and lipoic acid have been implicated in several redox and transport processes in different cell types, and with thiol : disulphide interchange proving a common feature of transduction pathways, there remain a wide variety of possible roles for the enzyme and its cofactor in the bloodstream form of *T.b.brucei*.

8.4 A general plasma membrane location for DHlipDH ?

In addition to the anaerobes *C.cylindrosporum* and *E.acidaminophilum* and bloodstream form *T.b.brucei*, the DHlipDH of *Thermoplasma acidophilum* appears to be associated with the plasma membrane of the cell (Danson, 1988). Rat adipocytes, which possess functional mitochondria, may also have a proportion of their DHlipDH in this extra-mitochondrial position (Danson, 1988). These findings beg the question as to whether a plasma membrane DHlipDH is a generalised phenomenon in most cell types, or whether it could be restricted to cells carrying out certain mechanistically similar processes, such as those involving particular thiol : disulphide interchange reactions.

One further implication of the discovery of a plasma membrane DHlipDH concerns the autoimmune disease, Primary Biliary Cirrhosis (PBC). In this disease, antibodies are produced which are directed mainly against components of the 2-oxo acid dehydrogenase complexes (Krams *et al.*, 1989). In particular, antibodies are reactive against the lipoyl domains of the E2 and protein X components, but they have also been detected against the E1-α polypeptide (Fregeau *et al.*, 1990). Immunisation with purified recombinant transacetylase yielded anti-mitochondrial antibodies (AMAs), but did not precipitate the disease, indicating that other factors such as genetic susceptibility must be involved (Krams *et al.*, 1989). Indeed, AMAs may only be produced as a result of tissue damage. One major drawback of the theory that antibodies cause the disease has always been that the autoantigens are intra-mitochondrial, and are therefore not exposed to antibody-producing cells. With these new discoveries of plasma membrane DHlipDHs, it is possible that they may be accompanied by lipoyl-bearing proteins and E1-like enzymes, and these might become exposed on the cell surface under certain conditions, eliciting an antibody response. In this context, Fusey *et al.* (1990) have already suggested a bacterial aetiology for PBC, and Uzoegwu *et al.* (1987) have looked for proteins reactive with PBC sera in various endoparasites. Stercorarian trypanosomes and procyclic cells of *T.b.brucei* contained antigenic proteins, but bloodstream forms of *T.b.brucei* showed no significant reactivity. The possible antigenic aetiology of PBC remains uncertain, but further studies with bacteria and

parasites may yet demonstrate that unusually located "mitochondrial" antigens elicit the initial antibody response.

8.5 Towards an understanding of DHlipDH in *T.b.brucei*

Several questions remain to be answered concerning DHlipDH in *T.b.brucei*. These concern the situation in the procyclic cell, intracellular targetting, and the genetic origin of the DHlipDH(s).

8.5.1 DHlipDH in procyclic cells

Whilst DHlipDH has been localised solely to the plasma membrane of the bloodstream form cell of *T.b.brucei*, the situation in the procyclic cell remains unclear (see section 4.4). This is because it was not possible to show by subcellular fractionation whether a small proportion of the DHlipDH was present at the plasma membrane of the cell. If there is a plasma membrane-DHlipDH in these cells, this would be very unusual, as there are few enzymes located in both the mitochondrion and the plasma membrane of an organism. If there is no plasma membrane DHlipDH in procyclics, then presumably this DHlipDH only has a function in the bloodstream form. Therefore, the possible functions of the enzyme are narrowed down to those which occur in one form, but not the other. Indeed, several membrane processes are very different between the two forms, with receptor-mediated endocytosis stopping in the procyclic cells, VSG being swapped for procyclin, and glucose transport changing (see section 1.2.2).

8.5.2 Intracellular targetting

With the small size of the eukaryotic mitochondrial genome, it is thought that the majority of constituent polypeptides are encoded in the nucleus and are synthesized on free ribosomes in the cytosol (see De Marcucci *et al.*, 1988). The trypanosomes are no exception in this respect, with the kinetoplast DNA encoding only cytochromes, ATPase and a few other polypeptides (Simpson, 1987). In other eukaryotes, nuclear-encoded polypeptides are targetted to the mitochondrion by means of an N-terminal signal peptide, which may also serve to regulate uptake, maintain hydrophobic proteins in their soluble state, interact with soluble cytosolic recognition factors, and prevent premature aggregation. Such extensions have been demonstrated for the mammalian PDHC and 2OGDHC components, with pre-E3 having an extra 15-20 amino acids (M_r = 1500-3500) in each case (see 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 ($t_{1/2}$ approx. = 5-10 min; Hunter & Lindsay, 1986). The dimeric DHlipDH then interacts with the E2 or protein X components of the 2-oxo acid dehydrogenase complexes.

Targetting to the plasma membrane is reasonably well understood for secretory and integral membrane proteins, but for those loosely associated with the membrane, the position remains unclear. It is presumed, however, that interaction with cytoplasmic factors such as chaperones is essential for correct processing and targetting (see Rhee & Hunter, 1990; Ellis & van der Vies, 1991).

8.5.3 The genetics of DHlipDH

In *T.b.brucei*, there are two basic models which can be considered for the encoding of the enzyme, namely that the plasma membrane and mitochondrial DHlipDHs could be encoded by one gene or by two separate genes.

The one gene model: If there were one gene for DHlipDH in *T.b.brucei*, then the decisive event for protein targetting must occur either at the DNA, RNA or protein level. Some form of genetic rearrangement at the DNA level could occur to ensure that the correct leader sequence is attached to the active gene. Indeed, the gene could be transposed, or copied, in the bloodstream form cell, to an active expression site, such as one of those for VSG production. These expression sites contain several developmentally regulated genes (see van der Ploeg, 1987; Revelard *et al.*, 1990).

Alternatively, messenger RNA splicing may mediate differential targetting. An example of this is the mitochondrial and cytosolic fumarases of *Saccharomyces cerevisiae*, which are encoded by a single nuclear gene, FUM1 (Wu & Tzagoloff, 1987). Almost every imaginable pattern of alternative splicing has been reported in different organisms, and the process is often associated with stage-specific expression (Maniatis, 1991), making it a clear possibility for the trypanosomal DHlipDH.

The two gene model: With two genes for DHlipDH, one could contain the mitochondrial leader, and the other a signal for the plasma membrane. The citrate synthases of *S.cerevisiae* are examples of mitochondrial and non-mitochondrial proteins encoded by separate homologous genes (Rosenkrantz *et al.*, 1986). These differ markedly in their N-termini, with CIT1 having a 38-residue mitochondrial target sequence, and CIT2 having a different 20-amino acid N-terminal extension, the function of which is unknown. There are also examples of homologous genes in *T.b.brucei*. Glycosomal and cytosolic phosphoglycerate kinase genes, together with a third homologous gene, are encoded in a tandem array in a multicistronic transcription unit (Gibson *et al.*, 1988). This is processed into individual mRNAs by splicing and addition of mini-exons.

8.6 Future work

The beauty of the trypanosomal DHlipDH system is that one can study the enzyme in two separate cellular locations within one organism, but at different stages in its lifecycle. This allows studies at both the genetic and protein levels which are not possible in other organisms, and provides an excellent model system for looking at developmental regulation.

8.6.1 Enzymological studies

Within the procyclic cell, it remains to be seen whether a DHlipDH is present at the plasma membrane. Immunocytochemical localisation and digitonin-permeabilisation studies should provide an answer to this question (see section 4.4).

Purification of DHlipDH from the bloodstream form cell remains a priority, as protein sequence data are vital to genetic investigations on the enzyme. It will also be useful to purify the DHlipDH from the mitochondria of procyclics in order to gain further sequence data for genetic analysis.

8.6.2 Genetic studies

Probably the best route to structural and functional studies on the trypanosomal DHlipDH(s) is via cloning and sequencing of the gene(s) for the enzyme(s).

With the use of oligonucleotide probes, it should be possible to identify the gene(s) for DHlipDH in the genome of *T.b.brucei*. A probe derived from the N-terminal amino acid sequence of the bloodstream form DHlipDH, when applied to genomic digests should identify the correct gene by Southern analysis. If the one gene model were true, this gene might contain an upstream mitochondrial leader sequence separated from the gene by a splice or recombination site. With the two gene model, some other signal for the plasma membrane might be present, but there would be no mitochondrial leader. This is not a watertight method for distinguishing between the models, but the use of homologous probes, based upon conserved sequences from other DHlipDHs, should help to identify the one or two genes involved.

During the writing of this thesis, a gene encoding a DHlipDH has been identified in the genome of *T.b.brucei* using homologous probes (A.J.Else, University of Bath, personal communication). These were a 43mer constructed from part of the FAD domain (*E.coli* residues 40 to 54), and a 29mer from part of the central domain (*E.coli* residues 318 to 327), of DHlipDH (see Carothers *et al.*, 1989 for sequence alignments). The N-terminal sequence of the bloodstream form DHlipDH will be a useful tool in determining whether this gene encodes a plasma membrane DHlipDH. Indeed, any protein sequence data from either the plasma membrane or mitochondrial enzymes would be very helpful in this respect.

8.6.3 Structural and functional studies

Once the gene for the bloodstream form DHlipDH has been identified and cloned, it should be possible to express the enzyme in *E.coli*, as a prelude to structural studies such as nuclear magnetic resonance or X-ray crystallography. Antibodies to the pure enzyme can be raised and used for immunocytochemical localisation and rapid purification through immunoprecipitation.

It should also be possible to use the gene for functional studies. Specific mutations could be made *in vitro* to produce inactive enzyme. The mutant gene could be recombined back into the genomes of live trypanosomes by homologous recombination, so that it replaces the existing gene. The effect of this mutation upon the survival of the cell and several cellular functions could be assessed (see Eid & Sollner-Webb, 1991). The same effect would be produced by making antisense RNA to a particular region of the gene, and introducing this into the cell, so that it binds to the DHlipDH gene and blocks its transcription (see Kinsman & Kinsman, 1988).

8.6.4 Developmental regulation

Another interesting direction for future research into DHlipDH in *T.b.brucei* could be to look at the developmental switching of the enzyme in its different locations. A technique for differentiating bloodstream to procyclic cells *in vitro* has been developed by Overath *et al.* (1987). Differentiation is triggered by the addition of citrate/*cis*-aconitate to the growth medium, and alteration of the temperature from 37°C to 26°C. Using this method, Durieux *et al.* (1991) followed alterations in carbohydrate catabolism and citric acid cycle enzymes over a 28 day time period. Whilst citrate synthase, isocitrate dehydrogenase (NAD^+), succinate dehydrogenase and fumarase were not detectable in bloodstream trypomastigotes, they all appeared after 24 h differentiation, except citrate synthase, which took 48 h to appear. The activities of all of the citric acid cycle enzymes then increased steadily up to 28 days post-stimulation. It would be very interesting to follow DHlipDH activities and locations and mRNA levels with this method, through which a greater understanding of switching events could be gained. There are problems, however, with the procedure, as significant levels of citrate synthase and NAD^+-dependent isocitrate dehydrogenase were detected in *in vitro*-produced procyclics. Citrate synthase activity is very low (Jenkins *et al.*, 1988), and NAD-IDH is not present in "true" procyclics (Fairlamb & Opperdoes, 1986). This suggests that the metabolic switching *in vitro* is not properly regulated, perhaps because the environment of the cells does not have the correct stimulating and modulating factors.

With current gene cloning and enzymological approaches to investigating the unusual DHlipDH of bloodstream form trypomastigotes, it should not be long before both the structure and function of this enzyme have been elucidated. The trypanosome, however,

remains a confounding organism to those who attempt to study it. With its unique combination of metabolic laziness and creative complexity, it still manages to evade attack from mammalian and insect hosts and drug designers around the world. The plasma membrane DHlipDH is perhaps a minor peculiarity when compared to the variable surface glycoprotein or glycosomal compartmentation, but even such small discoveries may prove essential to the war against this debilitating parasite, and to the gaining of a greater understanding of biological systems.

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