Glycolysis in *Crithidia fasciculata*

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by Ruth Denise Hale

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To Mum and Dad

SUMMARY

The trypanosomatid flagellate Crithidia fasciculata is an insect parasite, closely related to the pathogenic trypanosomes which cause sleeping sickness (Trypanosoma brucei brucei complex) in Africa and Chagas' disease (Trypanosoma cruzi) in South America.

In bloodstream form T.brucei the energy for the organism is derived totally from the breakdown of glucose via the glycolytic pathway, terminating with the production of pyruvate. The rate of glycolysis is about 50 times that of mammalian cells. Glycolysis is therefore an important chemotherapeutic target. The metabolism of C.fasciculata was investigated to determine whether pyruvate could be detected as an end-product of glucose metabolism. A detectable level of pyruvate efflux from the cells was found and some work was carried out investigating this process. The level of pyruvate efflux varied considerably depending on the culture conditions used. Antimycin A, an inhibitor of the electron transport chain, was found in some cases to greatly stimulate the efflux of pyruvate from the cell. The possibility of a specific transport system for pyruvate across the cell membrane was investigated by using a potential inhibitor, α-cyano-4-hydroxycinnamate.

Glycolysis in trypanosomatids is unique in that the glycolytic enzymes are contained within a membrane-bound compartment, the glycosome. The possibility of chemotherapeutic attack against either individual glycolytic enzymes, or against the glycolytic unit as a whole is therefore very attractive.

This project has involved a closer study of one of the key enzymes of glycolysis, hexokinase, which is located in the glycosome. Hexokinase from C.fasciculata has been purified 50-fold by subjecting total cell homogenates to ammonium sulphate precipitation, followed by hydrophobic interaction chromatography and ion-exchange chromatography. The final preparation had a subunit molecular mass of 50,000 on SDS-polyacrylamide electrophoresis. Gel filtration indicated that the native enzyme was a hexamer. Glucose-6-phosphate did not inhibit the enzyme.

The purified hexokinase from C.fasciculata was used to raise antiserum in rabbits, which was then used in an attempt to study the location and incorporation of hexokinase in the glycosome. Western blot analysis of total cell homogenates showed a single major polypeptide of molecular mass 50,000. The compartmentation of hexokinase in C.fasciculata was confirmed by selective digitonin extraction of whole cells. Immunoprecipitation was carried out using polypeptides labelled in vivo with [^{35}S]-methionine. This technique was used to study the effect of potential processing inhibitors on uptake of hexokinase by the glycosome. No evidence was found for the accumulation of hexokinase in the cytosol, in either a mature form or as an unprocessed precursor. In vitro translation of mRNA from C.fasciculata indicated that hexokinase is translated at its mature subunit size.

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ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CIE	crossed immunoelectrophoresis
CM-cellulose	carboxymethyl cellulose
CNS	central nervous system
CoA	coenzyme A
DEAE-cellulose (DE52)	diethylaminoethyl cellulose
DHAP	dihydroxyacetone phosphate
DNA	deoxyribonucleic acid
cDNA	complementary DNA
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
EEO	electroendosmosis
FAO	Food and Agriculture Organization of the United Nations
FPLC	fast protein liquid chromatograph
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HK	hexokinase
K_m	Michaelis constant
M_r	molecular mass
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)

$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NBT	nitroblue tetrazolium
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PEG	polyethylene glycol
pI	isoelectric point
PMS	phenazine methosulphate
PMSF	phenylmethylsulphonyl fluoride
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
mRNA	messenger RNA
RRL	rabbit reticulocyte lysate
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SHAM	salicylhydroxamic acid
TCA (precipitation)	trichloroacetic acid
TCA cycle	tricarboxylic acid cycle
TEMED	N,N,N',N' tetramethylethylenediamine
TLCK	Nα-p-tosyl-L-lysine chloromethyl ketone
Tris	Tris (hydroxymethyl) aminomethane
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TS	Tris saline

UK5099	α-cyano-β-(1 phenylindol-3-yl) acrylate
V_{max}	maximal rate of reaction
WBB	western blot buffer
WHO	World Health Organization

Enzymes

glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

hexokinase (EC 2.7.1.1)

lactate dehydrogenase (EC 1.1.1.27)

malic enzyme (EC 1.1.1.40)

pyruvate kinase (EC 2.7.1.40)

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INTRODUCTION

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1.1.1 Trypanosomiases and Their Control

The pathogenic trypanosomes are responsible for serious diseases in both man and domestic animals throughout large regions of Africa and South America. Thousands of people die every year as a result of infection (Ellis, 1985). The report of a joint WHO Expert Committee and FAO expert consultation (WHO, 1979) stated that "trypanosomiases in Africa are still one of the most serious threats to the health of man and a serious obstacle to the development of agricultural industry". In Africa trypanosomes cause not only human sleeping sickness (Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense), but also nagana in cattle (Trypanosoma brucei brucei), surra in horses and camels (Trypanosoma evansi) and dourine in horses (Trypanosoma equiperdum). Untreated the disease is fatal due to invasion of the central nervous system (CNS) and its consequent destruction (Fairlamb, 1982). It was estimated that about 35 million people and approximately 25 million cattle are exposed to the risk of infection (WHO, 1979). More recent reports suggest that 45 million people are at risk (Goodwin, 1985). The high incidence of the disease means that breeding of domestic livestock throughout tropical Africa is impossible. Over 3 million cattle die each year and the rearing of domestic cattle, sheep and goats is impossible in 10 million square kilometres of Africa. Consequently animal trypanosomiases cause severe nutritional and economic problems. Animals in S.America and East Africa also provide a reservoir for those trypanosomes pathogenic to man.

In South America T.cruzi infects an estimated 10 million people, causing Chagas' disease. There is no effective treatment. Only in the very early stages when it often goes unnoticed is effective treatment possible. Young children are particularly likely to die in the acute febrile stages, whereas adults tend to survive to the chronic phase of the illness. Then the parasite damages heart muscle and autonomic nerve tissue, causing respectively congestive heart failure and dilation of the oesophagus and colon (Fairlamb, 1982).

In South America about 120,000 new cases of Chagas' disease are reported every year in Brazil alone. In Africa there is an annual incidence of about 20,000 cases of human sleeping sickness every year, and the disease constantly threatens to reach epidemic proportions. The figure has more than doubled since 1979 due to epidemics in Cameroon, Sudan and Uganda (Ellis, 1985; Goodwin, 1985).

There are several ways to approach the problem of trypanosomiasis. The political climate and environmental conditions in the countries which suffer most are responsible to a large extent for the problems which arise. In South America rehousing and destruction of the mud huts which people inhabit would also destroy the habitat in which the insect vectors thrive (Gutteridge and Coombs, 1977). The population and development of jungle areas also increases the problem. Civil unrest in several African countries in recent years has given rise to outbreaks of sleeping sickness in epidemic proportions due to the breakdown of diagnostic and surveillance facilities (Ellis, 1985; Goodwin, 1985).

The spread of infections can be controlled by the use of insect vector control methods. However, the use of these methods can only maintain certain defined areas in a vast continent and is only effective if regular respraying with insecticides is carried out, which may not be cost-effective.

The potential for a widespread immunization programme is hindered by the ability of the African trypanosomes to vary the antigenic determinants of their coat protein. This means that the likelihood of developing a suitable vaccine in the near future is not high, although a considerable amount of research is being carried out in this field. The stercorarian trypanosome T.cruzi is able to evade the host immune response by multiplying and developing intracellularly (Gutteridge and Coombs, 1977; Goodwin, 1985).

These methods for the control of trypanosomiases, as well as having limitations, are of use only in the prevention of the disease. The only way of dealing with infections that have already taken place is to use chemotherapy.

1.1.2 Chemotherapy

There is no totally suitable drug available for treatment of any of the trypanosomiases. The trypanocidal drugs currently available have several disadvantages. There are only a few of them, they have serious side effects, and there is a problem of widespread drug resistance (Fairlamb et al, 1977).

The drugs available for treatment of African trypanosomiases fall into two groups. The first of these includes the non-metallic, organic preparations suramin and pentamidine.

Drugs in this group can only be used in the early stages of the disease before invasion of the CNS occurs as they will not cross the blood-brain barrier. There is a danger of possibly fatal side-effects (WHO, 1979). The second group of drugs used in the later stages of the disease, when there is CNS involvement, are the more toxic arsenicals and nitrofurazone. Melarsoprol is the only drug giving a reasonable chance of a cure when the CNS is involved and the infection is by T.b.rhodesiense. In late stages of infection with T.b.gambiense it is the preferred drug from a number of arsenicals. However, it is extremely dangerous, causing mortality in 2-5% of cases. There is also a problem with drug resistance, and the solvent used is an irritant. When melarsoprol is ineffective nitrofurazone, which is also toxic and causes side-effects, is used (WHO, 1979). A drug having widespread veterinary use is Berenil, which is related to pentamidine. Although this can be used in the treatment of early stages of human trypanosomiasis, it has never been registered for use in man. The use of these drugs for chemoprophylaxis is also limited. In areas where T.b.gambiense infection is high suramin and pentamidine, preferably the latter, may be used to give a maximum of six months protection. In areas with a high incidence of T.b.rhodesiense chemoprophylaxis is not recommended because of the higher risk of drug resistance with this subspecies (WHO, 1979; Gutteridge, 1985).

For Chagas' disease there are no drugs registered for chemoprophylactic use. Two drugs, nifurtimox and benznidazole were introduced in the late 70's for treatment of acute and chronic infections. Nifurtimox (Lampit) can control acute cases

but is less effective against chronic infections (about 90%). Benznidazole (Radanil) is effective against more than 80% of acute and chronic cases (Gutteridge, 1985).

1.1.3 Drug Development

The limited number of drugs available, with their associated problems of drug resistance and side-effects, means that new drugs, both for curative and prophylactic use, are urgently required for both human and animal forms of trypanosomiases. "Highly active compounds of low toxicity that are easily administered and cheap to produce are needed" (WHO,1979). Most of the drugs currently available arose as a result of a random screening programme rather than fundamental research. However, the results from this are not rewarding and the costs are high. The countries involved are not economically well-off and pharmaceutical companies are therefore unwilling to become involved in developing drugs (Gutteridge and Coombs, 1977). In the treatment of African trypanosomiases, no new drug has advanced beyond the experimental stage since 1960. It has been estimated that to obtain one or two interesting compounds 10,000 have to be screened, at a cost of about £100 million over 10 years, and of these there is only a 10% chance of successful development (WHO, 1979; Gutteridge, 1985).

The alternative to this random approach is a more rational one, dependent on a good knowledge of the biochemistry of both parasite and host (Gutteridge and Coombs, 1977). For many of the drugs currently available, the details of their mode of action are uncertain. Some are thought to inhibit energy metabolism,

others to interfere with DNA synthesis (Gutteridge, 1985). A clearer understanding of the metabolism of the protozoa is required, not only to elucidate the mode of action of currently available drugs and to improve them, but also to enable more efficient targeting of drugs to specific points of the metabolism. Greater knowledge would enable the design of drugs exploiting areas of the parasite's metabolism which differ from that of the hosts. For many years vast sums of money have been spent on random screening programmes, whilst until recently very little has been invested in fundamental research. These two avenues of approach are now considered by the World Health Organisation to be of equal importance (WHO, 1979).

1.2 Trypanosomes - The Organisms

1.2.1 Historical Background

The first recorded observation of insect parasites was Anthony van Leeuwenhoek's description of 'animalcules' in the gut of a horsefly, when writing to Robert Hooke at the Royal Society in 1680 (Dobell, 1932). These organisms were originally thought to be Leptomonas but, in view of later observations on horsefly flagellates, they were very probably Blastocrithidia, Crithidia or Trypanosoma (Wallace, 1966). Leeuwenhoek was therefore probably the first man to see a trypanosome.

During the mid 19th century, trypanosomes were seen in fish, frogs, moles and rats and it was soon realised that all vertebrates, invertebrates and even some plants were liable to harbour parasitic protozoa of the family Trypanosomatidae (Hoare, 1972; Goodwin, 1985). Kent (1880) established the genera

Leptomonas and Herpetomonas, and also recognised the similarity between insect parasites and the trypanosomes of mammals. In 1898 Ross (1906) found parasites in mosquitoes and observed that they were found attached to the intestinal wall, but that when disturbed they became free, elongated, and swam actively. Between 1902 and 1904 Léger published a number of descriptions of trypanosomatids from various diptera and proposed the genus name Crithidia.

Subsequent to this there was a period of some confusion regarding the taxonomy of the family. There were queries over whether some or even all of the insect trypanosomes previously observed were in fact stages in the life-cycles of the digenetic trypanosomes or Leishmania (Wallace, 1966). The importance of trypanosomes as pathogens was recognised towards the end of the 19th century by Lewis and Evans in India and by Bruce in Africa (Hoare, 1972). The invasion of man into sylvatic zoonotic cycles became an additional source of food for the blood-sucking arthropods and an accidental host for the parasites they carried. African sleeping sickness was transmitted from antelopes to man and his domestic stock by tsetse flies. Chagas' disease was transmitted to man from the wild animals of South America by reduviid bugs which rapidly adapted to feeding and breeding in human dwellings. Both forms were more pathogenic in their new hosts (Goodwin, 1985). With the increased knowledge of the pathogenic trypanosomes, the insect trypanosomatids could be distinguished more easily, and Wenyon (1926) began to untangle the web of confusion regarding the taxonomy. However, the true Crithidia species were still wrongly assigned to the sub-genus

Strigomonas (Lwoff and Lwoff, 1931). Work on the morphology and nutrition of the various genera continued into the early half of this century, when the genera were unequivocally established, and the genus Crithidia was correctly distinguished (Wallace, 1943; 1966).

1.2.2 Crithidia fasciculata as a Model Organism

C.fasciculata is often used as a model organism for its pathogenic relatives. It has many practical advantages which make it suitable as a model eukaryote as well as a model trypanosomatid (Glassberg et al, 1985). It grows rapidly in vitro, in either complex or defined media, in bulk quantities if required. When plated on agar it forms colonies from single cells, mutants can be isolated, and it is cloneable (Gottlieb et al, 1972; Goldberg et al, 1974). C.fasciculata is biochemically related to the pathogenic trypanosomes but is cheaper and safer to use than these organisms.

The use of trypanosomatids that can be grown in vitro and utilised in drug studies is very important in the possible elucidation of drug action in pathogenic trypanosomes (Hill and Hutner, 1968). C.fasciculata is sensitive to at least some trypanocides (Gutteridge, 1969; Hill and Hutner, 1968; Goldberg et al, 1974). Previously, C.fasciculata has been used as an in vitro test system for screening and studying drug action. Newton (1962) used C.oncopelti as a test system, but this organism is now known to be unsuitable due to the presence of endosymbionts. The arguments put forward for its use apply better to C.fasciculata.

The disadvantages of using C.fasciculata are clear, and some have considered it unsuitable as a model organism (Gottlieb et al, 1972). Morphologically, C.fasciculata is distinct from T.brucei (Gutteridge and Coombs, 1977) and bloodstream form T.brucei differs biochemically in some aspects of metabolism. Bloodstream form T.brucei is insensitive to cyanide and has no active cytochrome system, while in contrast C.fasciculata and the insect vector and culture forms of T.brucei are cyanide-sensitive, have active cytochrome systems, and have an altered kinetoplast-mitochondrion complex (Bowman and Flynn, 1976; Vickerman, 1985). These differences might influence drug sensitivity and make C.fasciculata unsuitable for assessing the mode of action of drugs (Gottlieb et al, 1972).

In spite of biochemical and morphological differences, there are considerable similarities in the intermediary metabolism of C.fasciculata and the pathogenic trypanosomes. In particular the location of the glycolytic enzymes in a membrane-bound organelle, the glycosome, is an apparently universal feature of the trypanosomatids (Opperdoes, 1985). Studies on glycolysis in C.fasciculata may therefore reveal information which is applicable to trypanosomes in general, and particularly bloodstream form T.brucei which is totally dependent on glycolysis. Any information may be applied to a more rational approach to drug design or screening.

1.2.3 Classification

The phylum Protozoa has recently been reclassified and upgraded from a phylum to a sub-kingdom (Molyneux and Ashford,

1983). The family Trypanosomatidae (Doflein, 1901) is classified as follows :-

SUBKINGDOM	Protozoa
PHYLUM	Sarcomastigophera
SUBPHYLUM	Mastigophera
CLASS	Zoomastigophera
ORDER	Kinetoplastida
SUBORDER	Trypanosomatina
FAMILY	Trypanosomatidae

The genera into which the trypanosomatidae is divided (Fig. 1.1) are all real and separable groups, although species differences may be less certain (Wallace, 1966). The genus Leptomonas is the simplest and most generalized of the genera, and each of the others differs in some particular manner. The genus Crithidia was defined in 1902 by Léger for the species Crithidia fasciculata from the mosquito Anopheles malculipennis. He emphasized the stubby, truncate shape 'en grain d'orge ophat'. In 1903 he described two more species, again noting the barley-corn shape. During the general confusion over the taxonomy of the various genera, however, confusion arose when the genus Crithidia was wrongly characterised as having an undulating membrane. It was due to this that it became generally accepted that 'crithidial' forms of parasites were merely immature stages of the trypanosomes in vertebrates. Lwoff and Lwoff (1931) proposed Strigomonas as a subgenus of Leptomonas to include the truly distinct Crithidia. It was not until 1943 that the error was corrected when Wallace isolated C.fasciculata in culture and corrected the description of the genus Crithidia.

1.2.4 Morphology

Crithidia are monoxenous parasites of insects, most frequently parasitizing the orders Diptera and Hemiptera. The

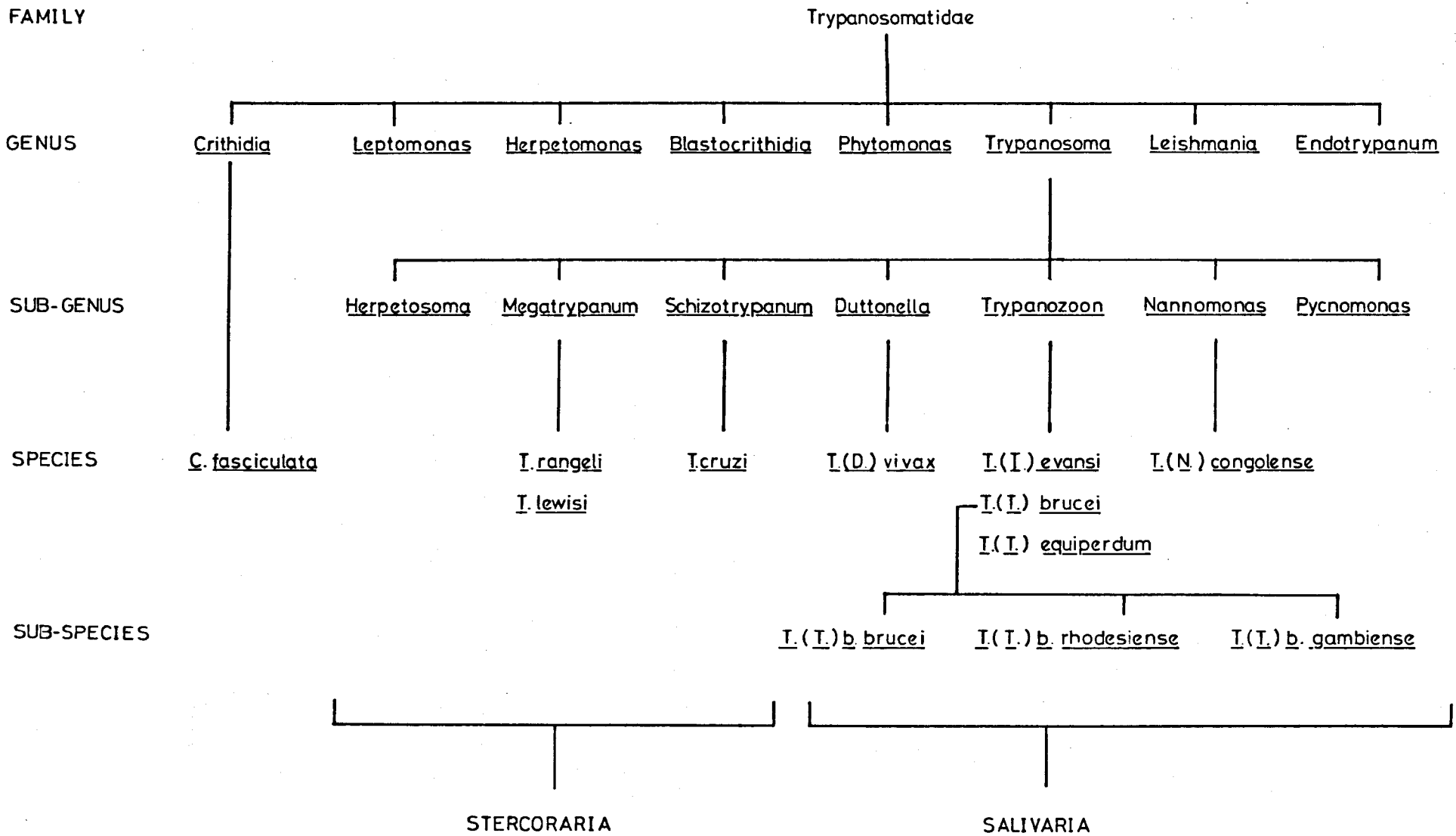

Figure 1.1 **Classification of the Family Trypanosomatidae**

genus is defined by the presence of choanomastigote (barley-corn) forms with a single free flagellum (Molyneux and Ashford, 1983). They are relatively small, the body usually being shorter and wider than other genera. The anterior end is truncate and the body is often constricted in the anterior third to produce a vase-like shape. The flagellar pocket is wide, the mouth occupying most of the anterior end. The kinetoplast, a DNA-containing extension of the mitochondrion, lies at the base of the flagellum, is large, lateral and sometimes near or beside the nucleus. The single anterior flagellum moves in a circular motion. The length of Crithidia species is usually around 4-13 μm with a flagellum of 7-14 μm (Wallace, 1966; Molyneux and Ashford, 1983).

Morphologically C.fasciculata is most similar to the epimastigote stages of the T.brucei life-cycle. The T.brucei group of African trypanosomes includes some stocks which are pathogenic to man (T.b.rhodesiense and T.b.gambiense), and some which are non-infective (T.b.brucei). They are however morphologically indistinguishable, and all subject to the alterations in morphology which the progression of the life-cycle induces. They range from the long slender forms present in the mammalian host, which later develop into the short stumpy forms that multiply when ingested by the insect vectors, to the much longer forms, lacking a protein coat, present in the insect mid-gut (procyclic forms) (Gutteridge and Coombs, 1977; Vickerman, 1985).

1.2.5 Life-Cycle

The life-cycle of an individual haemoflagellate is relatively simple. A longitudinal fission is followed by growth. When fission occurs the kinetoplast divides, followed by the nucleus. A new flagellum arises from near one of the daughter kinetoplasts (Wallace, 1966). C.fasciculata is found in Anopheles and Culex mosquitoes throughout the world. Parasites are usually located in the mid and hind gut of the insect, often clustered together, or attached like the pile of a carpet to the intestinal wall. C.fasciculata in mosquitoes has a free-living stage in water, when it is transmitted by passing between larvae. This results in later infection of adults as the parasites are carried through from larval to adult stages (Molyneux and Ashford, 1983).

There are two main groups of mammalian trypanosomes, divided according to their mode of transmission. They are the Salivaria (eg. T.brucei) which are inoculated through the fly's salivary glands, and the Stercoraria (T.cruzi) which are transmitted by faecal contamination of skin lesions and mucous membranes (Fairlamb, 1982). T.brucei, a typical salivarian trypanosome, multiplies in the blood and other fluids of man, game and cattle. It is one of several species transmitted by the tsetse fly in Africa. Transmission is cyclical and involves ingestion of short stumpy forms by the blood-sucking tsetse, development into procyclic trypomastigotes, migration from gut to salivary glands of the insect (epimastigote stage) and development into infective metacyclic trypomastigotes. These are inoculated into the bloodstream of the vertebrate host during feeding. There

they develop into the long slender trypomastigote forms which migrate in the bloodstream and may invade the CNS. Bloodstream forms show polymorphism between long slender and short stumpy forms. The short stumpy forms are thought to be a preadaptation to survival in the insect mid-gut. Long slender forms do not multiply when ingested by the fly, they either die or change into stumpy forms (Gutteridge and Coombs, 1977; Kilgour, 1980; Vickerman, 1980).

T.cruzi, the causative agent of Chagas' disease in South America, multiplies inside the muscle tissue of the heart and alimentary tract of man. Whilst growing and dividing intracellularly the parasites are amastigotes. The motile trypomastigotes then emerge into the body fluids. They do not divide, but serve to spread the infection either by entering new cells and multiplying as amastigotes again, or by being picked up by the insect vector, the blood-sucking reduviid bug, whilst it is feeding. The parasites multiply and develop in the insect gut in the epimastigote form, and the cycle is completed when they are deposited in the insects faeces as infective metacyclic trypomastigotes (Gutteridge and Coombs, 1977; Kilgour, 1980; Vickerman, 1985).

1.3 The Biochemistry of Trypanosomes

1.3.1 The Importance of Glycolysis to Trypanosomes

The glycolytic pathway is a primary source of energy in trypanosomes, particularly so in bloodstream form T.brucei where glucose is the most important exogenous substrate. These organisms are totally dependent on energy produced from the

glycolytic breakdown of glucose from the host bloodstream. Enormous quantities of glucose are consumed, as much as their own dry body weight every hour. Glucose metabolism proceeds at an extremely high rate (85nm glucose/min/mg protein) and this is directly coupled to ATP production (Fairlamb and Bowman, 1980). The rate of respiration in bloodstream form T.brucei is about 50 times higher than that in its mammalian host. There are no energy stores in T.brucei and therefore the organism cannot survive for more than a few minutes without the carbon supply necessary to maintain ATP levels (Opperdoes, 1983). The removal of exogenous substrate results in a rapid loss of respiratory activity and motility, and the cells disintegrate (Ryley, 1956). Similarly any substance interfering directly with energy production will have a rapid trypanocidal effect (eg. the combination of salicylhydroxamic acid (SHAM) and glycerol completely blocks glycolysis and eliminates trypanosomes from the bloodstream of an infected host rapidly (Clarkson and Brohn, 1976; Fairlamb et al, 1977)). Any drug acting by rapidly blocking glycolysis will also shorten the required duration time of treatment and thereby lessen the possibility of drug resistance building up. The high rate of glycolysis is required to maintain the generation time of the trypanosome, therefore even an incomplete inhibition may slow down cell division sufficiently to allow the host to overcome the infection by means of its own defence systems (Opperdoes, 1983).

The maintenance of such a high glycolytic flux may be helped by the compartmentation of glycolytic enzymes within a membrane-bound organelle, which has been termed 'the glycosome'

(Opperdoes and Borst, 1977). The glycolytic enzymes themselves may individually exhibit properties differing from their soluble host counterparts, which could be exploited chemotherapeutically. Alternatively, it is possible that the glycolytic unit as a whole may be a potential target since not only is it enclosed within a membrane-bound organelle, but the enzymes themselves may be associated into a multi-enzyme complex within the glycosome (Oduro et al, 1980a&b; Opperdoes and Nwagwu, 1980). In most eukaryotic systems the glycolytic enzymes are thought to form only weak interactions with each other (Mowbray and Moses, 1976; Ottaway and Mowbray, 1977). The assembly of the proteins into the glycosome is also a subject of some interest.

The glycosome is, however, not a specialized adaptation to the high glycolytic flux in bloodstream form T.brucei as it is also present in insect vector stages of T.brucei, and has been shown to be a characteristic feature of the major genera of the Trypanosomatidae (Opperdoes, 1985). It is possible therefore that studies on glycolysis and the glycosome, in C.fasciculata, may be of relevance, not only in the treatment of African trypanosomiases, but also for dealing with infections of T.cruzi and Leishmania.

1.3.2 Structural and Biochemical Peculiarities

Over the last few years rapid advances have been made in understanding the metabolism of the trypanosomes and in identifying areas for potential attack. These organisms have a number of structural and biochemical features which are peculiar

to themselves and thus make attractive chemotherapeutic targets. There are two structural features which are of biochemical importance, and which are also apparently unique to the Kinetoplastida. The first of these is the glycosome, the membrane-bound organelle enclosing glycolytic enzymes. The second is a single, often highly branched, mitochondrion which extends into a specialized structure, the kinetoplast (Borst and Hoeijmakers, 1979; Englund, 1981; Stuart, 1983). The kinetoplast is located at the base of the flagellum and contains a gigantic network of catenated circles of DNA. There is a single network in each cell, representing 5-30% of the total cellular DNA, and composed of a mixture of many mini-circles and fewer maxi-circles (Fairlamb *et al*, 1978). The maxi-circles seem to have a similar role to that of conventional mitochondrial DNA in higher eukaryotes (Borst *et al*, 1979). The mini-circles, which make up 90-95% of the network mass, are thought not to be transcribed, and their function is unknown (Englund, 1981). However, some evidence for the expression of mini-circle DNA from *C.fasciculata* in *E.coli* has been found (Schlomai and Zadok, 1984; 1986) and examination of the DNA sequence of mini-circle in *C.fasciculata* suggests that expression of some small proteins is feasible (Sugisaki and Ray, 1987).

1.3.3 The Glycosome

The 'glycosome' was the term given to the microbody-like organelles originally discovered to be the location of all the glycolytic enzymes necessary for the conversion of glucose and glycerol into 3-phosphoglycerate in bloodstream form *T.brucei*

(Opperdoes and Borst, 1977). Since then these organelles have been identified in insect vector forms of T.brucei (Opperdoes et al, 1981; Broman et al, 1982) as well as all the major representatives of the Trypanosomatidae: T.cruzi (Taylor et al, 1980; Cannata et al, 1982), Leishmania (Coombs et al, 1982) and Crithidia (Taylor et al, 1980; Opperdoes et al, 1977a; Opperdoes, 1981). This apparently unique feature is in contrast to the situation in other mammalian and eukaryotic cells where the glycolytic reactions occur in the cytosol (Ottaway and Mowbray, 1977), although some enzymes may be associated with particulate fractions, eg. brain hexokinase is associated with the mitochondrion (Crane and Sols, 1953) and some glycolytic enzymes are actin-bound in muscle (Masters, 1981).

Since the discovery of the glycosome and its apparent glycolytic function it has been found that several other enzyme activities, not directly involved in glycolysis, are located within the organelle (Hart et al, 1984; Opperdoes, 1985). They include adenylate kinase activity (Opperdoes et al, 1981); two enzymes involved in pyrimidine biosynthesis (Hammond and Gutteridge, 1980; Hammond et al, 1981; Pragobol et al, 1984); enzymes involved in the purine salvage pathway (Gutteridge and Davies, 1982); enzymes involved in ether lipid synthesis (Opperdoes, 1984); and the enzymes involved in carbon dioxide fixation and oxidation of NADH (phosphoenolpyruvate carboxykinase and malate dehydrogenase) (Opperdoes et al, 1981; Cannata et al, 1982; Opperdoes and Cottem, 1982; Broman et al, 1983). With the discovery of such diverse enzyme activities in the glycosome it has become accepted that it has a less

specialized role than first envisaged, and that it is important for metabolic functions other than glycolysis. It is in fact more like a conventional microbody such as the peroxisomes and glyoxysomes, although it is unique amongst microbodies in including glycolytic enzymes.

Morphologically the glycosome resembles all other microbodies present in eukaryotic cells. It is bounded by a single membrane, and appears in sections to be round or oval shaped. Often it has a crystalloid core and an electron-dense matrix (Opperdoes et al, 1984). Although morphologically similar to the peroxisomes and glyoxysomes, no enzymes typical of these organelles have been discovered in the trypanosomatids except for catalase in some species of Crithidia and Leptomonas (Muse and Roberts, 1973; Opperdoes et al, 1977a; Souto-Padron and de Souza, 1982) and peroxidase in T.cruzi (Docampo et al, 1976).

A detailed study of the glycosomes in bloodstream form T.brucei has been carried out (Opperdoes et al, 1984). One homogenous class of microbodies was found with an average diameter of 0.27μm and occupying about 4.3% of the total cell volume. A few of the organelles exhibited a dense core, similar to that seen in peroxisomes. When present the core had a multilamellar appearance. It was calculated that there were an average of 230 glycosomes present in each T.brucei cell. The single membrane was found to contain only two major phospholipids, phosphatidylcholine and phosphatidylethanolamine, in a ratio of approximately 2:1 (Opperdoes et al, 1984). In procyclic T.brucei phosphatidylinositol and phosphatidylserine

were also found (Hart et al, 1984). No evidence was found for the presence of DNA in the glycosome (Opperdoes et al, 1984).

Analysis of the glycolytic enzymes within the organelle has shown that during the life-cycle of T.brucei distinct changes occur, not only in the enzyme complement, but also in the specific activities of enzymes which are present in insect vector and bloodstream forms (Hart et al, 1984). There is a marked decrease in the glycolytic capacity of the glycosome upon transformation to the insect vector forms. Thus it would appear that repression of mitochondrial activity and size in T.brucei is accompanied by an increase in glycolytic activity, which compensates for loss of energy production from the mitochondrial respiratory chain, and takes advantage of the glucose-rich environment of the host bloodstream. In bloodstream forms the specific activities of hexokinase, phosphoglucose isomerase, phosphofructokinase, fructose-bisphosphate aldolase, glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase are all higher. The activities of triose phosphate isomerase, glycerol-3-phosphate dehydrogenase and glycerol kinase remain the same. The activity of adenylate kinase increases on transformation to insect vector forms, as does that of phosphoenolpyruvate carboxykinase and malate dehydrogenase. Finally, the enzyme phosphoglycerate kinase, which is located in the glycosome of bloodstream form T.brucei, is found in the cytosol after transformation to insect vector forms (Hart et al, 1984).

1.3.4 The Biochemistry of Energy Production

The oxidation of glucose is an important source of energy in trypanosomes, although they may also derive energy from other substrates. In insect mid-gut stages of T.brucei there is a high rate of oxidation of the amino acid proline (Bowman and Flynn, 1976; Vickerman, 1985). C.fasciculata is also capable of oxidising amino acids as an alternative to glucose. It has been reported that amino acids are the preferred energy source for this organism (Marr et al, 1977), although there is now evidence that conflicts with this view, with glycolysis being the primary energy source (Cazzulo et al, 1985). In bloodstream form T.brucei energy is derived from the oxidation of glucose from the host bloodstream. Glycolysis, and its associated reactions, is therefore the main focus of attention when considering possibilities for chemotherapeutic intervention.

The metabolism of glucose in the trypanosomes occurs basically by the classic Embden-Meyerhof pathway (Grant and Fulton, 1957). Aerobic glycolysis occurs with the breakdown of glucose to pyruvate, the fate of which is determined by the species or morphology of the organism (Bowman and Flynn, 1976).

In T.brucei there is a significant alteration in metabolism which coincides with the transition between insect vector stages and bloodstream forms. In long slender bloodstream forms glycolysis proceeds from the breakdown of glucose and terminates with the production of pyruvate (Ryley, 1956; Flynn and Bowman, 1973; Brohn and Clarkson, 1978). The organism does not possess the enzymes necessary for the further metabolism of pyruvate and it is lost into the host bloodstream. Mitochondrial biogenesis

is repressed, no cytochromes are present and a functional TCA cycle is lacking (Fulton and Spooner, 1959; Flynn and Bowman, 1973). There is no lactate dehydrogenase (Dixon, 1966) and therefore reducing equivalents produced during glycolysis are reoxidised by the combined action of a glycerol-3-phosphate : dihydroxyacetone phosphate shuttle and glycerol-3-phosphate oxidase using molecular oxygen (Fairlamb and Bowman, 1977) (Fig. 1.2). Net ATP synthesis occurs in the cytoplasm with the conversion of 3-phosphoglycerate to pyruvate (Opperdoes and Borst, 1977). The shuttle involves a NAD^+-dependent glycerol-3-phosphate dehydrogenase located in the glycosome, and is linked to a unique cyanide-insensitive terminal oxidase (Opperdoes et al, 1977a). Glycerol-3-phosphate oxidase has been localized to the mitochondrion (Opperdoes et al, 1977a) and appears to operate without the need for pyridine nucleotide cofactors or cytochromes in bloodstream form T.brucei. There is no evidence for the coupling of oxidative phosphorylation of ADP to the enzyme (Grant and Sargent, 1960; 1961). It has a very high activity and specificity for glycerol-3-phosphate (Bowman and Flynn, 1976). Although insensitive to cyanide, the oxidase can be inhibited by salicylhydroxamic acid (SHAM) (Fairlamb and Bowman, 1977). Inhibition by SHAM mimics the anaerobic glycolytic pathway where glucose is degraded into equimolar amounts of pyruvate and glycerol (Grant and Fulton, 1957; Brohn and Clarkson, 1980) and there is still a net ATP synthesis (Opperdoes et al, 1976). The compartmentation of enzymes within the glycosome allows for the accumulation of high levels of glycerol-3-phosphate and ADP. Each mole of triose phosphate

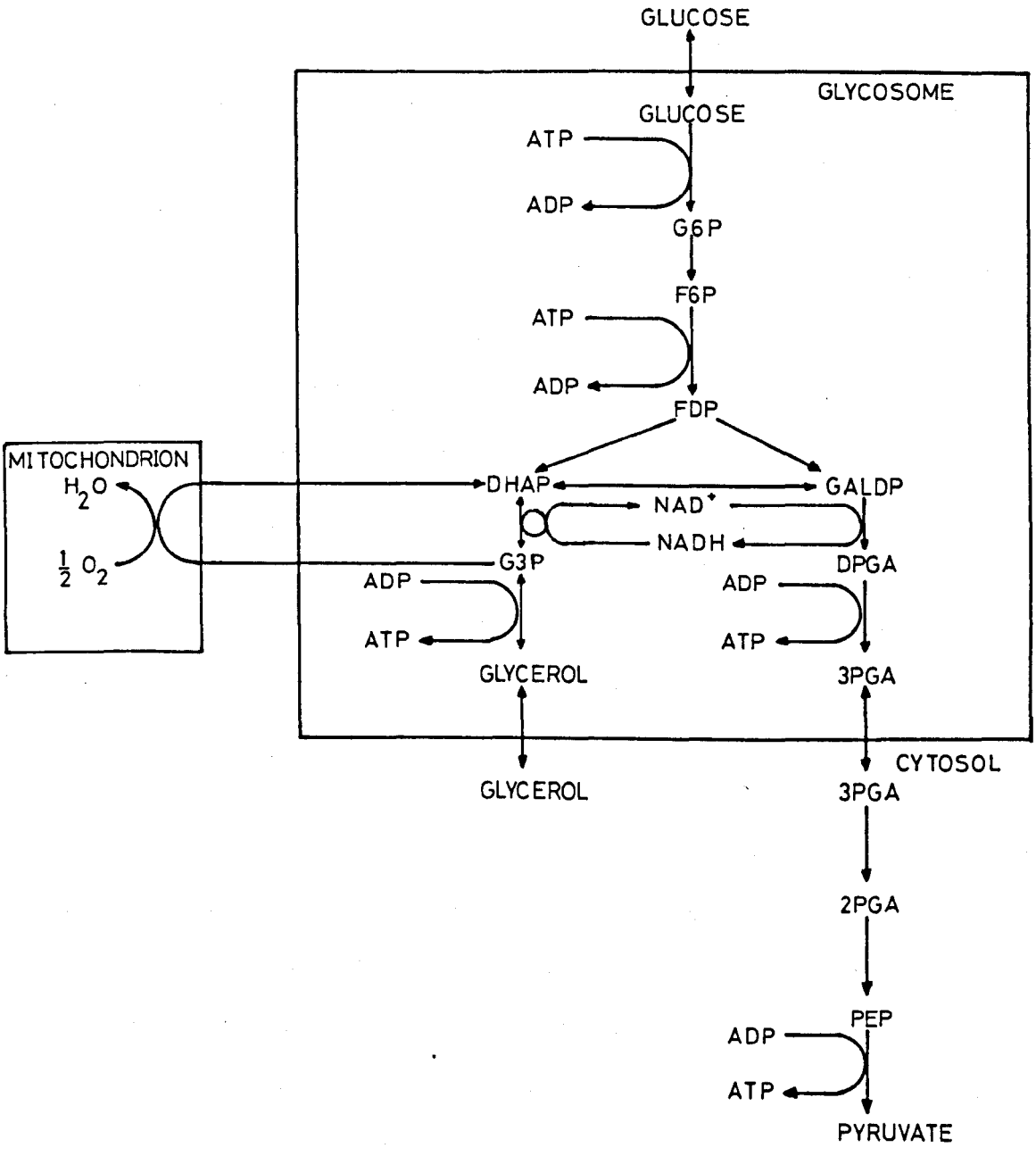

Figure 1.2 **Compartmentation of Glycolysis in Bloodstream Form T.brucei**

Abbreviations:- G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; FDP: fructose-1,6-diphosphate; DHAP: dihydroxyacetone phosphate; GALDP: glyceraldehyde phosphate; G3P: glycerol-3-phosphate; DPGA: diphosphoglycerate; 3PGA: 3-phosphoglycerate; 2PGA: 2-phosphoglycerate; PEP: phosphoenolpyruvate.

converted to pyruvate leads to the formation of one mole of NADH from NAD^+. This in turn is reoxidised to produce glycerol-3-phosphate from dihydroxyacetone phosphate. Glycerol is formed from the reversed action of glycerol kinase accompanied by ADP phosphorylation. Net ATP synthesis occurs, as before, in the cytoplasm with the formation of pyruvate from 3-phosphoglycerate (Opperdoes, 1983).

During the transition from long slender bloodstream form T.brucei to short stumpy forms, preadaptation to survival in the insect mid-gut begins (Fairlamb, 1982). New enzymes begin to appear in the mitochondrion, which develops immature, plate-like cristae (Brown et al, 1973), and some pyruvate can be further metabolized to succinate, acetate and a trace of carbon dioxide (Bowman, 1974). There is a full complement of TCA cycle enzymes present (Ryley, 1962), although citrate synthetase and succinate dehydrogenase levels are very low, meaning the cycle is unable to operate (Flynn and Bowman, 1973). Finally a complete cytochrome chain appears and respiration becomes partly cyanide-sensitive (Njogu et al, 1980; Bowman, 1974). With the completion of the transition to insect vector forms mitochondrial biogenesis is fully active, in contrast to the state in bloodstream forms. There is a very highly developed mitochondrion with all the enzymes necessary for an active TCA cycle.

In insect vector stages glucose is metabolised and at the same time carbon dioxide fixation occurs and high levels of succinate are produced from the cell (Ryley, 1962; Bowman, 1974). The transformation to insect vector forms brings about

not only changes in mitochondrial morphology and enzyme complement, but also alterations in the enzyme activities involved in the glycolytic pathway (Hart et al, 1984). While the activity of the dihydroxyacetone phosphate:glycerol-3-phosphate shuttle is reduced, there are elevated levels of both phosphoenolpyruvate carboxykinase and malate dehydrogenase (Opperdoes et al, 1981). It has been proposed that these two enzymes are responsible for the reoxidation of glycolytically reduced NAD^+ in insect vector forms (Opperdoes and Cottem, 1982; Broman et al, 1983) (Fig. 1.3). Phosphoenolpyruvate carboxykinase is located in the glycosome and is responsible for the fixation of carbon dioxide into oxaloacetate. Malate produced in the glycosome by the action of malate dehydrogenase is probably transported through the membrane to the cytosol where it can be converted into other metabolites. The presence of a 'malic' enzyme in the cytosol could explain the production of pyruvate from malate since there is no detectable pyruvate kinase in insect vector stages of T.brucei (Opperdoes and Cottem, 1982). This means that pyruvate cannot be formed by the conventional route from phosphoenolpyruvate.

In T.cruzi the pathways involved in carbohydrate metabolism are less clear, but both epimastigotes and trypomastigotes appear to resemble the state in insect vector stages of T.brucei (Bowman et al, 1963). Carbon dioxide, succinate and acetate are produced as end-products (Ryley, 1956; Bowman et al, 1963; Rogerson and Gutteridge, 1980). The metabolic pathways involved are probably the same as those in T.brucei, with the TCA cycle operating aerobically (Shaw et al, 1964; Docampo et al, 1978).

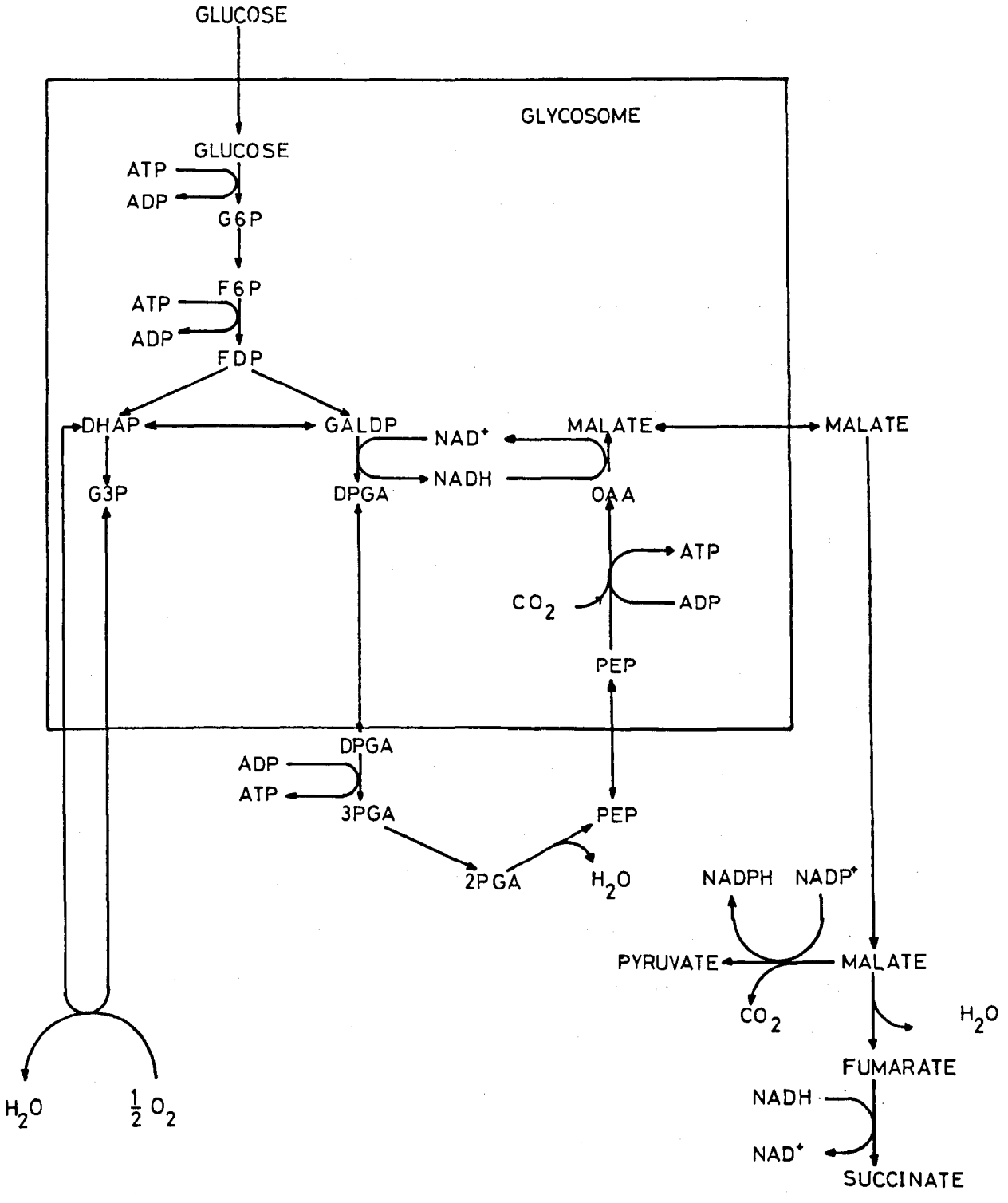

Figure 1.3 **Compartmentation of Glycolysis and Carbon Dioxide Fixation in Procyclic T.brucei**

Abbreviations:- G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; FDP: fructose-1,6-diphosphate; DHAP: dihydroxyacetone phosphate; GALDP: glyceraldehyde phosphate; G3P: glycerol-3-phosphate; DPGA: diphosphoglycerate; 3PGA: 3-phosphoglycerate 2PGA: 2-phosphoglycerate; PEP: phosphoenolpyruvate; OAA: oxaloacetate.

The presence of the glycerol-3-phosphate shuttle has not been demonstrated, and there is no evidence for a glycosomal glycerol-3-phosphate dehydrogenase (Cannata and Cazzulo, 1984a). The evidence is available that the enzymes required are present for the fixation of carbon dioxide to occur as described for T.brucei (Cataldi de Flombaum et al, 1977). There are both a glycosomal phosphoenolpyruvate carboxykinase (Cannata et al, 1982) and a cytosolic 'malic' enzyme (Cazzulo et al, 1980). There is a glycosomal malate dehydrogenase for the reoxidation of glycosomal NADH (Cannata and Cazzulo, 1984b).

The metabolic pathways involved in glucose catabolism in C.fasciculata appear to combine those operating in both bloodstream forms and insect vector forms of T.brucei. There is a glycerol-3-phosphate shuttle (Bacchi et al, 1968; Reynolds, 1975) with a glycosomal glycerol-3-phosphate dehydrogenase (Opperdoes et al, 1977a). There is also a pyruvate kinase (Taylor et al, 1980). The enzymes for carbon dioxide fixation, a glycosomal phosphoenolpyruvate carboxykinase (Cazzulo et al, 1980; Cannata et al, 1982) and a cytosolic, NADP-dependent, malic enzyme (Cazzulo et al, 1980), are present. The levels of phosphoenolpyruvate carboxykinase and malate dehydrogenase are both very high, and the use of carbon-13 nuclear magnetic resonance analysis has indicated that phosphoenolpyruvate is the primary acceptor for carbon dioxide fixation, leading to succinate formation (De los Santos et al, 1985).

In T.brucei insect vector forms, the fixation of carbon dioxide coupled to formation of oxaloacetate from phosphoenolpyruvate in the glycosome, leads to an additional ADP

phosphorylation (Opperdoes and Cottem, 1982). In order to compensate for this, and maintain a balance of ADP phosphorylation and ATP dephosphorylation in the glycosome (net ATP synthesis occurring in the cytosol) there is a shift in the location of the final glycosomal, glycolytic reaction. Upon transition from bloodstream forms to insect vector forms phosphoglycerate kinase is no longer present in the glycosome but is instead found in the cytosol (Opperdoes and Borst, 1977; Opperdoes *et al*, 1981). In *Crithidia* this enzyme is located both in the glycosome and in the cytosol (Opperdoes, 1981), which may indicate that the pathways found in both bloodstream and insect vector forms of *T.brucei* are operative in this organism, or that cultures are heterogeneous, i.e. contain more than one form.

1.4 Research Strategy

1.4.1 Pyruvate Production

The end-product of the aerobic metabolism of glucose in bloodstream form *T.brucei* is mainly pyruvate. Pyruvate cannot be metabolised further and is released into the host bloodstream in large quantities due to the rapid flux of glucose through the glycolytic pathway (Brohn and Clarkson, 1978). As many as 98% of the carbon atoms are released from the organism in the form of pyruvate (Grant and Fulton, 1957).

In other trypanosomatids, such as *C.fasciculata* and *T.cruzi*, glucose oxidation proceeds further giving a wider range of end-products. In the case of *C.fasciculata* succinate is the main end-product (eg. Marr, 1973; Cosgrove, 1959), with a variety of additional end-products such as pyruvate, ethanol and lactate

(Cosgrove, 1959; Marr et al, 1977; Schwartz, 1961). More recently a report from Cazzulo et al (1985) stated that the end-products of glucose degradation in C.fasciculata were succinate, ethanol, acetate and pyruvate.

In the present study C.fasciculata has been investigated to determine whether or not pyruvate could be detected in the incubation medium, as an end-product of glucose metabolism, at significant levels. The capacity of both exponential and stationary phase cells for pyruvate production has been examined. The effect of altering the exogenous carbohydrate source provided on production of pyruvate has also been investigated.

The catabolism of glucose follows the basic glycolytic pathway of Embden and Meyerhof through to the production of phosphoenolpyruvate (De los Santos et al, 1985). However, after this stage the pathways involved in producing the various metabolites are less certain. The production of succinate in C.fasciculata is proposed to occur by part of the TCA cycle operating in reverse (Klein et al, 1975; Marr et al, 1977). Oxaloacetate is formed by carbon dioxide fixation on phosphoenolpyruvate (Bacchi et al, 1970; Marr et al, 1977) and then either oxaloacetate or malate presumably enters the TCA cycle and then follows the reverse route via fumarase to fumarate, which is then reduced to succinate by the reverse action of succinic dehydrogenase (fumarate reductase) (Klein et al, 1975).

I have investigated the effect of including respiratory inhibitors in the incubation medium to determine whether there

was any effect on the level of pyruvate produced by the cell, which might indicate which pathways predominate in C.fasciculata.

1.4.2 Pyruvate Transport

Bloodstream form T.brucei produce large amounts of pyruvate which are subsequently lost from the cell into the surrounding medium. The manner in which pyruvate crosses the membrane is unknown. It may occur by a simple process of passive diffusion. However, the large quantities involved and the low pK_a of pyruvate suggest that a carrier system may be located in the membrane. In several eukaryotic systems evidence has been found for the presence of a carrier specific for the transport of carboxylic acids, such as pyruvate and lactate, across the cell membrane. Transport systems appearing to have similar properties have been found in rat hepatocytes (Monson et al, 1981a; 1981b), human erythrocytes (Halestrap and Denton, 1974) and tumour cells (Spencer and Lehninger, 1976). In each case it was possible to inhibit the passage of pyruvate and/or lactate across the cell membrane using specific inhibitors.

Erythrocytes efflux lactate quantitatively since they lack the enzymes necessary to metabolize it any further. Similarly, Ehrlich Ascites tumour cells metabolize glucose at a high rate producing lactate which is released from the cells in large amounts. It has been proposed that any compound arresting lactate transport would have an adverse effect on tumour cells by blocking glycolysis. By using inhibitors to almost completely block lactate transport glycolysis was inhibited. Prevention of

lactate leaving the cell led to an increase in intracellular levels of lactate and a consequent lowering of intracellular pH (Johnson et al, 1980).

If a similar specific carrier was found to exist in the cell membrane of T.brucei for pyruvate efflux, there would be a similar potential for inhibiting glycolysis. Blocking the carrier would cause a build-up of intracellular pyruvate, which could not be metabolized any further. Increasing levels of pyruvate could cause a drop in intracellular pH and thus inhibit glycolysis. I have used an inhibitor which successfully blocked transport of pyruvate and lactate in erythrocytes and tumour cells in an attempt to block the efflux of pyruvate from C.fasciculata.

1.4.3 Hexokinase

Hexokinase is one of the key glycolytic enzymes and is responsible for the initial step in the catabolism of glucose. No evidence has been found for the regulation of glycolytic flux, of the type found in most eukaryotic cells, involving hexokinase, phosphofructokinase and pyruvate kinase (Marr and Berens, 1977; Flynn and Bowman, 1980; Nwagwu and Opperdoes, 1982; Cronin and Tipton, 1985). The only regulatory mechanism would seem to be a rate-limiting first step. This could be caused by limited transport of glucose into the cell (Gruenburg et al, 1978) or by the phosphorylation of glucose being governed by the accessibility of glucose to hexokinase (Nwagwu and Opperdoes, 1982). In T.brucei there is a considerable drop in the level of hexokinase upon transition from bloodstream forms

to procyclic forms, the enzyme undergoing a 15-fold reduction (Hart et al, 1984). Hexokinase is essential for glucose phosphorylation and must have an important role in maintaining the required level of glycolysis. In addition, the trypanosomal hexokinase is of interest because it is one of the glycosomal enzymes (Opperdoes and Borst, 1977).

It is possible therefore that there may be distinct differences between the properties of the glycosomal enzymes of trypanosomes and the glycolytic enzymes of other eukaryotes. A much more detailed knowledge of the structure and properties of the parasite enzymes is needed. It may then be possible to exploit these differences, and the differences in the structural organisation of metabolism between host and parasite, making it possible to devise novel inhibitors of glycolysis in trypanosomes. An understanding of the individual enzymes properties may also help to explain the high rate of glucose utilization. This may be due to the intrinsic properties of the individual enzymes, to the interactions between those enzymes, or to the compartmentation of the enzymes within the glycosome.

In order to gain more detailed information about the glycolytic enzymes, the first step is their purification. Hexokinase was selected for its key role in glycolysis and has been purified from crude cell sonicates of C.fasciculata to homogeneity as judged by SDS-polyacrylamide electrophoresis. The purified enzyme has been used to study the physical nature of the protein, and details of the kinetic properties of the enzyme have been investigated. The effect of substrate and reaction product on the hexokinase reaction have been investigated to

determine whether they play an allosteric role in regulation of glycolysis similar to that found in mammalian cells.

Any information derived from the study of glycosomal enzymes in C.fasciculata will not only be of intrinsic biochemical interest, but may also be related to the nature of the enzymes in the pathogenic species and therefore be of chemotherapeutic value. Any way of interfering with the phosphorylation of glucose and the subsequent energy-producing steps, would have a lethal effect on the survival of T.brucei in the host bloodstream (Opperdoes, 1983).

1.4.4 Glycosome Biogenesis and Protein Topogenesis

Little is known about the mechanism of assembly of the glycosome and its enzyme complement. There is no machinery, such as DNA and ribosomes, for the synthesis of enzymes inside the glycosome (Opperdoes et al, 1984). The proteins must be synthesised in some other location and then either transferred into preexisting organelles or have glycosomal membranes formed de novo around them. The direction of proteins into the glycosome rather than any other of the cells organelles or the cytosol would require a signal or address label of some kind, a 'topogenic sequence'. This process of translocation could be a possible target for attack, since anything interfering with the accumulation of enzymes into the glycosome would have a potentially lethal effect on the cell. If the enzymes are translated as precursors in the cytosol it might be possible to inhibit the processing of these precursors and prevent uptake into the glycosome. Signal sequences incorporated in the protein

sequence would be suitable targets for drug design. Alternatively any compound interfering with the integrity of the glycosomal membrane or the formation of new glycosomal membranes would disrupt glycolysis.

I have used hexokinase, purified from C.fasciculata, as a tool for the study of the biosynthesis of the glycosome and glycosomal enzymes. The purified hexokinase protein has been used to raise antiserum, which has been used to further characterise the enzyme. The antiserum has been used as a tool to look at the intracellular location of hexokinase and also to investigate how the protein is incorporated into the glycosome. Immunoblotting and immunoprecipitation techniques have been used in conjunction with the antiserum to identify those polypeptides which are recognized by it. A protein destined for inclusion in a cellular organelle may be initially located in the cytosol in a form that may or may not be enzymatically active. In order to investigate the cellular location of hexokinase, as both an active enzyme and in a form recognised by the antiserum, differential extraction of cells with digitonin has been carried out. Using this technique any inactive precursor present in the cytosol could be identified. Since the glycosomal proteins must cross the glycosomal membrane or be enclosed by the membrane, an agent causing perturbation of the membrane or preventing membrane synthesis might disrupt the glycosomal unit. A potential inhibitor of membrane synthesis has been used to investigate its effect on compartmentation of hexokinase. A precursor protein could be formed as a larger molecular mass species which is processed upon entry into the glycosome. The

use of a range of protease inhibitors might help in indicating a proteolytic role in this processing step. Alternatively, a precursor might be susceptible to proteolytic degradation in the process of extracting the cells. Again, a range protease inhibitors has been used during the extraction to investigate this possibility.

Finally, a comparison of the *in vitro* translation product of mRNA from *C.fasciculata* with the mature hexokinase protein has been carried out in order to determine whether any alteration in molecular mass occurs after translation.

Chapter 2

METHODS

2.1 Organism

Crithidia fasciculata was obtained from Professor D. Lloyd, University College, Cardiff, Wales.

Leishmania donovani (strains L9 and L710) cell extracts were obtained from Dr. M. Chance, School of Tropical Medicine, Liverpool.

2.2 Maintenance

The organism was routinely maintained in one of two simple, undefined media. The first, CF1, comprised 2% (w/v) Bacto-peptone (Difco), 0·1% (w/v) neutralized liver digest (Oxoid) and 0·0025% (w/v) haemin (Sigma). The latter was added from a 10 mg/ml stock solution in 50% (v/v) triethanolamine. Glucose was autoclaved separately as a stock 40% (w/v) solution and added aseptically to the media to a final concentration of 1% (w/v).

The alternative medium for growth, CF3, was identical to the above with the exception of the liver digest, which was replaced with 0·25% (w/v) yeast extract (Difco). Cultures were maintained by transfer of a 3% (v/v) inoculum to 50ml of medium in 250ml flasks, and incubated at 30°C on an orbital shaker operating at 180 r.p.m..

Where indicated in the text a semi-defined medium, CF2, (based on that of Ellenbogen and Hutner, 1967) was used, consisting of 0·25% (w/v) yeast extract, 0·001% (w/v) haemin (from stock solution as above) and 10% (v/v) amino acid cocktail (see below). The pH was altered to 8·2 with 4M NaOH before autoclaving. Glucose was added as above. The amino acid

cocktail, stored at -20°C, was made up as a 50ml HEPES buffered stock solution containing :-

Uracil	5 mg
L-methionine	125 mg
D,L-aspartic acid	250 mg
L-lysine HCl	250 mg
L-arginine HCl	250 mg
L-glutamic acid	1000 mg
Glycine	250 mg
HEPES	200 mM

2.3 Large Scale Cultures

For large scale cultivation of C.fasciculata, a 2% (v/v) inoculum from an overnight culture was added to 500ml of CF3 medium in a 2·5L flask. Generally between 2·5 and 5 litres of culture were used for each large scale preparation. Cells were incubated on an orbital shaker at 30°C and 120 r.p.m. for 36 hours (until late exponential phase).

2.4 Cell Harvesting

All steps were carried out at 4°C unless otherwise indicated. All centrifugation steps were carried out in a Sorvall RC5-B centrifuge. Cells were initially harvested by centrifugation for 10 minutes at 6000 r.p.m. (5875g) in a GSA rotor. Subsequent centrifugations during washing steps were for 10 minutes at 8000 r.p.m. (7719g) in a SS34 rotor. Cells were washed twice by resuspending in PBS (about 100mls/10x0·5L culture) and once in 0·1M Tris-HCl, pH 7·4 (about 50ml/5L initial culture).

BUFFERS :-

PBS - Phosphate buffered saline
0·137M NaCl
0·007M K_2HPO_4
0·003M KH_2PO_4

2.5 Cell Counting

Cell counts were made using a Thoma haemocytometer at x400 magnification. The number of organisms in the total ruled area were counted (total volume 0·1 mm^3). All counts were carried out in duplicate and were only accepted when the number of organisms was between 30 and 100.

2.6 Cell Disruption

Cells were disrupted by sonication of 10 ml batches at 12 microns peak-to-peak for 4x15s bursts with 15s intervals between each burst (MSE 150 Watt Ultrasonic Disintigrator, 9·5 mm end diameter titanium probe). Unbroken cells and subcellular debris were removed by centrifugation for 30 minutes at 15,000 r.p.m. (27,138g) in a Sorvall SS34 rotor.

2.7 Ammonium Sulphate Precipitation

Ammonium sulphate (Analar grade) was used to precipitate proteins from the crude cell sonicates. All steps were carried out at 4°C. Ammonium sulphate was added slowly, with stirring, to the cell sonicate to give a final concentration of 1·8M. This solution was allowed to equilibrate for 30 minutes before precipitated proteins were removed by centrifugation for 30 minutes at 15,000 r.p.m. (27,138g) in a SS34 rotor. The concentration of ammonium sulphate in the supernatant was increased to 2·6M. After a 30 minute equilibration period the precipitated proteins were removed by repeating the above centrifugation step. Pellets and supernatants were kept for analysis.

2.8 Chromatography

Unless otherwise indicated chromatography experiments were carried out at 4°C. Protein concentration in eluates was monitored by its absorbance at 280nm.

2.9 Phenyl-Sepharose Chromatography

Phenyl-Sepharose was obtained from Pharmacia Fine Chemicals. The matrix was equilibrated with 100mM Tris-HCl, pH 7·4, containing 1·2M ammonium sulphate. A column size of 2·6x15 cm was used, onto which a 10 ml sample was loaded. Sample application, washing with equilibration buffer and the running of ammonium sulphate and ethanediol gradients were all performed at a flow rate of 10 cm hr^{-1}. The gradients comprised a total volume of approximately 350 ml and 5 ml fractions were collected.

2.10 Ion-exchange Chromatography

Ion-exchange chromatography was carried out using the anion exchanger DEAE-cellulose (DE52; Whatman, Kent). The matrix was equilibrated with 50mM Tris-HCl, pH 8·0. A column size of 2·6x20 cm was used, and a flow rate of 10 cm hr^{-1} was used for sample application and elution. Fractions of 5ml volume were collected.

A smaller DE52 column of 7x1·5 cm, equilibrated in the same buffer, was used at room temperature where indicated. Buffer and samples were run onto the column under gravity and 1·5ml fractions were collected.

Samples containing salt were desalted prior to use by passage through a column of Sephadex G-25 (Pharmacia).

2.11 Gel Filtration

Native molecular masses were determined on a column (1x60 cm) of Sephacryl S-300 (Pharmacia). The column was equilibrated in 0·1M Tris-HCl, pH 7·4, 0·5M NaCl. The sample was applied in a volume of 1 ml and eluted with the same buffer. A flow rate of 0·2ml min^{-1} was maintained and fractions of 0·85ml volume were collected. The void volume was determined with Blue Dextran and the column was calibrated with a range of marker proteins as indicated in the results.

2.12 Sample Concentration

Dilute enzyme containing solutions were concentrated by vacuum filtration through Millipore CX-30 ultrafilters (30,000 MW cut-off) (Millipore (U.K.) Ltd., Harrow, Middlesex.).

2.13 Pyruvate Efflux

Pyruvate production by intact cells was determined using mid- to late-exponential phase C.fasciculata. Cells were harvested and washed twice, gently, in PBS (Sorvall SS34 rotor at 8000 r.p.m. (7719g) for 10 minutes). Cells were then resuspended in a HEPES-buffered salts medium (HEPES-buffer) to a final concentration of $5x10^8$ cells ml^{-1}. Experiments were started by the addition of 100µl of the above cell suspension to 0·9mls of HEPES-buffer, in a universal bottle, giving a final concentration of $5x10^7$ cells ml^{-1}. The cells were incubated at 30°C, with shaking, in the presence of 20mM glucose or glycerol and inhibitors where indicated in the results. At the end of the 30 minute incubation period the experiment was terminated by

microcentrifugation of the cells for 1 minute at 15,000g, and 100µl aliquots of the supernatants were assayed for pyruvate.

Results were corrected for pyruvate carried over into the incubation mixture by carrying out control experiments to determine pyruvate present at zero time. When inhibitors were used, control incubations including the inhibitor solvent were carried out.

BUFFERS :-

PBS - Phosphate buffered saline
see section 2.4

HEPES-buffer - HEPES buffered salts
20mM HEPES
5mM KCl
80mM NaCl
1mM $MgSO_4$
pH 7·4

2.14 Enzyme Assays

Assays were carried out in either a Pye Unicam SP1800 or a LKB Biochrom Ultrospec 4051 spectrophotometer. Cuvettes with a path length of 10mm and 1ml volume were used throughout. In all assays extinction coefficients at 340nm, for both NADH and NADPH, of $6{\cdot}22 \times 10^3$ $lmol^{-1}cm^{-1}$ were assumed.

2.15 Pyruvate Assay

Pyruvate was assayed by measuring the change in absorbance at 340nm due to the oxidation of NADH caused by the conversion of pyruvate to lactate by lactate dehydrogenase, based on the method of Czok and Lamprecht (1974). The final 1ml assay volume contained :- 0·12 mM NADH; 0·5M triethanolamine-HCl, pH 7·6; 5 mM EDTA. The sample to be assayed was added and mixed and the absorbance at 340nm noted. Lactate dehydrogenase (6·4 units;

bovine muscle, Sigma) was added and mixed, and the change of absorbance at 340nm noted after a constant value was reached (<1 minute). The concentration of pyruvate was calculated using the extinction coefficient for NADH at 340nm.

2.16 Hexokinase Assay

Except where indicated otherwise, the spectrophotometric determination of hexokinase was carried out using a coupled enzyme assay adapted from that of Bergmeyer (1974). Glucose was converted to glucose-6-phosphate by the action of hexokinase in the presence of ATP. Glucose-6-phosphate so formed was oxidised by glucose-6-phosphate dehydrogenase, and the concomitant reduction of $NADP^+$ was monitored at 340nm. The assay mix contained 15mM glucose; 20mM $MgCl_2$; 20mM Tris-HCl, pH 7·6; 0·01mM EDTA; 0·9mM $NADP^+$; and 1·3 units of glucose-6-phosphate dehydrogenase (Leuconostoc mesenteroides; Sigma) in a final volume of 1ml. A sample volume of up to 100µl was used and the volume in the cuvette was made up to 1ml with distilled water when necessary. The reaction was started by the addition of 33µl of ATP (32·5mM, neutralized to pH 7 approx. with 1M NaOH) to give a final concentration of 1mM. The reaction rate was monitored at 340nm for approximately 5 minutes. Control assays were carried out using distilled water in place of enzyme sample.

This assay was adapted to give a simple colorimetric test for hexokinase activity in samples such as column chromatography eluates. The same reagents were used as above, with the addition of nitroblue tetrazolium (NBT) and phenazine methosulphate

(PMS). The assay solution contained 100µl of each of the following :- 0·15M D-glucose; 0·2M $MgCl_2$; 9mM $NADP^+$; Glucose-6-phosphate dehydrogenase (13U/ml); 32·5 mM ATP; 1mg/ml NBT; and 40µl of 1mg/ml PMS. Test samples of 20µl were dispensed into microtitre dishes and 20µl of the assay solution was added to each sample. The plate was covered and kept in low light as both PMS and NBT are light sensitive. Samples with a very high level of hexokinase activity developed a blue-black precipitate almost immediately. Samples containing less activity turned blue more slowly. [Reagents for the above assays were stored at -20°C with the exception of glucose-6-phosphate dehydrogenase which was stored at 4°C.]

For product inhibition studies and where indicated in the results, an alternative spectrophotometric assay was used, based on the method of Racagni and Machado de Domenech, (1983). ATP produced by the hexokinase reaction was coupled to the reactions of pyruvate kinase and lactate dehydrogenase on phosphoenolpyruvate, and the concomitant oxidation of NADH was monitored. The final assay volume of 1ml in each cuvette included 0·2mM NADH; 5mM phosphoenolpyruvate; 6·25 units of lactate dehydrogenase; 4 units of pyruvate kinase; 20mM $MgCl_2$; 20mM Tris-HCl, pH 7·6, and 1 mM ATP. The sample to be assayed was added, mixed and the absorbance at 340nm determined. The reaction was started by the addition of glucose to a final concentration of 15mM, and the rate of absorbance change at 340nm was monitored.

2.17 Malic Enzyme

Malic enzyme was assayed by its action on L-malate causing a reduction of $NADP^+$ (Cazzulo et al, 1977). The change in absorbance at 340nm was measured. The 1ml final reaction volume included 50mM Tris-HCl, pH 7·6; 5mM L-malate; 0·12mM $NADP^+$; and 1mM $MgCl_2$. The reaction was started by adding sample containing enzyme activity, and the rate of change in absorbance measured after 1 minute.

2.18 Lactate Assay

Lactate was assayed by measuring the reduction of NAD^+ caused by the action of lactate dehydrogenase. The assay mix included 0·1M Tris-HCl, pH 7·8, 0·01M semicarbizide, 0·8mM NAD^+ and up to 100 µl of sample in a final volume of 1ml. The absorbance at 340nm was measured and then 20 units of lactate dehydrogenase was added, mixed and the change in absorbance noted.

2.19 Ethanol Assay

Ethanol was assayed using a Boehringer Mannheim test kit (UV-method for the determination of ethanol in foodstuffs). The kit used a coupled assay involving alcohol dehydrogenase and aldehyde dehydrogenase.

2.20 Enzyme Kinetics

The apparent K_m values of hexokinase for glucose and ATP were determined using the Lineweaver-Burk method (Lineweaver and Burk, 1934). The standard enzyme assay solutions were used

except for glucose and ATP which were used at the concentrations indicated in the relevant results sections.

2.21 Protein Assay

Protein was assayed using the Lowry method with Folin-phenol reagent (Lowry et al, 1951). Reagent A: 1% (w/v) $CuSO_4.5H_2O$; reagent B: 2% (w/v) sodium potassium tartrate; and reagent C: 2% (w/v) Na_2CO_3 in 0·1M NaOH, were prepared in advance. Immediately prior to use reagent D was prepared by diluting each of reagents A and B 100-fold in reagent C. Each sample was assayed in duplicate by placing 0·5ml in test tubes, adding 2·5ml reagent D, mixing, and allowing to stand at room temperature for ten minutes. Folin and Ciocalteu's phenol reagent (BDH) was diluted to 30% (v/v) in distilled water and 0·3mls added to each tube and mixed rapidly. After an incubation period of 30 minutes at room temperature, the absorbance of each sample was read at 500nm. A standard curve was prepared using 0-1mg/ml bovine serum albumin (Sigma, RIA grade).

If low levels of protein were anticipated or interfering compounds such as Tris-HCl were known to be present an adaptation of the above method was used (Bensadoun and Weinstein, 1976). Interfering compounds were removed by precipitation and the assay was more sensitive to low levels of protein. The assay was performed in 1·5ml microcentrifuge tubes and protein samples were made up to 1ml volumes. Sodium deoxycholate (10µl of 2% (w/v)) was added to each sample and the tubes were then left at room temperature for 15 minutes. Trichloroacetic acid (300µl of 26% (w/v)) was added to each

sample and mixed, they were then centrifuged at 15,000g for 4 minutes at 4°C. The supernatants were removed and each pellet resuspended by vortex mixing in 1ml of Lowry copper tartrate reagent (reagent D) as used above. Folin and Ciocalteu's reagent was prepared by diluting 1:1 with distilled water and 100µl was added to each sample and mixed rapidly. After standing for 45 minutes at room temperature, the absorbance of each sample was read at 660nm. A standard curve was prepared using 0-100µg/ml bovine serum albumin (RIA grade).

2.22 Polyacrylamide Gel Electrophoresis

Protein samples were characterized by polyacrylamide gel electrophoresis in the presence of the dissociating agent sodium dodecyl sulphate (SDS-PAGE). The discontinuous buffer system of Laemmli was used (Laemmli, 1970). Vertical slab gels were prepared from a stock solution of 30% (w/v) acrylamide/ 0.8% (w/v) N,N'-bis-methylene acrylamide. The 10% acrylamide separating gel contained final concentrations of 0·375M Tris-HCl, pH 8·8, and 0·1% (w/v) SDS. The gels were polymerized chemically by the addition of 0·1% (w/v) ammonium persulphate and 0·1% (v/v) TEMED. A 6% acrylamide stacking gel was used containing final concentrations of 0·125M Tris-HCl, pH 6·8, and 0·1% (w/v) SDS and it was polymerized with 0·1% (w/v) ammonium persulphate and 0·2% (v/v) TEMED. The running buffer, pH ~8·3, was 0·05M Tris-HCl, 0·384M glycine and 0·1% (w/v) SDS. Samples for analysis were prepared in buffer containing 0·062M Tris-HCl, pH 6·8; 1% (w/v) SDS; 20% (v/v) glycerol; 0·001% (w/v) bromophenol blue as tracking dye and 5% (v/v) 2-mercaptoethanol.

Proteins were denatured by incubation at 100°C for 5 minutes. Electrophoresis was carried out in a vertical gel electrophoresis apparatus operating at either 40V constant voltage overnight (16 hours) or at 120V (60V until the tracking dye entered the separating gel) for about 6 hours.

Gels were stained in a solution of 0·1% (w/v) Coomassie Brilliant Blue R250 in 10% (v/v) acetic acid and 50% (v/v) methanol, for at least 4 hours. Destaining was by repeated washing in 10% (v/v) acetic acid, 5% (v/v) methanol until the background was clear.

When all or part of a gel was to be used for the detection of radiolabelled polypeptides by fluorography, a polyacrylamide gel support medium (Gel-Bond PAG film, FMC Corp., Marine Colloids Division, Bioproducts Dept.) was used. The film was fixed to one of the glass electrophoresis plates using silicon grease and Triton X-100. A stock acrylamide solution using 1% (w/v) acrylaide (Miles Scientific), in place of N,N'-bis-methylene acrylamide, was used in preparing the separating gel in order to bond the gel to the film. The stacking gel was prepared as usual. The stock solution containing 1% (w/v) acrylaide was equivalent to the usual acrylamide/bis mixture according to the suppliers, although the results suggested a lower percentage than expected. Gels to be stained were placed in the normal Coomassie stain, but only for 2 hours since destaining was less rapid with only one surface of the gel available for diffusion of stain.

2.23 Size Reduction of SDS-Polyacrylamide Gels

Visability of faint bands was improved by shrinking gels using polyethylene glycol (PEG) to extract water (Palumbo and Tecce, 1983). A 40% (w/v) solution was prepared by dissolving PEG 6000 in warm water. The gel was placed in the solution and gently agitated occasionally. After about 30 minutes the solution was changed and the gel was allowed to continue to shrink until its minimum size had been reached (about 4 hours). About a 6-fold reduction in size was possible leading to an intensification of all bands on the gel. The gel could then be placed in isopropyl alcohol and gently agitated until the gel was hard. [However, if this step was carried out the gel was brittle and did not remain flat.] Alternatively gels were sealed in polythene.

2.24 Fluorography

Gels to be subjected to fluorography were transferred to 10% (w/v) trichloroacetic acid for 30 minutes, after electrophoresis, to fix the proteins. The gels were washed twice, for 15 minutes each, in distilled water then soaked in a solution of 5% (v/v) glycerol and 1M sodium salicylate for 2 hours (Chamberlain, 1979). Gels were dried using an infra-red lamp for 1-2 hours or until they had become a uniform white, opaque film. Radiolabelled bands were detected using preflashed Kodak X-Omat AR film. Film was exposed to the gel for at least 48 hours at -70°C. Developing was carried out using Ilford PQ Universal developer (10% (v/v)) for 5 minutes, a 3% (v/v) acetic acid stop bath for 1 minute and then Ilford Hypam fixer

(5% (v/v)) for 10 minutes. The film was then washed for 15 minutes in running water and allowed to dry.

Subsequently an improved method of fluorography, using 2,5-diphenyloxazole (PPO) as the fluor, was used (Skinner and Griswold, 1983). SDS-polyacrylamide gels were run in the conventional manner and prefixed in acetic acid for 30 minutes. Gels were then transferred to a 20% (w/v) solution of PPO in acetic acid for 90 minutes, followed by 30 minutes in water. The gels, infused with the scintillant, were placed on a filter paper backing, covered with 'Cling-film', and dried on a gel dryer (Bio-rad) at 70°C for two hours. The gel dryer was allowed to cool before releasing the vacuum. The dried gels were exposed to preflashed Fuji X-ray film at -70°C as before, and developed using 20% (v/v) developer, at 20°C, for 5 minutes, with a one minute water wash and 2 minutes in 20% (v/v) fixer.

2.25 Immunization Protocol for Production of Antiserum

Hexokinase purified from C.fasciculata was used to raise antiserum in two male New Zealand white albino rabbits according to the following immunization schedule.

Day 1: Each rabbit was injected with 0·5mls of protein antigen solution containing 0·5 mg/ml protein in PBS, emulsified with an equal volume of Freund's complete adjuvant (Sigma Chemical Co.). [The protein solution was mixed with the adjuvant by shaking, and by repeatedly drawing the mixture in and out of a hypodermic syringe fitted with a 19 gauge needle, until a thick white emulsion had formed.] The emulsion was injected subcutaneously

into multiple sites on the back of the rabbit (50µl at each site).

Day 22: A second, booster injection of protein antigen was given to each rabbit as on day 1, with the exception that incomplete Freund's adjuvant (Sigma Chemical Co.) was used in place of the complete adjuvant.

Day 43: The rabbits were bled by making a nick in the marginal ear vein. Blood was collected in a glass beaker, at least 10ml from each rabbit.

Day 62: Blood was collected again as on day 43.
Antiserum was prepared from the blood by stirring it gently and then leaving it overnight, at 4°C, to allow the serum to separate from the clotted blood. The serum was pipetted carefully off the clot and cleaned by centrifuging gently for 1 minute at 15,000g. It was stored as 1ml aliquots at -20°C.

2.26 Immunoelectrophoresis

Rocket immunoelectrophoresis and crossed immunoelectrophoresis were used to analyse the antiserum prepared using the above protocol. Tris-barbitone electrophoresis buffer was used throughout. Agarose (Sigma, Type 1: Low EEO) was prepared as a 1% (w/v) solution in the above buffer and kept at 55°C. Agarose was added to the required amount of antibody, in a test tube at 55°C, and gently mixed. Immunoelectrophoresis was carried out on Gel-Bond film (FMC Corp., obtained through Sigma) cut to the required size (5x5 cm). The antibody/agarose solution was poured carefully onto the Gel-Bond (0·11 ml/cm^3), on a level surface, and allowed to set.

Wells (10µl) were cut towards one end of the gel and the agarose removed from the wells by suction. The gel was placed on a water cooled platen (15°C) fitted in a Shandon 600 X-100 electrophoresis tank. The appropriate protein antigens (up to 10µl) were loaded into the wells using a Gilson micropipette. The electrode chambers were filled with Tris-barbitone buffer, (500ml in each chamber). Strips of 3MM filter paper, to be used for the wicks, were cut to the width of the gel, or gels if several were being run side by side, and soaked in the buffer (6 layers/wick). The wicks covered 0·5 cm of the gel at each end, and a firm contact along the edge of the gel was ensured. The free ends of the wicks were trailed into the electrode buffer reservoirs. The electrophoresis tank was covered and electrophoresis was carried out at 30V overnight (~16 hours).

BUFFERS :-

Tris-barbitone buffer
Prepared as 5X stock :-
0·61M 5'5-Diethyl Barbituric Acid
1·83M Tris base
pH 8·6

dilute X5 prior to use

2.27 Crossed Immunoelectrophoresis (CIE)

Antigens present in crude protein samples were initially separated by electrophoresis into a 1% (w/v) agarose gel (10x15 cm on a glass plate). Bovine serum albumin, a fast moving protein, with 1% (w/v) bromophenol blue dye bound to it, was also run on the gel as a marker protein. Electrophoresis was carried out in the Shandon electrophoresis tank using the same buffer and 8 layers of 3MM filter paper for each wick. Proteins

were electrophoresed into the gel at 250V for about 45 minutes, or until the blue dye had migrated 3-4 cm from the wells. The agarose gel was then cut into strips, each strip containing the separated antigens from one well. Each strip was transferred to the end of a piece of Gel-Bond film, so that the second dimension electrophoresis of antigens would be at right angles to the first. Agarose containing antibody was prepared and poured onto the remaining area of Gel-Bond film as described for Rocket gels. The film was transferred to the electrophoresis tank and electrophoresis was performed overnight as described for Rocket gel electrophoresis.

2.28 Staining of Immunoelectrophoresis Gels

Unprecipitated protein was removed by washing with distilled water. The gel was then covered with several layers of filter paper and pressed under a heavy weight for 10 minutes, renewing the top layers of filter paper after 5 minutes. The gel was then soaked for 15 minutes in 0·1M NaCl, pressed for a further 10 minutes, soaked for 15 minutes in distilled water, and pressed again for 10 minutes. Finally the gel was allowed to dry to a smooth film, using an infrared heat lamp to speed up the process. Staining was carried out for 5 minutes in a solution of 5% (w/v) Coomassie Brilliant Blue R250 in 45% (v/v) ethanol and 10% (v/v) glacial acetic acid. Destaining was carried out by 2-3 rapid washes in 45% (v/v) ethanol, 10% (v/v) acetic acid.

Detection of hexokinase activity in the gels was carried out using the nitroblue tetrazolium/phenazine methosulphate assay solution previously described. The gels were covered with the

solution and allowed to incubate in the dark at room temperature until a blue colour developed in the gel.

2.29 Western Blotting (Immuno Blotting)

The method was based on the method of Towbin et al (1979). Proteins were separated by SDS-polyacrylamide gel electrophoresis, as previously described, at high voltage for about 6 hours. On completion of electrophoresis the portion of the separating gel required for transfer was cut away from the remainder and kept moist with western blot buffer (WBB). A sheet of nitrocellulose (Schleicher and Schüll, BA85) was cut to the size of the gel and placed carefully onto it, making sure no air bubbles were trapped. The gel and nitrocellulose were sandwiched between 3 sheets each side of 3MM filter paper soaked in WBB buffer. This sandwich was placed between two Scotch-Brite pads in a plastic grid support, and slotted into the transfer apparatus (Biorad-TransBlot cell) [with the nitrocellulose filter nearest to the anode (+ve)]. The tank was filled with cold (4°C) WBB and placed on a magnetic stirrer. The tank was covered and connected to a power pack operating at 30V (120mA) overnight (16 hours).

On completion of transfer the gel and nitrocellulose sheet were carefully separated. The gel could be transferred to Coomassie blue stain to check for efficient transfer of proteins if required. The nitrocellulose filter was transferred to a plastic sandwich box and covered with 30mls of BSA-binding buffer to block the remaining binding sites on the nitrocellulose. This was placed on an orbital shaker (Luckham)

for at least three hours. The buffer was removed and the nitrocellulose filter was washed with 30mls of Tris-saline (TS buffer) for 15 minutes, repeated once. Antiserum was diluted in TSA buffer (0·5mls in 30mls) and the nitrocellulose filter covered with this solution and left on the shaker overnight (16 hours) at room temperature. The diluted antiserum was then removed and the filter washed with five changes of 30mls of TS buffer, over a 2-3 hour period. The nitrocellulose sheet was transferred to 30mls of TSA buffer containing either 1μCi [^{125}I]-protein A or 3μCi [^{125}I]-donkey anti-rabbit serum, and placed on the shaker for at least 2 hours or overnight if possible. The filter was then washed with five changes of TS buffer over a 3 hour period. The filter was dried by placing it on 3MM filter paper and heating at 65°C for 15 minutes, or until the nitrocellulose was dry, when radiolabelled bands were detected by autoradiography.

After transfer of protein from an SDS-polyacrylamide gel to the nitrocellulose, a reversible staining procedure was used to check for efficient transfer of proteins (Towbin and Gordon, 1984). The nitrocellulose was placed in a solution of 6M urea for 10 minutes, then transferred to a solution of phenol red (50mg/ml in 1 mM HCl) for a further 10 minutes. The filter was washed briefly in 1mm HCl. Protein bands stained yellow. The stain was removed by washing in TS buffer, before continuing with the protocol as described above.

BUFFERS :- WBB - Western blot buffer
20mM Tris base
150mM glycine
20% (v/v) methanol
0·1% (w/v) SDS

TS buffer - Tris-saline
Made up as 20X solution :-
1M Tris, pH 7·5
3M NaCl
Dilute X20 before use

BSA binding buffer
TS buffer
3% (w/v) BSA (Sigma, RIA grade)
5mM $MgCl_2$
pH 7·5

TSA buffer
20mM Tris-HCl, pH 7·6
0·876% (w/v) NaCl
5mg/ml BSA (Sigma, RIA grade)
0·05% (w/v) sodium azide

2.30 Autoradiography

Nitrocellulose filters for autoradiography were attached to thin cardboard and covered in 'Cling-film' before placing in an X-ray cassette with Kodak X-Omat S film. The cassette was kept at -70°C and the film was exposed for at least 48 hours before developing. Developing was carried out for 5 minutes in Ilford PQ Universal developer (1 in 5), followed by a water rinse and then fixed for 2 minutes in Ilford Hypam fixer (1 in 5). The film was washed and dried as described for fluorography.

2.31 Radiolabelling

In vivo labelling of cell polypeptides was carried out with [^{35}S]-methionine. A 50 ml overnight culture, grown in the semi-defined medium previously described, was centrifuged at 8,000 r.p.m. (7719g) in a Sorvall SS34 rotor for 10 minutes. The cells were resuspended in the same medium, lacking methionine when [^{35}S]-methionine was to be added, to a final density of $5x10^7$ cells ml^{-1}. Aseptic conditions were maintained throughout.

The cells were preincubated for at least 30 minutes, at 30°C, in a shaking water bath, in duplicate 10ml samples, each in a 100ml flask. When inhibitors were included they were added at the beginning of the preincubation period. After preincubation 50μCi [^{35}S]-methionine was added to one of each duplicate pair of flasks and incubation continued at 30°C in a shaking water bath. Samples were taken as required for immunoprecipitation or for determination of radioactive incorporation into cell protein. The non-radiolabelled flask was used for checking cell viability and for non-radioactive cell sampling when required.

The incorporation of radioactivity into protein was determined by taking 100μl samples at regular time intervals. Each sample was used to load 20μl onto each of four filters (Whatman; GF/C, 2·4cm diameter) using a Gilson P20 micropipette, and the filters were allowed to dry at room temperature. Two of the filters from each set of four were then transferred directly to scintillation vials to determine total counts while the remaining two were placed in test tubes and the protein on the filters was precipitated with trichloroacetic acid. To each tube, 2ml of trichloroacetic acid was added and left for 10 minutes with occasional vortex mixing. The liquid was discarded and this step was repeated once more with trichloroacetic acid and then with absolute ethanol. The filters were removed and allowed to dry before transferring to scintillation vials. The filters were covered with non-aqueous liquid scintillant (BDH Chemicals Ltd.: Scintran Cocktail O in toluene) and the vials were capped and placed in a liquid scintillation counter

(Beckman LS1801 Liquid Scintillation System) programmed to detect S-35.

2.32 Triton Extraction

Cell extracts were prepared using Triton X-100 to disrupt membranes. Samples of cells (1ml, $5x10^7$ cells ml^{-1}) were placed in 1ml microcentrifuge tubes and centrifuged for 1 minute, 15,000g. The supernatants were discarded and the cells washed by resuspending in 1ml PBS and centrifuging again. The cells were then resuspended in 1ml PBS containing 1% (v/v) Triton X-100 and incubated for 10 minutes at room temperature, or until microscopic examination revealed that the majority of the cells had been disrupted. Whole cells and cell debris were removed by centrifugation for two minutes, 15,000g, and the supernatants were used as required. Preparation of Leishmania Triton-X100 extracts for western blot analysis was carried out in a similar manner.

2.33 Digitonin Extraction

Samples of cells were washed and resuspended in PBS as described for Triton extraction. Digitonin was prepared as a 40mg/ml stock solution in dimethyl formamide, and was added to give the required final concentration in a 1ml sample volume. Cells were incubated at 30°C for the required time and the supernatants were obtained by centrifugation at 15,000g for 2 minutes. When particulate fractions were required, pellets were resuspended in 1ml PBS and remaining particulate enzyme activity was released by extracting again with 2mg/ml digitonin,

incubating for 2 minutes. Extracts were kept on ice. Control experiments indicated that no enzyme release was caused by the solvent, dimethyl formamide, alone.

2.34 Immunoprecipitation

Immunoprecipitation of antibody/antigen complexes, based on the method of Johnstone and Thorpe, (1982), was carried out using protein A from S.aureus, Cowan 1 strain (Sigma). A 10% (w/v) suspension of protein A was prepared in IP/Triton Buffer and washed twice in the same buffer by centrifugation at 15,000g for 2 minutes in a microcentrifuge at 4°C. The protein A was resuspended in the same volume of IP/Triton Buffer. A 10% (w/v) BSA solution was prepared and 10µl BSA solution per 300µl protein A suspension was added and the solution kept on ice.

Radiolabelled cell extracts were prepared either by digitonin treatment or by Triton X-100 extraction. To each 1ml cell extract, in 1·5 ml microcentrifuge tubes, 100µl of the BSA/protein A suspension was added and mixed gently for 30 minutes at 4°C. This step removed non-specific absorbents in the cell extract. The protein A was removed by centrifugation for 4 minutes, 15,000g at 4°C. From each supernatant 300µl was transferred to a new microcentrifuge tube and 650µl of IP/Triton Buffer was added and 10µl of BSA solution. The protein A pellets were discarded as radioactive waste. To each tube, 10µl of hexokinase antiserum was added, mixed thoroughly, and incubated at room temperature for 30 minutes. When controls with normal rabbit serum were carried out, a second 300µl aliquot of the supernatant was taken and treated in the same way. After the

incubation period 50µl of the protein A suspension was added and mixed at regular intervals, over 1 hour, at 4°C. The tubes were centrifuged for 2 minutes, 15,000g, at 4°C and the supernatants discarded. The pellets, which included any polypeptides recognised by the antiserum, were washed by resuspending in 1·0 ml IP/Triton Buffer, vortex mixing until the pellet was thoroughly resuspended, and then centrifuging again as before. The washing procedure was repeated using IP/SDS/Triton Buffer and then again using IP Buffer. The supernatants were all discarded and the pellets were either used immediately or stored at -20°C until they could be analysed. Each pellet was resuspended in 50 µl SDS-PAGE sample buffer, boiled for 5 minutes, and subjected to SDS-polyacrylamide gel electrophoresis followed by fluorography to detect radiolabelled bands.
For direct counting of radioactivity in pellets, they were resuspended in 100µl IP/Tris buffer and loaded onto filters (Whatman; GF/C, 2·4cm diameter). After drying the filters were placed in scintillation vials and counted in a Beckman liquid scintillation counter.

BUFFERS :-

IP buffer
10mM Tris-HCl, pH 7·3

IP/Triton buffer
10mM Tris-HCl, pH 7·3
0·5% (w/v) Triton X-100
0·5M NaCl

IP/SDS/Triton buffer
10mM Tris-HCl, pH7·3
0·5% (w/v) SDS
0·5% (w/v) Triton X-100
0·5M NaCl

2.35 RNA Isolation

RNA was isolated from a late exponential phase culture of C.fasciculata based on the method of Russell et al (1984). About $1x10^{10}$ cells were washed twice in TWB, then resuspended in 20 mls of ice-cold lysis buffer and gently shaken to lyse the cells. This suspension was phenol extracted twice (1:1), using phenol/chloroform/isoamyl alcohol (24:24:1) equilibrated in lysis buffer. RNA was precipitated from the aqueous phase with freezing ethanol and pelleted. DNA was removed by repeated extraction with 3M $NaCH_3COO$ (pH5·5), leaving behind the insoluble RNA. RNA was resuspended in dd.H_2O to give a final concentration of 3-4 mg/ml. The mRNA was purified by isolating poly(A)$^+$ RNA using two cycles of oligo dT cellulose chromatography (Weeks and Collis, 1976). RNA was again precipitated using freezing ethanol and 3M $NaCH_3COO$, pH 5·5, pelleted, then resuspended to give a final concentration of 0·5-1·0 mg/ml.

2.36 In Vitro Translation

The mRNA isolated as above was translated in a rabbit reticulocyte (RRL) in vitro translation system (Pelham et al, 1976). The translation mix included 40µl RRL, 10µCi [^{35}S]-methionine and 3µl RNA, it was mixed by vortexing in a 0·5 ml microcentrifuge tube. The tubes were placed in a 30°C water bath for 90 minutes. Incorporation of radioactive methionine was determined by trichloroacetic acid precipitation of 1µl samples and counting as previously described. A negative

control with no RNA and a positive control using tobacco mosaic virus mRNA were also carried out.

2.37 Chemicals

Chemicals were of the highest grade available, and were obtained from the major supply firms. Biochemicals were obtained mainly from Sigma Chemical Co., Poole, Dorset and Boehringer, Mannheim. Radiochemicals were obtained from Amersham International plc., Amersham, Bucks..

Chapter 3

RESULTS

PYRUVATE PRODUCTION AND TRANSPORT

3.1 Pyruvate Production

C.fasciculata was grown in CF1 medium, harvested and resuspended in buffered salts. The production of pyruvate was measured using 20mM glucose as the carbon source (Table 3.1). Pyruvate was produced at an easily detectable level, 107 nmoles/30 min/$5x10^7$ cells. Parallel assays carried out using 20mM glycerol, in place of the glucose, showed that the level of pyruvate produced was significantly reduced, to approximately 25% of the value for glucose. The production of pyruvate could be directly related to the exogenous substrate provided, since incubations with no carbohydrate provided in the incubation medium showed that no significant amounts of pyruvate were produced during the 30 minute incubation period.

3.2 Pyruvate Production Related to Growth Phase

It has been suggested in the past that C.fasciculata uses amino acids preferentially over glucose during early phases of growth, and that consumption of glucose does not commence until late in the logarithmic stage and in the stationary phase of growth (Marr et al, 1977). A comparison was made of the capacity for pyruvate production by cells harvested at intervals throughout the growth curve of C.fasciculata (Fig. 3.1). This indicated that most pyruvate was produced by cells from mid-exponential phase (Table 3.2). No pyruvate was produced by lag phase cells, and the capacity for pyruvate production dropped as the end of the exponential phase was approached and the cells went into the stationary phase.

3.3 Effect of Electron Transport Inhibitors on Pyruvate Production

Antimycin A inhibits mitochondrial respiration by blocking the electron transport chain between cytochromes b and c. When 10μg/ml antimycin A was included in the incubation medium there was a large increase in the level of pyruvate produced. With glucose as substrate there was a 6·7 fold increase in pyruvate produced (Table 3.3(a)). When glycerol was provided as the substrate, under otherwise identical conditions, pyruvate production was lower, as expected, in the incubation without inhibitor but the addition of antimycin A still caused a 3·0 fold increase in pyruvate production (Table 3.3(a)).

When cells for the experiments were prepared from cultures grown up with glycerol rather than glucose (Table 3.3(b)), there was an increase in the efficiency of the cells for pyruvate production from glycerol compared to that from glucose, from 27% to 52%. However there was not such a large stimulation of pyruvate production by antimycin A. With glucose as substrate there was a 2·7 fold increase and with glycerol as substrate only a 1·3 fold increase.

The presence of salicylhydroxamic acid (SHAM) had no effect on pyruvate production in the presence or absence of antimycin A.

3.4 Lactate and Ethanol Production

Assays for lactate production, carried out under similar conditions to those under which pyruvate was produced, indicated that there was no detectable lactate production during the 30

minute incubation period (within the limits of the assay, <2 nmol/min/$5x10^7$ cells).

The level of ethanol in the incubation mixture was 251 nmol/$5x10^7$ cells at zero time. After 30 minutes incubation, with conditions as for pyruvate production, the level had not significantly altered (249 nmol/$5x10^7$ cells). After 60 minutes incubation the level of ethanol had decreased by 22% to 195 nmol/$5x10^7$ cells. Further starvation led to an even greater reduction in the ethanol concentration.

3.5 Effect of Culture Medium on Pyruvate Production

After several months, during which time a change from CF1 to CF3 medium for routine sub-culturing had been made, the original experiments to detect pyruvate were repeated. In contrast to the original results, there was almost no pyruvate production using either glucose or glycerol (Table 3.4). There was no stimulation of pyruvate production by antimycin A.

When the cells were transferred back into the original growth medium (CF1) and the experiments repeated, pyruvate was produced (Tables 3.5 and 3.6). Repeated sub-culturing was not necessary, the alteration taking place as soon as the change in medium was carried out. However, the level of pyruvate produced, using the same experimental conditions as in the original work, was much higher, typically 4-5 times as high.

In contrast to the original work, antimycin A was now found to have an inhibitory effect, almost completely abolishing pyruvate production (96·5% inhibition). The use of ethanol as a solvent for the inhibitor was also found to have an adverse

effect on the cells (Table 3.6). Ethanol alone inhibited pyruvate production to 11·3% of its normal value. Methanol was used as an alternative solvent and was found to have no effect on pyruvate production in control incubations (Table 3.5).

3.6 Effect of a Cyanocinnamic Acid Derivative on Pyruvate Production

The transport of pyruvate and lactate by intact cells and mitochondria in several other systems is believed to occur by means of a specific membrane carrier. Evidence for the presence of this carrier in human erythrocytes and rat liver mitochondria was found when it was shown that α-cyano-4-hydroxycinnamic acid caused the specific inhibition of pyruvate and lactate transport (Halestrap and Denton, 1974).

To investigate the possibility of such a carrier existing in C.fasciculata for the transport of pyruvate, α-cyano-4-hydroxycinnamic acid was included in incubations to determine its effect on pyruvate production (Fig. 3.2). At low concentrations (<400μM) there was no apparent effect. At 600μM inhibitor there was over 70% inhibition of pyruvate production, and at concentrations greater than 800μM inhibition was complete.

SUBSTRATE	PYRUVATE [μmol/30min./5 x 10^7 cells]	
		-CONTROL
glucose	0·136	0·107 ± 0·008
glycerol	0·056	0·056 ± 0·003
none	0·023	0

Table 3.1 Pyruvate Production by C.fasciculata.

Pyruvate production was determined for cells incubated with either glucose, glycerol or no exogenous substrate. Cells for the experiments were cultured in CF1 medium with glucose provided. Values were corrected for the presence of pyruvate carried over into the incubation by using a zero time control (0·027 μmol/5 x 10^7 cells). Results are expressed as the mean and standard deviation of four determinations.

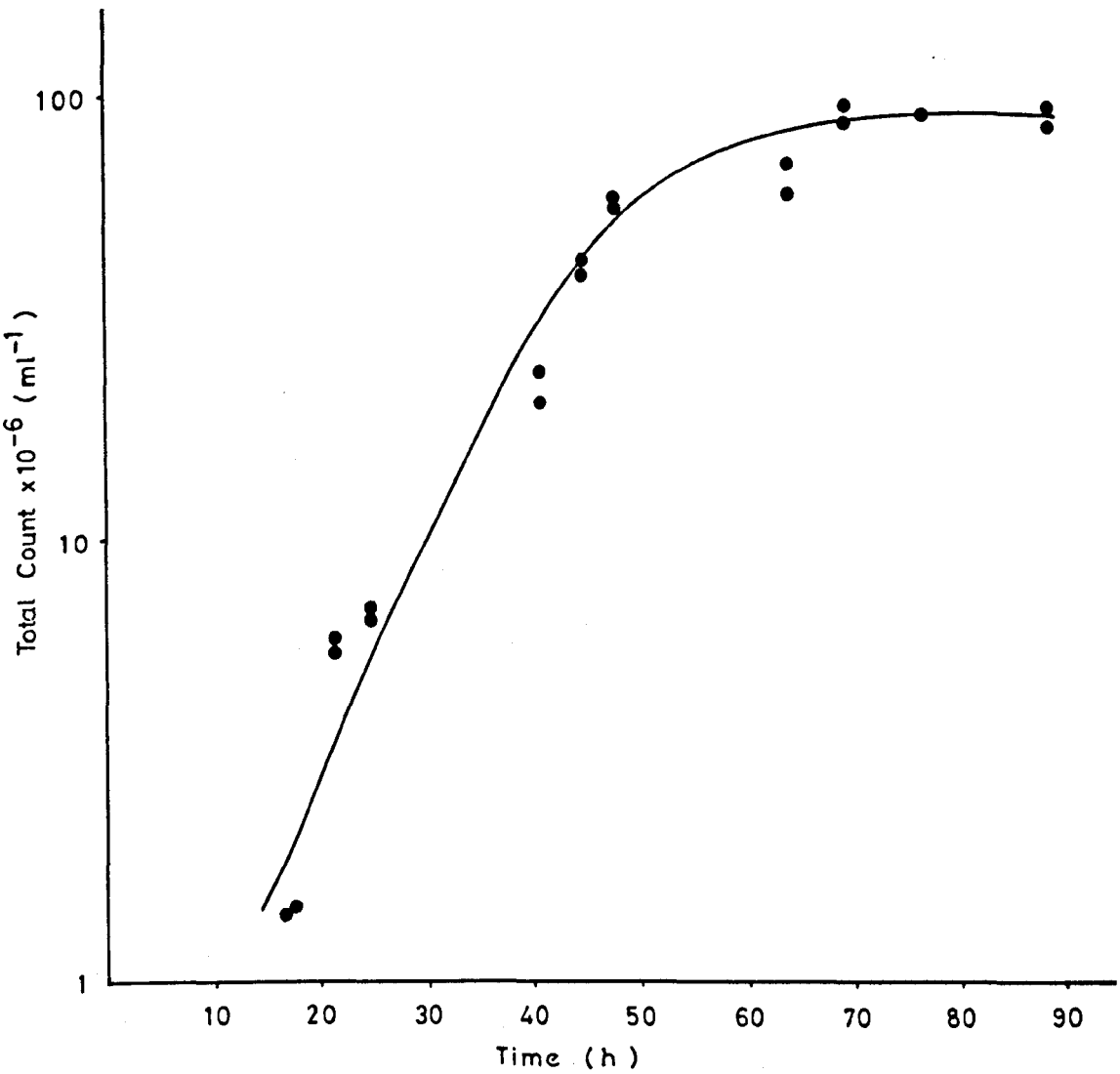

Figure 3.1 C.fasciculata: Growth Curve in CF1 Medium

A 1 ml inoculum from a late exponential phase CF1 culture was used to start 100 ml cultures in CF1 medium with glucose. At the times indicated in Table 3.2 cells were harvested from a culture and the capacity of the cells for pyruvate production using glucose as substrate was determined (see Table 3.2).

TIME	PYRUVATE
[hours]	[μmol/30min./ 5 x 10^7 cells]
18	n.d.
38	0·144 ± 0·005
57	0·112 ± 0·006
88	0·072 ± 0·003

Table 3.2 Pyruvate Production Related to Growth Phase.

Cells were sampled at intervals through the growth cycle (see Fig. 3.1) and the capacity of the cells for pyruvate production was determined using glucose as substrate. Values are corrected for the presence of pyruvate in the original cultures. Results are expressed as the mean and standard deviation of four determinations. n.d. : not detectable

(a)

SUBSTRATE	PYRUVATE [μmol/30min./5 x 10^7 cells]	
	STANDARD	+ ANTIMYCIN A
glucose	0·106 ± 0·010 (100)	0·712 ± 0·026 (672)
glycerol	0·028 ± 0·004 (26)	0·084 ± 0·008 (300)*

(b)

SUBSTRATE	PYRUVATE [μmol/30min./5 x 10^7 cells]	
	STANDARD	+ ANTIMYCIN A
glucose	0·109 ± 0·006 (100)	0·291 ± 0·008 (267)
glycerol	0·057 ± 0·009 (52)	0·073 ± 0·012 (128)*

Table 3.3 The Effect of Antimycin A on Pyruvate Production by C.fasciculata.

Pyruvate production was determined as usual (standard) and in the presence of Antimycin A. Antimycin A was added as a 1 mg/ml solution in ethanol to give a final concentration of 10 μg/ml Antimycin A and 1% (v/v) ethanol. Ethanol alone at 1% (v/v) had no effect. Incubations were carried out in the presence of either glucose or glycerol as indicated. Cells for the experiments were cultured in CF1 medium with added glucose (a) or glycerol (b). Figures in brackets are percentages, relating the level of pyruvate production to the standard result from glucose with no inhibitor added (100%). Figures in brackets marked * are percentages related to the level of pyruvate produced from glycerol. Results are expressed as the mean and standard deviation of four determinations.

SUBSTRATE	PYRUVATE [μmol/30min./5 x 10^7 cells]	
	STANDARD	+ ANTIMYCIN A
glucose	0·013 ± 0·006	0·006 ± 0·004
glycerol	0·007 ± 0·002	-

Table 3.4 Pyruvate Production from Cells Cultured in CF3 Medium.

Pyruvate production was determined using either glucose or glycerol as indicated. When antimycin A was included in the incubation it was added as a 1 mg/ml solution in ethanol, to give a final concentration of 10 μg/ml antimycin A and 1% (v/v) ethanol. Cells for the experiments were cultured in CF3 medium with glucose. Results are expressed as the mean and standard deviation of four determinations.

ADDITIONS		PYRUVATE
		[μmol/30 min./5 x 10^7 cells]
ANTIMYCIN A	METHANOL	
-	-	0·402 ± 0·020
+	+	0·014 ± 0·002
-	+	0·414 ± 0·016

Table 3.5 The Effect of Antimycin A and Methanol on Pyruvate Production.

Cells were harvested from cultures in CF1 medium with added glucose. Pyruvate production was determined, with glucose, in the standard manner and in the presence of antimycin A and/or methanol as indicated. Antimycin A was added, from a 1 mg/ml solution in methanol, to give a final concentration of 10 μg/ml and 1% (v/v) methanol. Methanol alone was added to give a final concentration of 1% (v/v). Results are expressed as the mean and standard deviation of four determinations.

ADDITIONS		PYRUVATE
		[μmol/30 min./5 x 10^7 cells]
ANTIMYCIN A	ETHANOL	
-	-	0·443 ± 0·015
+	+	0·012 ± 0·003
-	+	0·050 ± 0·007

Table 3.6 The Effect of Antimycin A and Ethanol on Pyruvate Production.

Cells were harvested from cultures in CF1 medium with added glucose. Pyruvate production was determined, with glucose, in the standard manner and in the presence of antimycin A and/or ethanol as indicated. Antimycin A was added, from a 1 mg/ml solution in ethanol, to give a final concentration of 10 μg/ml and 1% (v/v) ethanol. Ethanol alone was added to give a concentration of 1% (v/v). Results are expressed as the mean and standard deviation of four determinations.

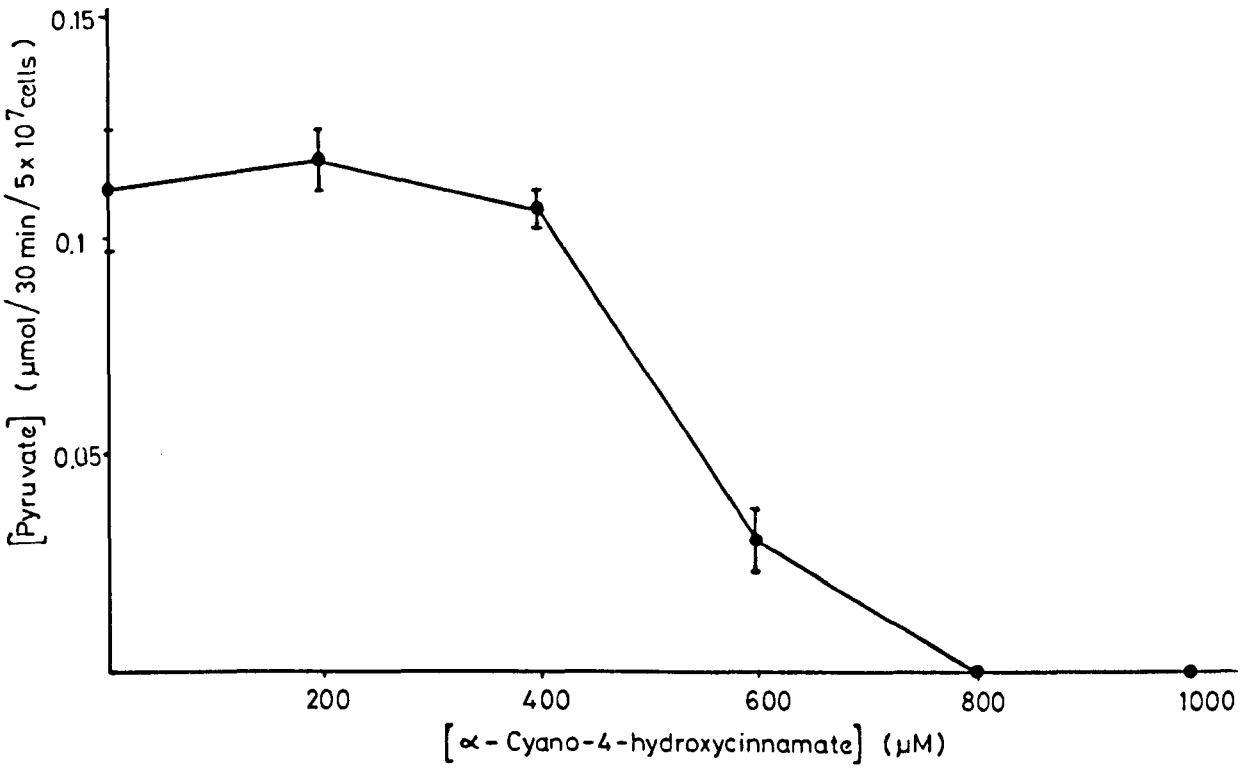

Figure 3.2 **The Effect of α-Cyano-4-Hydroxycinnamate on Pyruvate Production by C.fasciculata.**

Cells were harvested from cultures grown in CF1 medium with glucose. Pyruvate production was determined as usual and incubations were carried out with a range of α-cyano-4-hydroxycinnamate concentrations. The inhibitor was added from a solution in ethanol to give a final concentration in the range 0-1 mM. Points plotted are the mean, and show the standard deviation, of four determinations.

Chapter 4

DISCUSSION

PYRUVATE PRODUCTION AND TRANSPORT

4.1 End-Products of Glucose Catabolism

The catabolism of glucose in trypanosomatids has long been known to take place by a process termed 'aerobic fermentation' (Von Brand, 1951). The oxidation of glucose is rapid and incomplete, giving rise to carbon dioxide and a number of organic acids, the type and quantity of which varies from one species to another, and between morphological forms (Ryley, 1956). Aerobic fermentation is characterized by its lack of a Pasteur effect. The Pasteur effect is a characteristic of most organisms including mammalian cells (Krebs, 1972). It involves a rapid drop in the rate of glucose consumption upon transition from anaerobiosis to aerobiosis. In trypanosomes this does not occur; in contrast the rate alters very little. The organic acids produced from glucose degradation may vary from pyruvate, to pyruvate and glycerol, or may include lactate, acetate and some alcohols. In many cases though the major end-product is succinate (Marr, 1973).

In the case of C.fasciculata, all reports agree that succinate is the predominant end-product of aerobic glucose catabolism (eg. Hunter, 1960; Cosgrove, 1959; Marr, 1973). The same is also true for anaerobic metabolism (Schwartz, 1961). However the number and significance of products reported in addition to succinate varies considerably. Cosgrove (1959) found that large amounts of ethanol and trace amounts of pyruvate were produced. Marr (1973) reported that succinate and pyruvate were produced using glycerol as the substrate. Using glucose as the substrate (1974), he found that ethanol was produced in addition. Anaerobic metabolism has been reported to give rise to

succinate, carbon dioxide, ethanol and lactate, but no pyruvate (Schwartz, 1961) and much more recently ethanol, succinate, and the previously unrecorded glycerol (De los Santos et al ,1985). Cazzulo et al (1985) reported recently that aerobically C.fasciculata produces succinate, ethanol, acetate and pyruvate. It is apparent that variation in the minor end-metabolites does occur.

My own experiments indicated that C.fasciculata produced pyruvate, no lactate, and possibly ethanol. Assays for succinate were not carried out but it was assumed to be the main end-product. These results fit with the general pattern well, although in some of the reports pyruvate was found only in trace amounts.

4.2 Glycolysis Related to Growth Phase

The importance of aerobic fermentation of glucose to growth in C.fasciculata has been questioned in the past, and it has been suggested that amino acids form the preferred growth substrate (Marr et al, 1977). Marr (1974) reported that neither glucose nor glycerol utilization began until well into the exponential phase of growth, when amino acid consumption began to decline, and succinate accumulated only in late exponential phase. When the presence of metabolites in cell sonicates was measured (Marr et al, 1977) glucose consumption was found not to begin until the exponential phase was almost complete, and oxaloacetate and acetate were the first glycolytic metabolites detected. The level of glucose decreased and succinate and pyruvate appeared only after the end of the exponential phase.

Pyruvate was not demonstrated in significant amounts in mid-exponential phase. Ammonia, an indicator of amino acid consumption, was produced in large quantities in the lag phase and at only a slightly lower level in the early exponential phase.

My results for pyruvate production from cells sampled at stages throughout the growth cycle, give evidence conflicting with this view. It was found that the capacity of cells for pyruvate production was maximal using mid-exponential phase cells, and declined towards stationary phase. Since, under the experimental conditions used, pyruvate is presumably derived from the catabolism of glucose, it would seem that glycolysis (as judged by pyruvate production) was capable of occurring most actively in mid-exponential phase rather than stationary phase cells as Marr reported. It is possible, however, that glycolysis was occurring in stationary phase cells but that pyruvate was not an end-product of glycolysis at this stage, perhaps being metabolised further.

Cosgrove (1959) reported that in the absence of exogenous carbohydrate, respiration occurred using an endogenous nitrogenous compound, indicated by the excretion of NH_3. This was probably due to amino acid consumption. He found however that utilisation of sugars completely abolished respiration due to this endogenous substance. Recently further evidence has been published which supports the relative importance of glucose consumption over amino acid consumption (Cazzulo et al, 1985). Glucose consumption was found to be maximal during the exponential phase, and most active during mid-exponential phase.

The rate of production of metabolites closely followed the consumption of glucose. Glucose was exhausted before the end of the exponential phase even when the concentration was increased. In complete contrast to the work of Marr, NH_3 was produced only after glucose had been exhausted, and continued through the stationary phase, suggesting that amino acid consumption was not important until glucose consumption had ceased.

4.3 Lactate and Ethanol Production

My results for the production of ethanol suggest that, since there was no alteration in the concentration of ethanol over a 30 minute incubation period, the effect was either artefactual or the production was at equilibrium with the utilisation of ethanol. Longer periods of incubation resulted in a decrease in the level of ethanol present, and complete starvation of the cells overnight resulted in a large reduction in the level of ethanol in the medium. This suggests that ethanol is produced by the cells but that possibly, as glucose is consumed and nears exhaustion, ethanol is utilised as an alternative carbohydrate source. Cosgrove (1959) reported that C.fasciculata was able to consume a number of alcohols, including ethanol, in the absence of exogenous substrate. Cazzulo et al (1985) reported that there was an early increase in ethanol production, but that the level began to decrease again in mid-exponential phase as glucose levels in the medium began to fall. This again suggested that the cells were consuming the ethanol produced earlier in the growth cycle.

There was no evidence that lactate was produced as an end-product of glucose metabolism. A similar observation was made by Cazzulo et al (1985).

4.4 Effect of Substrate on Pyruvate Production

The type of exogenous substrate provided was found to have an effect on the rate at which pyruvate was produced. The rate was higher with glucose than with glycerol. Glycerol catabolism is assumed to occur in part by the same route as that of glucose, entering the glycolytic pathway at the level of glycerol-3-phosphate. The higher rate of pyruvate production from glucose compared to that from glycerol would then be a direct stoichiometric result of 1 mole of glucose producing 2 moles of phosphoenolpyruvate, whilst 1 mole of glycerol will produce only 1 mole of phosphoenolpyruvate. Theoretically the rate of pyruvate production from glycerol would be half the rate when glucose was the substrate.

The results obtained indicate that in fact the rate of production from glycerol was even lower than this. This could be explained by the entry of triose phosphate from glycerol into the glycolytic pathway being limited by the rate at which glycerol was phosphorylated. Low activity of the enzymes for the conversion of glycerol to the triose phosphate could be responsible for this. When the cells had been cultured in medium with glycerol rather than glucose provided as exogenous substrate, the relative rate of pyruvate production from glycerol in experiments increased, which may indicate that

induction and/or repression of enzymes occurs depending on the exogenous substrate provided.

The presence of the enzymes necessary for the introduction of glycerol as glycerol-3-phosphate into the glycolytic pathway, suggests that it ought to be possible for the reverse route to occur with glycerol being produced as an end-product under the right conditions. The production of glycerol by C.fasciculata had not been reported previously but De los Santos et al (1985) found, using C-13 nuclear magnetic resonance analysis, that glycerol was produced anaerobically, and they suggested that this would reflect the role of glycerol-3-phosphate dehydrogenase in the reoxidation of glycolytic NADH (Bacchi et al 1968). This would suggest a similarity with the metabolic pathways present in bloodstream form T.brucei.

4.5 Metabolism of Phosphoenolpyruvate and CO_2 Fixation

The metabolic pathways which occur after the initial breakdown of glucose to phosphoenolpyruvate are not so clearly understood. In the case of bloodstream form T.brucei, pyruvate is known to be the only major product as there are no enzymes for its further metabolism. However, the accumulation of succinate in the growth medium by many other trypanosomes must require the presence of other enzyme systems. The production of succinate depends on carbon dioxide fixation, first shown by Bowman et al (1963) in T.cruzi. Although the details of this process were not understood, it was proposed very early on that the pathway for this production was a reversal of part of the

TCA cycle: malate -> fumarate -> succinate (Baernstein, 1953(a); 1953(b)).

Ryley (1962) suggested that the source of malate was from carboxylation of pyruvate to oxaloacetate, which was reduced by malate dehydrogenase. Since then a number of reports on the mechanism of CO_2 fixation have appeared. Bacchi et al (1970) found that there were at least two ways in which oxaloacetate production could occur. The first was by the action of phosphoenolpyruvate carboxykinase, which was readily reversible, and the second was by the action of pyruvate carboxylase. Carbon dioxide fixation by malic enzyme to produce malate was not found, this enzyme catalysing the reverse reaction only with pyruvate being produced from malate. The most likely route for CO_2 fixation was that involving phosphoenolpyruvate carboxykinase, which would also involve net ATP production. The high malate dehydrogenase activity would then reduce oxaloacetate to malate and also serve to reoxidise NADH. Marr (1974) confirmed that the major pathway in C.fasciculata was from phosphoenolpyruvate to oxaloacetate and malate, and found that production of pyruvate by pyruvate kinase was of secondary importance. The V_{max} of pyruvate kinase was about 100-fold lower than that of phosphoenolpyruvate carboxykinase. Klein et al (1975) also found that phosphoenolpyruvate carboxykinase was the major CO_2 fixing enzyme in C.fasciculata, and also that it was highly active in culture forms of T.b.brucei (grown in defined medium including HCO_3^-). The activity of pyruvate carboxylase, which was present in C.fasciculata, was very much lower than the CO_2 fixing activity of phosphoenolpyruvate carboxykinase.

Labelling experiments carried out with $NaH^{14}CO_3$ indicated a sequence of metabolite production consistent with a oxaloacetate -> malate -> fumarate -> succinate pathway (Fig. 4.1).

4.6 Involvement of TCA cycle Enzymes in Glucose Catabolism

Although indirect evidence for the production of succinate from malate has been found, the actual enzymes have not been localized. Fumarate reductase activity (NADH-dependent reversal of succinate dehydrogenase) has only been found in the mitochondrial fraction, so malate may enter the mitochondrion for the final reactions leading to the production of succinate (Klein et al, 1975; Marr et al, 1977).

There is evidence that the TCA cycle operates in C.fasciculata (Hunter, 1960; Toner and Weber, 1972) but there is no evidence that it is fully functional aerobically, and able to cope with the flux of reduced coenzyme, while glucose consumption is occurring. Marr et al (1977) found that citrate, isocitrate and 2-oxoglutarate, which are other products of a fully functional TCA cycle, were not present or present at very low concentrations. Cazzulo et al (1985) found that the level of citrate synthase, an indicator of TCA cycle activity, increased 4-fold in cultures after glucose had been exhausted, suggesting that repression of cycle activity occurs during glycolysis.

4.7 Involvement of the Glycosome in CO_2 Fixation

The discovery of the glycosome has led to the further elucidation of the method of regulating CO_2 fixation and avoiding wasteful recycling of C4-dicarboxylic acids, formed by

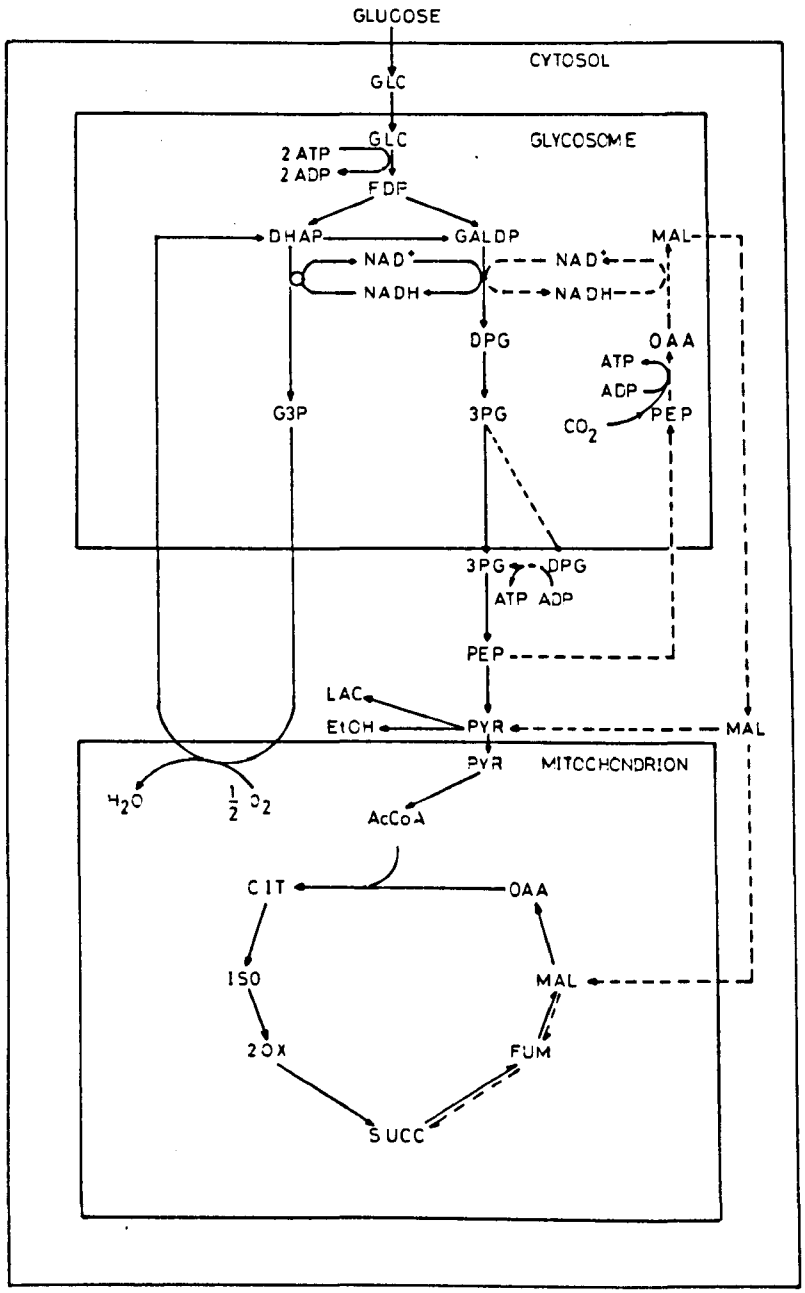

Figure 4.1 **Compartmentation of Glycolysis and Associated Pathways in C.fasciculata**

Abbreviations:- GLC: glucose; FDP: fructose-1,6-diphosphate; DHAP: dihydroxyacetone phosphate; GALDP: glyceraldehyde phosphate; G3P: glycerol-3-phosphate; DPG: diphosphoglycerate; 3PG: 3-phosphoglycerate; PEP: phosphoenolpyruvate; OAA: oxaloacetate; MAL: malate; PYR: pyruvate; LAC: lactate; EtOH: ethanol; AcCoA: acetyl CoA; CIT: citrate; ISO: isocitrate; 2OX: 2-oxoglutarate; SUCC: succinate; FUM: fumarate; - - - - - :- carbon dioxide fixation and succinate formation pathway.

phosphoenolpyruvate carboxykinase, back to C3-monocarboxylates by malic enzyme. Phosphoenolpyruvate carboxykinase is a glycosomal enzyme, responsible for CO_2 fixation in the glycosome, producing oxaloacetate which is used by malate dehydrogenase for the reoxidation of NADH in the glycosome (Cataldi de Flombaum et al, 1977; Cazzulo et al, 1980; Opperdoes and Cottem, 1982). Malic enzyme is involved in the decarboxylation of malate in the cytosol producing pyruvate. The process of CO_2 fixation is thus also responsible for the reoxidation of glycosomally produced NADH as an alternative to the glycerol-3-phosphate shuttle (Opperdoes et al, 1981; Opperdoes and Cottem, 1982).

De los Santos et al (1985), using C-13 NMR analysis of glucose metabolism in C.fasciculata, confirmed that production of phosphoenolpyruvate is followed by its carboxylation to oxaloacetate. This is then converted into succinate by the action of malate dehydrogenase, fumarase, and either fumarate reductase or succinate dehydrogenase. The possibility of pyruvate acting as a CO_2 acceptor to form oxaloacetate was ruled out, the only C3 acceptor detected being phosphoenolpyruvate.

4.8 Effect of Antimycin A on Pyruvate Production

I found that when measuring pyruvate production from whole cells of C.fasciculata, there was a significant stimulation in the level produced when antimycin A was present (Hale and Miller, 1983). Antimycin A has a powerful inhibitory effect on mitochondrial respiration, blocking electron transport between cytochromes b and c.

There are two possible pathways from phosphoenolpyruvate, either via carbon dioxide fixation in the glycosome into succinate and pyruvate, or to pyruvate by the action of pyruvate kinase in the cytosol (Fig. 4.1). The pathway which it has been claimed predominates in C.fasciculata during glucose consumption is that involving carbon dioxide fixation (Marr, 1974). This process does not involve oxidative phosphorylation. Malate produced in the glycosome is probably not metabolized any further in this organelle. No evidence was found for the presence of a glycosomal fumarase in procyclic T.brucei (Opperdoes and Cottem, 1982). This supports the hypothesis that malate leaves the glycosome and is either converted into pyruvate in the cytosol by the malic enzyme, or enters the mitochondrion for reduction to fumarate and then to succinate. The production of succinate is not thought to be linked to oxidative phosphorylation (Bacchi et al, 1970), rather fumarate probably acts as a terminal electron acceptor in a similar manner to that found in helminths (Saz and Lescure, 1969). Reduction of fumarate occurs by the reverse action of succinate dehydrogenase acting as fumarate reductase (Klein et al, 1975). Marr et al (1977) could not demonstrate a cytosolic fumarate reductase in C.fasciculata. The reversal of succinate dehydrogenase could involve substrate level phosphorylation and ATP synthesis, and is thought to be NADH-dependent (Klein et al, 1975). A mitochondrial fumarate reductase, which is NADH dependent, has also been reported in T.cruzi (Cannata and Cazzulo, 1984a; Boveris et al, 1986). However De los Santos et al (1985) concluded that fumarate reductase activity was

probably NADH independent in C.fasciculata on the basis of labelling patterns when using C-13 NMR analysis.

One possible hypothesis for the effect of antimycin A in C.fasciculata is that normally both pathways, CO_2 fixation on phosphoenolpyruvate and pyruvate formation by pyruvate kinase, are occurring, but that inhibition of oxidative phosphorylation by antimycin A causes an increased flux from glycolysis through the CO_2 fixation pathway. This increased flux would lead to a build-up of malate in the cytosol, which would then be either converted into pyruvate by the malic enzyme or converted into succinate in the mitochondrion. Malate has been shown to enter the mitochondrion readily (Toner and Weber, 1972). An increase in the production of both pyruvate, as was found, and succinate would be expected. A similar effect was found by Stoppani et al (1980) working with T.cruzi. They found that the presence of antimycin A caused an increase in succinate production from [1-^{14}C]-glucose, while there was a concomitant decrease in labelling of glutamate and alanine due to reduced TCA cycle activity.

An alternative explanation for the effect of antimycin A on pyruvate production is that CO_2 fixation does not occur, either because of a lack of HCO_3^- and CO_2 in the medium, or because the organisms were deficient in the required enzymes. A deficiency in CO_2 fixation would imply that pyruvate is formed only by the action of pyruvate kinase, entering the conventional TCA cycle for further metabolism. Antimycin A would inhibit the TCA cycle by blocking oxidative phosphorylation and would therefore cause a build-up of pyruvate which could not be metabolized further.

This theory is unlikely since TCA cycle activity is thought to be very low when active glycolysis is occurring (Cazzulo et al, 1985) and there is no evidence in any trypanosomatid that it is capable of dealing with the large amounts of pyruvate produced (Klein et al, 1975). It would also assume that glycosomal NADH is kept balanced by means of the glycerol-3-phosphate shuttle, using the mitochondrial glycerol-3-phosphate oxidase. However, the glycerol-3-phosphate oxidase is thought to be linked to cytochrome-mediated electron transfer, unlike the situation in bloodstream form T.brucei where it acts independently (Bowman and Flynn, 1976). This means that antimycin A would block NADH reoxidation and therefore also block pyruvate production. NADH reoxidation by malate dehydrogenase would not occur if CO_2 fixation was not occurring. The only other means of NAD^+ regeneration would be by using a similar method to anaerobic bloodstream form T.brucei, which produce equimolar amounts of pyruvate and glycerol. Glycerol-3-phosphate is converted into glycerol instead of entering the shuttle. Glycerol production has been shown to occur anaerobically in C.fasciculata (De Los Santos et al, 1985) therefore this method of NADH reoxidation is feasible. However, experiments with glycerol provided in place of glucose also showed some stimulation in pyruvate production under the influence of antimycin A. This implies that NAD^+ regeneration could not occur by the production of glycerol since this reaction would be pushed in the opposite direction.

A further possibility is that under normal conditions CO_2 fixation was occurring, but that formation of succinate in the mitochondrion by part of the TCA cycle operating in reverse is

not independent of oxidative phosphorylation, and is therefore blocked by antimycin A. This would lead to all malate, formed by CO_2 fixation, being diverted into pyruvate by the action of malic enzyme in the cytosol. This is unlikely since there is no evidence for a link with oxidative phosphorylation (Bacchi et al, 1970) and succinate is known to accumulate anaerobically (Schwartz, 1961; De Los Santos et al, 1985).

4.9 Effect of Altered Growth Conditions

After the medium used for routine sub-culture of C.fasciculata had been changed from CF1 to CF3 there was a change in the results obtained from pyruvate production experiments. Very little pyruvate was produced and antimycin A did not cause an increase. When cells were cultured in CF1 medium again the results for pyruvate production did not revert to the original pattern. In contrast a much higher level of pyruvate was now produced and antimycin A inhibited this production. This suggested that an irreversible alteration in the metabolism of the cells had occurred.

Antimycin A caused the inhibition of pyruvate production which suggests that pyruvate production was linked to oxidative phosphorylation by the electron transport chain. This implies that pyruvate was formed by the action of pyruvate kinase on phosphoenolpyruvate rather than by CO_2 fixation to malate and then pyruvate. Marr (1974) reported that pyruvate kinase had a V_{max} 100-fold lower than that of phosphoenolpyruvate carboxykinase, and proposed that of the two possible paths from phosphoenolpyruvate to either oxaloacetate or pyruvate,

oxaloacetate production was predominant owing to the higher activity of phosphoenolpyruvate carboxykinase over pyruvate kinase. He found that the build-up of oxaloacetate by this enzyme would in turn cause the inhibition of the malic enzyme preventing formation of pyruvate by this route. However, it is now known that oxaloacetate is produced inside a membrane-bound compartment, the glycosome, where it is converted to malate, which is exported into the cytosol. The physical separation of oxaloacetate and malic enzyme would prevent the inhibitory effect on the enzyme. The glycosomal membrane forms a permeability barrier to some glycolytic intermediates (Visser and Opperdoes, 1980) and equilibration of the glycosomal pool with the cytosolic pool is slow compared to the high glycolytic flux (Visser *et al*, 1981).

Bacchi *et al* (1970), whilst dismissing the importance of the pyruvate kinase reaction in an investigation into carbon dioxide fixation in *C.fasciculata*, did produce evidence that both reactions from phosphoenolpyruvate, to oxaloacetate and to pyruvate, were occurring and that pyruvate was produced three times as fast as oxaloacetate. Pyruvate kinase is found in cells actively consuming glucose as Cazzulo *et al* (1985) found. They found that pyruvate kinase was like hexokinase, in that both enzymes were at their highest levels in *C.fasciculata* during glucose consumption. After glucose was exhausted pyruvate kinase levels fell.

If pyruvate was produced by pyruvate kinase, then the implication is that, during maintenance in CF3 medium, an alteration in the metabolism of phosphoenolpyruvate had occurred

which was not reversible since renewed maintenance in CF1 did not cause a reversion to the original situation. The alteration may have occurred because the difference in composition of the medium caused increased or decreased expression of some enzymes. It is possible that a reduction in expression of mitochondrial proteins such as the cytochromes or TCA cycle enzymes occurred. The production of oxaloacetate from phosphoenolpyruvate is dependent on a high level of HCO_3^- in the growth medium in order for CO_2 fixation from phosphoenolpyruvate to occur. If the alternative medium (CF3) used for the growth of C.fasciculata was deficient in HCO_3^-, or caused lower expression of the enzymes required for CO_2 fixation and succinate formation, then the pyruvate kinase reaction would dominate. The work by De los Santos et al (1985), using C-13 NMR analysis, indicated that addition of sodium bicarbonate to the incubation mixture shifted the balance between carboxylation of phosphoenolpyruvate leading to succinate and pyruvate decarboxylation leading to ethanol. The ethanol:succinate ratio of 2·0 in the absence of bicarbonate, changed to a ratio of 0·6 in its presence. The labelling patterns indicated that ethanol was produced from pyruvate from the pyruvate kinase reaction and not from pyruvate produced by malic enzyme acting on malate.

One explanation then, for the changes observed, is that for some reason a reduction in the CO_2 fixation pathways took place in CF3 medium which was continued upon return to CF1 medium, leaving the pyruvate kinase reaction to predominate. If it is assumed that TCA cycle activity is not high enough to cope with the large glycolytic flux then there would be a build-up of

pyruvate. Reoxidation of glycosomal reducing equivalents, which normally occurs during the formation of malate from oxaloacetate when CO_2 fixation occurs, would be carried out by the glycerol-3-phosphate/dihydroxyacetone phosphate shuttle, operating with the glycosomal glycerol-3-phosphate dehydrogenase and the mitochondrial glycerol-3-phosphate oxidase. Antimycin A would inhibit the shuttle and therefore also inhibit pyruvate production.

There was very little pyruvate produced in experiments using CF3 grown cells, compared to when using the CF1 grown cells, which implies either that it was metabolised further or possibly that glycolysis was not important when cells were grown in this medium. It could be converted to ethanol by the action of an alcohol dehydrogenase. Ethanol has been shown to be an end-product in some cases. De Los Santos et al (1985) found that pyruvate produced by the pyruvate kinase reaction was converted to ethanol. Although assays for ethanol production in the CF3 grown cells were not carried out, there was a very noticeable smell of ethanol in cultures grown up in this medium.

The higher level of pyruvate production in CF1 medium cultured cells is presumably a reflection of the difference in medium composition, possibly giving rise to a decrease in ethanol production from pyruvate. This may occur because of a lower expression of the enzymes necessary for the conversion in the CF1 medium. Alternatively it may be that there is a much lower mitochondrial activity in CF3 grown cells which is not capable of dealing with the required reoxidation of reducing equivalents produced by the high glycolytic flux. The alcohol

dehydrogenase step in the conversion of pyruvate to ethanol also serves to regenerate reducing equivalents. This step may therefore be a convenient way for the cell to cope with the high glycolytic flux, although, if this reaction occurs in the cytosol and mitochondrial shuttle activity is reduced, it does not explain how the balance of reducing equivalents is maintained in the glycosome. One possibility is the production of glycerol from glycerol-3-phosphate as an alternative to using the shuttle. The transfer from CF3 to CF1 medium may give rise to increased mitochondrial activity, which is able to cope with a higher rate of regeneration of reducing equivalents, and therefore the alcohol dehydrogenase step is no longer required.

It was interesting to find that the use of ethanol as a solvent for antimycin A was not possible in the later experiments on CF1 grown cells. Whereas previously ethanol was found to have no noticeable effect on pyruvate production in control experiments, it was now found to have a severe inhibitory effect. This suggests that ethanol may have been having some sort of regulatory feedback effect on glycolysis, possibly at the level of pyruvate kinase which is a non-reversible step. This would fit with the hypothesis that the production of pyruvate was occurring by the pyruvate kinase pathway rather than by the carbon dioxide fixation pathway. Methanol, which was used as an alternative solvent to ethanol for experiments using antimycin A, did not have any observable effect in control experiments. This was as expected since, unlike ethanol, methanol is not a metabolic product in

C.fasciculata, neither can it be utilised by the cell (Cosgrove, 1959).

4.10 Variations in Results

It is apparent, both from my own results and those of other people, that considerable variation in the results obtained from similar experiments can occur. Some of the differences are only minor variations in end-products of glycolysis while others are more important. The work of Marr suggested that amino acids were more important than glucose during the exponential phase of growth. In contrast to this the results I obtained suggested the converse to be true, with glucose the preferential energy source during exponential growth. The work of Cazzulo et al (1985) has provided additional support for this case. Cazzulo et al suggested that some of the variation may have been due to the the growth curve of Marr having a long lag phase, possibly due to his having used a stationary starter inoculum for his cultures. Cazzulo et al used an exponential phase starter culture, which gave rise to a very short lag phase similar to that which I obtained. In some of his experiments Marr appears to have used sonicated samples of whole cells, which would therefore include intracellular metabolites.

The most likely explanation for the variations found between different reports, is that differences in the strains of C.fasciculata and in experimental conditions used are responsible. Different strains may have a different enzymic make-up, giving rise to alterations in the metabolic pathways which predominate. It is also likely that differences in the

composition of media used may have a strong influence. The levels of bicarbonate and CO_2 may determine whether or not the pathways of carbon dioxide fixation are operating. As most of the work has been carried out using undefined media it is difficult to assess the influence of bicarbonate content on the results obtained.

4.11 A Carrier for Pyruvate Transport

The possibility that a membrane transport system exists in some cells, specific for pyruvate and other monocarboxylic acids, has long been suspected, although the positive evidence for this has not always been available. It was first suggested that pyruvate enters the mitochondrion on a specific carrier by Papa et al (1971). Subsequently support for this proposal was obtained when it was discovered that α-cyano-4-hydroxycinnamate was a specific inhibitor of mitochondrial and cell membrane pyruvate transport (Halestrap and Denton, 1974). α-Cyanocinnamate and its derivatives have been shown to be specific, potent and reversible inhibitors of mitochondrial pyruvate transport, and it is thought that they act by reacting with essential thiol groups on the carrier (Halestrap, 1976a). Monocarboxylates, the anions of weak and often lipophilic acids, were long thought to cross membranes by non-ionic diffusion of the undissociated acid via the lipid domain of the membrane (Deuticke, 1977). Most unsubstituted α-hydroxy and α-oxo-substituted aliphatic monocarboxylates penetrate the erythrocyte membrane to a considerable extent by non-ionic diffusion, but pyruvate and lactate may be exceptions to this

rule (Deuticke et al, 1978). Deuticke et al (1982) found evidence that the transport of lactate across the erythrocyte membrane was almost totally due to the presence of a specialized carrier. Only 5% of transport occurred by non-ionic diffusion and 5% was due to the classical anion exchange system.

The mechanism of transport is not electrogenic since changes in membrane potential have no effect (Deuticke, 1982). Electroneutrality is probably maintained by a coupled exchange against OH^-, or by H^+ cotransport (Deuticke et al, 1982; Dubinsky and Racker, 1978; Regen and Tarpley, 1978; 1980). The most likely of these two possibilities is the H^+ cotransport model (Regen and Tarpley, 1980; Halestrap, 1978; Deuticke, 1982). A similar mechanism has been proposed for lactate transport in Ehrlich Ascites tumour cells (Spencer and Lehninger, 1976).

It seems highly likely that similar specific monocarboxylate carriers may be operative in several other cell membranes where a specialized carrier has been proposed eg. intestinal epithelial cells (Lamers, 1975); cardiac muscle (Watts and Randle, 1967); skeletal muscle (Foulkes and Paine, 1961) and the blood-brain barrier (Oldendorf, 1973). More recently a monocarboxylate carrier has been proposed in rabbit heart sarcolemma (Mann, 1985) and a similar carrier possibly exists in the nerve endings of Torpedo marmorata (O'Regan, 1983).

4.12 Inhibition of Pyruvate Transport

Since bloodstream form T.brucei produce large amounts of pyruvate as an end-product of glycolysis, which has to cross the cell membrane in order to leave the cell and enter the host

bloodstream, the possibility of a specific carrier existing for this purpose has been investigated. α-Cyanocinnamic acid and its derivatives are known to inhibit transport of pyruvate and/or lactate across rat liver mitochondrial membranes and human erythrocytes (Halestrap and Denton, 1974); rat hepatocytes (Monson et al, 1981b); Ehrlich Ascites tumour cells (Spencer and Lehninger, 1976) and rat erythrocytes and thymocytes (Anderson et al, 1978).

The effect of α-cyano-4-hydroxycinnamate on pyruvate efflux from C.fasciculata has been investigated and it has been found that total inhibition is achieved using a concentration of 800μm (Hale and Miller, 1983). Work previously carried out using T.brucei (Miller, 1980) indicated that pyruvate efflux from this organism could be blocked using compound UK5099 [α-cyano-β-(1 phenylindol-3-yl) acrylate]. A 50% inhibition of pyruvate efflux was achieved with <200μM UK5099. The results did not indicate the concentration required for total inhibition, or if this was possible. The difference in the concentration required for inhibition was probably due to the differences in effectiveness of the various derivatives of α-cyanocinnamate. α-Cyanocinnamate, α-cyano-5-phenyl-2,4-pentadienoate and UK5099 are all much more powerful than α-cyano-4-hydroxycinnamate (Halestrap, 1975).

It has been reported that the effectiveness of these inhibitors in blocking mitochondrial pyruvate and lactate transport, is much greater than in blocking cell membrane transport (Halestrap and Denton, 1975). Halestrap and Denton (1974) found that less than 200μM α-cyano-4-hydroxycinnamate

inhibited rat liver mitochondrial pyruvate transport, however 2.5mM α-cyano-4-hydroxycinnamate was required for maximum inhibition of pyruvate and lactate transport across erythrocyte membranes (Halestrap, 1976b). The transport of pyruvate across tumour cell membranes (Spencer and Lehninger, 1976) required 2.5mM α-cyano-4-hydroxycinnamate for inhibition, and maximum inhibition of lactate transport across rat hepatocyte membranes was achieved by 2mM α-cyano-3-hydroxycinnamate.

Deuticke (1982) suggests that while α-cyanocinnamate derivatives are specific inhibitors of mitochondrial monocarboxylate transport, they are less selective in erythrocyte transport systems. It might therefore be expected that low concentrations of the inhibitor would cause a build-up of pyruvate due to the blockage of mitochondrial uptake and oxidation, which would therefore be lost from the cell, giving an apparent increase in pyruvate production in cells where mitochondrial oxidation of pyruvate is important. The results for pyruvate production from C.fasciculata in the presence of the inhibitor did not indicate any increase in production at low concentrations. This suggests that the mitochondrial oxidation of pyruvate produced from glucose catabolism is not important in cells when glycolysis is actively occurring.

The concentrations of α-cyano-4-hydroxycinnamate required to inhibit Crithidia pyruvate production was lower than the values reported for other cell membranes, but it is not possible to say whether this is due to a higher specificity for a pyruvate carrier in the membrane. The effect of the inhibitor could be due to it causing a block elsewhere in the reactions leading to

the formation of pyruvate. An inhibitory effect on one or more of the enzymes involved cannot be ruled out. Halestrap and Denton (1974) found that up to 200mM α-cyano-4-hydroxycinnamate did not inhibit any of the enzymes of pyruvate oxidation in rat liver mitochondria, and presumably acted by blocking mitochondrial uptake of pyruvate.

One characteristic of the coupled H^+/monocarboxylate carrier in other systems has been the marked effects of pH changes at one or both sides of the membrane (Deuticke, 1982). The efflux of pyruvate from preloaded mitochondria was stimulated by increased external pH (Halestrap, 1978), and the efflux of β-hydroxybutyrate from rat erythrocytes and thymocytes was inhibited by lowering the external pH (Regen and Tarpley, 1978). Altering the pH of the medium used to test for pyruvate production in C.fasciculata would give more information about a possible pyruvate carrier.

Previously a transport mechanism, across the cytoplasmic membrane of C.fasciculata, for α-aminoisobutyrate has been proposed (Midgley, 1978). However, this was thought to be an active mechanism relying on an electrochemical gradient maintained by an ATPase. This is therefore not likely to be linked to a monocarboxylate carrier such as that already described.

The need for the presence of a monocarboxylate carrier, particularly in T.brucei where such high levels of pyruvate pass into the host bloodstream, would seem to be high. Although at neutral pH monocarboxylates may exist in sufficient proportion in the undissociated form to be able to dissolve in the membrane

lipid, and thus penetrate the non-aqueous layer, it does not follow that this process is sufficient to provide an adequate transport rate (Mowbray, 1975). It therefore seems likely that some form of transport mechanism is involved in removing pyruvate from the trypanosome.

Chapter 5

RESULTS

HEXOKINASE PURIFICATION AND CHARACTERISATION

5.1 Hexokinase Levels During Growth

The levels of hexokinase activity were compared in cells from mid-exponential, late-exponential and stationary phase cultures of C.fasciculata (Fig. 5.1). Hexokinase activity in stationary phase cells was found to be about 20% of that in exponential phase cells (Table 5.1). There was little apparent variation in levels during the exponential phase.

5.2 Hexokinase purification

5.2.1 Preparation of Cell Sonicates

The preparation of crude cell extracts, for the purification of hexokinase from C.fasciculata, was carried out by sonication of whole cells. Hexokinase is a particulate, glycosomal enzyme (Taylor et al, 1980), but sonication was found to effectively solubilize the enzyme activity, which did not exhibit latency when cell extracts were incubated either prior to or during assays with 1% (v/v) and 0·1% (v/v) Triton X-100 respectively (Table 5.2).

5.2.2 Ammonium Sulphate Precipitation

The bulk of the hexokinase activity in crude cell extracts was found to precipitate between 1·8M and 2·6M ammonium sulphate (Table 5.3).

5.2.3 Phenyl-Sepharose Chromatography

Hexokinase was found to form strong hydrophobic interactions with Phenyl-Sepharose. The enzyme bound to a column of Phenyl-Sepharose when loaded in buffer containing 1·2M ammonium

sulphate. A non-linear decreasing gradient of 1·2M-0M ammonium sulphate was run, and at the same time a non-linear gradient, 0-10% (v/v) ethanediol, of increasing polarity was run. Hexokinase activity did not elute from the column until the ammonium sulphate concentration in the buffer was zero, and would not elute without the presence of 10% (v/v) ethanediol (Fig. 5.2). Under these conditions hexokinase activity eluted in a tight peak. Attempts to increase the purification factor by altering the conditions slightly were found to cause considerable broadening of the peak.

5.2.4 Ion-Exchange Chromatography (A)

Pooled and concentrated hexokinase fractions from Phenyl-Sepharose chromatography were applied to a column of DEAE-cellulose. The degree of binding to this matrix was found to be variable, depending on the stage of purification and the conditions used. A proportion of the activity applied always bound to the column. When hexokinase from Phenyl-Sepharose chromatography was applied to a column, more than 50% of the applied protein was eluted with the starting buffer, i.e. was not bound. The bulk of the hexokinase activity also eluted with this fraction (Fig. 5.3). Application of buffer containing 0·4M NaCl caused the elution of bound protein, and included in this was a small amount of hexokinase activity which was discarded.

5.2.5 Ion-Exchange Chromatography (B)

The fractions containing hexokinase activity from the above DEAE-cellulose chromatography were pooled and concentrated. A

repeat application of this sample to the same column under the same conditions resulted in a similar pattern of elution, with very little recoverable hexokinase activity binding to the matrix. However, it was found that by using a much smaller column and operating it at room temperature, a much greater proportion of the applied enzyme activity bound to the matrix and could be eluted with buffer containing 0·4M NaCl (Fig. 5.4).

5.2.6 Final Purification Criteria

Table 5.4 shows the increase in the purity of the hexokinase preparation at each step. The final preparation showed an increase in specific activity of 51 times that of the original crude cell extract (after sonication and centrifugation). The true figure is likely to be higher than this, as the level of protein in the solution was very dilute, and its measurement was limited by the sensitivity of the protein assay. There was also some loss in activity of the enzyme especially in the concentration of the final DEAE-cellulose fractions.

Protein samples from each step in the purification procedure were separated by SDS-polyacrylamide gel electrophoresis (Fig. 5.5), and the final preparation appeared to be purified to a single polypeptide band of apparent molecular mass 50,000.

5.3 Gel Filtration

The native molecular mass of hexokinase was determined by gel filtration on a Sephacryl S-300 column (Fig. 5.6). The determination was complicated by the fact that the enzyme appeared to bind to the matrix. Without 0·5M NaCl present in the

buffer, the enzyme bound totally to the column. Even when salt was present the enzyme still appeared to bind to some degree and often smearing of the activity was observed throughout the elution profile. When sufficient activity was applied, a peak of activity appeared with an average Kav of 0·188, indicating a molecular mass of about 290,000. A similar figure was obtained for hexokinase in a crude cell extract and in a partially purified sample.

5.4 pH Optimum

Using the glucose-6-phosphate dehydrogenase coupled assay for activity, the hexokinase appeared to have a broad pH specificity with activity remaining fairly constant between pH 7 and 9 (Fig. 5.7 & Fig. 5.8). When using the alternative assay with lactate dehydrogenase and pyruvate kinase, the enzyme appeared to have a more specific pH optimum (Fig. 5.9), being most active at pH 7·5.

5.5 The Effect of Glucose-6-Phosphate

Hexokinase enzymes in most other organisms undergo allosteric regulation by their reaction product glucose-6-phosphate. The inclusion of glucose-6-phosphate in the assay for hexokinase activity gave no indication that there was a similar inhibitory effect on the enzyme from C.fasciculata at a final concentration of either 0·5mM or 1·0mM (Table 5.5).

5.6 Enzyme Kinetics

The substrate affinity of hexokinase for glucose was determined using both purified enzyme and crude cell extracts. Lineweaver-Burk plots of enzyme activity against substrate concentration (Fig. 5.10 (a) & (b)) gave an apparent K_m of 33μM for both the crude cell extract and the purified enzyme. Lineweaver-Burk plots for ATP concentration (Fig. 5.11 (a) & (b)) gave a K_m for both the crude cell extract and the purified enzyme of 83μM.

Using purified hexokinase, the V_{max} for glucose was 30 units, and the V_{max} for ATP was 32 units, (Table 5.6).

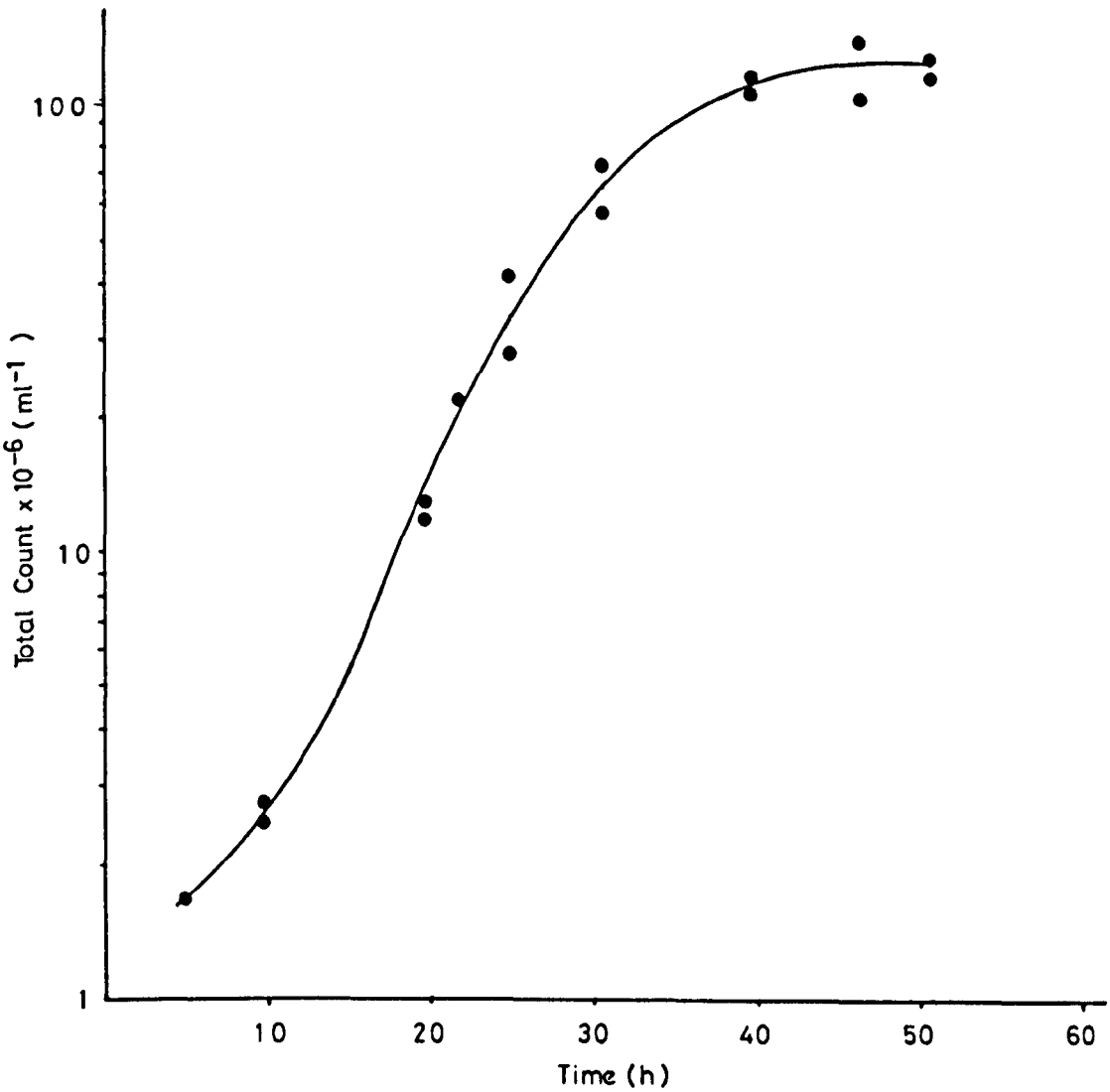

Figure 5.1 C.fasciculata : Growth Curve in CF3 Medium

A 2ml inoculum from a late-exponential phase CF3 culture of C.fasciculata was used to start 200ml cultures in CF3 medium with glucose. When indicated in Table 5.1, cells were harvested and hexokinase levels were determined.

TIME (hours)	HEXOKINASE ACTIVITY (units / $2x10^9$ cells)
22	8·360 ± 0·432
32	8·680 ± 0·321
45	1·865 ± 0·096

Table 5.1 Hexokinase Levels During Growth of C.fasciculata

Cells were harvested at intervals through the growth cycle (see Figure 5.1) at the times indicated, washed, counted and resuspended in Tris-HCl, pH 7·4 to give a cell density of $2x10^9$ cells/ml. Cells were disrupted by sonication and hexokinase activity was assayed.

One unit of activity is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose-6-phosphate / minute. Results are expressed as the mean and standard deviation of four determinations.

SAMPLE	HEXOKINASE ACTIVITY (units/ml)
Crude Cell Sonicate	0·788 ± 0·035
Crude Cell Sonicate + Triton X-100	0·819 ± 0·021

Table 5.2 Latency of Hexokinase Activity in Crude Extracts

Hexokinase activity was assayed both in the presence and absence of Triton X-100 at a final concentration of 0·1% (v/v). Results are expressed as the mean and standard deviation of four determinations. One unit of activity is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose-6-phosphate / minute.

AMMONIUM SULPHATE FRACTION	TOTAL HEXOKINASE ACTIVITY (units)
1·8M Precipitate	6·43 (1·31%)
1·8M Supernatant	483·39 (98·70%)
2·6M Precipitate	406·45 (83·00%)
2·6M Supernatant	35·05 (7·20%)

Table 5.3 Ammonium Sulphate Precipitation of Hexokinase

Ammonium sulphate precipitation was carried out on a crude cell-free extract of C.fasciculata. The total hexokinase activity in each fraction was determined by assaying in duplicate. Figures in brackets refer to the percentage of the total activity in the crude cell extract.

One unit of activity is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose-6-phosphate / minute.

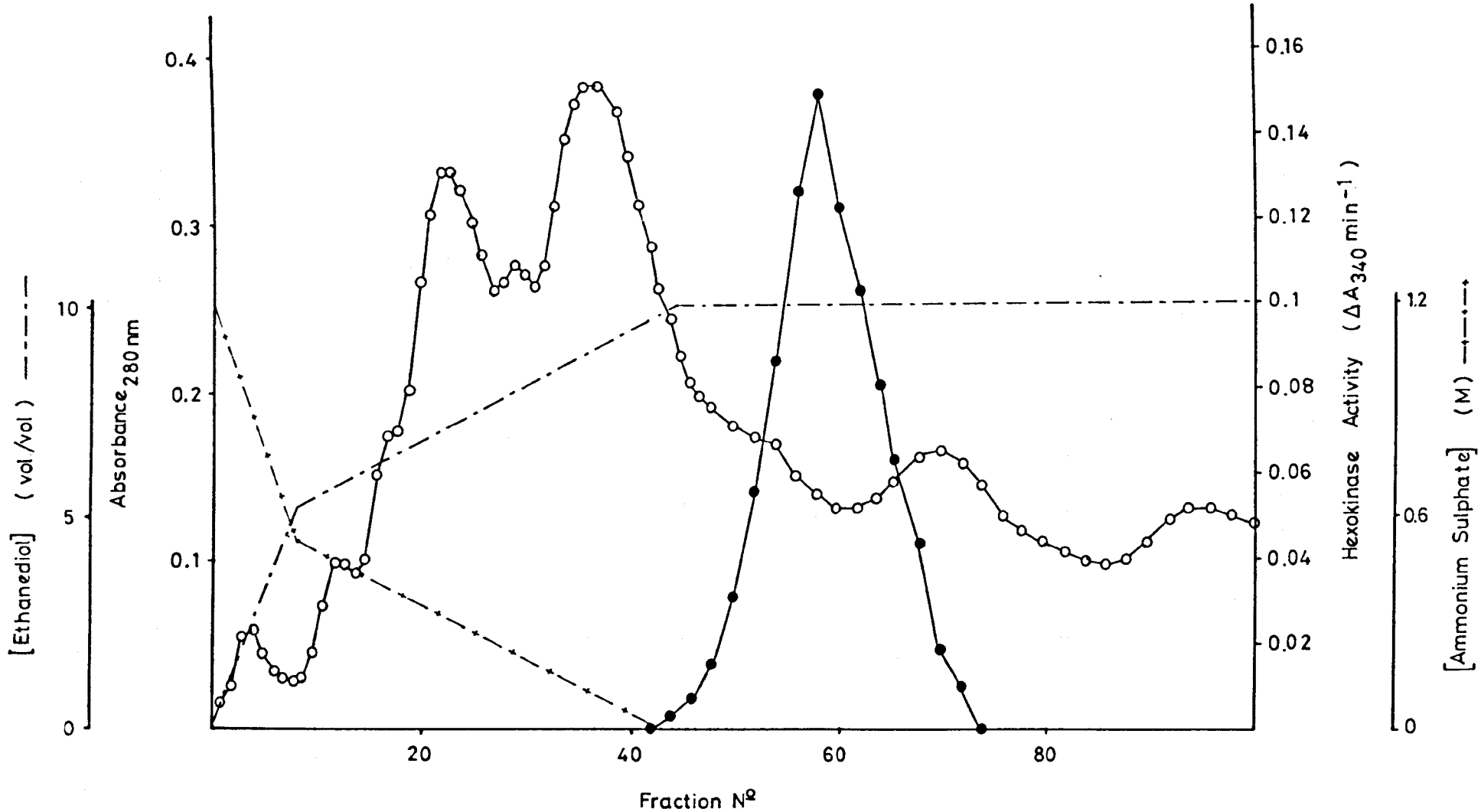

Figure 5.2 <u>Phenyl-Sepharose Chromatography</u>

A 10 ml sample (approx. 5 ml ammonium sulphate precipitated fraction diluted in equilibration buffer) was loaded onto the column. Protein (absorbance at 280nm): —○—○— ; hexokinase: —●—●—. Fractions 52-68 were pooled.

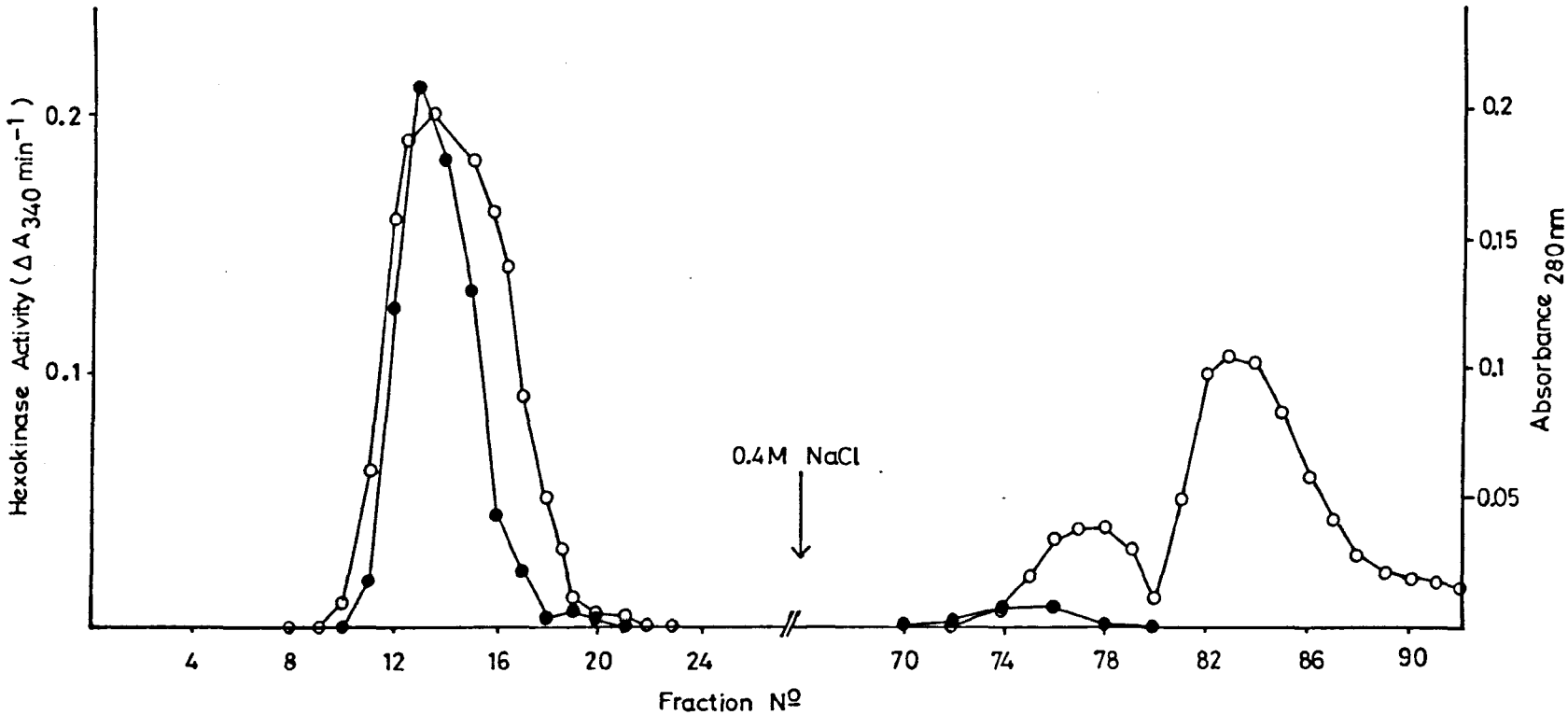

Figure 5.3 <u>Ion-Exchange Chromatography (A)</u>

The concentrated hexokinase pool from Phenyl-Sepharose chromatography was loaded onto a column of DEAE-cellulose. The elution buffer was changed to one including 0·4M NaCl at fraction 50. Protein (absorbance at 280nm): —○—○—; hexokinase: —●—●—. Fractions 11-17 were pooled.

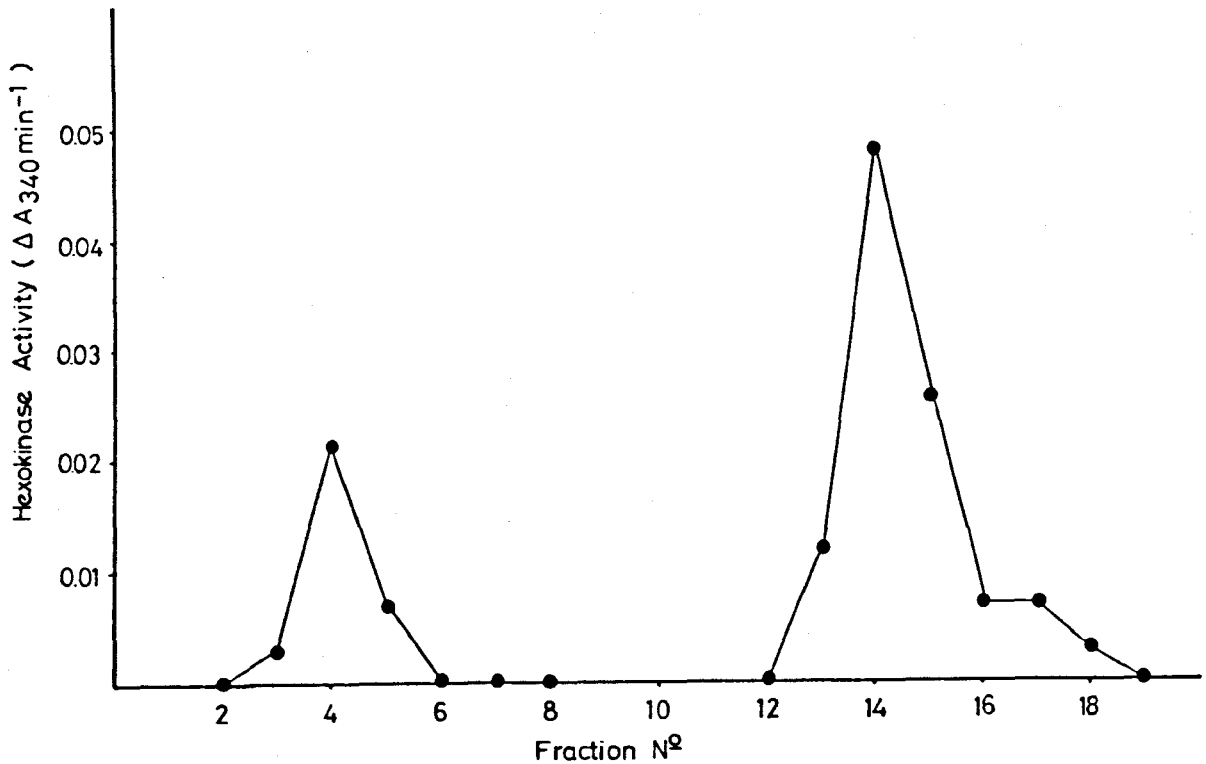

Figure 5.4 **Ion-Exchange Chromatography (B)**

The concentrated hexokinase pool from ion-exchange chromatography (A) was loaded in 0·5ml sample volumes onto a second column of DEAE-cellulose. The elution buffer was changed to one containing 0·4M NaCl where indicated. Fractions 13-17 were pooled.

FRACTION	VOLUME (mls)	TOTAL PROTEIN (mgs)	TOTAL ACTIVITY (units)	SPECIFIC ACTIVITY (units/mg)	PURIFICATION (fold)	YIELD (%)
Crude Cell-Free Extract	35.0	1200	680	0.57	(1)	(100)
1.8-2.6M Ammonium Sulphate Cut	4.5	400	572	1.43	2.52	84
Phenyl-Sepharose Pool	72.0	118	510	4.32	7.62	75
Phenyl-Sepharose Pool:- Concentrated	9.6	87	463	5.32	9.37	68
Ion-Exchange Chromatography (A) Pool	42.0	38	399	10.49	18.50	59
Ion-Exchange Chromatography (A) Pool:- Concentrated	10.4	39	323	8.28	14.61	48
Ion-Exchange Chromatography (B) Pool	[40.0	1.67	48.53]*	29.03	51.20	30
Ion-Exchange Chromatography (B) Pool:- Concentrated	[6.0	1.69	33.97]*	20.08	35.42	21

Table 5.4 **Purification of Hexokinase**

Figures shown [ ]* are total of 5 column runs, purification factor is adjusted accordingly.

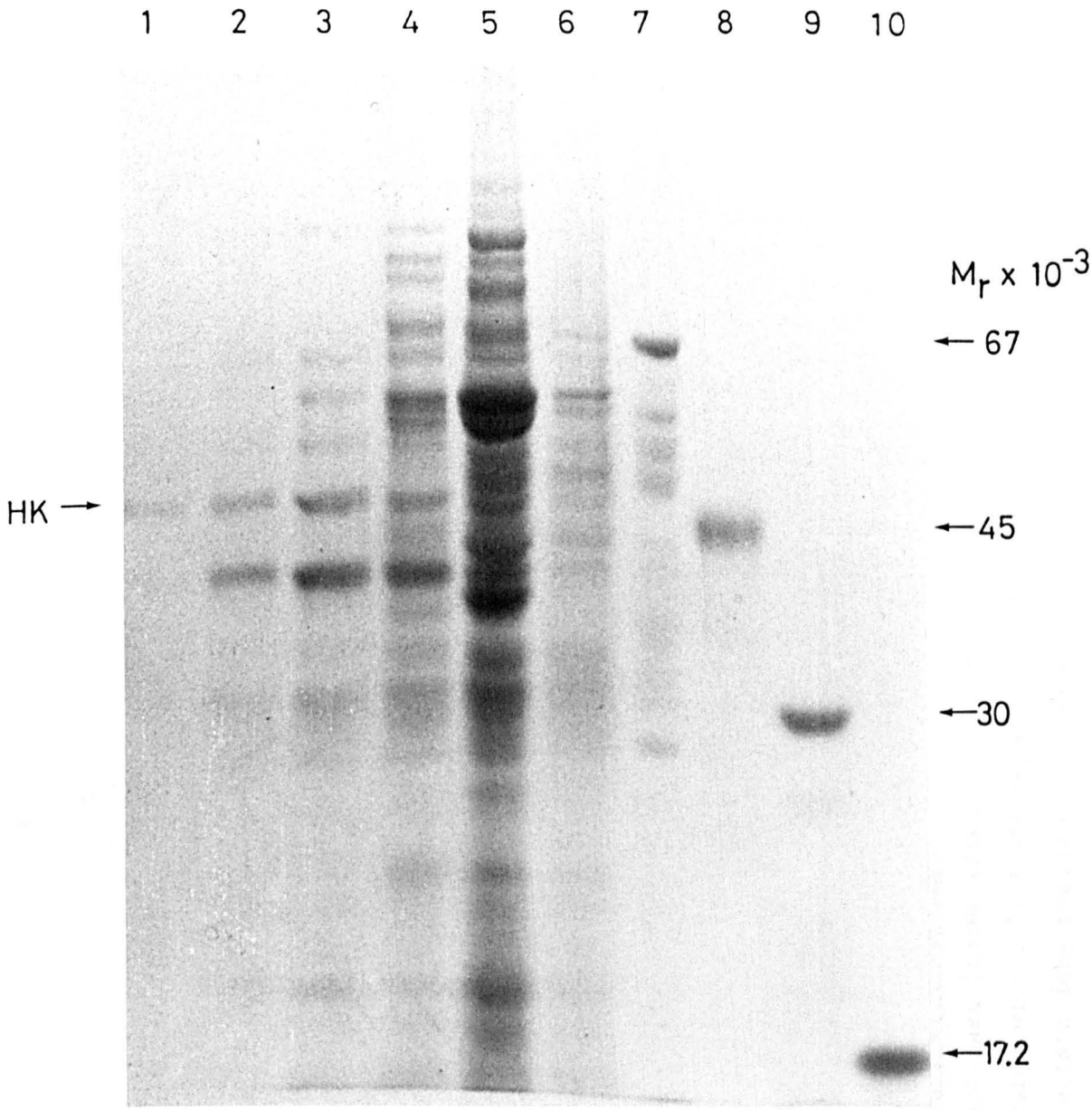

Figure 5.5 **SDS-Polyacrylamide Gel Electrophoresis of Hexokinase Purification**

Samples from each step of the purification procedure were separated by SDS-PAGE. Track 1: purified hexokinase (50µl); track 2: post ion-exchange chromatography (A) (50µl, 1:5); track 3: post ion-exchange chromatography (A) (50µl); track 4: post Phenyl-Sepharose chromatography (50 µl, 1:15); track 5: ammonium sulphate precipitated protein (50µl, 1:20); track 6: crude cell-free extract (50µl, 1:50). Tracks 7-10:- marker proteins: BSA, ovalbumin, carbonic anhydrase and myoglobin respectively.

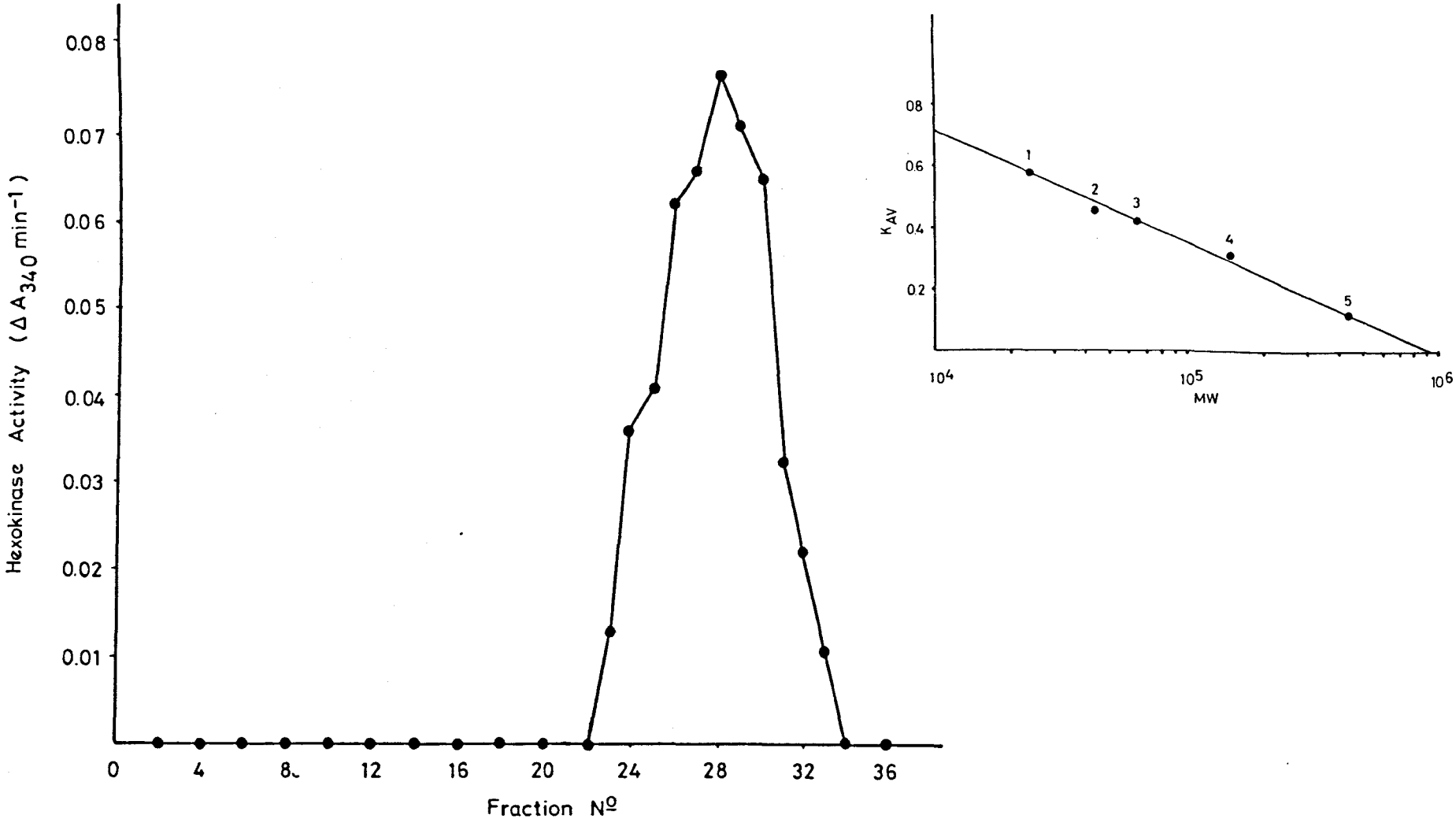

Figure 5.6 **Gel Filtration Chromatography of Hexokinase**

A partially purified sample of hexokinase (taken after ion-exchange chromatography (A)) was separated on Sephacryl S-300. The column was calibrated with : 1. trypsinogen (M_r 24,000); 2. ovalbumin (M_r 45,000); 3. bovine serum albumin (M_r 67,000); 4. lactate dehydrogenase (M_r 145,900); 5. ferritin (M_r 440,000).

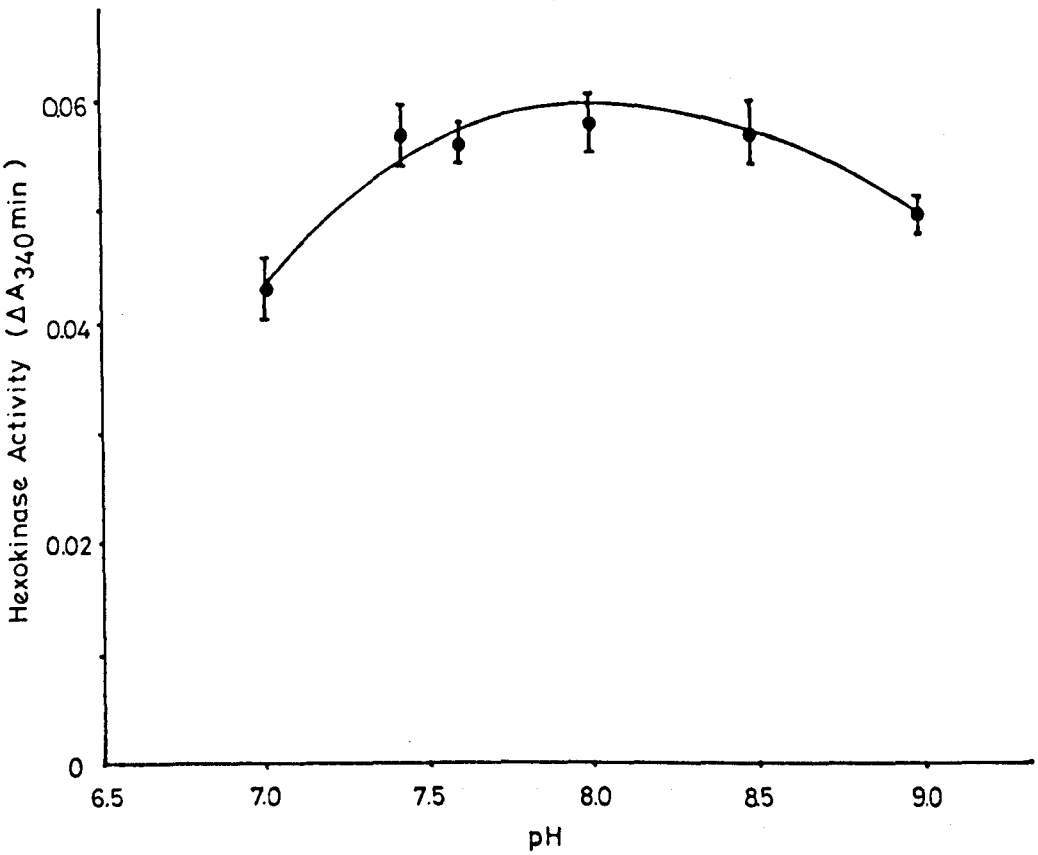

Figure 5.7 **pH Optimum of Hexokinase - Crude Extract**

Hexokinase activity in a crude cell-free extract was determined using the glucose-6-phosphate dehydrogenase coupled assay, varying the pH of the Tris-HCl buffer in the assay mix between pH 7·0 and 9·0.

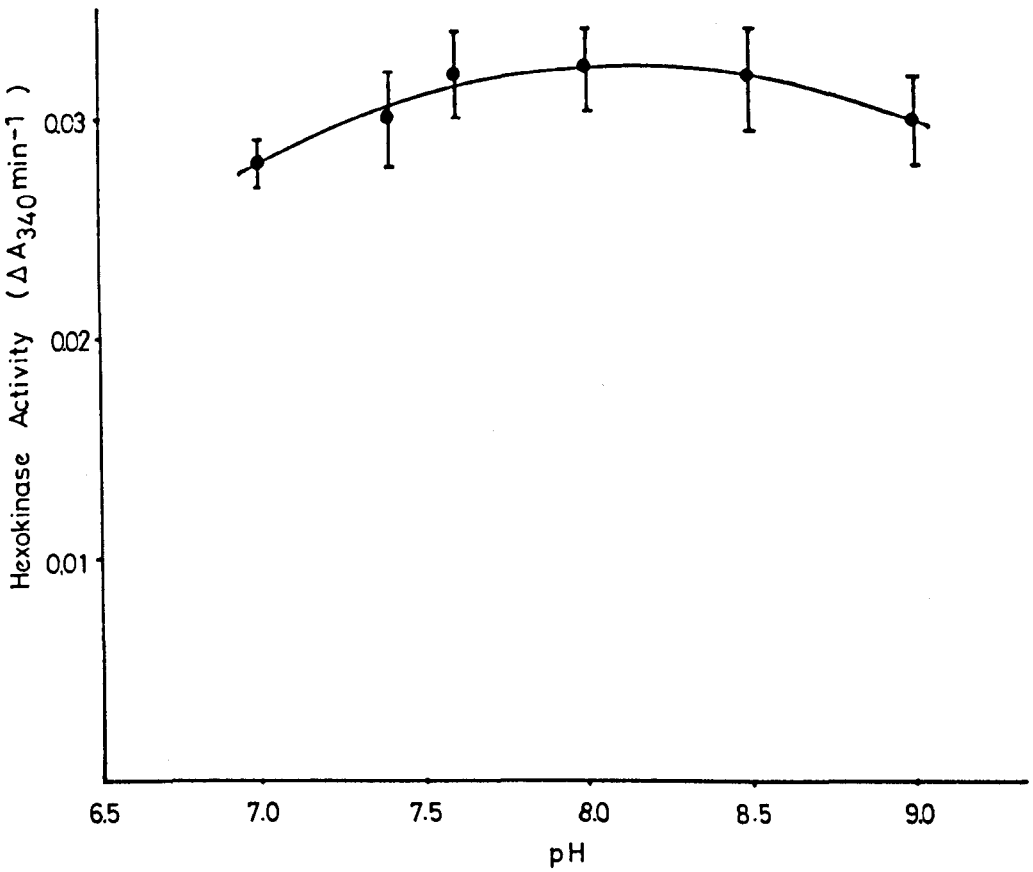

Figure 5.8 **pH Optimum of Hexokinase - Purified Sample**

The hexokinase activity of a purified sample was determined using the glucose-6-phosphate dehydrogenase coupled assay, varying the pH of the Tris-HCl buffer in the assay mix between pH 7·0 and 9·0.

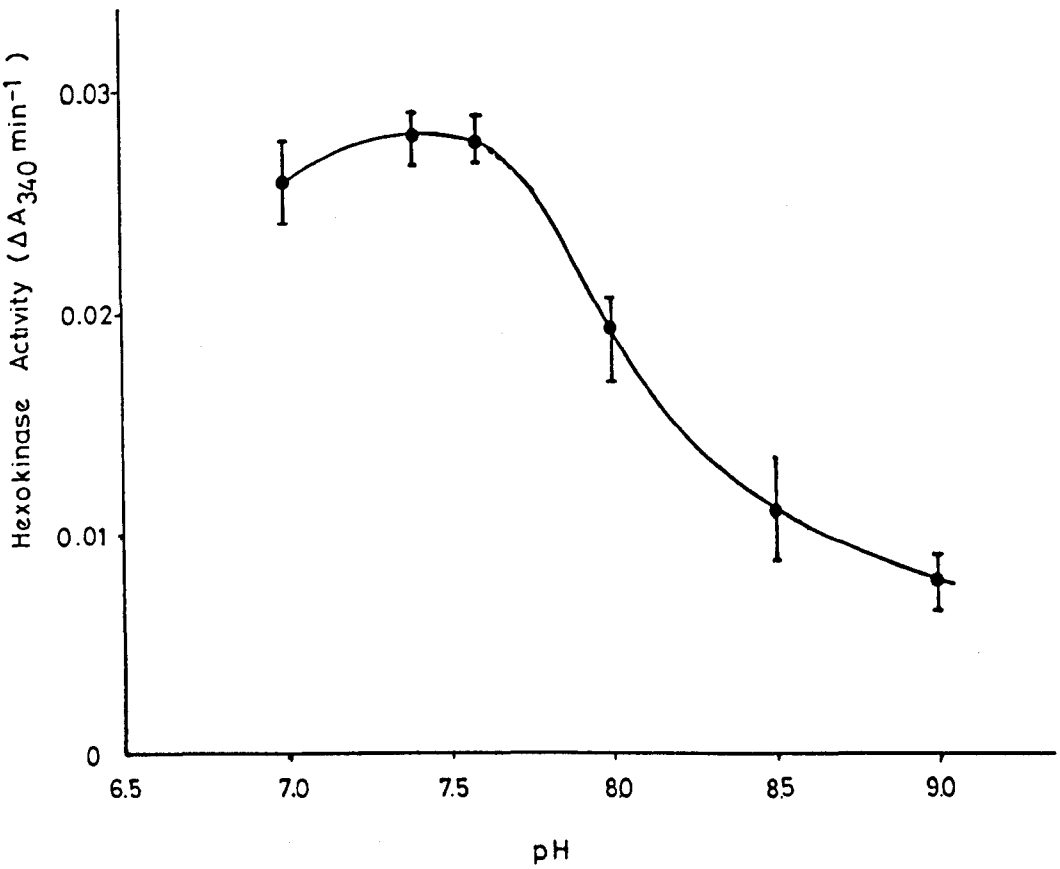

Figure 5.9 **pH Optimum of Hexokinase - Alternative Assay**

The hexokinase activity of a purified sample was determined using the pyruvate kinase / lactate dehydrogenase coupled assay, varying the pH of the Tris-HCl buffer in the assay mix between pH 7·0 and 9·0.

GLUCOSE-6-PHOSPHATE	HEXOKINASE (units)	
	CRUDE ENZYME	PURIFIED ENZYME
0	1·02 ± 0·031	0·298 ± 0·030
0·5mM	1·07 ± 0·045	-
1·0mM	-	0·297 ± 0·020

Table 5.5 Product Inhibition of Hexokinase

Hexokinase activity was determined by the pyruvate kinase / lactate dehydrogenase coupled assay. Glucose-6-phosphate was included in the assay where shown and at the concentrations indicated. Results are expressed as the mean and standard deviation of four determinations. One unit of activity is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose-6-phosphate / minute.

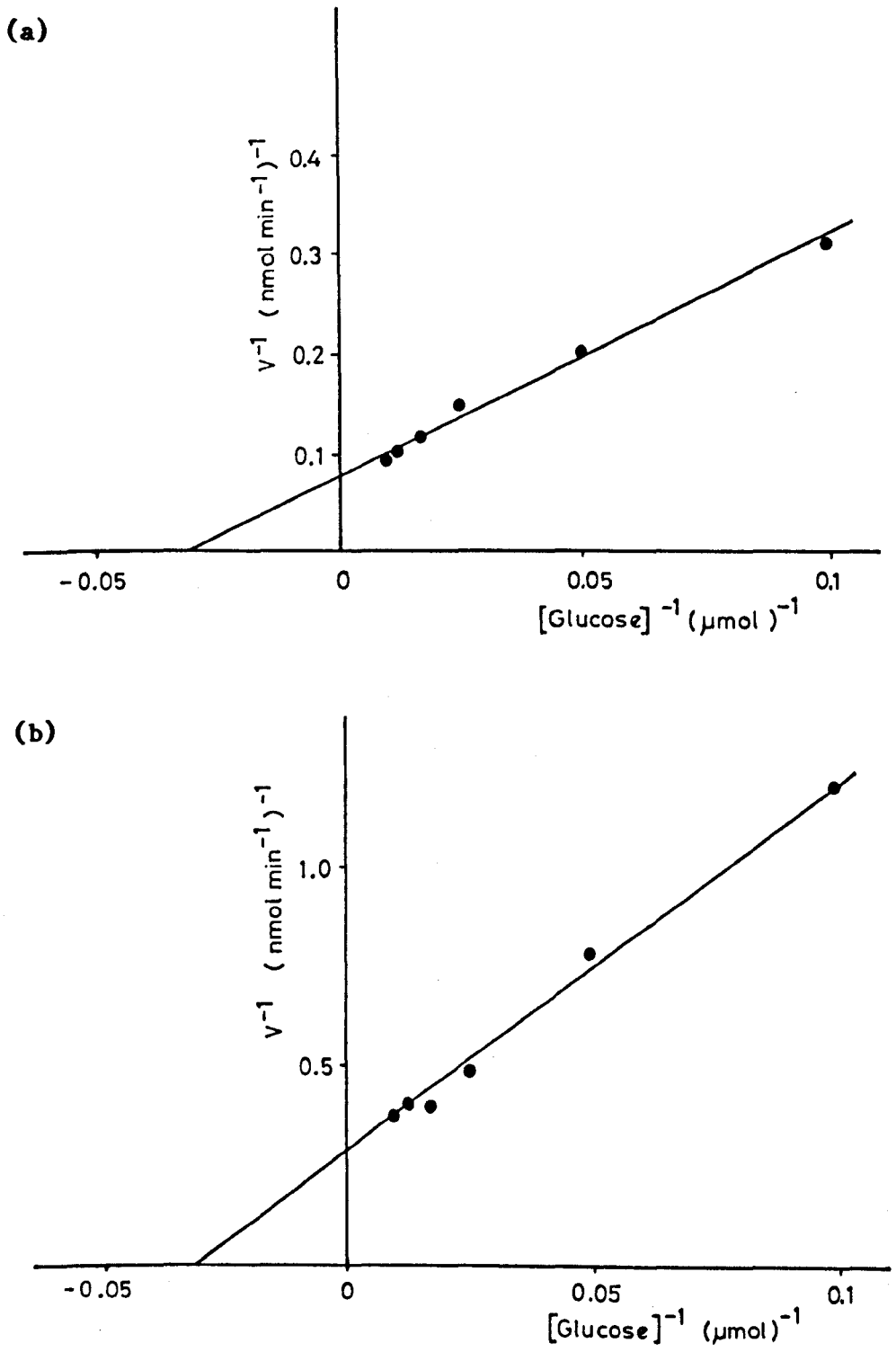

Figure 5.10 **Lineweaver-Burk Plot - Glucose**

Hexokinase activity was determined using the standard glucose-6-phosphate dehydrogenase coupled assay and using glucose concentrations from 10-100 μM. The activity of hexokinase in a crude cell-free extract (a) and in a purified sample (b) was determined.

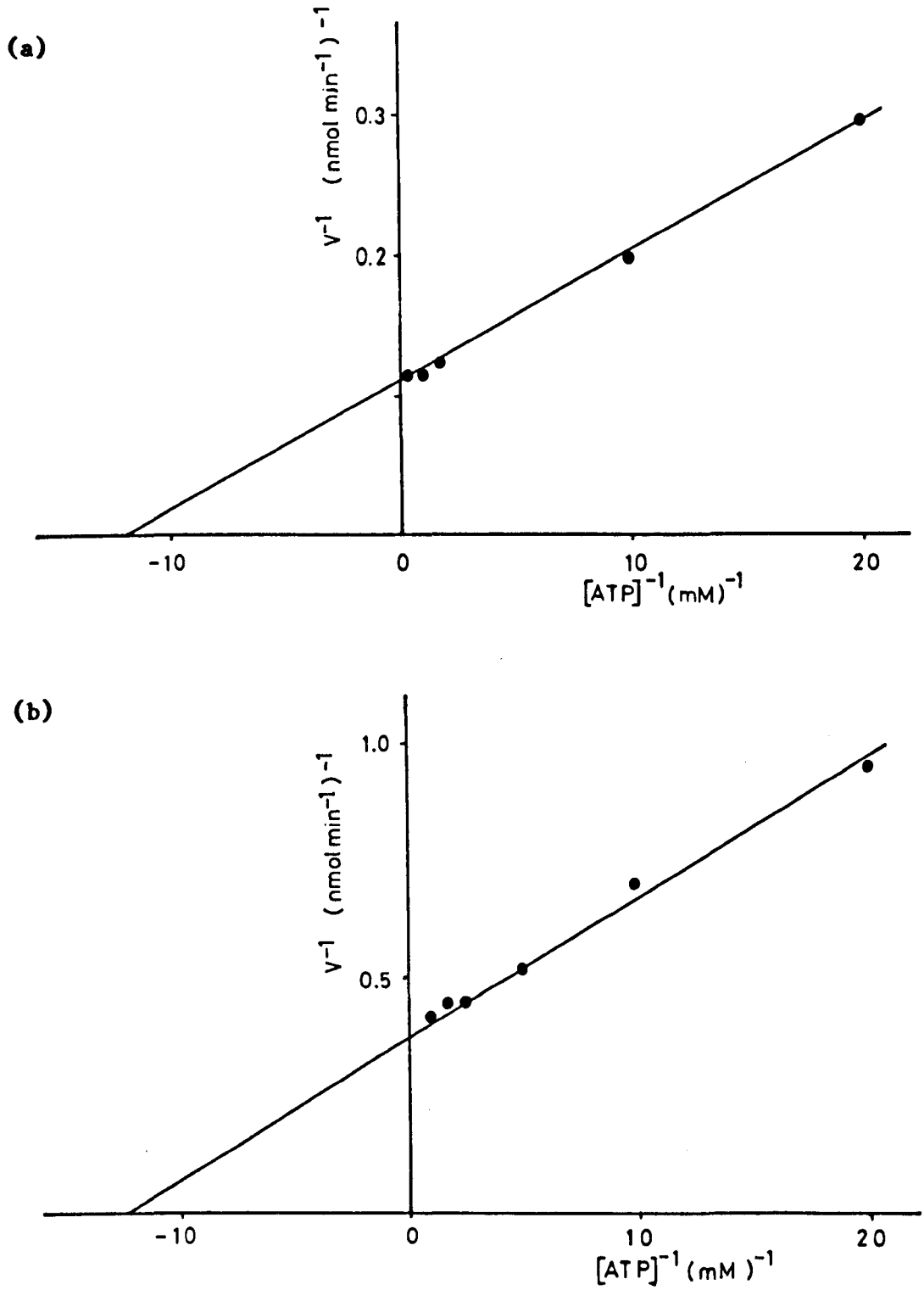

Figure 5.11 **Lineweaver-Burk Plot - ATP**

Hexokinase activity was determined using the standard glucose-6-phosphate dehydrogenase coupled assay and using ATP concentrations from 0·05-1·0mM. The activity of hexokinase in a crude cell-free extract (a) and in a purified sample (b) was determined.

(a)

	K_m	V_{max} (units/mg)
glucose	33·33 μM	0·554
ATP	83·33 μM	0·568

(b)

	K_m	V_{max} (units/mg)
glucose	33·33 μM	29·75
ATP	83·33 μM	31·80

Table 5.6 Hexokinase - Kinetic Parameters

Values for K_m and V_{max} were obtained from Lineweaver-Burk plots (figures 5.10 and 5.11), (a): hexokinase in a crude cell-free extract; (b): purified hexokinase. One unit of activity is defined as the amount of enzyme catalysing the formation of 1 μmole of glucose-6-phosphate / minute.

Chapter 6

DISCUSSION

HEXOKINASE PURIFICATION AND CHARACTERISATION

6.1 Variation in Hexokinase Levels

The results obtained for the variation in level of hexokinase in cells throughout the growth cycle are consistent with those results obtained for the production of pyruvate, i.e. the capacity for pyruvate production was much greater in exponentially grown cells and diminished towards stationary phase. Whilst the level of hexokinase per cell remained reasonably constant during the exponential phase of growth, stationary phase cells underwent a 4·5 fold reduction in the level of hexokinase present. This suggests that the consumption of glucose via the glycolytic pathway is much more important during exponential growth than during the stationary phase. These results together with those for pyruvate production support the suggestion that glycolysis is important to exponentially growing cells, and contradict the evidence from Marr *et al* (1977) that amino acid consumption is the primary energy source in the exponential phase with glucose consumption not occurring until late exponential and stationary phase growth.

Work by Cazzulo *et al* (1985) into glucose metabolism in *T.cruzi* and *C.fasciculata* drew conclusions similar to those I have put forward. They found that hexokinase levels in both *T.cruzi* and *C.fasciculata* were highest during glucose consumption. Glucose consumption was found to be maximal during exponential phase growth, while amino acid incorporation did not begin until glucose was exhausted. The activity of citrate synthase, an enzyme of the TCA cycle, increased 4-fold after glucose exhaustion, suggesting repression of cycle activity

during active glycolysis. All these facts together point to the conclusion that the preferred energy source of C.fasciculata during exponential growth is the glycolytic breakdown of glucose.

6.2 Purification Procedure

The purification of hexokinase from C.fasciculata was attempted with a view to identifying novel characteristics of the trypanosomal enzyme which could be exploited for the development of a suitable means of chemotherapeutic attack. A major difference from the hexokinase of mammals and other organisms is the location of the glycolytic enzymes within a membrane-bound organelle, the glycosome (Taylor et al, 1980). The trypanosomatids are the only known organisms where this is the case. This major difference may cause the glycolytic enzymes from trypanosomes to exhibit differences from their counterparts in other eukaryotic cells, that render classical purification procedures insufficient.

Since hexokinase is located within a membrane-bound organelle, the initial step in preparing cell extracts for purification was to solubilize the enzyme activity. Many of the glycolytic enzymes, including hexokinase, have been shown to exhibit latency, although there is conflicting evidence as to the efficiency of the methods employed for releasing activity. McLaughlin (1981) found that hexokinase and other enzyme latencies in T.rhodesiense were released by 200mM KCl but not by sonication or by 0·25% (v/v) Triton-X100. In contrast Opperdoes et al (1977b) found that α-glycerophosphate dehydrogenase, a

glycosomal enzyme, could be released by Triton-X100 but not Cl^- unless the glycosomes were first disrupted. Misset and Opperdoes (1984) in the purification of T.brucei glycolytic enzymes used Triton-X100 to solubilize the glycolytic enzymes from a glycosomal preparation, prepared using cells disrupted by silicon carbide grinding.

The differences observed by these workers are probably due to the experimental techniques used. McLaughlin (1981) used glass beads to disrupt his cells and also a low ionic strength buffer. Misset et al (1986) have found that under conditions of low ionic strength most of the glycolytic enzymes tend to aggregate in the absence of a bounding membrane. Elevated ionic strength promotes dissociation and individual behavior of each enzyme (Opperdoes and Nwagwu, 1980; Oduro et al, 1980b). I found that hexokinase exhibited no significant latency in cell extracts of C.fasciculata. This is probably due to the use of sonication rather than the more gentle silicon carbide grinding to disrupt cells. The effect of sonication on whole cells is presumably harsh enough to disrupt glycosomal as well as cell membranes. The inability of McLaughlin to demonstrate the release of glycosomal activities by sonication could be due to the very low ionic strength buffer used and to the cells having been previously broken up by grinding.

The methods classically used in the purification of hexokinase from other organisms include ion-exchange chromatography on DEAE-cellulose, ammonium sulphate precipitation and affinity chromatography. Yeast hexokinase has been separated into two molecular forms on DEAE-cellulose, which

could be eluted with a salt gradient (Colowick, 1973). More recently two hexokinases, from rabbit red blood cells and rabbit reticulocytes, have been purified by their capacity to bind to DEAE-Sephadex and be eluted with a potassium chloride gradient, followed by ammonium sulphate fractionation and affinity chromatography (Fornaini et al, 1982). Hexokinase II from rabbit skeletal muscle has been purified by binding to DEAE-cellulose and eluting with a potassium chloride gradient, followed by affinity chromatography (Easterby and Quadri, 1982). Similar procedures have been developed for hexokinases from Drosophila melangaster (Lee, 1982), Ascaris suum (Supowit and Harris, 1982) and for the glucokinase of S.mutans (Porter and Chassy, 1982).

The purification of C.fasciculata hexokinase was carried out in part by using two of the above mentioned techniques, ammonium sulphate precipitation and ion-exchange chromatography on DEAE-cellulose. The use of affinity chromatography on reactive blue-2-Sepharose was not possible as the enzyme bound irreversibly to the matrix. When using ammonium sulphate precipitation, the C.fasciculata hexokinase differed considerably in its behavior from the enzymes of eukaryotic organisms. Hexokinase could not be precipitated from crude cell extracts within narrow ammonium sulphate concentration limits. Instead a wide ammonium sulphate cut was necessary in order to precipitate the bulk of the activity.

The behaviour of the C.fasciculata enzyme on DEAE-cellulose was also unusual in that only very limited binding of the enzyme to the matrix was found (depending on the conditions used). Application of a crude cell extract to a DEAE-cellulose column

resulted in about 50% of the hexokinase activity binding to the column and being released by a salt gradient (Miller, unpublished work). However, if this step was performed after the crude cell extract had been partially purified by ammonium sulphate precipitation or Phenyl-Sepharose chromatography, the bulk of the hexokinase activity passed through without binding to the matrix. The first ion-exchange step in the procedure was therefore used as a method of removing other contaminating proteins, while the bulk of the hexokinase activity passed straight through. In contrast to this, when DEAE-cellulose chromatography was used as a final step in the purification, the hexokinase activity was found to bind to the column. Work carried out on a monoQ ion-exchange column, on an FPLC, to separate partially purified hexokinase (Miller, unpublished work) indicated that the bound and non-bound hexokinase fractions were not substantially different, since the effect was reversible, with either fraction producing further bound and non-bound fractions when rechromatographed.

It is possible that this effect occurs as a result of enzyme aggregation. It has been reported that elevated ionic strength leads to dissociation and individual enzyme behavior of glycosomal enzymes, whilst conditions of low ionic strength cause them to aggregate (Opperdoes and Nwagwu, 1980; Oduro *et al*, 1980b; Misset *et al*, 1986). This suggests that the hexokinase from *C.fasciculata* does not bind to DEAE-cellulose when it is behaving as an individual enzyme, but that under certain conditions it aggregates either with other proteins or with itself, and that in this form it binds to the matrix. This

binding may not be an ionic effect but simply the effect of the protein aggregates precipitating out on the column matrix. The use of salt gradients to elute the bound enzyme would cause dissociation of the aggregates into individual enzymes due to the increased ionic strength. The substantial binding of hexokinase from crude cell sonicates to the DEAE-cellulose column may have been due to the close association of glycolytic enzymes (Oduro et al, 1980b; Opperdoes and Nwagwu, 1980; Aman et al, 1985). The concentration of protein in these extracts may have meant that the ionic strength of the buffer was not sufficient to dissociate the enzyme complex completely. The binding of hexokinase activity to the matrix in the final purification step could also be a non-specific effect caused by low ionic strength and low protein concentration leading to precipitation of the enzyme onto the column. There was a tendency for a precipitate to form in the solution when concentrating the hexokinase fractions from the previous step. Misset et al (1986) reported that recovery of T.brucei hexokinase activity from a CM-cellulose column was low due to reduced solubility of hexokinase at low ionic strength and the formation of a precipitate when concentrating the hexokinase activity to apply to the column.

The most satisfactory separation of hexokinase was obtained by hydrophobic interaction chromatography on Phenyl-Sepharose. This illustrates the bizarre nature of the glycolytic enzymes of the trypanosomes compared to their counterparts in other organisms. Hexokinase bound very firmly to the hydrophobic matrix and could only be eluted by using two gradients, one of

decreasing ammonium sulphate concentration, and one of increasing ethanediol concentration. The hexokinase could not be eluted from the column until the ethanediol gradient had commenced. The concentration of hexokinase fractions, after Phenyl-Sepharose chromatography, by ultrafiltration, was a very prolonged process, taking several hours, since the ethanediol made the solution very viscous. This, and non-specific absorption to the filter, may have caused some loss in enzyme activity. Loss of enzyme activity was particularly noticeable in the final concentration step.

The purity of the final hexokinase preparation was judged by its apparent homogeneity on SDS-polyacrylamide gel electrophoresis. A single polypeptide band was visible after staining the gels. However, the purification factor obtained did not appear very high and may indicate that substantial loss of activity was occurring during the purification. Improvements to the procedure are obviously required. This is the first reported purification of hexokinase from C.fasciculata. A number of attempts have been made by other research groups to purify hexokinase from various trypanosomes and Leishmania species. In most cases only partial purifications have been carried out, which probably reflects the difficulty in using classical purification techniques in purifying glycosomal enzymes.

Risby and Seed (1969) partially purified hexokinase from African trypanosomes and T.equiperdum using sonication, ammonium sulphate precipitation and DEAE-Sephadex chromatography. Berens et al (1980) carried out a partial purification of hexokinase from Leishmania donovani and Leishmania braziliensis using DEAE-

cellulose chromatography and a salt gradient. Racagni and Machado de Domenech (1982) reported a partial purification of hexokinase from T.cruzi using ammonium sulphate precipitation and DEAE-cellulose chromatography with a potassium chloride gradient. They reported finding different molecular forms using ion-exchange chromatography although no details of this finding are given.

The usefulness of Phenyl-Sepharose chromatography in the purification of glycosomal proteins is further illustrated in its use by Mottram and Coombs (1985). They used it as an initial step in the purification of phosphoenolpyruvate carboxykinase and malate dehydrogenase, two glycosomal enzymes, from Leishmania mexicana mexicana. They also reported that hexokinase from this organism had been purified during this procedure, although with a very low yield.

Recently a method for purifying nine glycolytic enzymes, including hexokinase, from bloodstream form T.brucei has been published (Misset et al, 1986). An important step in the simultaneous purification of these enzymes was the exploitation of their hydrophobic nature using Phenyl-Sepharose chromatography. It is possible, therefore, that this method will be of significant value as an initial step in the purification of any glycosomal enzyme in trypanosomes or Leishmania. Hexokinase in T.brucei was purified to homogeneity by further chromatography on CM-cellulose and ATP elution. The purification factor achieved using this method was very high although the yield was very low, lower than any of the other enzymes purified in the procedure.

6.3 Molecular Mass and Subunit Structure

Hexokinase from C.fasciculata was found to have an apparent subunit molecular mass of 50,000 as judged by SDS-polyacrylamide gel electrophoresis. Western blot analysis confirmed that the antiserum raised against the enzyme recognised a 50,000 molecular mass component. The native molecular mass of the hexokinase was determined, by gel filtration on Sephacryl S-300, to be 290,000. The tendency for hexokinase activity to bind to the column was probably due to the slightly polar polysaccharide matrix which would attract the hydrophobic enzyme. The molecular mass of the native enzyme suggests that the mature protein is a hexamer of six 50,000 molecular mass subunits. The report on the purification of T.brucei hexokinase (Misset et al, 1986) indicated a similar finding, with a native molecular mass of 295,000 and a subunit molecular mass of 51,000. These are the only reported examples of hexameric hexokinases, therefore it seems likely that this is a feature unique to the trypanosomes.

The only report of a molecular mass for a leishmanial hexokinase is that of Mottram and Coombs (1985). They reported that gel filtration chromatography on Sephadex G-100 gave a molecular mass of 83,000 for the enzyme from L.m.mexicana. It was suggested that the unexpectedly low molecular mass for malate dehydrogenase in the same separation could have been caused by the conditions of the column run, which may have caused dissociation of the enzyme with retained catalytic activity, so the same may also have been true for hexokinase. The native and subunit molecular masses of the leishmanial hexokinase have therefore yet to be established unequivocally.

It might be expected that since the glycosome is a shared feature of the trypanosomatids, there would be some similarities in the structure of similar glycosomal enzymes of different species. However, there have been reports giving different molecular masses for another glycosomal enzyme, phosphofructokinase, in different species. In T.brucei this enzyme is reported by Cronin and Tipton (1985) to have a native molecular mass of 220,000 with a subunit mass of 49,000. Misset et al (1986) were in close agreement with this, with corresponding figures of 196,000 and 50,000 respectively. But, the enzyme in T.cruzi is reported to have a native and subunit molecular mass of only 17,000 (Aguilar and Urbina, 1986).

The molecular mass and subunit structure of the trypanosomatid hexokinase appears to be unique. There is no other known hexameric hexokinase, those of C.fasciculata and T.brucei are the only two described to date. There are four different mammalian hexokinase isozymes, designated A,B,C and D or I, II, III and IV, and their distribution varies from tissue to tissue and also depends on the organism (Colowick, 1973; Ureta, 1982). All of the mammalian hexokinases described so far have been monomeric. Types A, B and C have a broad tissue distribution and have similar native and subunit molecular masses in the region of 100,000 (Purich et al, 1973; Ureta, 1982). Type D hexokinase, also known as glucokinase, is found in the liver (Colowick, 1973). This isozyme differs considerably from the other three with a molecular mass in the region of 50,000 (Ureta, 1982).

The hexokinases of non-vertebrate organisms have subunit molecular masses in the region of 50,000. Their native molecular masses may be either 50,000 or 100,000. Drosophila hexokinase has a molecular mass of about 50,000 (Ureta, 1982; Lee, 1982), as does that of wheatgerm and Asterias (Ureta, 1982). Saccharomyces and Neurospora hexokinases are dimers of about 100,000 (Ureta, 1982; Purich et al, 1973; Colowick, 1973). The subunit structure of the trypanosomal hexokinase, therefore, more closely resembles that of the yeast enzyme than the mammalian hexokinases, although the enzyme is hexameric rather than dimeric.

6.4 pH Optimum

Hexokinase appeared to have a fairly broad pH optimum using the glucose-6-phosphate dehydrogenase coupled assay, and a more specific optimum, of about pH 7·5, using the pyruvate kinase/lactate dehydrogenase coupled assay. However, as both hexokinase assays were coupled enzyme assays, it is possible that the pH of the solution would have some effect on the efficiency of the coupling enzymes, and thereby affect the result to some extent. This probably explains the different pH profiles obtained using the two different methods. The narrow pH range for optimum hexokinase activity found with the pyruvate kinase / lactate dehydrogenase coupled assay is thus most probably due to the effect of pH on the coupling enzymes, rather than a direct characteristic of hexokinase which had a much broader optimum pH range using the glucose-6-phosphate dehydrogenase coupled assay.

Nwagwu and Opperdoes (1982) reported that T.brucei hexokinase had a similarly broad pH range with a maximum at pH 7·8. They used a similar glucose-6-phosphate dehydrogenase coupled assay system. The hexokinase from T.cruzi has been characterized by Racagni and Machado de Domenech (1983) and they found it had a pH optimum between 7·4 and 8·2, using both the standard and alternative assays. Seed and Baquero (1965) found that the hexokinase from T.rhodesiense and T.gambiense had a broad pH optimum between 7·0 and 9·0. Risby and Seed (1969) tested partially purified hexokinases from T.brucei, T.rhodesiense, T.gambiense and T.equiperdum and found they had a broad pH range with pH optima between 6·5 and 7·0. In the latter two reports hexokinase activity was assayed by measuring the reduction in glucose concentration. This type of assay excludes any effect of pH on externally added enzymes for coupling systems. However, an assay such as this, where the disappearance of a substance is measured, involves measuring the difference between two large values. A more accurate result is obtained when measuring the appearance of a product, as is the case for the coupled enzyme assays. This type of assay also prevents the build up of products potentially detrimental to the activity of the enzyme.

The overall picture for trypanosomal hexokinases appears to be one of a very broad pH range, with an optimum in the region of 7·5 to 8·0. This figure is not dissimilar to that of Baker's yeast hexokinase (Sols et al, 1958) which has a pH maximum of 7·5 and displays half maximal activity at pH 5·4 and pH 9·4. Other hexokinases with broad pH ranges are those from

Rhodoturula glutinis (Mazón et al, 1975) and Streptococcus mutans (Porter and Chassy, 1982).

The isoelectric point of hexokinase from C.fasciculata was not determined. However, its behaviour on the anion exchange matrix, DE52, at pH 8·0, suggests that it has a high pI compared to most previously described hexokinases. The enzyme from T.brucei has been reported to have a pI of 10·2 (Misset et al, 1986). This value is much higher than the value reported for yeast and mammalian hexokinases. Rabbit erythrocyte hexokinase has a pI of 6·2-6·3 (Fornaini et al, 1982), the rat muscle enzyme has a pI of 5·7-6·2 (Easterby and Quadri, 1982) and rat brain has a pI of 6·0 (Chou and Wilson, 1972). Yeast hexokinases similarly have low isoelectric points (Misset et al, 1986). The pI of some T.cruzi strains has also been found to be high, in the region 9·40-9·55 (Ebert, 1982). This suggests that a high isoelectric point may be a characteristic of trypanosomal hexokinases in general.

6.5 Glucose-6-Phosphate Inhibition

In 1954 Crane and Sols reported that mammalian brain hexokinase is extremely sensitive to glucose-6-phosphate inhibition. It is now known that all mammalian hexokinase isozymes are sensitive to glucose-6-phosphate inhibition with the exception of type IV, the liver enzyme, which is glucose-6-phosphate insensitive at physiological concentrations (Anderson et al, 1971; Ureta, 1982). In a number of organisms hexokinase is involved in the regulation of glycolysis. Hexokinase, together with phosphofructokinase and pyruvate kinase, act as

regulatory enzymes, responding to various activators and inhibitors depending on the source. The most common effector is the product, which acts as an inhibitor (Krebs, 1972). In mammalian cells glucose-6-phosphate acts as an inhibitor of hexokinase, and the glucose-6-phosphate level is determined by phosphofructokinase. An increase in phosphofructokinase activity causes a decrease in the glucose-6-phosphate concentration and thus reduces inhibition of hexokinase with a resultant increase in glucose consumption.

The hexokinase from yeast cells does not have a similar allosteric reaction, there is no glucose-6-phosphate inhibition, and it is thought unlikely that glucose-6-phosphate plays a role in regulation (Purich et al, 1973). In this respect hexokinase from C.fasciculata resembles the enzyme from yeast cells. There was no evidence that glucose-6-phosphate had any effect on the enzyme. This is also a characteristic of several other trypanosomal hexokinases. Glucose-6-phosphate had no effect on T.brucei hexokinase even at a concentration of 17 mM (Nwagwu and Opperdoes, 1982). However, in this paper the reported method for assaying hexokinase was one coupling activity to glucose-6-phosphate dehydrogenase, which would presumably remove added glucose-6-phosphate before it could have any inhibitory effect. Hexokinase from T.cruzi was also insensitive to glucose-6-phosphate (Urbina and Crespo, 1984; Racagni and Machado de Domenech, 1983). Seed and Baquero (1965) found that T.rhodesiense and T.gambiense hexokinases were insensitve while Risby and Seed (1969) found that the enzymes of T.brucei, T.gambiense, T.rhodesiense and T.equiperdum were sensitive to

glucose-6-phosphate. However the inhibition in this case was only 4 to 6%, and is therefore probably not significant. The hexokinases of Leishmania donovani and Leishmania braziliensis were similarly found to have kinetic and regulatory characteristics resembling the mammalian type IV hexokinase rather than the other isozymes (Berens et al, 1980).

6.6 Enzyme Kinetics

Hexokinase from C.fasciculata had a fairly high affinity for its substrate (glucose), with an apparent K_m of 0·03 mM; the K_m for ATP was 0·08 mM. The K_m reported for T.brucei hexokinase was even lower, 0·017 mM, while the K_m for ATP was 0·116 mM (Nwagwu and Opperdoes, 1982). These figures are of a similar order of magnitude to those for C.fasciculata and suggest a high affinity of the trypanosomal hexokinases for glucose. The hexokinase of T.cruzi had only a slightly lower affinity for glucose, with a K_m of 0·09 mM, and a K_m for ATP of 0·14 mM in the report of Racagni and Machado de Domenech, (1983) and a K_m for glucose of 0·04 mM in the report of Urbina and Crespo (1984). Earlier work reported a K_m for glucose in T.rhodesiense and T.gambiense of 0·28 mM (Seed, J.R. and Baquero, M.A., 1965) and in T.rhodesiense 0·4 mM (Risby and Seed, 1969). The slightly lower affinity for glucose indicated by these results may be due to the different method of hexokinase assay used and differences in experimental conditions. The glucose affinity of leishmanial hexokinase also appears to be high. In L.donovani the K_m for glucose was 0·06 mM and in L.braziliensis 0·1 mM, while for ATP

the K_m values were 0·02 and 0·04 mM respectively (Berens et al, 1980).

The variation in glucose affinity between the various trypanosomatids cannot be directly compared due to variations in experimental conditions. It is possible that in bloodstream form T.brucei, which relies totally on glycolysis for energy, that hexokinase has a higher affinity for glucose than in other trypanosomatids which are not totally reliant on the glycolytic pathway for their energy requirements. However, C.fasciculata hexokinase had a K_m for glucose almost as low as that of T.brucei, so it is more likely that a high affinity for glucose is a general characteristic of trypanosomatid hexokinases.

The hexokinases of yeasts tend to have K_m values in the region of 0·1 mM (Colowick, 1973). Of the mammalian hexokinases, isozyme III has the greatest affinity for glucose, with a K_m in the order 10^{-6} M (Colowick, 1973) to 10^{-5} M (Ureta, 1982). Hexokinases I and II have slightly lower affinities, with K_m values of the order 10^{-5} and 10^{-4} M respectively (Colowick, 1973; Ureta, 1982) Mammalian hexokinase IV has a very much higher K_m value for glucose eg. 12 mM (Colowick, 1973) and 4·5 mM (Ureta, 1982). Hexokinase III, the low K_m isozyme, is generally inhibited by its substrate, glucose, at concentrations greater than 0·1 mM in rats or 1 mM in humans (Balinsky and Fromm, 1978). The hexokinase of C.fasciculata was not inhibited by excess glucose, and the trypanosomal hexokinases generally do not appear to be inhibited by excess glucose (eg. Nwagwu and Opperdoes, 1982; Racagni and Machado de Domenech, 1983).

6.7 Conclusions

In conclusion, the characteristics of hexokinase from C.fasciculata, and those reported for other trypanosomal hexokinases, indicate that the closest similarity is with the yeast hexokinases. This is true in respect of subunit molecular mass and lack of glucose-6-phosphate regulation. However the hexameric structure of the native protein appears to be unique. The enzyme bears little resemblance to the mammalian hexokinases in respect of structure and kinetics, and may therefore be an ideal target for chemotherapeutic attack.

Chapter 7

RESULTS

THE USE OF HEXOKINASE ANTISERUM

7.1 Rocket and Crossed Immunoelectrophoresis

The determination of the specificity of the antiserum, prepared from purified hexokinase, by immunoelectrophoresis, proved to be difficult. Precipitin lines were often not visible, or stained very faintly when using either a crude cell extract obtained by sonication or the purified enzyme. Using Triton X-100 extracts of cells gave more visible but less clearly defined rockets. Testing for hexokinase activity was not successful as staining was located only in the gel surrounding the wells. Fig. 7.1 shows that the purified protein formed one well defined rocket (lane 3) and a crude antigen sample (lanes 1 & 2) also produced a rocket as well as some staining around the wells. The rockets produced by Triton extracts (lanes 4 & 5) are not so well defined and much more densely stained.

Crossed immunoelectrophoresis of both purified hexokinase and crude protein samples produced a single very faint precipitin arc (not photographically reproducible). The results of crossed immunoelectrophoresis of Triton extracts (Fig. 7.2) show a very clearly visible precipitin arc, but there are also some areas of diffuse staining.

7.2 Western Blot Analysis

Western blot analysis of crude cell extracts and partially purified preparations separated by SDS-PAGE was carried out (Figs. 7.3 & 7.4). A single major component was visible on the autoradiographs with a molecular mass of 50,000. This band corresponded to the single band visible on SDS-polyacrylamide gels of purified hexokinase preparations.

There are other faint bands visible in Fig. 7.3 as well as the major 50,000 molecular mass band. They may be artifactual since their presence and molecular mass varies depending on the nature of the sample. Alternatively, they may reflect a minor recognition of a cell component which is concentrated during the purification process and is not present in sufficient levels in crude cell extracts to be recognised.

7.3 Analysis of Leishmania Cell Extracts

Triton extracts of two strains of Leishmania donovani (L9 & L710) were used and hexokinase activity was confirmed by spectrophotometric assay. There was no recognition of any antigens using rocket immunoelectrophoresis. The extracts were subjected to SDS-polyacrylamide gel electrophoresis and then analysed by western blotting (Fig. 7.4). There was no apparent recognition of any proteins in the Leishmania extract by C.fasciculata hexokinase antiserum.

7.4 Digitonin Extraction of Hexokinase Activity

Digitonin interacts specifically with the 3-hydroxy steroids of the plasma membrane (Zuurendonk and Tager, 1974). The use of low concentrations of the detergent will selectively permeabilise the cytoplasmic membrane, releasing cytosolic enzymes such as malic enzyme (Cazzulo et al, 1980), whilst internal membranes including that of the glycosome remain intact. Higher concentrations will also permeabilise the glycosomal membrane, releasing the glycosomal enzymes. A concentration of 0·1 mg/ml digitonin solubilized 47% of malic

enzyme activity but <1% of hexokinase activity. Digitonin at 0·25mg/ml released 70% malic enzyme activity but only 40% of the hexokinase activity (Fig. 7.5)

For subsequent experiments it was found that a more efficient separation of cytosolic and particulate enzymes could be obtained by using a constant digitonin concentration (0·05mg/ml), and increasing the length of incubation at 30°C. This released a greater percentage of cytosolic enzymes and a much lower percentage of particulate enzymes (Fig. 7.6). After 3 minutes incubation 75% of the malic enzyme activity was released compared with <2% of hexokinase activity.

7.5 Radiolabelling Proteins

Labelling of proteins was carried out with [^{35}S]-methionine. Radioactivity incorporated into TCA precipitatable protein was found to increase linearly for at least 4 hours. Lack of glucose in the medium resulted in a 50% drop in the rate of incorporation (Fig. 7.7).

Radiolabelled proteins recognised by the hexokinase antiserum were identified by immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis and fluorography. In addition to the 50,000 molecular mass band presumed to represent hexokinase there were also a number of other bands in the immunoprecipitates of either Triton X-100 or digitonin extracts. Two bands always appeared to coprecipitate with hexokinase, one of 64,000 and one of 32,000.

Immunoprecipitation of Triton X-100 extracts of the cells labelled with and without glucose present, gave a slightly

different pattern of bands on SDS-PAGE fluorographs, although the 50,000 band was not affected (Fig. 7.8). Immunoprecipitates of cells radiolabelled in the absence of glucose showed a decrease in intensity of the 64,000 band and the increase in intensity of a 62,000 band.

7.6 Digitonin Extraction of Radiolabelled Proteins

Confirmation that the 50,000 molecular mass component recognised by the antiserum was found in the same fraction as the hexokinase activity after digitonin extraction was obtained by using [^{35}S]-methionine labelled cells. The proteins of interest in the digitonin extracts were identified by their reaction with the antiserum, and were separated from the radiolabelled mixture by immunoprecipitation. Analysis of immunoprecipitates by SDS-PAGE, followed by fluorography, showed that the main 50,000 band was not released by low digitonin concentrations (Fig. 7.9) and was therefore probably not a cytosolic enzyme.

7.7 The Effect of Protease Inhibitors

Protease inhibitors were used for two reasons. Firstly, they were included in the incubation medium for *in vivo* radiolabelling experiments to establish whether they had any effect on processing of hexokinase. Secondly, they were included during digitonin extraction of radiolabelled cells to determine whether proteolytic degradation of protein was occurring during the extraction procedure.

7.7.1 The Effect of TLCK

TLCK (Nα-tosyl-L-lysine chloromethylketone) is a chloromethyl ketone derivative which inhibits some serine proteases as well as many cysteine proteases (North, 1982). It has been shown to be an effective inhibitor of proteolytic activity in several trypanosomatids (Toruella et al, 1981; Cazzulo, 1984; North et al, 1983). The effect of TLCK on amino acid incorporation in C.fasciculata has been investigated and also the possibility of TLCK acting as a processing inhibitor, preventing incorporation of proteins into the particulate fraction.

The presence of 0·5mM TLCK was found to inhibit incorporation of [^{35}S]-methionine into total cell protein by at least 40%. The fluorograph of immunoprecipitated digitonin extracts shows that TLCK reduced the radiolabelled bands present in cytosolic extracts, so that virtually no bands were present (Fig. 7.10). There was no apparent reduction in the radiolabelled bands immunoprecipitated from the particulate fraction.

The percentage of the total labelled protein which immunoprecipitated from the 10 minute particulate fraction was also assessed by drying immunoprecipitates onto filters and scintillation counting. This indicated that 7·50% of the counts were immunoprecipitated from the particulate fraction of control cells, compared to 9·75% in TLCK treated cells.

7.7.2 The Effect of PMSF

PMSF (phenylmethylsulphonyl fluoride) was also found to inhibit proteolytic activity in trypanosomes (Toruella et al, 1981; Cazzulo, 1984). The inclusion of 0·2mM PMSF had no effect either on incorporation of radiolabel into TCA precipitatable protein or on the distribution of label in immunoprecipitates of digitonin extracts.

7.7.3 The Effect of a Protease Inhibitor Cocktail

A cocktail of protease inhibitors including 20 μg/ml of each of leupeptin, chymostatin, pepstatin A, aprotinin and antipain was included in the incubation with radiolabelled methionine. There was a less than 10% reduction in incorporation of label into protein when the protease inhibitors were present. However, the fluorograph of immunoprecipitated digitonin extracts suggests that there is less immunoprecipitatable protein in cytosolic extracts of treated cells, and considerably less in particulate fractions (Fig. 7.11).

7.7.4 The Effect of 1,10 Phenanthroline

A number of processing proteases have been shown to be inhibited by 1,10 phenanthroline eg. processing of the E.coli M13 coat protein and processing of synthetic substrates in dog pancreas endoplasmic reticulum (Heinrich, 1982).

When included in radiolabelling incubations this inhibitor had an instantly lethal effect at a concentration of 1mM. At 0·1mM the effect of the inhibitor was slower, taking about 30 minutes to act.

7.8 The Effect of Cerulenin

Cerulenin is an antibiotic which inhibits growth of fungi and some Gram-positive bacteria by interfering with lipid metabolism (Nomura et al, 1972; Jacques, 1983). It may also act by causing membrane perturbation thus preventing protein synthesis. The effect of cerulenin on C.fasciculata was tested to see if incorporation of protein into the glycosome could be prevented, leading to an accumulation of precursor hexokinase in the cytosol. This might occur as a result of inhibition of glycosomal membrane synthesis and/or protein processing.

At 3μg/ml cerulenin there was no noticeable effect on incorporation of radiolabel into total cell protein or on the distribution or level of immunoprecipitated protein. At 30μg/ml incorporation of [^{35}S]-methionine into total cell protein was inhibited by over 49% but there was no apparent effect on the distribution of label between cytosolic and particulate fractions (Fig. 7.12). Table 7.1 shows that the percentage of total counts in immunoprecipitates was only slightly different in cytosolic and particulate fractions of cerulenin treated cells compared to normal cells.

Digitonin extraction was also carried out using a cocktail of protease inhibitors in all the buffers used for extraction in an attempt to prevent possible proteolytic degradation of precursor proteins. There was no suggestion on the fluorograph that any additional bands of higher molecular mass appeared in the presence of protease inhibitors (Fig. 7.12).

Direct counting indicated that the percentage of total counts in immunoprecipitates of cytosolic fractions was affected

very little by the presence of protease inhibitors (Table 7.1). However the presence of protease inhibitors caused a considerable drop in the percentage of total counts detected in particulate fractions, especially in cerulenin treated cells.

7.9 mRNA Isolation and in vitro Translation

In vitro translation of poly A mRNA from C .fasciculata was carried out in a rabbit reticulocyte lysate system. After a 90 minute incubation period incorporation of [^{35}S]-methionine into TCA precipitatable protein was found to be 55,000 cpm/µl.

Immunoprecipitation of a 20µl sample of in vitro translation products with hexokinase antiserum indicated that the 50,000 molecular mass component was present and that there was no apparent additional band of higher molecular mass (Fig. 7.13).

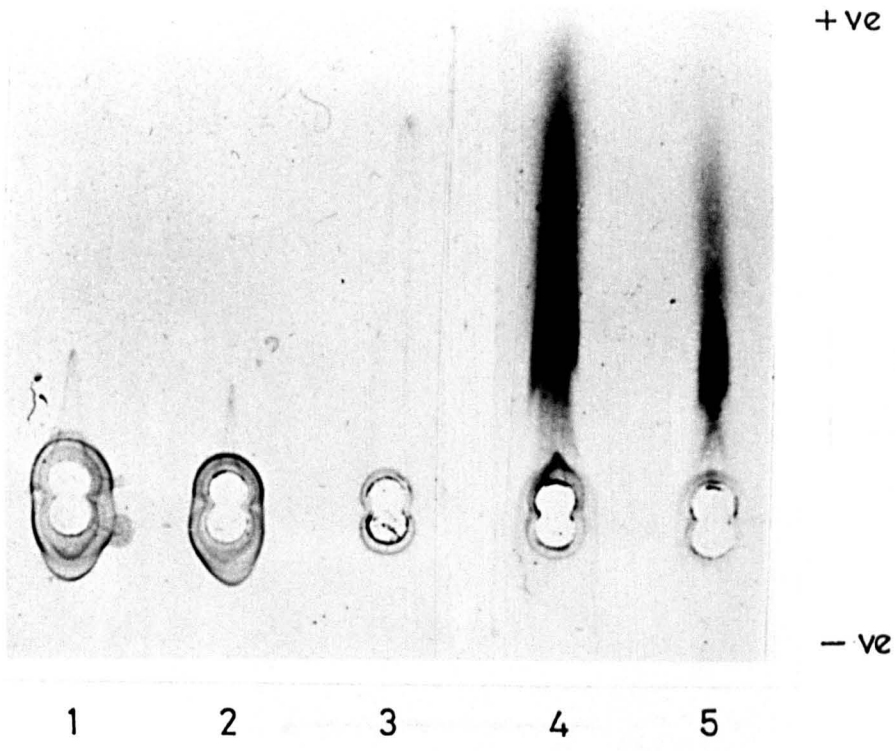

Figure 7.1 **Rocket Immunoelectrophoresis**

Rocket immunoelectrophoresis of C.fasciculata cell extracts was carried out with 200µl of hexokinase antiserum included in the gel. Well 1: 10µl crude cell-free extract; Well 2: 5µl crude cell-free extract; Well 3: 10µl purified hexokinase; Well 4: 10µl Triton X-100 extract: Well 5: 5µl Triton X-100 extract. Actual gel size = 5x5cm.

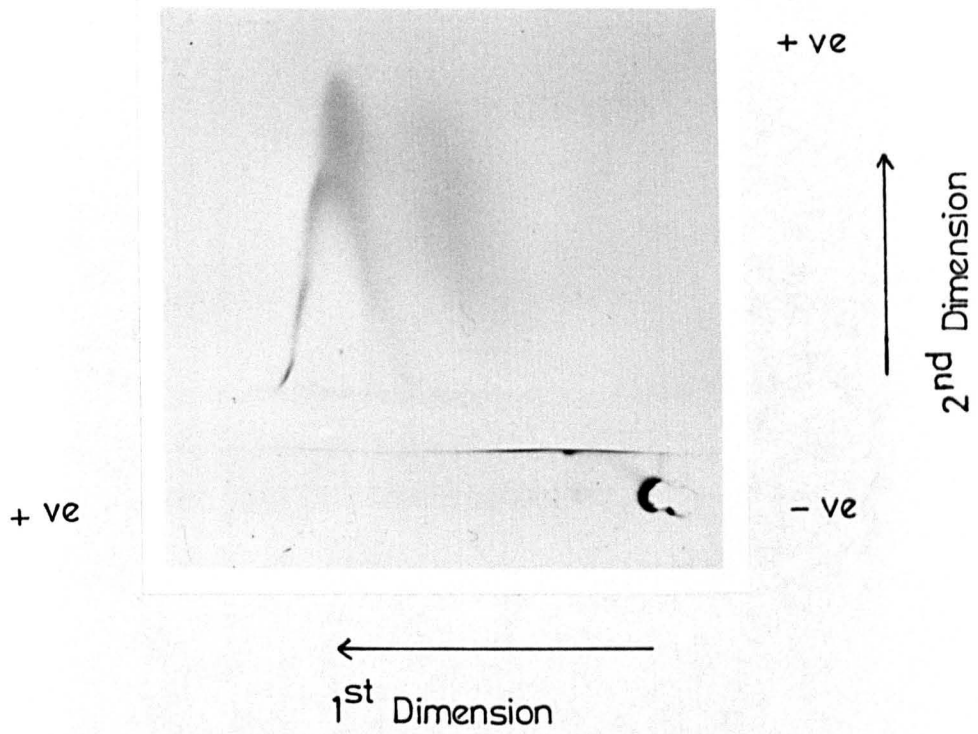

Figure 7.2 **Crossed Immunoelectrophoresis**

The first dimension electrophoresis of 10µl Triton X-100 cell extract was carried out into agarose. The second dimension immunoelectrophoresis was into agarose including 50µl hexokinase antiserum. Actual gel size = 5x5cm.

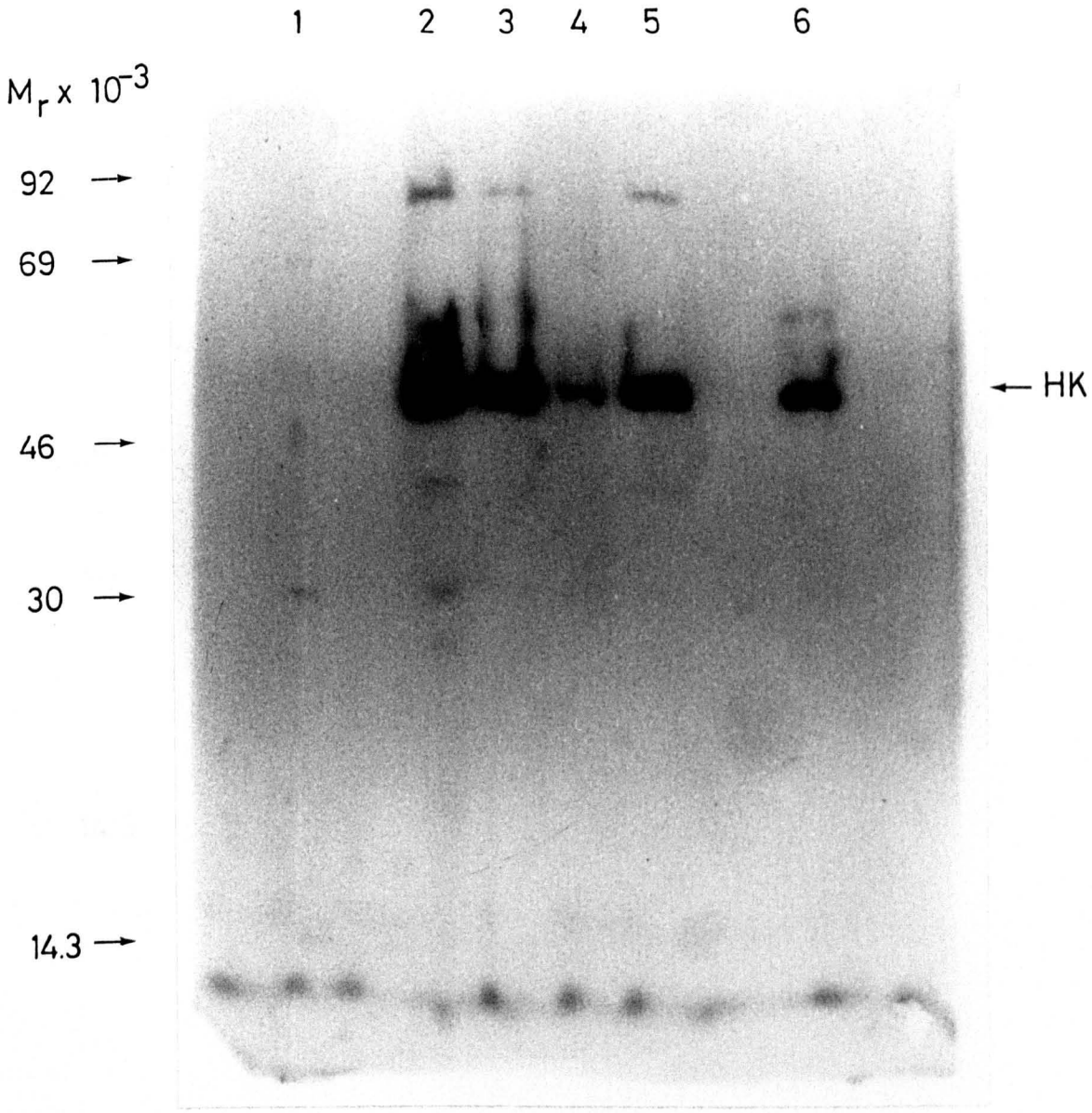

Figure 7.3 **Western Blot of C.fasciculata Extracts**

Polypeptides from crude cell-free extracts and partially purified hexokinase which were recognised by the antiserum were identified by using [^{125}I]-protein A. Track 1: Marker Proteins:- phosphorylase b, 92,500; BSA, 69,000; ovalbumin, 46,000; carbonic anhydrase, 30,000; lysozyme, 14,300. Track 2: concentrated Phenyl-Sepharose eluate (50µl). Track 3: as track 2 (30µl). Track 4: Phenyl-Sepharose eluate (trace). Track 5: Phenyl-Sepharose Eluate (50µl); Track 6: DEAE-cellulose eluate-1:10 (50µl).

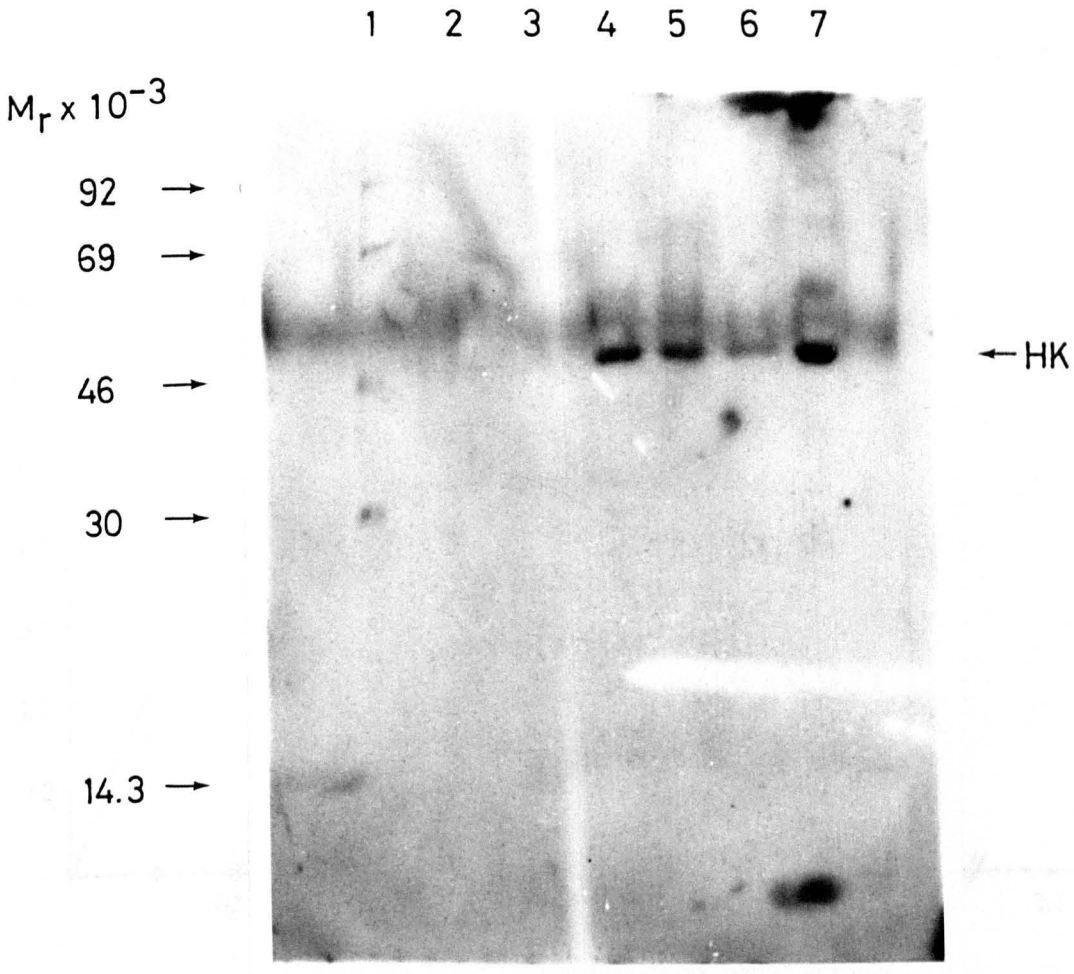

Figure 7.4 Western Blot of Leishmania and Crithidia Extracts

Crude extracts of C.fasciculata and L.donovani were analysed by western blotting with hexokinase antiserum followed by [^{125}I]-protein A. Track 1: Marker proteins as Figure 7.3. Tracks 2 and 3: Triton X-100 extracts of L.donovani strains L9 and L710 respectively (50µl). Track 4: Digitonin (2mg/ml) extract of C.fasciculata (50µl). Track 5: Triton X-100 extract of C.fasciculata. Tracks 6 and 7: Crude cell-free extract of C.fasciculata, 1:10 and 1:50 respectively (50µl).

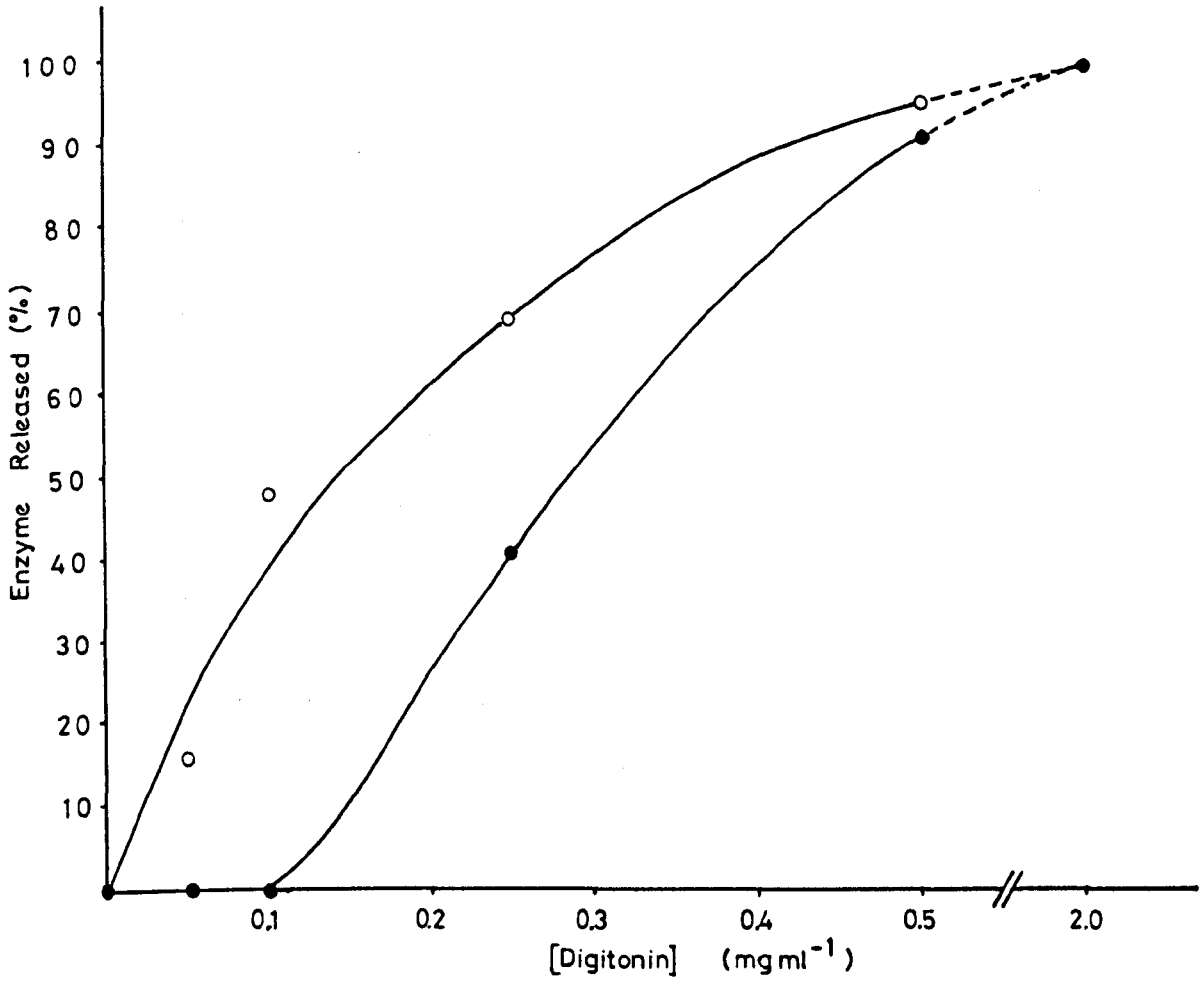

Figure 7.5 **Selective Release of Enzymes by Digitonin**

Digitonin extraction of cells was carried out using the concentrations indicated and incubating at 30°C for 1 minute. Hexokinase and malic enzyme activity were determined in each extract and results are expressed as percentage of the total activity in the cells as determined by further extraction with 2·0 mg/ml digitonin, for 2 minutes, for each sample (i.e. activity in the initial solubilized fraction was added to activity released by the second digitonin extraction to give total activity).

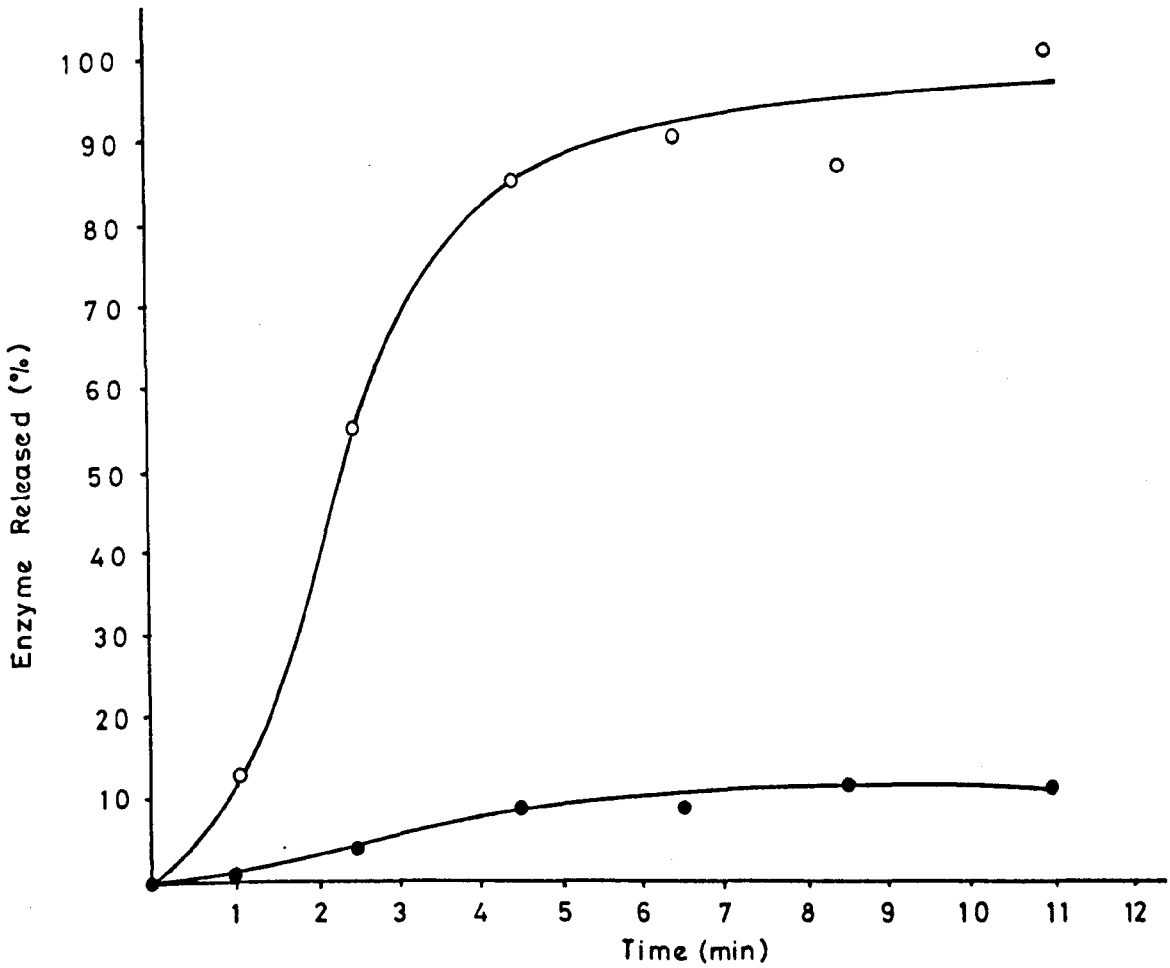

Figure 7.6 **Selective Enzyme Release by Digitonin and Time**

A concentration of 0·05 mg/ml digitonin was used, and incubations were carried out for the times indicated. Results are expressed as the percentage of total activity released. Total activity was determined by further extraction with 2·0 mg/ml digitonin, incubating for 2 minutes.

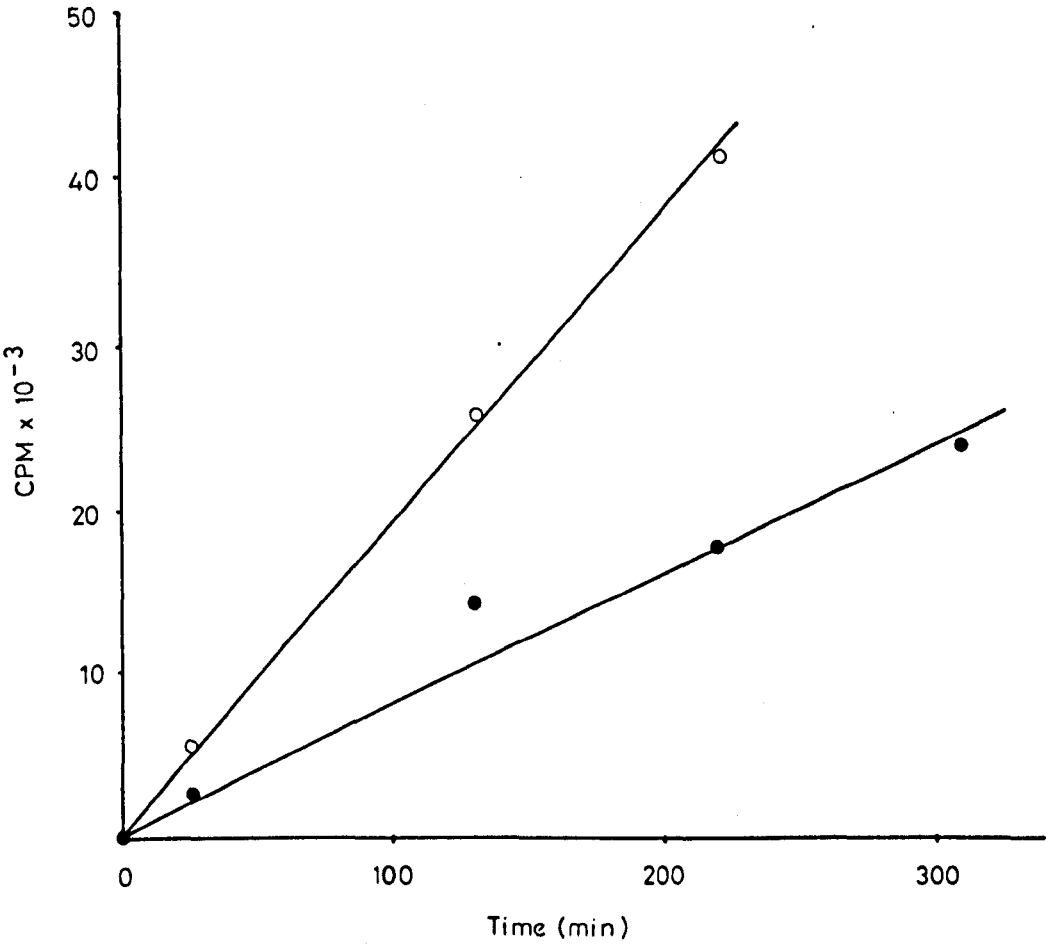

Figure 7.7 **Incorporation of Radiolabelled Methionine by C.fasciculata**

Radiolabelling of cells with [^{35}S]-methionine was carried out both in the presence and absence of glucose. A nominal 58 µCi was added to each 10 ml culture ($5x10^7$ cells/ml). Results are expressed as radiolabel (cpm) incorporated into TCA precipitatable protein (duplicate 20µl samples were precipitated).

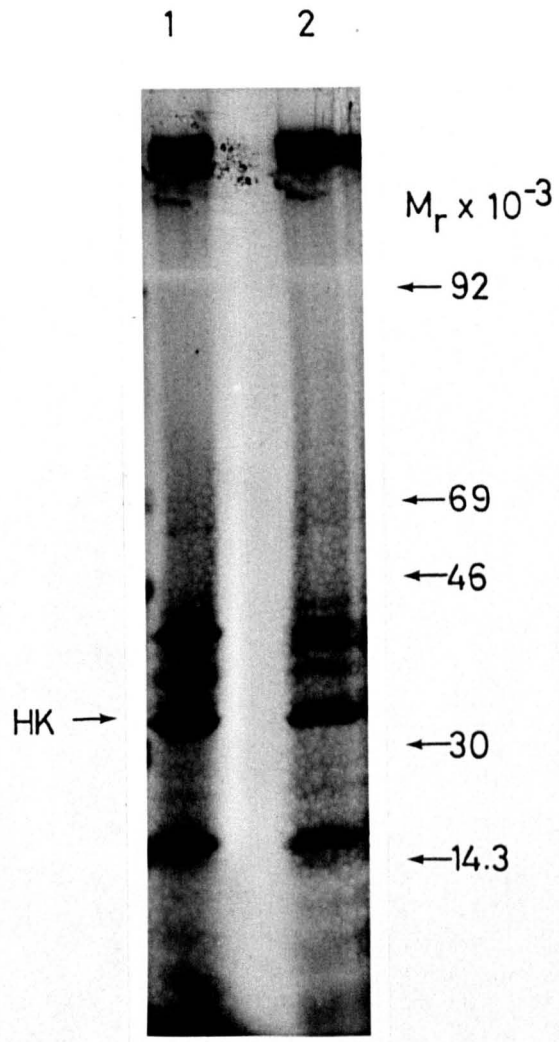

Figure 7.8 **Effect of Glucose on the Immunoprecipitation Pattern**

Immunoprecipitates were prepared from Triton X-100 extracts of *in vivo* radiolabelled *C.fasciculata* (3 hour incubation). Track 1: Immunoprecipitated polypeptides from cells labelled in the presence of glucose. Track 2: Immunoprecipitated polypeptides from cells labelled in the absence of glucose. Marker proteins as for Figure 7.3.

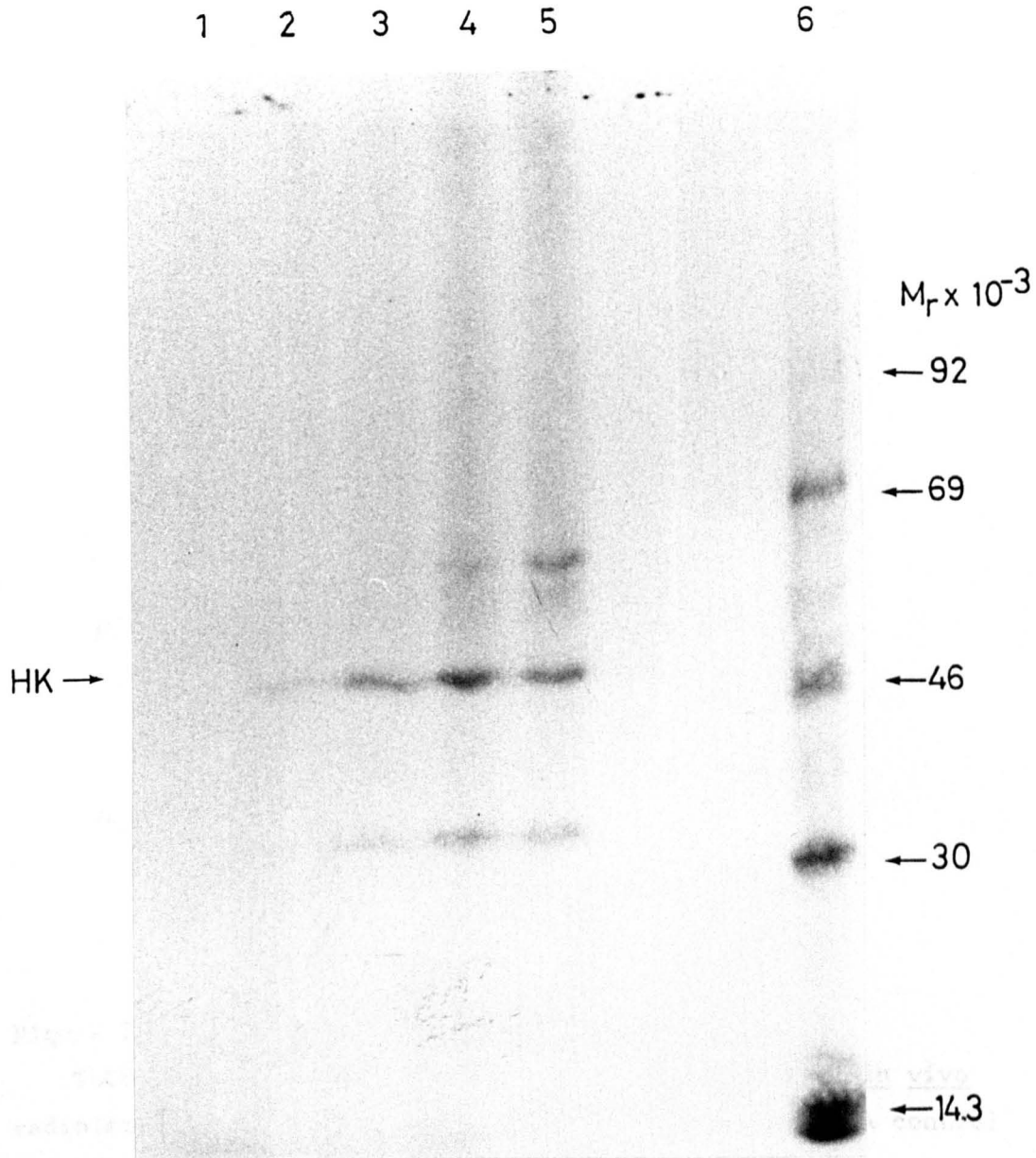

Figure 7.9 **Selective Digitonin Extraction and Immunoprecipitation of C.fasciculata Hexokinase**

Radiolabelled proteins in C.fasciculata were extracted with a range of digitonin concentrations and immunoprecipitated with hexokinase antiserum. Tracks 1-4: Protein released after 1 minutes incubation with 0·1, 0·25, 0·5 and 2·0 mg/ml respectively. Track 5: Pellet from 2·0 mg/ml extraction resuspended and extracted again with 2·0 mg/ml digitonin for 5 minutes. Track 6: Marker proteins as for Figure 7.3.

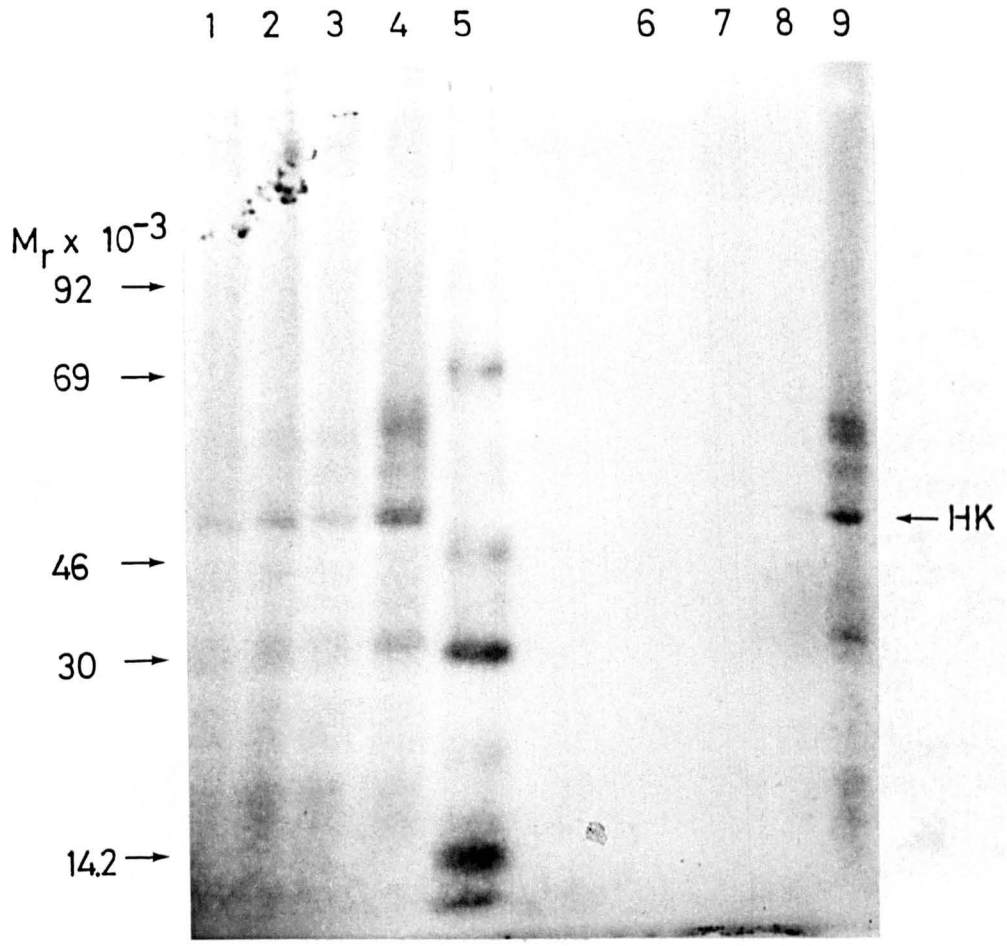

Figure 7.10 **The Effect of TLCK on Immunoprecipitates**

TLCK (0·5mM) was included in the incubation during *in vivo* radiolabelling of *C.fasciculata* with [^{35}S]-methionine. A control incubation with no added inhibitor was carried out simultaneously. Immunoprecipitation was carried out on 1ml samples taken after 2·5 hours incubation. Cytosolic fractions of cells were obtained by incubation with 0·05 mg/ml digitonin for the indicated times. Particulate fractions were obtained by resuspending pellets after initial extraction, with 0·05 mg/ml digitonin for the times indicated, and further extracting with 2·0 mg/ml digitonin for 2 minutes. Tracks 1-4: control incubation; Tracks 6-9: TLCK incubation. Tracks 1 and 6: 4 minute cytosolic fraction. Tracks 2 and 7: 6 minute cytosolic fraction. Tracks 3 and 8: 10 minute cytosolic fraction. Tracks 4 and 9: 10 minute particulate fraction. Track 5: Marker proteins as for Figure 7.3.

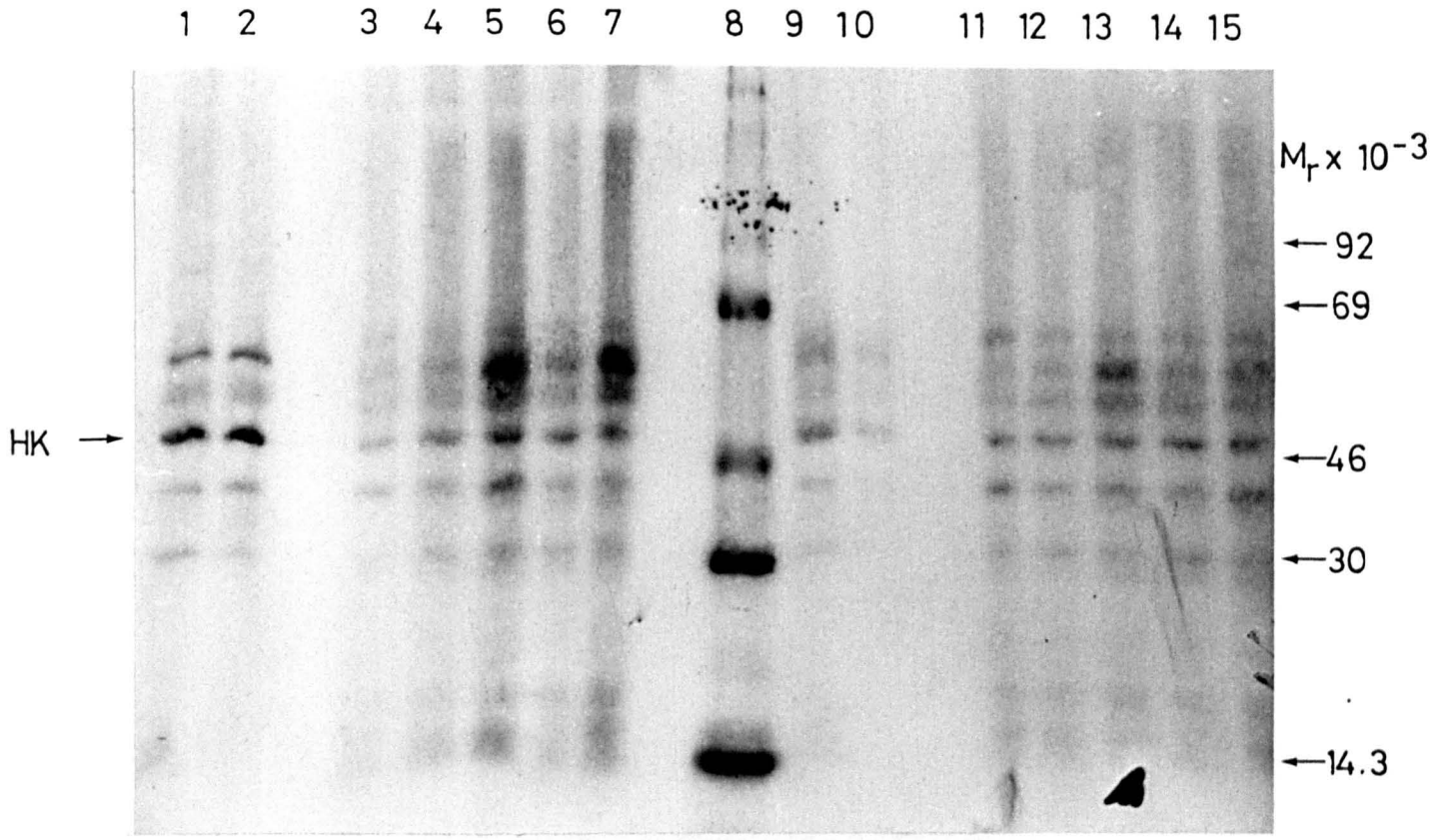

Figure 7.11 **The Effect of Protease Inhibitors on Immunoprecipitates**

A cocktail of 20 µg/ml of each of leupeptin, antipain, pepstatin A, aprotinin, and chymostatin was included when in vivo radiolabelling cells. A control incubation with no inhibitors present was carried out simultaneously. Immunoprecipitation was carried out on 1 ml samples taken after 2·5 hours incubation. Cytosolic fractions of cells were obtained by incubation with 0·05 mg/ml digitonin for the indicated times. Particulate fractions were obtained by resuspending pellets after initial extraction with 0·05 mg/ml digitonin for the times indicated, and further extracting with 2·0 mg/ml digitonin for 2 minutes. Tracks 1-7: Control incubation. Tracks 9-15: Protease inhibitor incubation. Tracks 1 and 9: 2 minute particulate fraction. Tracks 2 and 10: 10 minute particulate fraction. Tracks 3-7: 2,3,4,5 and 10 minute cytosolic fractions respectively. Tracks 11-15: as tracks 3-7. Track 8: Marker proteins as Figure 7.3.

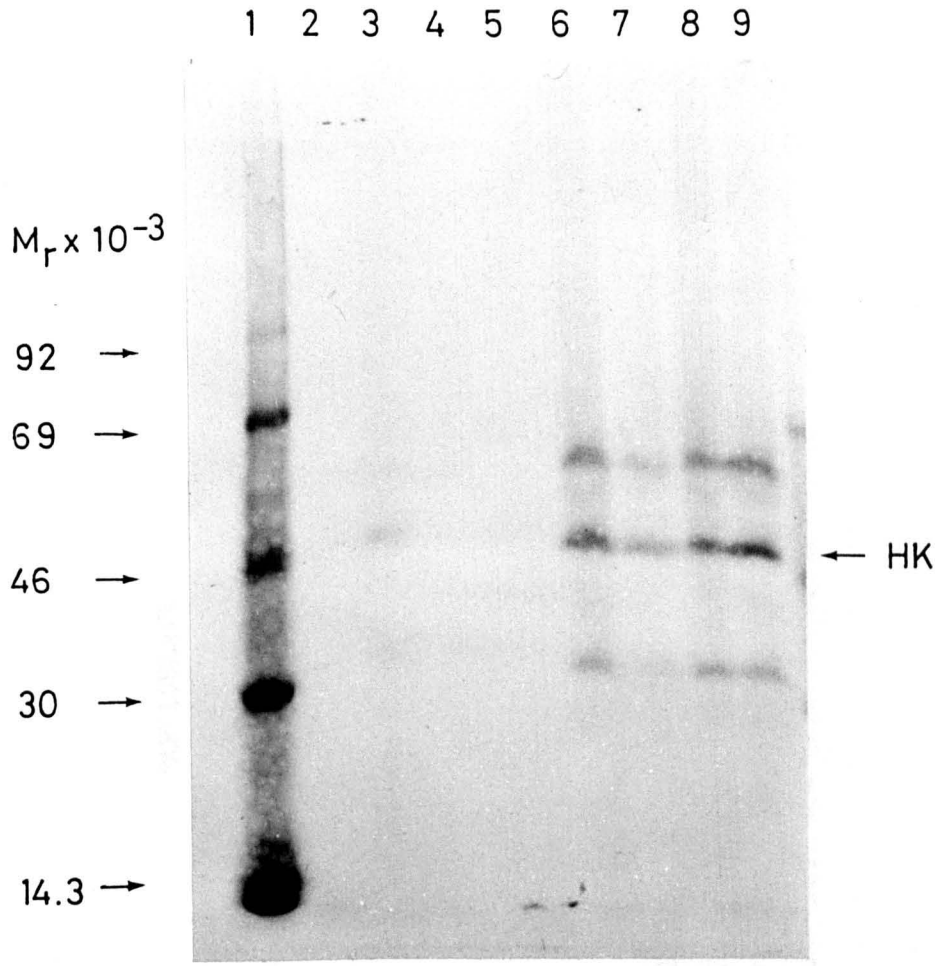

Figure 7.12 **The Effect of Cerulenin on Immunoprecipitates**

Cells were exposed to 30μg/ml cerulenin during in vivo radiolabelling with [^{35}S]-methionine for 2·5 hours. Digitonin extraction was carried out in standard buffers and also in buffer including a cocktail of protease inhibitors. Cytosolic fractions were obtained by 2 minutes incubation with 0·05 mg/ml digitonin. Particulate fractions were obtained by 10 minutes incubation with 0·05 mg/ml digitonin to remove cytosolic proteins, followed by 2 minutes incubation of the resuspended pellet with 2·0 mg/ml digitonin to extract particulate proteins. Track 1: Marker proteins as for Figure 5.3. Track 2: Cytosolic fraction, control incubation. Track 3: Cytosolic fraction, control incubation, extraction in protease cocktail buffer. Track 4: Cytosolic fraction, cerulenin treated cells. Track 5: Cytosolic fraction, cerulenin treated cells, extraction in protease cocktail buffer. Tracks 6-9: Particulate fractions, otherwise as tracks 2-5.

	CYTOSOLIC (% total labelled protein)		PARTICULATE (% total labelled protein)	
	control	+cerulenin	control	+cerulenin
normal extraction buffer	0·9	0·4	6·2	5·7
extraction buffer + protease inhibitors	1·2	0·4	4·5	2·8

Table 7.1 **The Effect of Cerulenin and Protease Inhibitors on Immunoprecipitates**

Particulate and cytosolic digitonin fractions of *in vivo* radiolabelled cells were obtained as detailed in figure 7.12. Duplicate immunoprecipitates to those used in figure 7.12 were dried onto filters and subjected to scintillation counting. The percentage of total radiolabelled, TCA precipitatable, protein which immunoprecipitated was determined for each fraction.

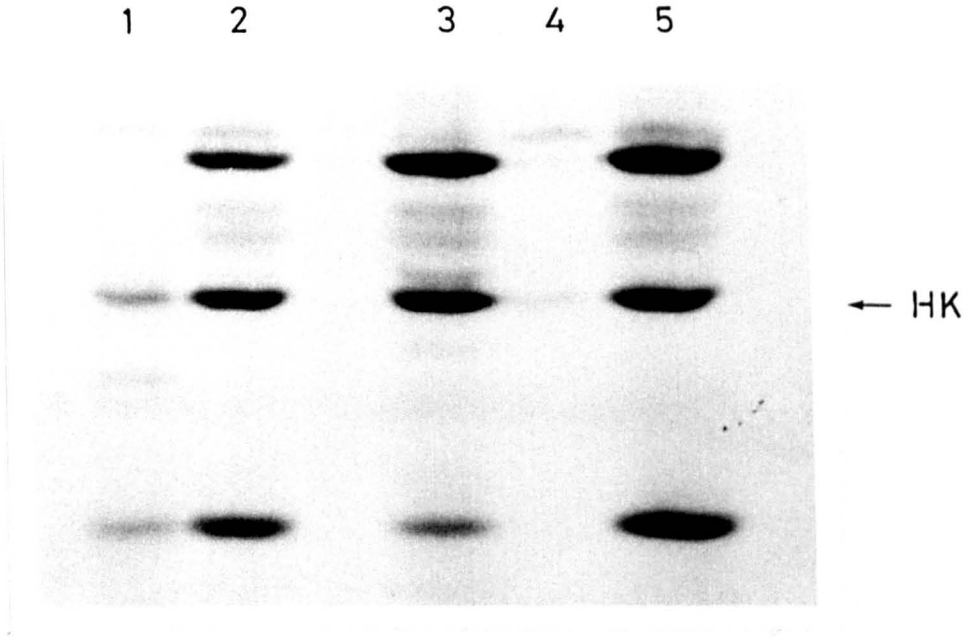

Figure 7.13 **Immunoprecipitation of *in vitro* Translation Products**

After *in vitro* translation, immunoprecipitation was carried out on a 20µl sample of the *in vitro* translation products resuspended to give a volume of 1ml. Immunoprecipitation was also carried out on extracts of *in vivo* radiolabelled *C.fasciculata*. Immunoprecipitates were separated by SDS-PAGE and identified by fluorography. Track 1: Immunoprecipitate of *in vitro* translation products. Track 2: Triton X-100 extract. Tracks 3-5: Digitonin extracts. Track 3: 10 minute particulate fraction. Track 4: 2 minute cytosolic fraction. Track 5: 2 minute particulate fraction. Cytosolic fractions were obtained by incubating cells with 0·05 mg/ml digitonin for the times indicated. Particulate fractions were obtained by resuspending pellets after extraction with 0·05 mg/ml digitonin for times indicated, to remove cytosolic proteins, and further extracting with 2·0 mg/ml digitonin for 2 minutes.

Chapter 8

DISCUSSION

HEXOKINASE ANTISERUM AND ITS USES

8.1 Homogeneity of the Enzyme Preparation

Antiserum against hexokinase from C.fasciculata was raised using an apparently homogeneous enzyme preparation as judged by SDS-polyacrylamide gel electrophoresis. Immunoelectrophoresis using this antiserum gave inconclusive results, particularly when using Triton X-100 extracts of cells. There may have been some precipitation of one or more other proteins by the antiserum, although the effects seen in the gels may have been due to protein aggregation and a non-specific reaction in the gel. Other proteins may have been associated with the hexokinase as complexes in the Triton X-100 extracts and not totally resolved during electrophoresis. Identification of hexokinase in precipitin complexes by the colorimetric enzyme assay was not successful, suggesting either that the enzyme was rendered catalytically inactive when immunoprecipitated or that hexokinase was not migrating into the gel. Risby and Seed (1969) also found that hexokinase activity in African trypanosomes was inhibited by its antiserum. A further possibility is that hexokinase has a high isoelectric point which prevented the enzyme from migrating into the antibody/agarose gel at the pH of the electrophoresis buffer (pH 8·6). Other trypanosomal hexokinases are known to have high isoelectric points (Misset et al, 1986; Ebert, 1982).

The Western blotting procedure was used to determine more clearly the specificity of the antiserum. One major band of molecular mass 50,000 was indicated, further confirming the subunit mass of the hexokinase. In a some cases a minor band of higher molecular mass was visible. However, the actual molecular

mass was variable depending on the sample, this may have been an artifact or reflected a minor recognition of another cell component.

8.2 Cross Reaction with Other Trypanosomal Hexokinases

As the enclosure of the glycolytic enzymes in glycosomes has proved to be a universal feature of the trypanosomatids, it seems likely that the enzymes themselves may exhibit interspecies similarities, and the properties of the enzymes, particularly from closely related species are likely to be the same. If this were the case, antiserum raised against a protein in one species might recognise the similar protein in another species. The antiserum raised against hexokinase in C.fasciculata has been used in an attempt to recognise a similar enzyme in a Leishmania species. There was no apparent cross-reaction between the antiserum to C.fasciculata hexokinase and Leishmania cell extracts. Mottram and Coombs (1985) found that hexokinase from Leishmania mexicana mexicana had a molecular mass of 83,000 after gel filtration chromatography. If this is the case for all Leishmania hexokinases then there must be a substantial difference between them and the trypanosomal hexokinases. This would be unexpected in view of the apparent similarity in regulatory and kinetic properties of the hexokinases from several trypanosomatids, and the similarity in structural properties of T.brucei and C.fasciculata hexokinases. A more comprehensive investigation of the properties of hexokinase from Leishmania is necessary before a satisfactory comparison can be made.

The lack of cross-reaction with the Crithidia antiserum may simply have been due to the low levels of hexokinase in the Leishmania extracts. It is also possible that only very minor changes in the amino acid composition of the enzyme in Leishmania would destroy the antigenic determinants recognised by the Crithidia antiserum. In the case of the mammalian hexokinases antisera against them are isozyme specific and antisera against the same isozyme in different vertebrates do not always cross-react. Hexokinase IV is highly preserved, but hexokinase I has antigenic determinants which vary from species to species (Ureta, 1982).

Cross-reactions have been observed previously with antisera to hexokinases from T.brucei, T.rhodesiense, T.gambiense and T.equiperdum, although these enzymes could be clearly distinguished from yeast on the basis of immunological specificity (Seed and Baquero, 1965; Risby and Seed, 1969). It has yet to be established whether or not there is a cross-reaction between Crithidia and Trypanosoma hexokinase antisera. It is possible that even though the hexokinases are structurally very similar, minor changes in the antigenic determinants would prevent a recognition.

8.3 Subcellular Location of Hexokinase

Digitonin disrupts membranes by interacting specifically with 3-hydroxy steroids. Zuurendonk and Tager (1974) developed a procedure for rapidly separating cytosolic from particulate fractions of rat liver cells using digitonin. Digitonin has been used previously in trypanosomatids: Visser and Opperdoes (1980)

used it to demonstrate enzyme latency in bloodstream trypomastigotes of T.brucei, and Kiaira and Njogu (1983) studied glycolysis in digitonin-permeabilised T.brucei. Cazzulo et al (1980) demonstrated the intracellular distribution of carbon dioxide fixing enzymes of C.fasciculata and T.cruzi by using digitonin to selectively disrupt membranes. Using this method they were able to demonstrate that malic enzyme is predominantly in the cytosolic fraction of the cell, whilst phosphoenolpyruvate carboxykinase is particulate.

In the present study, digitonin was used to separate C.fasciculata into particulate and cytosolic fractions using malic enzyme as a marker of cytosolic enzymes. Hexokinase activity was located predominantly in a particulate fraction of the cell, previously shown to be the glycosome (Taylor et al, 1980).

8.4 Immunoprecipitation

Confirmation that the hexokinase activity was located in the same subcellular fraction as the 50,000 polypeptide recognised by the antiserum was obtained by immunoprecipitation of in vivo radiolabelled cell extracts. Immunoprecipitation was not a very good indicator of the homogeneity of the antiserum towards hexokinase. There were a number of other polypeptide bands which separated out when immunoprecipitates were run on SDS-polyacrylamide gels. These bands were not previously identified by western blot analysis. Therefore it is likely that their presence in the immunoprecipitates is an artifact, or only indirectly linked to the recognition of hexokinase by the

antiserum. A certain amount of non-specific binding would be expected, although attempts were made to reduce this by preclearing the labelled cell extract with protein A, and the immunoprecipitate was washed extensively with both ionic and non-ionic detergents. The additional components in immunoprecipitates may result from coprecipitation of hexokinase and other associated glycolytic enzymes, especially since the buffers used were of low ionic strength (Oduro et al, 1980b; Aman et al, 1985; Misset et al, 1986). This would suggest that the affinity for hexokinase is strong enough to prevent the removal of these other proteins during the washing step.

The molecular masses of the two additional bands which always coprecipitated in immunoprecipitates were 32,000 and 64,000. A comparison with the molecular masses known for the glycolytic enzymes of T.brucei (Misset et al, 1986) indicates that the only enzyme reported to have a similar molecular mass to these coprecipitating polypeptides was one of 62,400 for glucose phosphate isomerase. It is possible that the molecular mass of this enzyme in C.fasciculata is similar, that is the 64,000 polypeptide is glucose phosphate isomerase. If this enzyme is associated with hexokinase in the glycosome it might give rise to the coprecipitation effect seen. The glucose phosphate isomerase and hexokinase of T.brucei have been shown, by cross-linking experiments, to be associated with each other (Aman and Wang, 1983). The molecular mass of the other coprecipitating polypeptide (32,000) does not appear to correspond closely to any of the T.brucei glycolytic enzymes. The enzymes closest to this value in T.brucei are triose

phosphate isomerase, glyceraldehyde phosphate dehydrogenase and glycerol-3-phosphate dehydrogenase. However the C.fasciculata polypeptide could be another glycosomal enzyme.

The slight alteration in the pattern of coprecipitating polypeptides when cells were radiolabelled in the absence of glucose may reflect an alteration in the expression of enzymes associated with glycolysis. This may have been induced by starvation conditions. The rate of incorporation of [^{35}S]-methionine into protein was reduced considerably in the absence of glucose which suggests that in the semi-defined medium provided the normal growth of cells was affected, a condition which may have caused the change in enzyme levels.

8.5 Protein Topogenesis

Various experiments were carried out to determine whether processing was involved in uptake of hexokinase by the glycosome. Immunoprecipitation was used to indicate whether or not there was any change in subcellular location or molecular mass of the enzyme. Any means of preventing hexokinase synthesis or uptake by the glycosome would be deleterious to the cell given its dependence on glycolysis. Hexokinase is essential as the first enzyme in the catabolism of glucose. Presumably a build-up of hexokinase in the cytosol would also seriously deplete the levels of cytosolic ATP.

The process of 'addressing' or 'sorting' a cell's proteins into their appropriate subcellular locations has been termed 'intracellular protein topogenesis' (Blobel, 1980). There are about 50,000 ribosomally synthesised proteins in a eukaryotic

cell some of which are destined for organelles such as the mitochondria or lysosomes, some for secretion and some for membranes such as the plasma membrane (Heinrich, 1982). Transport processes have to cope with translocation across single membranes, double membranes and integration into a membrane. Most of the work on translocation of proteins has been carried out on protein secretion and this work has given rise to two models in particular. The first of these is the signal hypothesis (Blobel and Dobberstein, 1975). The information for protein translocation is contained within a N-terminal signal sequence which binds to an integral membrane receptor which then forms a pore around the sequence and polypeptide chain elongation follows. The signal sequence is cleaved by the action of a protease. The second model is the membrane trigger hypothesis (Wickner, 1979). In this model certain proteins when encountering an amphipathic surface assemble into the membrane without the need for a pore. The protein is actually translocated through the bilayer rather than through a pore. A number of variants of these basic hypotheses exist, (Wickner and Lodish, 1985), with signal sequences playing an important part in protein topogenesis. The signal sequence is generally located at the N-terminus, especially in proteins targeted to the endoplasmic reticulum, mitochondria and chloroplasts (Schekman, 1985). Occasionally such proteins may also be dependent on additional sequence elements for complete transfer.

The study of microbody biogenesis has lagged behind studies of other cellular organelles, such as the mitochondria, because they are often poorly represented in cells. However, studies of

microbody biosynthesis have recently increased as technical difficulties have been overcome. Inducible biosynthesis of microbodies has provided greater scope for work eg. induction of microbody biosynthesis in mammalian liver (Lazarow and De Duve, 1976; Osumi and Hashimoto, 1984).

8.6 Microbody Biogenesis

Microbodies constitute a class of organelles, including peroxisomes and glyoxysomes, which is widespread amongst eukaryotic cells. They have characteristic morphological features, viz. they are bounded by a single membrane, they have no internal machinery for protein synthesis and often contain dense crystalline bodies (Trelease, 1984). In addition they have a high equilibrium density in sucrose of about $1{\cdot}23 g/cm^3$ and they carry out diverse, often inducible, metabolic functions (Borst, 1986). Considering the similarities in chemical, physical and ultrastructural properties, it is likely that the mode of biogenesis is similar for all of these organelles (Trelease, 1984; Kindl, 1982).

For a long time the main theory for the formation of microbodies involved the endoplasmic reticulum (ER) and was termed the ER-vesiculation model. This model proposed that microbodies arise as nascent organelles, budding off directly from the endoplasmic reticulum. Proteins were believed to be synthesised on the rough endoplasmic reticulum and either integrated into the ER membrane or into the lumen of the ER in the case of matrix proteins (Trelease, 1984). The proposed mechanisms of protein secretion are similar to the signal

hypothesis for secretory proteins (Blobel and Dobberstein, 1975; Sabatini et al, 1982) with a protease-sensitive N-terminal signal peptide leading to cotranslational translocation of proteins. Evidence at one time was strongly in favour of this theory, both from biochemical and morphological data (Lord, 1980; Beevers, 1982). However, evidence conflicting with this view has led to a consensus in favour of an alternative model (Lazarow, 1980). In this model proteins are synthesised on free polysomes and incorporated post-translationally into preexisting peroxisomes, or microbodies, from the cytosol (eg. Robbi and Lazarow, 1978). The endoplasmic reticulum plays no detectable role and new microbodies are thought to form by fission of preexisting ones (Lazarow et al, 1982). In addition most proteins are made in their mature forms and are not proteolytically processed during uptake (eg. Goldman and Blobel, 1978; Lazarow et al, 1982).There are a minor number of proteins that are synthesised as larger precursors and then proteolytically processed. Liver peroxisomal 3-ketoacyl-CoA thiolase is synthesised in vitro as a precursor with a molecular mass 5,000-8,000 larger than the in vivo labelled protein or the purified protein (Walk and Hock, 1978; Hock and Gietl, 1982; Riezman et al, 1980).

The information for directing peroxisomal proteins into peroxisomes lies within the amino acid sequence since no evidence has been found for glycosylation in any of the major proteins (Volki and Lazarow, 1982). Of the proteins found to have larger precursors there is no obvious common feature which might act as a microbody address label. There is no evidence

that the information in the extra piece is required for topogenesis. In those cases investigated processing of the precursor is not coupled to import into the microbody. The extra sequence could be involved in more efficient uptake or in regulating activity i.e. it could have a non-topogenic role (Borst, 1986).

8.7 The Glycosome as a Microbody

There are conflicting points of view over whether or not the glycosome should be classed as a microbody. De Duve (1982) does not consider the glycosome to belong to the same class as peroxisomes and glyoxysomes since it is the only organelle to include glycolytic enzymes. Lazarow and Fujiki (1985) consider microbodies to be peroxisomes and consider the glycosome to be an exception to this rule, since these organelles in some species of trypanosome do not contain catalase or peroxidases. Borst (1986) argues in favour of a relationship however, citing the accepted diversity in enzyme systems found in the microbodies of different genera. There are also strong similarities in the ultrastructural, physical and chemical properties of the organelles.

It was of interest to determine whether there are any points of similarity between the biogenesis of glycosomes and the known features of peroxisome biogenesis. Hexokinase is a glycolytic enzyme known to be segregated into the glycosome. The way in which glycosomal proteins are incorporated is not known. Information about this process would not only provide a comparison for peroxisome biogenesis, but also may provide the

basis of a valuable means of chemotherapeutic attack against the pathogenic trypanosomes.

8.8 Cerulenin

Cerulenin (2,3-epoxy-4-oxo-6,10-dodecadienoylamide) is an anti-fungal antibiotic from Cephalosporium caerulens. It inhibits growth of filamentous fungi and to a lesser extent gram positive and gram negative bacteria (Nomura et al, 1972). Cerulenin affects lipid metabolism, especially biosynthesis of sterols or fatty acids, probably by inhibiting the initial steps leading to fatty acid chain elongation from acetyl CoA (Nomura et al, 1972). Inhibition of secretion of protein by cerulenin has been shown in several bacteria. However, the mode of action of cerulenin in suppressing secretion may not be due to a direct effect on lipid synthesis. It has been suggested that, as an amphiphilic molecule, cerulenin may penetrate and interact with the lipid bilayer, inhibiting synthesis and secretion of proteins as a result of its physiochemical interaction with the membrane (Petit-Glatron and Chambert, 1981). Synthesis and secretion of glucosyl transferase in Streptococcus salivarius was prevented by cerulenin (Jacques, 1983). The role of cerulenin as an inhibitor of secretion in S.salivarius appeared to be due to its ability to perturb membrane structure rather than inhibit lipid synthesis directly. Perturbation of the membrane might inhibit secretion by disturbing the interactions between polysomes and membranes or the mechanism of translocation across the membrane (Jacques, 1983). Cerulenin was also found to inhibit secretion of staphylococcal α-toxin,

probably by altering membranes and thereby affecting the docking proteins that bind the signal peptide (Saleh and Freer, 1984). Similarly cerulenin inhibited protein secretion in B.subtilis (Mäntsälä, 1982).

The effect of cerulenin on protein synthesis and incorporation of hexokinase into the glycosome was investigated in C.fasciculata. Cerulenin could act by directly blocking lipid synthesis, thus inhibiting glycosomal membrane synthesis, or by causing membrane perturbation and inhibiting protein processing. At low concentrations cerulenin had no apparent effect on protein synthesis or on levels and subcellular location of hexokinase in immunoprecipitates. At high concentrations there was still no apparent effect on the level of immunoprecipitatable hexokinase in the particulate fraction of the cells, as seen on the fluorographs, although protein synthesis was inhibited by approximately 50%. Thus, if cerulenin was causing any perturbation of the glycosomal membrane it was not affecting translocation of hexokinase. One explanation for this could be that translocation of hexokinase into the glycosome does not require any membrane interaction for synthesis or processing, in contrast to the situation for bacterial secretory proteins. However, cerulenin may simply be ineffective in causing perturbation of the glycosomal membrane.

It did appear from direct counting of radiolabel in immunoprecipitates that incubation with cerulenin caused a small reduction in the level of immunoprecipitatable protein.The most obvious change was when protease inhibitors were included in the extraction buffers. This appeared to cause a considerable

reduction in the level of immunoprecipitatable protein in particulate fractions particularly from cells incubated with cerulenin.It is possible that the change in conditions, to include cerulenin and then protease inhibitors, affected the digitonin extraction process and was therefore responsible for the effect seen. The results obtained in this way did not appear to be mirrored by the intensity of the bands on the fluorograph. The accuracy of this method of determination is not known but there was probably no significant effect on protein topogenesis or processing.

Inhibition of lipid synthesis by cerulenin could prevent formation of glycosomal membranes, and could be particularly effective if the organelles were formed de novo rather than from fission of preexisting organelles. This might have some effect on the subcellular location of newly synthesised hexokinase. However, the results obtained gave no indication that this was the case. If glycosomes follow the same pattern of biosynthesis as other microbodies it is unlikely that they are formed de novo. Rather, newly synthesised enzymes probably enter preexisting organelles. New organelles are probably created by fission of glycosomes already present.

Whilst hexokinase synthesis did not appear to be affected by cerulenin, inhibition of general protein synthesis did occur. Nomura et al (1972) considered cerulenin to exert no direct influence on protein or nucleic acid biosynthesis in yeast. In bacterial cells inhibition of protein synthesis was also considered to be indirect, occurring as a result of disrupted binding of signal peptides to membrane receptors, thereby

preventing completion of protein synthesis (Saleh and Freer, 1984). From the results obtained for C.fasciculata it is not possible to rule out either a direct or indirect effect on protein synthesis.

8.9 The Effect of Protease Inhibitors

It was hoped that the inclusion of protease inhibitors would prevent proteolytic degradation of either newly synthesised protein or mature glycosomal protein. This would indicate whether or not hexokinase was synthesised as a larger precursor protein. When rat liver catalase was synthesised in in vitro translation systems the enzyme appeared to have a higher molecular mass than the purified form, suggesting that it was synthesised as a higher molecular mass precursor (Robbi and Lazarow, 1978; Goldman and Blobel, 1978). However, it was discovered that this difference was as a result of proteolytic degradation occurring when catalase was chemically purified. The in vivo radiolabelled and immunoprecipitated protein was indistinguishable from the in vitro translation product. The inclusion of a cocktail of protease inhibitors when digitonin extracting and cerulenin treating C.fasciculata cells did not give any indication that proteolytic degradation of hexokinase occurred at any stage.

Proteolytic enzymes play a role in the cotranslational transport of secretory proteins and the post-translational transport of polypeptides into organelles (North, 1982). Proteases have also been shown to be involved in processing of larger precursors (North, 1982). Post-translational processing

in Tetrahymena of pellicular proteins could be inhibited by TPCK leading to an increase in precursor protein (Collins and Wilhelm, 1978). Several protease activities have been identified in chloroplasts and mitochondria which cleave, posttranslationally, the cytoplasmically made polypeptide precursors to their mature sizes (Heinrich, 1982). EDTA was found to inhibit a mitochondrial processing protease (Mori et al, 1980) and iodoacetamide or N-ethyl-maleimide inhibited a chloroplast protease (Dobberstein et al, 1977). In these examples the processing proteases were found in the matrix of the mitochondria or the stroma of the chloroplasts. An enzyme of water melon glyoxysomes, g-malate dehydrogenase, is protease sensitive and processed upon entry into the organelle (Gietl and Hock, 1984). In vitro experiments showed that uptake by isolated glyoxysomes was better in the presence of various proteases. The results obtained led the authors to suggest that processing was occurring on the outside of the glyoxysome membrane, immediately prior to transfer into the organelle.

The use of various protease inhibitors known to inhibit protease activity in trypanosomes (Toruella et al, 1981; North et al, 1983; Cazzulo, 1984) in in vivo radiolabelling of C.fasciculata did not lead to any obvious build up of immunoprecipitatable radiolabelled polypeptides in cytosolic extracts. This could suggest that no proteolytic processing of hexokinase is necessary for uptake by the glycosome. However, in these experiments protease inhibitors were added externally to cells and access to the relevant protease may have been limited, particularly if processing was occurring inside the glycosomal

membrane rather than on the cytosolic side. Even if hexokinase was synthesised as a precursor requiring processing, it is not certain that processing would be necessary for glycosomal uptake. Of the few known examples of microbody proteins which are synthesised as larger precursors, the position of the extra piece is not known and it may be internal rather than a cleavable end sequence. There is no evidence that processing is coupled to import (Borst, 1986) in contrast to the import of mitochondrial proteins which can be blocked by inhibiting the processing protease (Schleyer and Neupert, 1985).

The significance of the effects seen on fluorographs, of reduced intensity of bands from immunoprecipitates of cells incubated with a protease cocktail, is unknown. The reduction was particularly noticeable in particulate fractions. It may simply be that the efficiency of digitonin extraction and immunoprecipitation were altered by incubating the cells with the inhibitors. The alternative is that the presence of the inhibitors was inhibiting synthesis of hexokinase and the other coprecipitating proteins although overall protein synthesis only appeared to be reduced by about 10%.

When the cells were incubated with TLCK a slightly different effect was seen after fluorography of immunoprecipitates, with reduced intensity of bands from cytosolic extracts and a possible increase in intensity of bands from the particulate fraction. The presence of TLCK appears to have pushed the enzymes into the glycosome rapidly, without allowing any enzyme to remain in the cytosolic pool. However, the effect could

equally be explained by the change in conditions affecting the efficiency of digitonin extraction.

8.10 In vitro Translation of mRNA

The development of cell-free systems that effectively translate mRNA was of great importance in the discovery of postulated pre-proteins (Heinrich, 1982). The *in vitro* translation of whole cell mRNA from *C.fasciculata* gave no indication that hexokinase was synthesised as a precursor significantly larger than the *in vivo* radiolabelled enzyme when they were compared in immunoprecipitates. This seems to confirm that hexokinase is synthesised as a mature polypeptide, and does not require proteolytic processing for glycosomal uptake. This conclusion rests on undetectable mobility differences between the putative precursor and the mature protein on SDS-polyacrylamide gels. It is possible that minor changes could occur which are undetectable on SDS gels.

8.11 Glycosome Protein Topogenesis

The results obtained, whilst giving no positive evidence of the exact process involved in hexokinase topogenesis, are consistent with the idea that topogenesis of glycosomal proteins is analogous to the processes occurring in other microbodies. Hexokinase appears to be synthesised as a mature polypeptide and transferred posttranslationally into the glycosome.

The experiments carried out so far give no information with regard to the fully assembled hexameric enzyme. Whilst hexokinase is synthesised as a mature subunit, the assembly of

these subunit polypeptides could occur in the cytosol or in the glycosome. If assembly occurs in the glycosome this could be the mechanism by which the topogenesis of hexokinase across the membrane is made irreversible, and it could also prevent wasteful consumption of ATP in the cytosol which would presumably occur if hexokinase was present in an active form prior to its translocation into the glycosome. Lazarow and De Duve (1971, 1973) proposed that catalase from rat liver peroxisomes is synthesised in the cytosol as a haem-less monomer and then enters the peroxisome where it aggregates with haem to an active tetrameric enzyme.

The alternative, that assembly of the mature hexokinase enzyme occurs in the cytosol, would require detrimental activity to be minimised by some other means, or for translocation to rapidly follow translation so that the cytoplasmic pool of hexokinase is minimal.Hexokinase assays of digitonin extracted cells indicated that in cytosolic extracts there was no activity detectable within the limits of the assay. Immunoprecipitation of radiolabelled polypeptides in cytosolic extracts indicated that there could be trace amounts of antigen present. This was probably contaminating protein leaking from the particulate fraction of the cell as the digitonin concentration increased. However, it could indicate a small pool of enzymatically inactive or undetectable hexokinase, which is nevertheless recognised by the antiserum.

The only other research carried out to date on topogenesis of glycosomal proteins has been on T.brucei enzymes using cDNA clones isolated using homologous yeast or mammalian probes. Four

enzymes have been isolated in this manner and sequenced, but it has not been possible to isolate the hexokinase gene in the same way. Comparisons of cytosolic and glycosomal isozymes of the same enzyme, when they exist, have also been carried out to determine the changes needed for redirection of proteins from the cytosol into the glycosome (Misset and Opperdoes, 1987; Misset *et al*, 1987). Phosphoglycerate kinase, which has both a cytosolic and a glycosomal isozyme in *T.brucei*, has been sequenced and 95% homology found between the two (Osinga *et al*, 1985) indicating that little alteration is required to redirect the enzyme into the glycosome. The glycosomal phosphoglycerate kinase has a C-terminal extension of 20 amino acids, it is moderately hydrophobic and amino acid additions and substitutions give it a net gain of 12 positive charges over its cytosolic counterpart. Of the other enzymes sequenced; fructose bisphosphate aldolase (Clayton, 1985), glyceraldehyde phosphate dehydrogenase (Michels *et al*, 1986) and triose phosphate isomerase (Swinkels *et al*, 1986), none have a significant C-terminal extension and only aldolase has an N-terminal extension (of ten amino acids).

There are no analogous internal sequences where the *T.brucei* enzymes differ from their counterparts in other eukaryotes. The only common feature found so far is a high ratio of basic over acidic amino acids and a high net positive charge. The high positive charge of glycosomal enzymes may be for delivery into the organelles or for function inside. *In vitro* translation of three enzymes from *T.brucei*; aldolase, glycerol-3-phosphate dehydrogenase and glyceraldehyde phosphate dehydrogenase,

indicated that their subunit masses were identical to the native enzymes (Misset et al, 1986). This suggests that in these cases at least there is no processing upon entry into the glycosome.

The physical and sequence data now available for the T.brucei glycosomal enzymes strongly suggest that they have no cleavable signal sequence and are not subject to processing. Rather they are likely to have internal signals which direct protein topogenesis (Misset et al, 1986). The internal signals may be formed by clustering of positive charges as a result of secondary and tertiary folding of the polypeptide chain (Misset et al, 1986). Analysis of the 3-dimensional structure of glycosomal phosphoglycerate kinase, triose phosphate isomerase and glyceraldehyde phosphate dehydrogenase by Wierenga and Hol has revealed two positively charged areas 40 Å apart which may have a role in glycosomal binding (Wierenga et al, 1984; Wierenga et al, 1987). It is not clear whether other microbody enzymes have similar high positive charges which feature in protein topogenesis (Osinga et al, 1985; Borst, 1986). It has been suggested that there may be a link between these positively charged 'hotspots' and the anti-trypanosomal activity of the drug suramin which has two negatively charged areas about 40 Å apart (Misset et al, 1987; Wierenga et al, 1987).

8.12 Conclusions

In conclusion hexokinase from C.fasciculata, in common with some of the glycosomal enzymes from T.brucei, appears to be synthesised in its mature form, entering the glycosome posttranslationally with no apparent processing. The mRNAs

coding for several of the T.brucei enzymes are translated on free cytosolic ribosomes (Misset et al, 1986; Hart et al, 1987). These facts suggest that similarities exist between glycosomal and peroxisomal topogenesis. The signals involved are uncertain, but in glycosomes may be linked to the unusually high positive charges of the enzymes.

BIBLIOGRAPHY

Aman, R. A. and Wang, C. C. (1983). Cross-Linking of the Glycolytic Enzymes in Trypanosoma brucei Glycosomes. Fed. Proc. **42**, 1979.

Aman, R. A., Kenyon, G. L. and Wang, C. C. (1985). Cross-Linking of the Glycosomes of Trypanosoma Brucei. J. Biol. Chem. **260**, 6966-6973.

Anderson, B. L., Tarpley, H. L. and Regen, D. M. (1978). Characterization of β-Hydroxybutyrate Transport in Rat Erythrocytes and Thymocytes. Biochim. Biophys. Acta **508**, 525-538.

Anderson, J. W., Herman, R. H., Tyrell, J. B. and Cohn, R. M. (1971). Hexokinase : A Compartmented Enzyme. Am. J. Clin. Nut. **24**, 642-650.

Bacchi, C. J., Ciaccio, E. I., Kaback, D. B. and Hutner, S. H. (1970). Oxaloacetate Production Via Carboxylations in Crithidia fasciculata Preparations. J. Protozool. **17**, 305-311.

Bacchi, C. J., Hutner, S. H., Ciaccio, E. I. and Marcus, S. M. (1968). O_2-Polarographic Studies on Soluble and Mitochondrial Enzymes of Crithidia fasciculata; Glycerophosphate Enzymes. J. Protozool. **15**, 576-584.

Baernstein, H. D. (1953a). Malic Dehydrogenase and Related Enzymes in the Culture Form of Trypanosoma cruzi. Exp. Parasitol. **2**, 380-396.

Baernstein, H. D. (1953b). The Enzyme Systems of the Culture Form of Trypanosoma cruzi. Ann. N.Y. Acad. Sci. **56**, 982-994.

Balinsky, D. and Fromm, H. J. (1978). Hexokinase III from Rana catesbeliana. Comp. Biochem. Physiol. **60B**, 71-75.

Beevers, H. (1982). Glyoxysomes in Higher Plants. In "Peroxisomes and Glyoxysomes. Ann N.Y. Acad. Sci." (Kindl, H. and Lazarow, P. B. eds.) Vol. 386, 243-253.

Bensadoun, A. and Weinstein, D. (1976). Assay of Proteins in the Presence of Interfering Materials. Analyt. Biochem. **70**, 241-250.

Berens, R. L., Deutsch-King, L. L. and Marr, J. J. (1980). Leishmania donovani and Leishmania braziliensis : Hexokinase, Glucose-6-Phosphate Dehydrogenase and Pentose Phosphate Shunt Activity. Exp. Parasitol. **49**, 1-8.

Bergmeyer, H. U. (ed.) (1974). "Methods in Enzymatic Analysis" 2edn.. Verlag Chemie, Weinheim, Academic Press. N.Y./London.

Blobel, G. (1980). Intracellular Protein Topogenesis. Proc. Natl. Acad. Sci. USA. **77**, 1496-1500.

Blobel, G. and Dobberstein, B. (1975). Transfer of Proteins Across Membranes. 1. Presence of Proteolytically Processed and Unprocessed Nascent Immunoglobulin Light Chains on Membrane-Bound Ribosomes of Murine Myeloma. J. Cell Biol. **67**, 835-851.

Blobel, G. and Dobberstein, B. (1975). Transfer of Proteins Across Membranes. 2. Reconstitution of Functional Rough Microsomes from Heterologous Components. J. Cell Biol. **67**, 852-862.

Borst, P. (1986). How Proteins Get Into Microbodies (Peroxisomes, Glyoxysomes, Glycosomes). Biochim. Biophys. Acta **866**, 179-203.

Borst, P. and Hoeijmakers, J. H. J. (1979). Kinetoplast DNA. Plasmid **2**, 20-40.

Boveris, A., Hertig, C. M. and Turrens, J. F. (1986). Fumarate Reductase and other Mitochondrial Activities in Trypanosoma cruzi. Mol. Biochem. Physiol. **19**, 163-169.

Bowman, I. B. R. (1974). Intermediary Metabolism of Pathogenic Flagellates. In "Trypanosomiasis and Leishmaniasis with Special Reference to Chagas' Disease". Ciba Foundation Symposium No. 20 (New Series) 225-284. Elsevier, Amsterdam.

Bowman, I. B. R. and Flynn, I. W. (1976). Oxidative Metabolism of Trypanosomes. In "Biology of the Kinetoplastida" (Lumsden, W. H. R. & Evans, D. A. eds.) Vol.1, pp 435-476. Academic Press, London.

Bowman, I. B. R., Tobie, E. J. and von Brand, Th. (1963). CO_2 Fixation Studies with the Culture Form of Trypanosoma cruzi. Comp. Biochem. Physiol. **9**, 105-114.

Brand, Von T. (1951). Metabolism of Trypanosomidae and Bodonidae in "Biochemistry and Physiology of Protozoa" (Lwoff, A., ed.) Vol.1, 177-234. Academic Press, N.Y..

Brohn, F. H. and Clarkson, A. B. Jr. (1978). Quantitative Effects of Salicylhydroxamic Acid and Glycerol on Trypanosoma brucei Glycolysis in vitro and in vivo. Acta Tropica **35**, 23-33.

Brohn, F. H. and Clarkson, A. B. Jr. (1980). Trypanosoma brucei brucei : Patterns of Glycolysis at 37°C in vitro. Mol. Biochem. Parasitol. **1**, 291-305.

Broman, K., Knupfer, A. L., Ropars, M. and Deshusses, J. (1983). Occurrence and Role of Phosphoenolpyruvate Carboxykinase in Procyclic Trypanosoma brucei brucei glycosomes. Mol. Biochem. Parasitol. **8**, 79-87.

Broman, K., Ropars, M. and Deshusses, J. (1982). Subcellular Location of Glycolytic Enzymes in Trypanosoma brucei Culture Form. Experientia **38**, 533-534.

Brown, R. C., Evans, D. A. and Vickerman, K. (1973). Changes in Oxidative Metabolism and Ultrastructure Accompanying Differentiation of the Mitochondrion in Trypanosoma brucei. Int J. Parasit. **3**, 691-704.

Cannata, J. J. B. and Cazzulo, J. J. (1984a). The Aerobic Fermentation of Glucose by Trypanosoma cruzi. Comp. Biochem. Physiol. **79B**, 297-308.

Cannata, J. J. B. and Cazzulo, J. J. (1984b). Glycosomal and Mitochondrial Malate Dehydrogenases in Epimastigotes of Trypanosoma cruzi. Mol. Biochem. Parasitol. **11**, 37-49.

Cannata, J. J. B., Valle, E., Docampo, R. and Cazzulo, J. J. (1982).Subcellular Localization of Phosphoenolpyruvate Carboxykinase in the Trypanosomatids Trypanosoma cruzi and Crithidia fasciculata. Mol. Biochem. Parasitol. **6**, 151-160.

Cataldi de Flombaum, M. A., Cannata, J. J. B., Cazzulo, J. J.and Segura, E. L. (1977). CO_2-Fixing Enzymes in Trypanosoma cruzi. Comp. Biochem. Physiol. **58B**, 67-69.

Cazzulo, J. J. (1984). Protein and Amino Acid Catabolism in Trypanosoma cruzi. Comp. Biochem. Physiol. **79B**, 309-320.

Cazzulo, J. J., Franke de Cazzulo, B. M., Engel, J. C. and Cannata, J. J. B. (1985). End Products and Enzyme levels of Aerobic Glucose Fermentation in Trypanosomatids. Mol. Biochem. Parasitol. **16**, 329-343.

Cazzulo, J. J., Juan, S. M. and Segura, E. L. (1977). The Malic Enzyme from Trypanosoma cruzi. J. Gen. Microbiol. **99**, 237-241.

Cazzulo, J. J., Valle, E., Docampo, R. and Cannata, J. J. B. (1980). Intracellular Distribution of Carbon Dioxide-Fixing Enzymes in Trypanosoma cruzi and Crithidia fasciculata. J. Gen. Micro. **117**, 271-274.

Chamberlain, J. P. (1979). Fluorographic Detection of Radioactivity in Polyacrylamide Gels with the Water-soluble Fluor, Sodium Salicylate. Analyt. Biochem. **98**, 132-135.

Chou, A. C. and Wilson, J. E. (1972). Purification and Properties of Rat Brain Hexokinase. Arch. Biochem. Physiol. **151**, 48-55.

Clarkson, A. B. Jr. and Brohn, F. H. (1976). Trypanosomiasis: An Approach to Chemotherapy by the Inhibition of Carbohydrate Metabolism. Science **194**, 204-206.

Clayton, C. E. (1985). Structure and Regulated Expression of Genes Encoding Fructose Bisphosphate Aldolase in Trypanosoma brucei. EMBO J. **4**, 2997-3003.

Collins, T. and Wilhelm, J. (1978). Post-Translational Proteolytic Cleavage of Pellicular Proteins in Tetrahymena. J. Cell Biol. **79**, 383a.

Colowick, S. P. (1973). The Hexokinases. In "The Enzymes" (Boyer, P. D. ed.) 3rd edn., Vol. IX. Academic press, N.Y./London.

Coombs, G. H., Craft, J. A. and Hart, D. T. (1982). A Comparative Study of Leishmania mexicana Amastigotes and Promastigotes. Enzyme Activities and Subcellular Locations. Mol. Biochem. Parasitol. **5**, 199-211.

Cosgrove, W. B. (1959). Utilization of Carbohydrates by the Mosquito Flagellate, Crithidia fasciculata. Can. J. Microbiol. **5**, 573-578.

Crane, R. K. and Sols, A. (1953). The Association of Hexokinase with Particulate Fractions of Brain and Other Tissue Homogenates. J. Biol. Chem. **203**, 273-292.

Crane, R. K. and Sols, A. (1954). The Non-Competitive Inhibition of Brain Hexokinase by Glucose-6-Phosphate and Related Compounds. J. Biol. Chem. **210**, 597-606.

Cronin, C. N. and Tipton, K. F. (1985). Purification and Regulatory Properties of Phosphofructokinase from Trypanosoma (Trypanozoon) brucei brucei. Biochem. J. **227**, 113-124.

Czok, R. and Lamprecht, W. (1974). Pyruvate, Phosphoenolpyruvate and D-Glycerate-2-Phosphate. In "Methods in Enzymatic Analysis". (Bergmeyer, H. U. ed.) Vol. 3, 1464-1468.

De Duve, C. (1982). Peroxisomes and Related Particles in Historical Perspective. In "Peroxisomes and Glyoxysomes. Ann. N.Y. Acad. Sci." (Kindl, H. and Lazarow, P. B. eds.) Vol. 386, 1-4.

Deuticke, B. (1977). Properties and Structural Basis of Simple Diffusion Pathways in the Erythrocyte Membrane. Rev. Physiol. Biochem. Pharmacol. **78**, 1-97.

Deuticke, B. (1982). Monocarboxylate Transport in Erythrocytes. J. Membrane Biol. **70**, 89-103.

Deuticke, B., Beyer,E. and Forst, B. (1982). Discrimination of Three Parallel Pathways of Lactate Transport in the Human Erythrocyte Membrane by Inhibitors and Kinetic Properties. Biochim. Biophys. Acta **684**, 96-110.

Deuticke, B., Rickert, I. and Beyer, E. (1978). Stereo Selective, SH-Dependent Transfer of Lactate in Mammalian Erythrocytes. Biochim. Biophys. Acta **507**, 137-155.

Dixon, H. (1966). Blood Platelets as a Source of Enzyme Activity in Washed Trypanosome Suspensions. Nature, London **210**, 428.

Dobberstein, B., Blobel, G. and Chua, N. H. (1977). In vitro Synthesis and Processing of a Putative Precursor for the Small Subunit of Ribulose-1,5-Bisphosphate Carboxylase of Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. USA. **74**, 1082-1085.

Dobell, C. C. (1932). "Anthony van Leeuwenhoek and his little Animals". 435pp. Harcourt Brace, N.Y..

Docampo, R., De Boiso, J. F., Boveris, A. and Stoppani, A. O. M. (1976). Localization of Peroxidase Activity in Trypanosoma cruzi microbodies. Experientia **32**, 972-975.

Docampo, R., De Boiso, J. F. and Stoppani, A. O. M. (1978). Tricarboxylic Acid Cycle operation at the Kinetoplast-Mitochondrion Complex of Trypanosoma cruzi. Biochim. Biophys. Acta **502**, 466-478.

Doflein, F. (1901). "Die Protozoen als Parasiten und Krankheit serreger, nach biologischen Gesichtspunkten dargestellt". 274pp. Fischer Jena.

Dubinsky, W. P. and Racker, E. (1978). The Mechanism of Lactate Transport in Human Erythrocytes. J. Membrane Biol. **44**, 25-36.

Easterby, J. S. and Quadri, S. S. (1982). Hexokinase Type II from Rat Skeletal Muscle. In "Methods in Enzymology" (Wood, W. A. ed.) Vol. 90, 11-15.

Ebert. F. (1982). The Identification of Two Main-Groups of Trypanosoma cruzi Stocks from Brazil by Their Isoenzyme Patterns of Isoelectrofocusing. Tropenmed. Parasitol. **33**, 140-146.

Ellenbogen, B. B. and Hutner, S. H. (1967). Cheap Partly Defined Media for Mass-Culturing Crithidia fasciculata. J. Protozool. **S14**, 12.

Ellis, C. J. (1985). The Trypanosomiases. Br. Med. J. **291**, 1524.

Englund, P. T. (1981). Kinetoplast DNA. In "Biochemistry and Physiology of Protozoa". (Levandowsky, M. and Hutner, S.H. eds.) 2nd ed. Vol. 4. Academic Press, N.Y., 333-384.

Fairlamb, A. (1982). Biochemistry of Trypanosomiasis and Rational Approaches to Chemotherapy. Trends Biochem. Sci. **7**, 249-253.

Fairlamb, A. H. and Bowman, I. B. R. (1977). The Isolation and Characterisation of Particulate sn-Glycerol-3-Phosphate Oxidase from Trypanosoma brucei. Int. J. Biochem. **8**, 659-668.

Fairlamb, A. H. and Bowman, I. B. R. (1980). Uptake of the Trypanocidal Drug Suramin by Bloodstream Forms of Trypanosoma brucei and Its Effect on Respiration and Growth Rate in vivo. Mol. Biochem. Parasitol. **1**, 315-333.

Fairlamb, A. H., Opperdoes, F. R. and Borst, P. (1977). New Approach to Screening Drugs for Activity Against African Trypanosomes. Nature **265**, 270-271.

Fairlamb, A. H., Weislogel, P. O., Hoeijmakers, J. H. J. and Borst, P. (1978). Isolation and Characterization of Kinetoplast DNA from Bloodstream Forms of Trypanosoma brucei. J. Cell Biol. **76**, 293-309.

Flynn, I. W. and Bowman, I. B. R. (1973). The Metabolism of Carbohydrate by Pleomorphic African Trypanosomes. Comp. Biochem. Physiol. **45B** 24-42.

Flynn, I. W. and Bowman, I. B. R. (1980). Purification and Characterisation of Pyruvate Kinase from Trypanosoma brucei. Arch. Biochem. Biophys. **200**, 401-409.

Fornaini, G., Dachà, M., Magnani, M. and Stocchi, V. (1982). Hexokinase from Rabbit Red Blood Cells. In "Methods in Enzymology" (Wood, W. A. ed.) Vol. 90, 1-10.

Foulkes, E. C. and Paine, C. M. (1961). The Uptake of Monocarboxylic Acids by Rat Diaphragm. J. Biol. Chem. **236**, 1019-1022.

Fulton, J. D. and Spooner, D. F. (1959). Terminal Respiration in Certain Mammalian Trypanosomes. Exp. Parasitol. **8**, 137-162.

Gietl, C. and Hock, B. (1984). Import of in vitro Synthesized Glyoxysomal Malate Dehydrogenase into Isolated Watermelon Glyoxysomes. Planta **162**, 261-267.

Glassberg, J., Miyazaki, L. and Rifkin, M. R. (1985). Isolation and Partial Characterization of Mutants of the Trypanosomatid Crithidia fasciculata and Their Use in Detecting Genetic Recombination. J. Protozool. **32**, 118-125.

Goldberg, B., Lambros, C., Bacchi, C. J. and Hutner, S. H. (1974). Inhibition by Several Standard Antiprotozoal Drugs of Growth and O_2 Uptake of Cells and Particulate Preparations of a Leptomonas. J. Protozool. **21**, 322-326.

Goldman, B. M. and Blobel, G. (1978). Biogenesis of Peroxisomes: Intracellular Site of Synthesis of Catalase and Uricase. Proc. Natl. Acad. Sci. USA. **75**, 5066-5070.

Goodwin, L. G. (1985). Trypanosomiasis: Introduction. Br. Med. Bull. 41, 103-104.

Gottlieb, M., Zahalsky, M. and Zahalsky, A. C. (1972). Crithidia as a Model Organism ? J. Parasitol. **58**, 1008-1009.

Grant, P. T. and Fulton, J. D. (1957). The Catabolism of Glucose by Strains of Trypanosoma rhodesiense. Biochem. J. **66**, 242-250.

Grant, P. T. and Sargent, J. R. (1960). Properties of L-α-Glycerophosphate Oxidase and its Role in the Respiration of Trypanosoma rhodesiense. Biochem. J. **76**, 229-237.

Grant, P. T. and Sargent, J. R. (1961). L-α-Glycerophosphate Dehydrogenase, a Component of an Oxidase System in Trypanosoma rhodesiense. Biochem. J. **81**, 206-214.

Gruenberg, J., Sharma, P. R. and Deshusses, J. (1978). D-Glucose Transport in Trypanosoma brucei : D-Glucose Transport is the Rate-Limiting Step of Its Metabolism. Eur. J. Biochem. **89**, 461-469.

Gutteridge, W. E. (1969). Some Effects of Pentamidine Di-isethionate on Crithidia fasciculata. J. Protozool. **16**, 306-311.

Gutteridge, W. E. (1985). Existing Chemotherapy and Its Limitations. Br. Med. Bull. **41**, 162-168.

Gutteridge, W. E. and Coombs, G. H. (1977). "Biochemistry of Parasitic Protozoa". Macmillan Press, London & Basingstoke.

Gutteridge, W. E. and Davies, M. J. (1982). Properties of the Purine Phosphoribosyltransferases of Trypanosoma cruzi. FEMS Lett. **13**, 207-212.

Hale, R. D. and Miller, P. G. G. (1983). Glycolysis in the Protozoan Crithidia fasciculata. Parasitol. **87**, xxv.

Halestrap, A. P. (1975). *The Mitochondrial Pyruvate Carrier :* Kinetics and Specificity for Substrates and Inhibitors. Biochem. J. **148**, 85-96.

Halestrap, A. P. (1976a). The Mechanism of the Inhibition of the Mitochondrial Pyruvate Transporter by α-Cyanocinnamate Derivatives. Biochem. J. **156**, 181-183.

Halestrap, A. P. (1976b). Transport of Pyruvate and Lactate into Human Erythrocytes. (Evidence for the Involvement of the Chloride Carrier and a Chloride Independent Carrier). Biochem. J. **156**, 193-207.

Halestrap, A. P. (1978). Pyruvate and Ketone-Body Transport Across the Mitochondrial Membrane : Exchange Properties, pH Dependence and Mechanism of the Carrier. Biochem. J. **172**, 377-387.

Halestrap, A. P. and Denton, R. M. (1974). Specific Inhibition of Pyruvate Transport in Rat Liver Mitochondria and Human Erythrocytes by α-Cyano-4-Hydroxycinnamate. Biochem. J. **138**, 313-316.

Halestrap, A. P. and Denton, R. M. (1975). The Specificity and Metabolic Implications of the Inhibition of Pyruvate Transport in Isolated Mitochondria and Intact Tissue Preparations by α-Cyano-4-hydroxycinnamate and Related Compounds. Biochem. J. **148**, 97-106.

Hammond, D. J. and Gutteridge, W. E. (1980). Enzymes of Pyrimidine Biosynthesis in Trypanosoma cruzi. FEBS Lett. **118**, 259-262.

Hammond, D. J., Gutteridge, W. E. and Opperdoes, F. R. (1981). A Novel Location for Two Enzymes of de novo Pyrimidine Biosynthesis in Trypanosomes and Leishmania. FEBS Lett. **128**, 27-29.

Hart, D. T., Baudhuin, P., Opperdoes, F. R. and De Duve, C. (1987). Biogenesis of the Glycosome in Trypanosoma brucei: the Synthesis, Translocation and Turnover of Glycosomal Polypeptides. EMBO J. **6**, 1403-1411.

Hart, D. T., Misset, O., Edwards, S. W. and Opperdoes, F. R. (1984). A Comparison of the Glycosomes (Microbodies) Isolated from Trypanosoma brucei Bloodstream Form and Cultured Procyclic Trypomastigotes. Mol. Biochem. Parasitol. **12**, 25-35.

Heinrich, P. C. (1982). Proteolytic Processing of Polypeptides During the Biosynthesis of Subcellular Structures. Rev. Physiol. Biochem. Pharmacol. **93**, 115-187.

Hill, G. C. and Hutner, S. H. (1968). Effect of Trypanocidal Drugs on Terminal Respiration of Crithidia fasciculata. Exp. Parasitol. **22**, 207-212.

Hoare, C. A. (1972). "The Trypanosomes of Mammals. A Zoological Monograph". Blackwell Scientific, Oxford. 749pp..

Hock, B. and Gietl, C. (1982). Cell-Free Synthesis of Watermelon Glyoxysomal Malate Dehydrogenase: A Comparison with the Mitochondrial Isoenzyme. In "Peroxisomes and Glyoxysomes. Ann. N.Y. Acad. Sci." Vol. 386, 350-361.

Honigberg, B. M. (1963). A Contribution to Systematics of the Non-Pigmented Flagellates. In "Progress in Protozoology. Proceedings of the First International Conference on Protozoology", Prague, 68-69.

Hunter, F. R. (1960). Aerobic Metabolism of Crithidia fasciculata. Exp. Parasitol. **9**, 271-280.

Jacques, N. A. (1983). Membrane Perturbation by Cerulenin Modulates Glucosyltransferase Secretion and Acetate Uptake by Streptococcus Salivarius. J. Gen. Microbiol. **129**, 3293-3302.

Johnson, J. H., Belt, J. A., Dubinsky, W. P., Zimniak, A. and Racker, E. (1980). Inhibition of Lactate Transport in Ehrlich Ascites Tumour Cells and Human Erythrocytes by a Synthetic Anhydride of Lactic Acid. Biochemistry **19**, 3836-3840.

Johnstone, A. and Thorpe, R. (1982). Affinity Chromatography and Immunoprecipitation. In "Immunochemistry in Practice". Chapter 10. Blackwell Scientific Publications.

Kent, W. S. (1880). "A manual of the Infusoria" Parts 1-3. 432pp. Bogue, London.

Kiaira, J. K. and Njogu, R. M. (1983). Comparison of Glycolysis in Intact and Digitonin-Permeabilized Bloodstream Trypomastigotes of Trypanosoma brucei. Int. J. Biochem. **15**, 1379-1383.

Kilgour, V. (1980). Trypanosoma: Intricacies of Biochemistry, Morphology and Environment. Int. J. Biochem. **12**, 325-332.

Kindl, H. (1982). The Biosynthesis of Microbodies (Peroxisomes, Glyoxysomes). Int. Rev. Cytol. **80**, 193-229.

Klein, R. A., Linstead, D. J. and Wheeler, M. V. (1975). Carbon Dioxide Fixation in Trypanosomatids. Parasitol. **71**, 93-107.

Krebs, H. A. (1972). The Pasteur Effect and the Relations Between Respiration and Fermentation. In "Essays in Biochemistry" Vol. 8 (Cambell, P. N. and Dickens, F. eds.) pp.1-34.

Laemmli, U. K. (1970). Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature (Lond.) **227**, 680-685.

Lamers, J. M. J. (1975). Some Characteristics of Monocarboxylic Acid Transfer Across the Cell Membrane of Epithelial Cells from Rat Small Intestine. Biochim. Biophys. Acta **413**, 265-276.

Lazarow, P. B. (1980). Properties of the Natural Precursor of Catalase: Implications for Peroxisome Biogenesis. In "Precursor Processing in the Biosynthesis of Polypeptides. Ann. N.Y. Acad. Sci." Vol. 343, 293-303.

Lazarow, P. B. and De Duve, C. (1971). Intermediates in the Biosynthesis of Peroxisomal Catalase in Rat Liver. Biochem. Biophys. Res. Commun. **45**, 1198-1204.

Lazarow, P. B. and De Duve, C. (1973). The Synthesis and Turnover of Rat Liver Peroxisomes. IV. Biochemical Pathway of Catalase Synthesis. J. Cell Biol. **59**, 491-506.

Lazarow, P. B. and De Duve, C. (1976). Fatty Acyl-CoA Oxidizing System in Rat-Liver Peroxisomes - Enhancement by Clofibrate, a Hypolipidemic Drug. Proc. Natl. Acad. Sci. USA. **73**, 2043-2046.

Lazarow, P. B. and Fujiki, Y. (1985). Biogenesis of Peroxisomes. Annu. Rev. Cell Biol. **1**, 489-530.

Lazarow, P. B., Robbi, M., Fujiki, Y., Wong, L. (1982). Biogenesis of Peroxisomal Proteins *in vivo* and *in vitro*. In "Peroxisomes and Glyoxysomes. Ann. N.Y. Acad. Sci." Vol. 386, 285-300.

Lee, C-Y. (1982). Hexokinase from *Drosophila melanogaster*. In "Methods in Enzymology" (Wood, W. A. ed.) Vol. 90, 16-21.

Léger, L. (1902). Sur un flagellé parasite de l'Anopheles malculipennis. Comptes Rendus des Séances de la Société de Biologie **54**, 354-356.

Lineweaver, H. and Burk, D. (1934). The Determination of Enzyme Dissociation Constants. J. Am. Chem. Soc. **56**, 658-666.

Lord, J. M. (1980). Biogenesis of Peroxisomes and Glyoxysomes. Sub-Cell. Biochem. **7**, 171-211.

Lowry, O. H., Rosebrough, N. H., Farr, A. L. and Randall, R.J. (1951). Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. **193**, 265-275.

Lwoff, M. and Lwoff, A. (1931). Recherches sur la Morphologie de Leptomonas oncopelti Noguchi et Tilden et Leptomonas fasciculata Novy, MacNeal et Toney. Archives de Zoologie Experimentale et Génerale **71**, Notes et Revue 21-37.

McLaughlin, J. (1981). Association of Adenylate Kinase with the Glycosome of Trypanosoma rhodesiense. Biochem. Int. **2**, 345-353.

Mann, G. E., Zlokovic, B. V.and Yudilevich, D. L. (1985). Evidence for a Lactate Transport System in the Sarcolemmal Membrane of the Perfused Rabbit Heart: Kinetics of Unidirectional Influx, Carrier Specificity and Effects of Glucagon. Biochim. Biophys. Acta **819**, 241-248.

Mäntsälä, P. (1982). Inhibition of Protein Secretion by Cerulenin in B.subtilis. J. Gen. Microbiol. **128**, 2967-2972.

Marr, J. J. (1973). Crithidia fasciculata: Regulation of Aerobic Fermentation by Malic Enzyme. Exp. Parasitol. **33**, 447-457.

Marr, J. J. (1974). Regulation of Aerobic Fermentation in Protozoans - III. Apparent Unimportance of Pyruvate Kinase in Carbohydrate Metabolism. Comp. Biochem. Physiol. **49B**, 531-545.

Marr, J. J. and Berens, R.L. (1977). Regulation of Aerobic Fermentation in Protozoans. VI. Comparative Biochemistry of Pathogenic and Non Pathogenic Protozoans. Acta Tropica **34**, 143-155.

Marr, J. J., Birenbaum, M. E. and Ladenson, J. H. (1977). Crithidia fasciculata : Appearance Kinetics of Intermediates and Regulation of Aerobic Fermentation. Exp. Parasitol. **42**, 322-330.

Masters, C. J. (1981). Interactions Between Soluble Enzymes and Subcellular Structure. CRC Crit. Rev. Biochem. **11**, 105-143.

Mazón, M. J., Gancedo, J. M. and Gancedo, C. (1975). Hexose Kinases from Rhodotorula glutinis. Identification and Properties of a Hexokinase and a Glucokinase. Arch. Biochem. Biophys. **167**, 452-457.

Michels, P. A. M., Poliszczak, A., Osinga, K. A., Misset, O., Van Beeumen, J., Wierenga, R. K., Borst, P. and Opperdoes, F. R. (1986). Two Tandemly Linked Identical Genes Code for the Glycosomal Glyceraldehyde Phosphate Dehydrogenase in Trypanosoma brucei. EMBO J. **5**, 1049-1056.

Midgley, M. (1978). The Transport of α-Aminoisobutyrate into Crithidia fasciculata. Biochem. J. **174**, 191-202.

Miller, P. G. G. (1980). The Intermediary Metabolism of African Trypanosomes. PhD Thesis, University of Cambridge.

Misset, O. and Opperdoes, F. R. (1984). Simultaneous Purification of Hexokinase, Class-I-Frutose-Bisphosphate Aldolase, Triosephosphate Isomerase and Phosphoglycerate Kinase from Trypanosoma brucei. Eur. J. Biochem. **144**, 475-483.

Misset, O. and Opperdoes, F. R. (1987). The Phosphoglycerate Kinases from Trypanosoma brucei. A Comparison of the Glycosomal and the Cytosolic Isoenzymes and Their Sensitivity Towards Suramin. Eur. J. Biochem. **162**, 493-500.

Misset, O., Bos, O. J. M. and Opperdoes, F. R. (1986). Glycolytic Enzymes of Trypanosoma brucei. Simultaneous Purification, Intraglycosomal Concentrations and Physical Properties. Eur. J. Biochem. **157**, 441-453.

Misset, O., Van Beeumen, J., Lambeir, A-M., Van Der Meer, R. and Opperdoes, F. R. (1987). Glyceraldehyde-Phosphate Dehydrogenase from Trypanosoma brucei. Comparison of the Glycosomal and Cytosolic Isoenzymes. Eur. J. Biochem. **162**, 501-507.

Molyneux, D. H. and Ashford, R. W. (1983). "The Biology of Trypanosoma and Leishmania, parasites of Man and Domestic Animals". Taylor and Francis, London.

Monson, J. P., Smith, J. A. Cohen, R. D. and Iles, R. A. (1981a). Lactate Entry Into Isolated Rat Hepatocytes : Evidence for a Transporter. Biochem. Soc. Trans. **9**, 401-402.

Monson, J. P., Smith, J. A. Cohen, R. D. and Iles, R. A. (1981b). Properties of the Hepatocyte Lactate Transporter : Effect of pH and Temperature, and Susceptibility to Inhibition. Biochem. Soc. Trans. **9**, 403.

Mori, M., Miura, S., Tatibana, M. and Cohen, P. P. (1980). Characterization of a Protease Apparently Involved in Processing of Preornithine Transcarbamylase of Rat Liver. Proc. Natl. Acad. Sci. USA. **77**, 7044-7048.

Mottram, J. C. and Coombs, G. H. (1985). Purification of Particulate Malate Dehydrogenase and Phosphoenolpyruvate Carboxykinase from Leishmania mexicana mexicana. Biochim. Biophys. Acta **827**, 310-319.

Mowbray, J. (1975). A Mitochondrial Monocarboxylate Transporter in Rat Liver and Heart and Its Possible Function in Cell Control. Biochem J. **148**, 41-47.

Mowbray, J. and Moses, V. (1976). The Tentative Identification in Escherichia coli of a Multienzyme Complex with Glycolytic Activity. Eur. J. Biochem. **66**, 25-36.

Muse, K. E. and Roberts, J. F. (1973). Microbodies in Crithidia fasciculata. Protoplasma **78**, 343-348.

Newton, B. A. (1962). The Effect of Quaternary Ammonium Trypanocides on Cell Division, Nucleic Acid and Protein Synthesis. In "Drugs, Parasites and Hosts. A Symposium on Relation Between Chemotherapeutic Drugs, Infecting Organisms and Hosts". (Goodwin, L. G. & Nimmo-Smith, R. H. eds.) Churchill, London.

Njogu, R. M., Whittaker, C. J. Hill, G. C. (1980). Evidence for a Branched Electron Transport Chain in Trypanosoma brucei. Mol. Biochem. Parasitol. **1**, 13-29.

Nomura, S., Horiuchi, T., Omura, S. and Hata, T. (1972). The Action Mechanism of Cerulenin 1. Effect of Cerulenin on Sterol and Fatty Acid Biosynthesis in Yeast. J. Biochem. **71**, 783-796.

North, M. J. (1982). Comparative Biochemistry of the Proteinases of Eukaryotic Microorganisms. Microbiol. Rev. **46**, 308-340.

North, M. J., Coombs, G. H. and Barry, J. D. (1983). A Comparative Study of the Proteolytic Enzymes of Trypanosoma brucei, T.equiperdum, T.evansi, T.vivax, Leishmania tarentolae and Crithidia fasciculata. Mol. Biochem. Parasitol. **9**, 161-180.

Nwagwu, M. and Opperdoes, F. R. (1982). Regulation of Glycolysis in Trypanosoma brucei : Hexokinase and Phophofructokinase Activity. Acta Tropica **39**, 61-72.

Oduro, K. K., Flynn, I. W. and Bowman, I. B. R. (1980a). Trypanosoma brucei : Activities and Subcellular Distribution of Glycolytic Enzymes from Differently Disrupted Cells. Exp. Parasitol. **50**, 123-135.

Oduro, K. K., Bowman, I. B. R. and Flynn, I. W. (1980b). Trypanosoma brucei : Preparation and Some Properties of a Multienzyme Complex Catalysing Part of the Glycolytic Pathway. Exp. Parasitol. **50**, 240-250.

Oldendorf, W. H. (1973). Carrier-Mediated Blood-Brain Barrier Transport of Short-Chain Monocarboxylic Acids. Am. J. Physiol. **224**, 1450-1453.

Opperdoes, F. R. (1981). in "Klein, R. A. & Miller, P. G. G., Workshop No.1. Alternative Metabolic Pathways in Protozoan Energy Metabolism. Parasitol. **82**, 1-30.

Opperdoes, F. R. (1983). Glycolysis as a Target for the Development of New Trypanocidal Drugs. In "Mechanism of Drug Action" (Singer, T. P., Mansour, T. E. & Undarzzo, R. N., eds.) pp. 121-131, Academic Press, Orlando.

Opperdoes, F. R. (1984). Localization of the Initial steps in Alkoxyphospholipid Biosynthesis in Glycosomes (Microbodies) of Trypanosoma brucei. FEBS Lett. **169**, 35-39.

Opperdoes, F. R. (1985). Biochemical Peculiarities of Trypanosomes, African and South American. Br. Med. Bull. **41**, 130-136.

Opperdoes, F. R. and Borst, P. (1977). Localization of Nine Glycolytic Enzymes in a Microbody-Like Organelle in Trypanosoma brucei : The Glycosome. FEBS Lett. **80**, 360-364.

Opperdoes, F. R. and Cottem, D. (1982).Involvement of the Glycosome of Trypanosoma brucei in Carbon Dioxide Fixation. FEBS Lett. **143**, 60-64.

Opperdoes, F. R. and Nwagwu, M. (1980). Subcellular Localization of Glycolytic Enzymes in the Glycosome of Trypanosoma brucei. In "The Host-Invader Interplay" (Van den Bossche, H. ed.) 683-686. Elsevier/North Holland Biochemical Press, Amsterdam.

Opperdoes, F. R., Baudhuin, P., Coppens, I., de Roe, C., Edwards, S. W., Weijers, P. J. and Misset, O. (1984). Purification, Morphometric Analysis, and Characterisation of the Glycosomes (Microbodies) of the Protozoan Haemoflagellate Trypanosoma brucei. J. Cell Biol. **98**, 1178-1184.

Opperdoes, F. R., Borst, P., Bakker, S. and Leene, W. (1977a). Localization of Glycerol-3-Phosphate Oxidase in the Mitochondrion and Particulate NAD^+-Linked Glycerol-3-Phosphate Dehydrogenase in the Microbodies of the Bloodstream Form of Trypanosoma brucei. Eur. J. Biochem. **76**, 29-39.

Opperdoes, F. R., Borst, P. and Fonck, K. (1976). The Potential Use of Inhibitors of Glycerol-3-Phosphate Oxidase for Chemotherapy of African Trypanosomiasis. FEBS Lett. **62**, 169-172.

Opperdoes, F. R., Borst, P. and Spits, H. (1977b). Particle-Bound Enzymes in the Bloodstream Form of Trypanosoma brucei. Eur. J. Biochem. **76**, 21-28.

Opperdoes, F. R., Markos, A. and Steiger, R. F. (1981). Localization of Malate Dehydrogenase, Adenylate Kinase and Glycolytic Enzymes in Glycosomes and the Threonine Pathway in the Mitochondrion of Cultured Procyclic Trypomastigotes of Trypanosoma brucei. Mol. Biochem. Parasitol. **4**, 291-309.

O'Regan, S. (1983). Uptake of Acetate and Proprionate by Isolated Nerve Endings from the Electric Organ of Torpedo marmorata and Their Incorporation into Choline Esters. J. Neurochem. **41**, 1596-1601.

Osinga, K. A., Swinkels, B. W., Gibson, W. C., Borst, P., Gerrit, H. V., Van Boom, J. H., Michels, P. A. M. and Opperdoes, F. R. (1985). Topogenesis of Microbody Enzymes : A Sequence Comparison of the Genes for the Glycosomal (Microbody) and Cytosolic Phosphoglycerate Kinases of Trypanosoma brucei. EMBO J. **4**, 3811-3817.

Osumi, T. and Hashimoto, T. (1984). The Inducible Fatty Acid Oxidation System in Mammalian Peroxisomes. Trends Biochem. Sci. **9**, 317-320.

Ottaway, J. H. and Mowbray, J. (1977). The Role of Compartmentation in the Control of Glycolysis. Current Topics in Cellular Regulation **12**, 107-208.

Palumbo, G. and Tecce, M. F. (1983). A Four- to Six-fold Enhancement in Sensitivity for Detecting Trace Proteins in Dye or Silver Stained Polyacrylamide Gels. Analyt. Biochem. **134**, 254-258.

Papa, S., Francavilla, A., Paradies, G. and Meduri, B. (1971). The Transport of Pyruvate in Rat Liver Mitochondria. FEBS Lett. **12**, 285-288.

Pelham, H. R. B. and Jackson, R. J. (1976). Efficient mRNA Dependent Translation System from Reticulocyte Lysates. Eur. J. Biochem. **67**, 247-256.

Porter, E. V. and Chassy, B. M. (1982). Glucokinase from Streptococcus mutans. In "Methods in Enzymology" (Wood, W. A. ed.) Vol. 90, 25-30.

Pragobol, S., Gero, A. M., Lee, C. S. and O'Sullivan, W. J. (1984). Orotate Phosphoribosyltransferase and Orotidylate Decarboxylase from Crithidia luciliae : Subcellular Location of the Enzymes and a Study of Substrate Channeling. Arch. Biochem. Biophys. **230**, 285-193.

Purich, D. L., Fromm, H. J. and Rudolph, F. B. (1973). The Hexokinases : Kinetic, Physical, and Regulatory Properties. In "Advances in Enzymology" Vol. 39, 249-326.

Racagni, G. E. and Machado de Domenech, E. E. (1982). Purification and Properties of Trypanosoma cruzi hexokinase. J. Protozool. **29**, 332. (A188).

Racagni, G. E. and Machado de Domenech, E. E. (1983). Characterization of Trypanosoma cruzi hexokinase. Mol. Biochem. Parasitol. **9**, 181-188.

Regen, D. M. and Tarpley, H. L. (1978). Effects of pH on β-Hydroxybutyrate Transport in Rat Erythrocytes and Thymocytes. Biochim. Biophys. Acta **508**, 539-550.

Regen, D. M. and Tarpley, H. L. (1980). Effects of pH on β-Hydroxybutyrate Exchange Kinetics of Rat Erythrocytes. Biochim. Biophys. Acta **601**, 500-508.

Reynolds, C. H. (1975). The NAD-Linked α-Glycerophosphate Dehydrogenase of Trypanosomes. Biochem. Biophys. Res. Commun. **67**, 538-543.

Riezman, H., Weir, E. M., Leaver, C. J., Titus, D. E. and Becker, W. M. (1980). Regulation of Glyoxysomal Enzymes During germination of Cucumber 3. In vitro Translation and Characterization of Four Glyoxysomal Enzymes. Plant Physiol. **65**, 40-46.

Risby, E. L. and Seed, J. R. (1969). Purification and Properties of Purified hexokinase from the African Trypanosomes and Trypanosoma equiperdum. J. Protozool. **16**, 193-197.

Robbi, M. and Lazarow, P. B. (1978). Synthesis of Catalase in Two Cell-Free Protein-Synthesizing Systems and in Rat Liver. Proc. Natl. Acad. Sci. USA. **75**, 4344-4348.

Rogerson, G. W. and Gutteridge. W. E. (1980). Catabolic Metabolism in Trypanosoma cruzi. Int. J. Parasitol. **10**, 131-135.

Ross, R. (1906). Notes on the Parasites of Mosquitoes found in India between 1895 and 1899. J. Hygiene **6**, 101-109.

Russell, D. G., Miller, D. and Gull, K. (1984). Tubulin Heterogeneity in the Trypanosome Crithidia fasciculata. Mol. Cell. Biol. **4**, 779-790.

Ryley, J. F. (1956). Studies on the Metabolism of the Protozoa. 7. Comparative Carbohydrate Metabolism of Eleven Species of Trypanosome. Biochem. J. **62**, 215-222.

Ryley, J. F. (1962). Studies on the Metabolism of the Protozoa. 9. Comparative Metabolism of Blood-Stream and Culture Forms of Trypanosoma rhodesiense. Biochem. J. **85**, 211-223.

Sabatini, D. D., Kreibich, G., Morimoto, T., Adesnik, M. (1982). Mechanisms for the Incorporation of Proteins into Membranes and Organelles. J. Cell Biol. **92**, 1-22.

Saleh, F. A. K. and Freer, J. H. (1984). Inhibition of Secretion of Staphylococcal α-Toxin by Cerulenin. J. Med. Microbiol. **18**, 205-216.

Santos, C. De Los, Buldain, G., Frydman, B., Cannata, J. J. B. and Cazzulo, J. J. (1985). Carbon-13 Nuclear Magnetic Resonance Analysis of [1-^{13}C] Glucose Metabolism in Crithidia fasciculata. (Evidence of CO_2 Fixation by Phosphoenolpyruvate Carboxykinase). Eur. J. Biochem. **149**, 421-429.

Santos, C. De Los, Frydman, B., Cazzulo, J. J. and Cannata, J. J. B. (1984). ^{13}C - Nuclear Magnetic Resonance Studies of Glucose Catabolism by Crithidia fasciculata. J. Protozool. **31**, 30A.

Saz, H. J. and Lescure, O. L. (1969). The Functions of Phosphoenolpyruvate Carboxykinase and Malic Enzyme in the Anaerobic Formation of Succinate by Ascaris lumbricoides. Comp. Biochem. Physiol. **30**, 49-60.

Schekman, R. (1985). Protein Transport - It's What's Up Front That Counts. Trends Biochem. Sci. **10**, 177.

Schleyer, M. and Neupert, W. (1985). Transport of Proteins Into Mitochondria : Translocational Intermediates Spanning Contact Sites Between Outer and Inner Membranes. Cell **43**, 339-350.

Schlomai, J. and Zadok, A. (1984). Kinetoplast DNA Minicircles of Trypanosomatids Encode for a Protein Product. Nucleic Acids Res. **12**, 8017-8028.

Schlomai, J. and Zadok, A. (1986). Kinetoplast DNA Minicircles of Trypanosomatids Encode for a Protein Product. J. Cell Bioc. **S10A**, 139 (C68).

Schwartz, J. B. (1961). Anaerobic Metabolism of Crithidia fasciculata. J. Protozool. **8**, 9-12.

Seed, J. R. and Baquero, M. A. (1965). The Characterization of Hexokinase from Trypanosoma rhodesiense and Trypanosoma gambiense. J. Protozool. **12**, 427-432.

Shaw, J. J., Voller, A. and Bryant, C. (1964). Intermediary Carbohydrate Metabolism of Four Species of Trypanosomatidae. Ann. Trop. Med. Parasit. **58**, 17-24.

Skinner, M. K. and Griswold, M. D. (1983). Fluorographic Detection of Radioactivity in Polyacrylamide Gels with 2,5-Diphenyloxazole in Acetic Acid and Its Comparison with Existing Procedures. Biochem. J. **209**, 281-284.

Sols, A., de la Fuente, G., Villar-Palasi, C. and Asensio, C. (1958). Substrate Specificity and some other Properties of Bakers Yeast Hexokinsase. Biochim. Biophys. Acta **30**, 92-101.

Souto-Padron, T. and de Souza, W. (1982). Fine Structure and Cytochemistry of Peroxisomes (Microbodies) in Leptomonas samueli. Cell Tissue Res. **222**, 153-158.

Spencer, T. L. and Lehninger, A. L. (1976). L-Lactate Transport in Ehrlich Ascites Tumour Cells. Biochem. J. 154, 405-414.

Stoppani, A. O. M., Docampo, R., De Boiso, J. F. and Frasch, A. C. C. (1980). Effect of Inhibitors of Electron Transport and Oxidative Phosphorylation on Trypanosoma Cruzi Respiration and Growth. Mol. Biochem. Parasitol. 2, 3-21.

Stuart, K. (1983). Kinetoplast DNA, Mitochondrial DNA with a Difference. Mol. Biochem. Parasitol. 9, 93-104.

Sugisaki, H. and Ray, D. S. (1987). DNA Sequence of Crithidia fasciculata Kinetoplast Minicircles. Mol. Biochem. Parasitol. 23, 253-263.

Supowit, S. C. and Harris, B. G. (1982). Hexokinase from Ascaris suum Muscle. In "Methods in Enzymology" (Wood, W. A. ed.) Vol. 90, 21-25.

Swinkels, B. W., Gibson, W. C., Osinga, K. A., Kramer, R., Veeneman, G. H., Van Boom, J. H. and Borst, P. (1986). Characterization of the Gene for the Microbody (Glycosomal) Triosephosphate Isomerase of Trypanosoma brucei. EMBO J. 5, 1291-1298.

Taylor, M. B., Berghausen, H., Heyworth, P., Messenger, N., Rees, L. J. and Gutteridge, W. E. (1980). Subcellular Localization of Some Glycolytic Enzymes in Parasitic Flagellated Protozoa. Int. J. Biochem. 11, 117-120.

Toner, J. J. and Weber, M. M. (1972). Respiratory Control in Mitochondria from Crithidia fasciculata. Biochem. Biophys. Res. Commun. 46, 652-660.

Toruella, M., Franke de Cazzulo, B. M., Engel, J. C., Ruiz, A. M., Segura, E. L. and Cazzulo, J. J. (1981). Trypanosoma cruzi and Trypanosoma rangeli : Glutamate Dehydrogenases and Proteolytic Activities. Comp. Biochem. Physiol. **70B**, 463-468.

Towbin, H. and Gordon, J. (1984). Immunoblotting and Dot Immunoblotting - Current Status and Outlook. J. Immunol. Methods 72, 313-340.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets : Procedure and Some Applications. Proc. Natl. Acad. Sci. USA **76**, 4350-4354.

Trelease, R. N. (1984). Biogenesis of Glyoxysomes. Annu. Rev. Plant Physiol. **35**, 321-347.

Urbina, J. and Crespo, A. (1984). Regulation of Energy Metabolism in Trypanosoma (Schizotrypanum) cruzi epimastigotes. I. Hexokinase and Phosphofructokinase. Mol. Biochem. Parasitol. **11**, 225-239.

Ureta, T. (1982). The Comparative Isozymology of Vertebrate Hexokinases. Comp. Biochem. Physiol. **71B**, 549-555.

Vickerman, K. (1985). Developmental Cycles and Biology of Pathogenic Trypanosomes. Br. Med. Bull. **41**, 105-114.

Visser, N. and Opperdoes, F. R. (1980). Glycolysis in Trypanosoma brucei. Eur. J. Biochem. **103**, 623-632.

Visser, N., Opperdoes, F. R. and Borst, P. (1981). Subcellular Compartmentation of Glycolytic Intermediates in Trypanosoma brucei. Eur. J. Biochem. **118**, 521-526.

Volki, A. and Lazarow, P. B. (1982). Affinity Chromatography of Peroxisomal Proteins on Lectin-Sepharose Columns. In "Peroxisomes and Glyoxysomes. Ann. N.Y. Acad. Sci." Vol. 386, 504-506.

Walk, R. A. and Hock, B. (1978). Cell-Free Synthesis of Glyoxysomal Malate Dehydrogenase. Biochim. Biophys. Res. Commun. **81**, 636-643.

Wallace, F. G. (1943). Flagellate Parasites of Mosquitoes with Special Reference to Crithidia fasciculata Léger, 1902. J. Parasitol. **29**, 196-205.

Wallace, F. G. (1966). The Trypanosomatid Parasites of Insects and Arachnids. Exp. Parasitol. **18**, 124-193.

Watts, D. J. and Randle, P. J. (1967). Evidence for the Existence of a Pyruvate Permease in Rat and Heart Muscle. Biochem. J. **104**, 51P.

Weeks, D. P. and Collis, P. S. (1976). Induction of Microtubule Protein Synthesis in Chlamydomonas reinhardi during Flagellar Regeneration. Cell **9**, 15-27.

Wenyon, C. C. (1926). "Protozoology. A manual for Medical men, Vetinarians and Zoologists." 2 Vol., 1563pp. Balliere, Tindall and Cox, London.

Wickner, W. T. (1979). The Assembly of Proteins into Biological Membranes: The Membrane Trigger Hypothesis. Annu. Rev. Biochem. **48**, 23-45.

Wickner, W. T. and Lodish, H. F. (1985). Multiple Mechanisms of Protein Insertion Into and Across Membranes. Science **230**, 400-407.

Wierenga, R. K., Hol, W. G. J., Misset, O. and Opperdoes, F. R. (1984). Preliminary Crystallographic Studies of Triosephosphate Isomerase from the Blood Parasite Trypanosoma brucei brucei. J. Mol. Biol. **178**, 487-490.

Wierenga, R. K., Swinkels, B., Michels, P. A. M., Osinga, K., Misset, O., Van Beeumen, J., Gibson, W. C., Postma, J. P. M., Borst, P., Opperdoes, F. R. and Hol, W. G. J. (1987). Common Elements on the Surface of Glycolytic Enzymes from Trypanosoma brucei May Serve as Topogenic Signals for Import into Glycosomes. EMBO J. **6**, 215-221.

World Health Organisation (WHO) (1979). The African Trypanosomiases. [Report of a Joint WHO Expert Committee and FAO Expert Consultation.][Rome 8-12 Nov. 1976]. WHO Technical Report Series **635**.

Zuurendonk, P. F. and Tager, J. M. (1974). Rapid Separation of Particulate Components and Soluble Cytoplasm of Isolated Rat-Liver Cells. Biochim. Biophys. Acta **333**, 393-399.

APPENDIX

Publications

The Work described in this thesis has been the subject of the following publication:-

Hale, R. D. and Miller, P. G. G. (1983). Glycolysis in the Protozoan Crithidia fasciculata. Parasitol. 87, xxv.