Studies on the Energy Metabolism of Herpetomonads

by

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ABBREVIATIONS

ADP	adenosine diphosphate
ALDO	aldolase
AMP	adenosine monophosphate
ATCC	American type culture collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BzPFR	benzoyl-prolyl-phenylanalyl-aranyl-p-nitro-analide
cAMP	cyclic adenosine monophosphate
CO_2	carbon dioxide
CoA	coenzyme A
CTP	cytidine triphosphate
Da	Daltons
DHAP	dihydroxyacetone phosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
f	flagellum
$FAD^+(H_2)$	flavine adenine dinucleotide (reduced)
F-1,6-P_2	frucose-1,6-bisphosphate
F-2,6-P2	fructose-2,6-bisphosphate
FR	fumarate reductase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
$GAPDH_c$	cytosolic GAPDH
$GAPDH_g$	glycosomal GAPDH

GDP	guanosine diphosphate
GK	glycerol kinase
$GlcNH_2$	glucosamine
GlcNAc	N-acetyl glucosamine
GMP	guanosine monophosphate
G-3-P	glycerol-3-phosphate
GPDH	glycerol-3-phosphate dehydrogenase
GPI	glycosylphosphatidyl inositol
GIPL	glycoinositol phospholipid
GPI-PLC	GPI-specific phospholipase C
GTP	guanosine triphosphate
HBSS	Hank's balanced salts solution
HCO_3^-	hydrogen carbonate
HDL	high density lipoprotein
Hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
HIFCS	heat inactivated foetal calf serum
HK	hexokinase
IMP	inosine monophosphate
k	kinetoplast
LDL	low density lipoprotein
LIT	liver infusion tryptose
LPG	lipophosphoglycan
m	microbody
Man	mannose
MDH	malate dehydrogenase

ME	'malic' enzyme
mRNA	messenger ribonucleic acid
mt	mitochondrion
n	nucleus
$NAD^+(H)$	nicotinamide adenine dinucleotide (reduced)
NAD-ICDH	NAD^+-specific isocitrate dehydrogenase
NADP(H)	nicotinamide adenine dinucleotide (reduced)
NADP-ICDH	NADP-specific isocitrate dehdrogenase
O_2	oxygen
PAGE	polyacrylamide gel electrophoresis
PC	pyruvate decarboxylase
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PFK	phosphofructokinase
3-PGA	3-phosphoglyceric acid
PGI	phosphoglucoseisomerase
PGK	phosphoglycerate kinase
PGK_c	cytosolic PGK
PGK_g	glycosomal PGK
P_i	inorganic orthophosphate
PI	phosphatidylinositol

PI-PLC	phosphatidylinositol-specific phospholipase C
PK	pyruvate kinase
PSP	promastigote surface protease
Q	quinone
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
SCFA	short-chain fatty acid
SDH	succinate dehydrogenase
SDS	sodium dodecyl sulphate
SHAM	salicylhydroxamic acid
TCA	tricarboxylic acid cycle
TEA	triethanolamine
TEMED	N,N,N',N'-tetramethylethylenediamine
TIM	triosephosphate isomerase
Tris	Tris(hydroxymethyl)aminomethane
TS	thymidylate synthase
UDP	uridine diphosphate
UMP	uridine monophosphate
UrPRTase	uracil phosphoribosyltransferase
UTP	uridine triphosphate
VSG	variant surface glycoprotein

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SUMMARY

Herpetomonas ingenoplastis is an intriguing and unusual trypanosomatid with many anaerobic features, including the lack of a complete cytochrome chain, the presence of an acristate mitochondrion and the ability to grow equally well under aerobic and anaerobic conditions.

In this study, I compared the glucose catabolism of *H. ingenoplastis* and the aerobic *H. muscarum* , with the aim of providing greater insight into the mechanisms whereby *H. ingenoplastis* manages to meet its energetic requirements under aerobic and anaerobic conditions. The results of this study showed that both *H. ingenoplastis* and *H. muscarum* can consume glucose. This correlates with the discovered presence of enzymes of both the Embden-Meyerhof pathway and the pentose-phosphate shunt. The activities of these enzymes are similar in the two organisms. In both species the specific activities of the Embden-Meyerhof pathway enzymes, hexokinase (HK), phosphoglucoseisomerase (PGI) and phosphofructokinase (PFK), were found to be much higher than the activities of two enzymes of the pentose-phosphate shunt, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase.

Glucose consumption varied with gaseous conditions. Anaerobic conditions caused a reduction in glucose consumption, compared with aerobic conditions, in the case of *H. muscarum.* The addition of CO_2 caused an increase in the rate of glucose consumption from that under argon (anaerobic) alone. Similarly for *H. ingenoplastis,* glucose consumption was reduced under anaerobic conditions compared with aerobic conditions and was stimulated by the presence of CO_2.

Herpetomonas muscarum was found to produce succinate and acetate as major excretory products of glucose catabolism, and ethanol and propionate

as minor excretory products. Compared with aerobic conditions succinate production increased under aerobic conditions in the presence of CO_2 by about 2-fold and up to 6-fold in the case of anaerobic conditions in the presence of CO_2. Acetate production increased slightly under the two conditions containing high CO_2. *Herpetomonas ingenoplastis* produced propionate as a major end product of glucose catabolism, along with succinate, acetate and ethanol. Ethanol production was found to be greatest under aerobic and aerobic plus CO_2 conditions, while succinate and propionate were found to be produced in the greatest quantities under anaerobic plus CO_2 conditions. The possibility that propionate production may help supply the energy needs of *H. ingenoplastis* is discussed.

One factor that may be important in the energy metabolism of both herpetomonads are the CO_2-fixation pathways. Two CO_2-fixing enzymes, 'malic' enzyme (ME) and phosphoenolpyruvate carboxykinase (PEPCK), were found at high activities in both organisms. This correlated with succinate production in the presence of CO_2.

There were differences between the two species with respect to the tricarboxylic acid (TCA) cycle enzymes. Notable was the presence of fumarate reductase (FR) and the absence of succinate dehydrogenase (SDH), in the case of *H. ingenoplastis.* Evidence produced suggests that the TCA cycle of *H. ingenoplastis* operates primarily in the reverse direction. *Herpetomomas muscarum* was found to have both FR and SDH, suggesting flux through the TCA cycle in both directions. Succinate dehydrogenase/fumarate reductase ratios of 5.02 and <0.08 for *H. muscarum* and *H. ingenoplastis*, respectively, suggest that *H. ingenoplastis* is an organism that prefers anaerobic conditions, while *H. muscarum* is a facultative anaerobe.

Differences between *H. muscarum* and *H. ingenoplastis* were also found

at the subcellular level with respect to studies on the possible presence of glycosomes in the two herpetomonads. Some evidence was obtained for the presence of glycosomes in *H. muscarum*, in that HK, PGI and malate dehydrogenase (MDH) all were recovered, in part, in the particulate fraction. The distribution of the enzymes of *H. ingenoplastis* was found to be very different, suggesting that either this organism lacks glycosomes or that the glycosomes are much more labile that those of *H. muscarum*. Both FR and SDH were found to be particulate in *H. muscarum* which is consistent with a location in the mitochondrial membrane, perhaps as part of the electron transport chain. The FR activity in *H. ingenoplastis* seemed to be approximately equally divided between the soluble and particulate fractions.

Pyruvate kinase is possibly the major site of glycolytic control in *H. ingenoplastis*. This enzyme requires $MgCl_2$ to function and is activated by fructose-1,6-bisphosphate (F-1,6-P_2) and fructose-2,6-bisphosphate (F-2,6-P_2). The enzyme has an apparent K_m for ADP of 1.3 mM, much higher than that of other trypanosomatids, and the activity shows sigmoid kinetics with respect to phosphoenolpyruvate (PEP) concentration. ATP acts as an allosteric inhibitor, while F-2,6-P_2 abolishes any inhibition by ATP. A pH optimum of 7.5 and subunit molecular weight of 63000 Da compares well with the isofunctional enzymes from other trypanosomatids.

The presence of excreted proteinases by both *H. muscarum* and *H. ingenoplastis*, and the presence of glutamate dehydrogenase, alanine aminotransferase and α–ketobutyrate dehydrogenase, suggests that both can catabolise amino acids, perhaps released through proteolytic degradation. The nature of the proteinases is unknown, although one, which was found to have optimal activity at pH 8.9, had characterisitics of a serine proteinase.

The results obtained show *H. ingenoplastis* to be an unusual trypanosomatid, with many characteristics of an anaerobe, that perhaps meets its energy requirements by fixing CO_2 via PEPCK and forming propionate *via* a reverse TCA cycle.

1.0 INTRODUCTION

1.1 The Trypanosomatidae

The Trypanosomatidae are a family of flagellated protozoa which includes the leishmanias and the trypanosomes. Various of these can infect humans causing a range of conditions from self healing sores to fatal diseases. Both the leishmaniases and the trypanosomiases are medically and economically important and are included as two of the six major diseases in the WHO Special Program for Research and Training in Tropical Diseases (TDR) (Molyneux and Ashford 1983).

1.1.1 The taxonomy of *Herpetomonas*

Trypanosomatidae belong to the order Kinetoplastida (Table 1). Members of the Kinetoplastida are distinguished by the presence of a single mitochondrion, usually extending the length of the cell body, which can either be a simple tube or a multi-branched structure. The mitochondrion contains a Feulgen-positive (DNA-containing), densely stained area (Vickerman 1976), which is termed the kinetoplast and is found near the basal bodies. The order Kinetoplastida contains two sub orders which are the Bodonina, which are largely free living, and the Trypanosomatina, which are probably all parasitic (Vickerman 1976). The Trypanosomatina contains one family, the Trypanosomatidae, which in turn contains genera that have either vertebrate and/or invertebrate hosts in their life cycles. The genera *Leptomonas, Herpetomonas, Crithidia, Blastocrithidia* and *Rhynchoidomonas* are monoxenous and largely parasitise insects, although some parasitise nematodes and worms (Vickerman 1976, Molyneux and Ashford 1983). The members of the genera *Endotrypanum, Leishmania* and *Trypanosoma* (excepting *T. equiperdum* which is adapted to sexual transmission) are digenetic with both a vertebrate and an invertebrate host. The members of the

genus *Phytomonas* are also digenetic but parasitise plants and are transmitted by plant sucking insects (Molyneux and Ashford 1983). Some *Phytomonas* sp. are pathogenic to plants, causing severe damage to many plants of economic importance e.g. coffee plants and coconut palms (Camargo *et al.* 1990).

The genera are distinguished on the basis of the different morphological forms found in the life cycle (Figure 1,Table 2) (Vickerman 1976, Molyneux and Ashford 1983). The forms are known as:

the promastigote, where the kinetoplast is located near the anterior end of the body;

the amastigote, which has the kinetoplast anterior to the nucleus and no emerging flagellum;

the opisthomastigote, with the kinetoplast posterior to the nucleus and a long flagellar pocket;

the epimastigote with the kinetoplast located anterior to the nucleus and the flagellum forming an undulating membrane along the body to the anterior end;

the trypomastigote in which the flagellum also forms an undulating membrane, however the kinetoplast is located posterior to the nucleus;

the choanomastigote, the 'barley corn' form, is distinguished by an anterior kinetoplast and a flagellum emerging from a broad flagellar pocket;

the sphaeromastigote, a rounded form with a free flagellum which forms an undulating membrane and is a transitional stage between amastigote and trypomastigote;

the paramastigote, an intermediate stage between the pro- and opisthomastigote, and has the kinetoplast and nucleus located close together

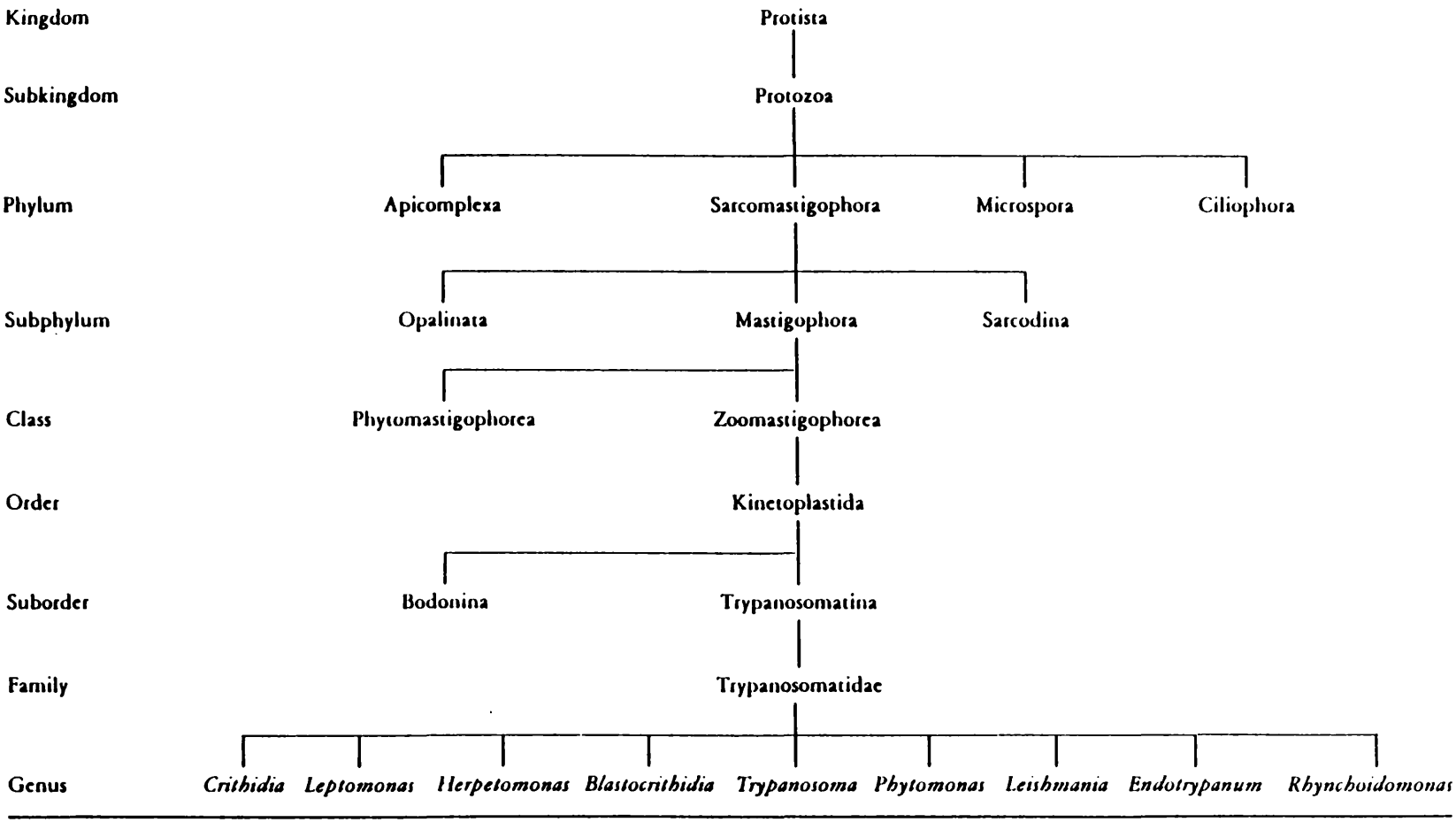


Table 1. Classification of Kinetoplastida (Molyneux and Ashford 1983).

Figure 1. Diagrammatic configuration of the morphological types of Trypanosomatidae (x1300). a, Promastigote. b, Opisthomastigote. c, Epimastigote. d, Trypomastigote. e, Choanomastigote. f, Amastigote. g, Paramastigote. h, Sphaeromastigote.

Abbreviations: Fl = flagellum; N = nucleus; k = kinetoplast. (Molyneux and Ashford 1983)

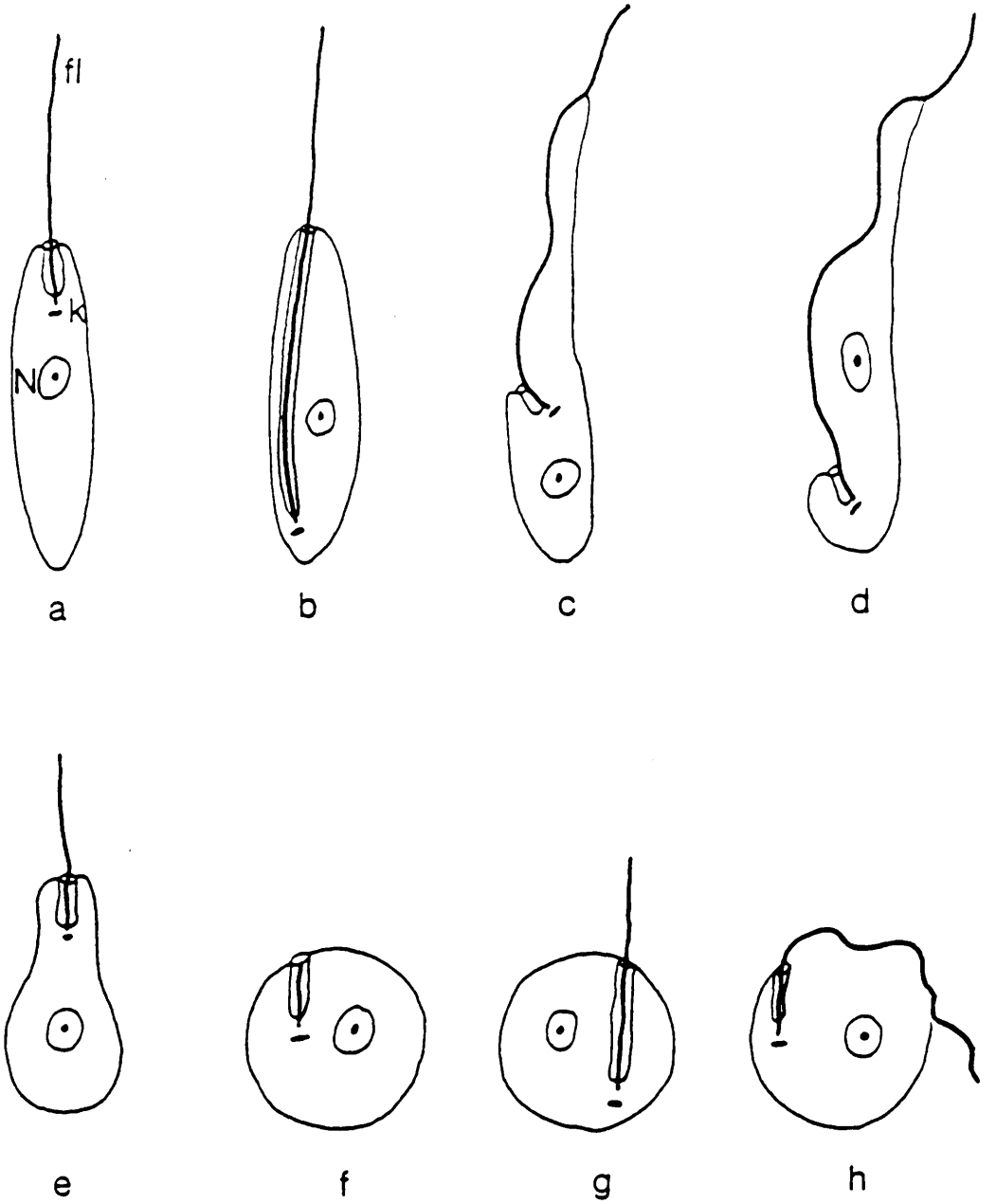


Table 2. Occurence of -mastigotes in genera of Trypanosomatidae (Molyneux and Ashford 1983)

	Pro-	Opistho-	Epi-	Trypo-	Choano-	A-	Para-	Sphaero-
Leptomonas	*					*		
Crithidia					*	*		
Herpetomonas	*	*					*	
Blastocrithidia	*		*			*		
Rhynchoidomonas				*				
Endotrypanum	*		*	*		*		
Phytomonas	*							
Trypanosoma	*		*	*		*		*
Leishmania	*					*	*	

and a flagellum emerging from the anterior end.

The members of the genus *Herpetomonas* are identified by having promastigote, opisthomastigote and paramastigote stages during the life cycle. A member of the genus was first discovered by Burnett in 1851 in the gut of the housefly *Musca domestica*, and was given the generic name *Herpetomonas* by Kent in 1880. It was next observed by Leidy in 1856 and in this case was named *Bodo muscarum*. The organism currently known as *Herpetomonas muscarum* has been referred to in the literature under a variety of names, e.g. *H. muscae domestica, Cercomonas muscae domestica,* and *Schedoacercomonas muscae domestica* (Wenyon 1926). Due to the fact that the promastigote form is the most common, members have often been identified as belonging to the genus *Leptomonas* and in 1952, Grasse suggested that the two genera be combined as *Leptomonas* (Molyneux and Ashford 1983). *Herpetomonas muscarum* has been described as having two distinct subspecies (Rogers and Wallace 1971) which are *H. m. muscarum* and *H. m. ingenoplastis* (referred to as *Herpetomonas muscarum* and *H. ingenoplastis* in this thesis for ease). Whereas *H. muscarum* is 10-12 μm long and exists as mono- and biflagellate promastigotes and opisthomastigotes, *H.ingenoplastis* is 20-30 μm long and the form that predominates is the biflagellate promastigote, although monoflagellate promastigotes and opisthomastigotes are also observed (Rogers and Wallace 1971). The occurrence of biflagellate forms is thought to be due to an initial rapid division of the cell at the flagellum (Molyneux and Ashford 1983).

1.1.2 Life cycle of *Herpetomonas*

Very little is known about the details of the life cycle of herpetomonads (Vickerman 1976). The most intriguing question concerns the

possible mode of transmission of members of this genus between hosts. It has been suggested that there is an encysted form, which may pass out with the faeces to be taken up by another fly (Wenyon 1926). However proof for the presence of cysts is not convincing and neither Rogers and Wallace (1971) nor Devlin (1990, personal communication), the latter results being preliminary work, could find evidence for cysts in flies or in cultures. It has also been found that it is possible to infect flies with flagellated forms (Wenyon 1926, Devlin 1990). In the adult fly, *H. muscarum* can occur in a variety of forms, in any part of the gut up to the opening of the proventriculus (Wenyon 1926). These could lead, by any one of three pathways (Figure 2), to the encysted form in the hindgut of the fly.

Flagellates of *H. muscarum* and *H. ingenoplastis* are also able to grow in the gut of the fly larvae, except for the Malpighian tubules, and in the case of *H. muscarum* are able to survive the pupal stage (Devlin 1990). The form they take in this stage is unknown, but they reappear as active promastigotes in the adult fly where they survive for a few days (Devlin 1990). Devlin (1990) suggested that while there is still no direct evidence for cysts in the life cycle, the presence of a 'stumpy' form in the hindgut of the larvae, similar to that described by Wenyon (1926) and the apparent absence of flagellates from the pupa, is consistent with an encysted form in development.

Of interest is Devlin's finding (1990) that *H. muscarum* survives less well in the adult *Colliphora vomitoria* compared to the larval form. This may reflect the difference in gut conditions. *Colliphora vomitoria* is not the natural host of *H. muscarum,* but even the natural host, *M. domestica,* is relatively resistant to *H. muscarum* with the flagellate surviving for only two days (Rogers and Wallace 1971). This compares with *H. ingenopolastis,* initially isolated from the blowfly *Phormia regina,* which survives for up to four days in the

experimental host *M. domestica. Herpetomonas muscarum* experimentally infected into adult *P. regina* only survived two days whereas *H. ingenoplastis,* produced heavy, lasting (13 days) infections (Rogers and Wallace 1971). Rogers and Wallace (1971) suggest the lack of lasting infections in *M. domestica* is perhaps due either to resistance mechanisms of the fly or to insufficient nutrients being available in it's gut.

1.2 Ultrastucture of the Trypanosomatidae

Members of the Trypanosomatidae have many features in common with other eukaryotes (Vickerman and Preston 1976) (Figures 3 and 4). The plasmalemma of *Trypanosoma* and *Leishmania* is a typical unit membrane of 2-4 nm in width, similar to that of other eukaryotic cells (Vickerman 1974). Beneath the plasmalemma is a complex array of sub-pellicular microtubules. The Trypanosomatidae are more richly endowed with these microtubules than any other flagellate group (Vickerman and Preston 1976). The microtubules completely surround the organism except for a gap where the flagellum is attached to the parasite body (Vickerman 1974). The sub-pellicular microtubules are believed to serve as a cytoskeleton providing support for the cell. They cause difficulty in some experimental work, for the cells are not easily lysed and usually it is necessary to use drastic methods such as grinding with abrasives, e.g. alumina (Mottram 1984) and silicon carbide (Opperdoes 1981), or incubating the cells with a detergent.

The flagellar apparatus provides the only apparent means of movement in the Kinetoplastida (Vickerman and Preston 1976) and is attached to the plasmalemma by plaques at the point of emergence (Molyneux and Ashford 1983). Although there is no recognisable difference in the structure of the membranes of the flagellum and the plasmalemma, in certain areas of the

Figure 2. Three methods by which the rounding-up (encystment) of *H. muscarum* takes place in the hind-gut of the house fly (Wenyon 1926).

1-3, retraction of the promastigote form.

4-8, rounding-up of the opisthomastigote form by looping of the body.

9-11, rounding-up of the opisthomastigote form by retraction of the body.

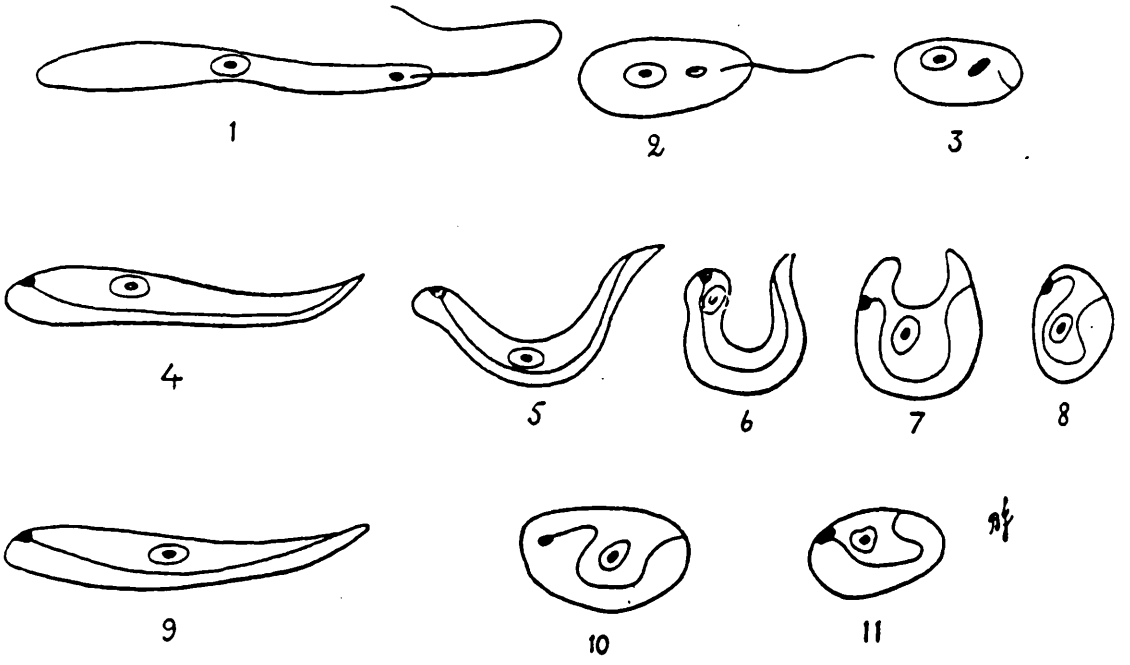


Figure 3. Transmission electron micrographs of sections of *H. muscarum*. Both micrographs demonstrate the abundance of plate-like cristae in the mitochondrion (mt), as well as the presence of microbodies (m) and the kinetoplast (k). The scale bars represent 0.5 μm.

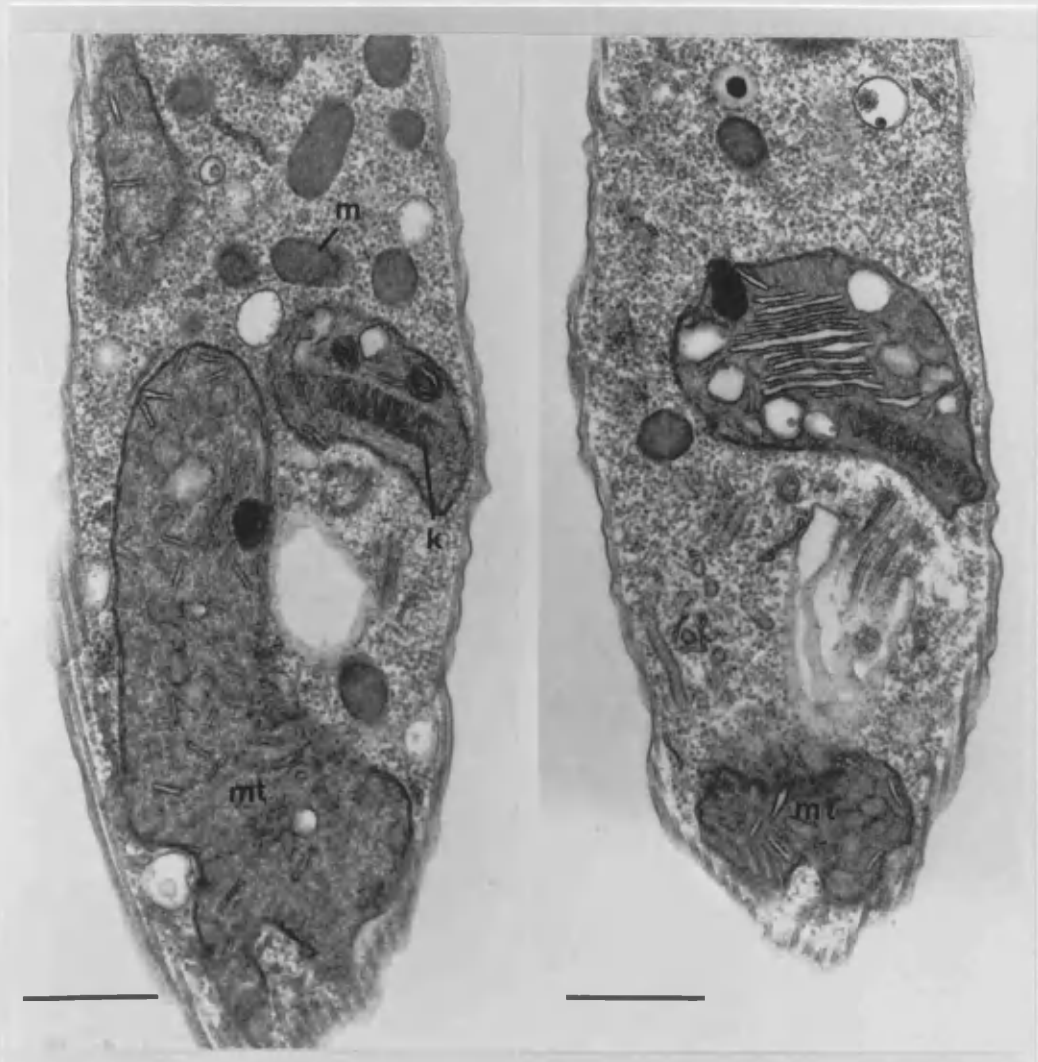
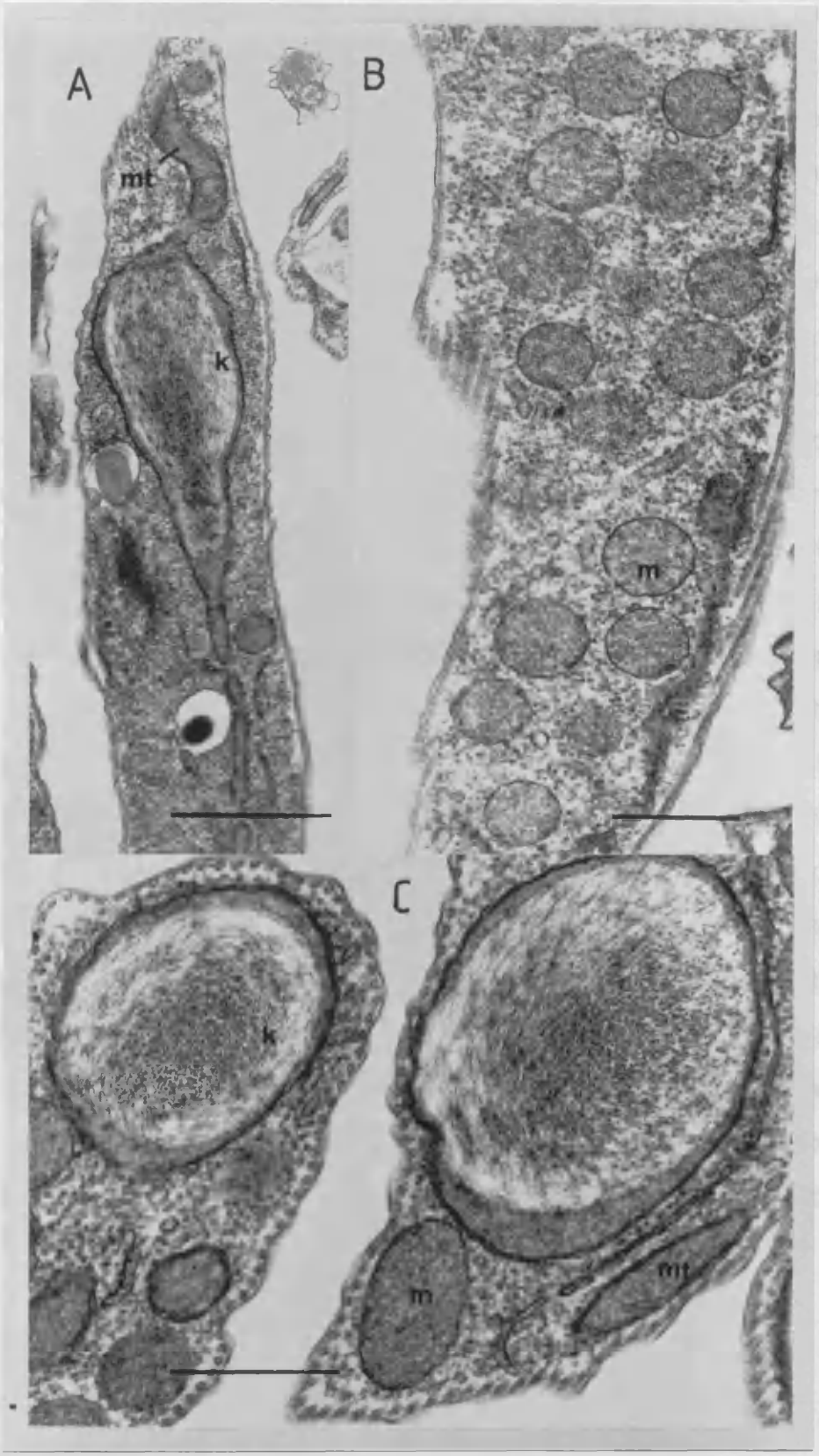


Figure 4. Transmission electron micrographs of sections of *H. ingenoplastis*. In figures A and B the type B kinetoplast (k) is clearly shown, contained within a mitochondrion (mt) that contains few cristae. Figure C demonstrates the abundance of microbodies. The scale bars represent 0.5 µm (A,C) and 1 µm (B).



insect foregut and hindgut, the flagellar membrane undergoes modification with junctional complexes (hemidesmosomes) forming between the flagellum and the gut lining, seen as electron dense bodies under the electron microscope (Molyneux and Ashford 1983).

The nucleus of trypanosomatids is that of a typical eukaryote, being surrounded by an envelope of two closely opposed 7 nm membranes, containing pores, 80-100 nm across, which link the nucleoplasm and cytoplasm. The outer membrane of the nucleus has ribososomes attached and it is continuous with the rough endoplasmic reticulum (RER), as in other eukaryotes. However, unlike higher eukaryotes where the nuclear envelope is absorbed into the endoplasmic reticulum at the metaphase stage of cell division, in the Kinetoplastida it remains intact throughout (Vickerman and Preston 1976). The endoplasmic reticulum, a system of membrane-bound, flattened sacks, known as cisternae and tubules, branches throughout the cytoplasm of the cell and is divided into two types. The RER, with ribosomes attached to the cytoplasmic side of the membrane, and the smooth endoplasmic reticulum, which lacks ribosomes. In the Kinetoplastida, the endoplasmic reticulum is found, in the main, as RER around the nucleus, especially between the nucleus and the flagellar pocket (Vickerman and Preston 1976). The endoplasmic reticulum is continuous with stacks of smooth membrane, known as the Golgi apparatus, where proteins are processed and packaged. Associated with the flagellar pocket, the Golgi apparatus of the Kinetoplastida is believed to be similar to that of other eukaryotes (Vickerman and Preston 1976). The kinetoplast, which is associated with the unitary mitochondrion, is the characteristic feature which gives the order its name. It is found near the basal body, from which the flagellum arises, and consists of catenated circles of DNA (Vickerman and

Preston 1976). The kinetoplast is found in two forms:

i) Type A is a transversely elongated structure, consisting of close fibrils, separated from its capsule envelope by mitochondrial matrix. *Crithidia fasciculata,* leishmanias, trypanosomes, and *H. muscarum* all have this type (Rogers and Wallace 1971, Wallace *et al.* 1973, Vickerman and Preston 1976).

ii) Type B is a loosely packed, fine fibril structure which fills the mitochondrion and is found in *C. luciliae, Blastocrithidia* sp. and *H. ingenoplastis* (Rogers and Wallace 1971, Wallace *et al.* 1973, Vickerman 1976).

Herpetomonas muscarum and *H. ingenoplastis* are described in the literature as two subspecies (Rogers and Wallace 1971, Wallace *et al.* 1973), yet there are remarkable differences between the structures of the kinetoplast-mitochondrial complexes of the two. Not only do they differ at the kinetoplast level, but the structures of the mitochondria are also very different. The mitochondrion of *H. muscarum* is well developed in that it is a branched structure and contains many plate-like cristae (Wallace *et al.* 1973, Hajduk 1980) while the mitochondrion of *H. ingenoplastis* has relatively few cristae and takes the form of a simple tube running down the length of the cell (Wallace *et al.* 1973, Hajduk 1980). These differences in the structure of the kinetoplast-mitochondrion complex are independant of the stage of the life cycle.

Structural differences in the kinetoplast-mitochondrion complex also exist in other trypanosomatids, but these are dependent on the life cycle stage. In the *T. cruzi* epimastigote stage, the kinetoplast is Type A, but in the insect trypomastigote it is Type B (Brack 1968). In *T. brucei* the kinetoplast stucture remains constant throughout its life cycle but the structure of the mitochondrion alters (Vickerman 1970, Molyneux and Ashford 1983,

Vickerman *et al.* 1988). The long slender bloodstream form has a simple tube-like mitochondrion with few cristae. On differentiating to the short stumpy form, there is an increase in the number of tubular cristae and the volume of the mitochondrion increases up to three fold. In the insect procyclic and epimastigote stages, the mitochondrion is branched, containing many plate-like cristae, while in the metacyclic form, the cristae are once again tubular. The changes in morphology in *T. brucei* are associated with the appearance of an active TCA cycle and respiratory chain in the procyclic and epimastigote forms (Vickerman 1976, Vickerman and Preston 1976). In *Leishmania* the mitochondrion remains cristate throughout its life cycle, corresponding with little variation in the mitochondrial metabolism (Marr 1980).

Another characteristic feature of the Trypanosomatidae is the presence of microbodies, known as glycosomes, which contain certain glycolytic enzymes. These were first discovered in the bloodstream form of *T. brucei* (Opperdoes and Borst 1977). Since then glycosomes have been discovered in every member of the Kinetoplastida analysed so far, including *T. cruzi, C. fasciculata, Leishmania,* and *Trypanoplasma borelli* (Opperdoes *et al.* 1977, Taylor *et al.* 1980, Hart and Opperdoes 1984,Opperdoes *et al.* 1988). Glycosomes have been purified from *T. brucei* to near homogeneity, with only 1% mitochondrial contamination, and have a diameter of 0.27 um (Opperdoes *et al.* 1984). *In situ* analysis showed the bloodstream forms of *T. brucei* to have approximately 230 glycosomes per cell, 8% of the cell protein, while electron microscopy revealed *L. m. mexicana* amastigotes to have 9-10 glycosomes per cell (Tetley *et al.* 1983). It is interesting to note morphological changes which occur in the glycosomes during the life cycle of *T. brucei.* In procyclic forms, glycosomes are bacilliform, while in long

slender and short stumpy bloodstream forms and metacyclics they are spherical (Vickerman 1985). The 'microbodies' of *H. muscarum* and *H. ingenoplastis* shown in figures 3 and 4 may be glycosomes.

Leishmania m. mexicana also contains lysosome-like organelles termed megasomes (Alexander and Vickerman 1975) which are absent in promastigotes. They are surrounded by a 10 nm thick membrane and are greater than 1.0 μm in diameter (Coombs *et al.* 1986). Megasomes contain lysosomal enzymes (Pupkis *et al.*1986) and may aid survival in the host macrophage (Coombs 1982). They appear to be absent from *L. donovani* and *L. major* amastigotes (Pupkis *et al.* 1986) and all other trypanosomatids.

1.3 Trypanosomatid metabolism

Trypanosomatids, like many other unicellular parasites, multiply rapidly; for example *H. ingenoplastis* grown in culture have a doubling time of about 18 hours, while *L. m. mexicana* promastigotes double every 12 hours in culture and amastigotes every 24 hours growing in macrophages *in vitro* (Mottram 1984). Because of this the parasites require large amounts of new cellular materials and energy (Von Brand 1979). An important general principle of metabolism is that the anabolic, or biosynthetic, and catabolic, or degradative, pathways are almost always distinct, both for energetic reasons and to facilitate control of metabolism (Stryer 1981). In eukaryotes this regulation and flexibility of metabolism is enhanced by compartmentation, for example in mitochondria. Catabolic processes form ATP (the principle donor of free energy in biological systems) and NADPH (used to reduce oxidised precursors) which are then consumed in cell motility, active transport and in anabolic processes, such as formation of lipid molecules for membranes. Clearly there has to be regulation of the

anabolic and catabolic processes and this is done in a variety of ways; at the level of synthesis and degradation of critical enzymes, the regulation of enzyme activities by substrates and/or products, and by altering enzyme activities through covalent modification.

Much is known about the metabolism of trypanosomatids, especially the pathogenic varieties, and also members of the genus *Crithidia*, especially *Crithidia fasciculata*, which are easily maintained *in vitro* and have been used as models for eukaryotic systems (McGhee and Cosgrove 1980).

1.3.1 Anabolic processes

In order for cells to function and multiply, small molecular weight precursors are formed into macromolecules which are then assembled into cellular structures such as the cytoskeleton and organelles. In biological systems there are four major classes of macromolecules; polysaccarides, nucleic acids, lipids and proteins. Polysaccarides, which serve as energy stores, e.g. glycogen, and parts of the cell structure e.g. cellulose, are believed to be absent from members of the Trypanosomatidae (Gutteridge and Coombs 1977). The metabolism of nucleic acids, proteins and lipids in trypanosomatids have some unique features which are the bases for a number of methods of chemotherapeutic attack.

1.3.1.1 Nucleic acids

The two main forms of nucleic acids are ribonucleic acid and deoxyribonucleic acids, which are used as the building blocks of RNA and DNA respectively, and are synthesised from purine and pyrimidine nucleotides. Purine and pyrimidine metabolism in trypanosomatids has been reviewed by Hammond and Gutteridge (1984) and Hassan and Coombs (1988).

1.3.1.1.1 Purine metabolism

Unlike mammalian cells, which can either synthesise purines *de novo* from glycine, formate, CO_2, glutamine and aspartate or, in some cases, salvage them from the bloodstream, trypanosomatids are unable to synthesise the purine ring *de novo* and must rely on the salvage pathways in order to meet the purine requirements (Jaffe and Gutteridge 1974). The only known exception to this is *C. oncopelti.* This incorporated radioactively labelled glycine into nucleic acid purines (Jaffe and Gutteridge 1974) but it contains 'bipolar bodies', which are thought to be endosymbiotic bacteria which may synthesise the purine ring *de novo* for the trypanosomatid (Gutteridge and Coombs 1977). Trypanosomes that have been found unable to produce purines *de novo* include *C. deanei* (Mundum and Roitman 1977), *T. mega* (Bone and Steinert 1956), *T. cruzi* (Fish *et al.* 1982), *L. donovani, L. braziliensis* (Marr *et al.* 1978) and *L. m. mexicana* (Hausen and Webster 1981).

Inosine monophosphate (IMP) has a central role in purine metabolism and is a precursor of adenine and guanine nucleotides. In most organisms, IMP is converted to AMP by adenylsuccinate synthetase and adenylsuccinate lyase, and to GMP by IMP dehydrogenase and GMP synthetase, and then to the respective nucleoside di- and triphosphates by the action of nucleoside monophosphate kinase and diphosphate kinase, respectively. AMP is converted back into IMP by AMP deaminase, or *via* 5'-nucleotidase to adenosine, which re-enters the salvage pathway. GMP reductase converts GMP into IMP. The adenylsuccinate synthetases of *L. donovani* and *T. cruzi* are similar to the those of other trypanosomatids but differ from those of humans in that they deaminate the IMP analogue allopurinol ribotide to succino-4-aminopyrazolo (3,4-delta) pyrimidine ribotide (Spector and Miller 1976,

Spector *et al.* 1979, 1982) which may be lethal to *Leishmania, T. cruzi, T. b. rhodesiense* and *T. b. brucei* (Hassan and Coombs 1988). In addition, GMP reductase of *L. donovani* is inhibited by allopurinol ribonucleotide, thiopurinol ribotide (Spector and Jones 1982) and formycin B 5'-monophosphate (Spector *et al.* 1984). While being potent antileishmanial chemicals *in vitro,* they are unlikely to have any role as drugs (Hassan and Coombs 1988). Related compounds, e.g. allopurinol, are, however, being tested as antileishmanial agents.

Due to the lack of a functional *de novo* purine synthesising pathway, trypanosomatids rely on the salvage of preformed purines. All trypanosomatids have purine phosphoribosyltransferases (PRTase) which catalyse the formation of 5'-purine ribonucleotides from purine bases and PRPP, and also kinases which phosphorylate nucleosides to form nucleotides. Nucleotidases, which hydrolyse nucleotides to nucleosides, and phosphorylases, which hydrolyse nucleosides to the purine bases and ribose-1-phosphate are absent from most trypanosomatids. Deaminases which have a role in the interconversion of purines are also present, in varying degrees, in some trypanosomatids.

As well as salvaging purines intracellularly, purines are also transported from the extracellular environment. Bloodstream form trypomastigotes of *T. b. brucei* and *T. congolense* have high affinity uptake systems for adenosine, guanosine, adenine and hypoxanthine (James and Born 1980). Okochi and coworkers (1983) have found that *T. vivax* has more than one transporter for adenosine, while *L. b. braziliensis* transports nucleosides in two ways (Hansen *et al.* 1982); diffusion at high concentrations and by a mediated process at low nucleoside concentrations. Two nucleotide transporters have been found in *L. donovani* promastigotes (Aronow *et al.* 1987) and *C. fasciculata* is believed to transport purine bases by two non-

energy dependant mechanisms, one being high affinity and low velocity and the other being low affinity and high velocity.

1.3.1.1.2 Pyrimidine metabolism

Trypanosomatids appear to be able to synthesise nucleotides *de novo* and also *via* salvage pathways.

The first three enzymes of the *de novo* pathway, carbamoylphosphate synthetase, aspartate transcarbamylase and dihydroorotase, are all cytosolic in trypanosomatids (Hassan and Coombs 1988). Whereas in mammals they exist as a multienzyme complex, it appears that in *C. luciliae* they exist as separate, unassociated proteins (Tampitag and Sullivan 1986). The next enzyme in the pathway is dihydroorotate dehydrogenase and in mammals occurs in the mitochondrion. In contrast, the isofunctional enzyme in the Kinetoplastida is cytosolic and consumes oxygen (Hammond and Gutteridge 1984). Evidence for *C. fasciculata* and *T. brucei* suggests that this enzyme is a flavoprotein oxidase (Pascal *et al.* 1983) and is therefore referred to as dihydroorotate oxidase. The last two enzymes which synthesis UMP from orotic acid, orotate phosphoribosyltransferase and OMP decarboxylase, are both particulate in trypanosomatids and have been discovered to be associated with the glycosomes of *T. brucei* trypomastigotes, *L. m. amazonensis* promastigotes (Hammond *et al.* 1981), *T. cruzi* epimastigotes ((Hammond and Gutteridge 1983) and *C. luciliae* (Pragobpol *et al.* 1984). They are believed to be on the outside of the glycosomal membrane with free access to the cytosol (Hammond and Gutteridge 1983). As isofunctional enzymes in mammals are cytosolic, these enzymes may present useful targets for chemotherapeutic attack.

The presence of uracilphosphoribosyltransferase (UrPRTase), uridine

phosphorylase and uridine nucleosidase suggests that uridine is converted to uracil by the actions of the latter two enzymes, and that UMP is then formed by the action of UrPRTase on uracil. Cytidine can also be converted into nucleic acids by all forms of *T. cruzi* (Gutteridge and Gaborak 1979) while thymidine, but not thymine, is used by trypanosomes for nucleic acid synthesis (Pizzi and Taliaferro 1960, Al Chalabi and Gutteridge 1977b, Cosgrove *et al.* 1979, Gutteridge and Gaborak 1979, Lafon *et al.* 1982, Mukkada *et al.* 1985). UMP formed is phosphorylated to UTP by the action of kinases, which is then converted into CTP by CTP synthetase (Hammond and Gutteridge 1984).

Little is known about the formation of deoxyribonucleic acids from the corresponding ribonucleic acids, although evidence suggests that there may be a ribonucleoside diphosphate reductase, converting the ribonucleoside diphosphate to the deoxyribonucleoside diphosphate Hassan and Coombs 1988). Deoxythymidylate is formed from deoxyuridylate and 5,10-methylenetetrahydrofolate by the enzyme thymidylate synthase (TS) forming dihydrofolate as a coproduct. The TS activity exists on a bifunctional protein in Trypanosomatidae with dihydrofolate reductase, which regenerates tetrahydrofolate (Al Chalabi and Gutteridge 1977a, Garret *et al.* 1984).

1.3.1.1.3 Replication and transcription

Little is known on the mechanism of replication in the nucleus of the Trypanosomatidae, although studies have been carried out on replication in the kinetoplast (reviewed in Simpson 1977, Ryan *et al.* 1988).

DNA polymerases have been found in *T. brucei* (Dube *et al.* 1979, Chang *et al.* 1980), *C. fasciculta* (Holmes *et al.* 1984) and in both promastigotes and amastigotes of *L. m. mexicana* (Chang *et al.* 1980). It

appears that these trypanosomes have two types of DNA polymerase; DNA polymerase A which has a high molecular weight and DNA polymerase B which is a low molecular weight type. The enzymes of *T. brucei* and *L. m. mexicana* were found to be distinct from the human enzymes using antisera specific for human DNA polymerases. The location of these enzymes in the cell is unknown. Kinetoplast DNA is one of the most unusual structures in nature (Simpson 1987) and is made of 5 x 10^3-10^4 minicircles of several classes, and 20-50 maxicircles, which contain the mitochondrial structural genes as well as several unidentified reading frames (Simpson 1987).

Minicircle replication involves the activity of topoisomerase II. This allows the detachment of covalently closed circles from the network and initiation of Cairns type replication, as found in *C. fasciculata* (Kitchen *et al.* 1984, 1985) and *T. equiperdum* (Ntambi and Englund 1985, Ntambi *et al.* 1986), with the newly synthesised strand consisting of small oligonucleotides, and the nicks being repaired after S-phase.

Maxicircle replication occurs at the same time as minicircle replication (Hajduk *et al.* 1984) but differs in that maxicircles replicate in network bound rolling-circles which can easily be seen under the electron microscope (Hajduk *et al.* 1984).

Topoisomerases have a central role in replication and have been purified from *T. cruzi* and *C. fasciculata* (Shloman *et al.* 1984, Douc-Rasy *et al.* 1986). They have been found to be ATP-dependent and genetic studies show that the TOP2 gene of *T. brucei* shows strong conservation with the human topoisomerase II and suggest that the nuclear and mitochondrial topisomerase II may come from the same gene (Strauss and Wang 1990).

Relatively little is known regarding general transcription in

trypanosomatids. The conserved region of maxicircle DNA transcribes the 9S and 12S RNAs, poly-adenylated RNAs and smaller guide RNAs (Jasmer *et al.* 1987) and its transcription is controlled and regulated during the life cycle. The maxicircle kDNA is the functional part of the mitochondrial genome and the organisation is similar in all trypanosomatids studied in that the conserved regions in *T. brucei, L. tarentolae* and *C. oncopelti* are completely transcribed (Stuart and Galvin 1982, Simpson *et al.* 1985, Tarassoff *et al.* 1987), the most abundant transcripts being for the 9S and 12S RNA molecules. Most trypanosomatid genes are arranged in tandem repeats and are transcribed as polycistronic mRNA molecules which are then processed to mature messages by trans-splicing and poly-adenylation (reviewed in Borst 1986, Boothroyd 1985). Little is known about initiation sites or the molecular basis for regulation (Shapira and Pedraza 1990) although the intergenic regions may be important. Studies on the the heat shock protein, HSP 83, of *L. m. amazonensis* have shown several areas in intergenic regions that partially resemble eukaryotic thermoregulated promotors in that they have dyad symmetry. However only one of these is partially homologous to the concensus heat shock element present upstream of all eukaryotic HSPs studied to date (Shapira and Pedraza 1990).

1.3.1.2 Proteins

While using the same twenty amino acids for protein synthesis as mammalian cells, the precise relationship between synthesis and uptake of the various amino acids has not been determined. In the case of *L. tarentolae* and *C. fasciculata,* ten and eleven amino acids, respectively, were found to be essential to growth (Von Brand 1973), while in *L. m. mexicana* amastigotes and promastigotes several amino acids were found to be

consumed in large quantities (Hart and Coombs 1982). Proline, however, was found to be excreted by *L. m. mexicana*, unlike culture forms of *T. brucei, L. donovani* and *L. b. braziliensis* which consume and catabolise proline (Krassner 1969, Evans and Brown 1972, Krassner and Flory 1972). Culture forms of *T. brucei, L.donovani, L. b. braziliensis* and *T. cruzi* take up and utilise many other amino acids (Steiger and Meshnick 1977). In the case of the two trypanosomes, Steiger and Meshnick (1977) found that threonine utilization predominated producing glycine. Both the leishmanias and the trypanosomes were also found to absorbe glutamate at very fast rates (Cross et al. 1975, Steiger and Meshnick 1977). Thus, for some trypanosomatids, at least, amino acids appear to represent an important energy source.

There has been much interest in the amino acid uptake mechanisms of trypanosomatids. In the case of promastigotes, it was found that the transport systems for proline and methionine required metabolic energy (Mukkada and Simon 1977, Law and Mukkada 1979). Studies by Lepley and Mukkada (1983) on *L. tropica* promastigotes using the non-metabolisable analogue of neutral amino acids, γ-aminoisobutyrate, showed that there is one common uptake mechanism for alanine, cysteine, glycine, methionine, proline and serine which is regulated by feedback inhibition and transinhibition.

As well as obtaining amino acids from the extracellular environment, evidence shows that trypanosomatids can also synthesise many amino acids. The glutamate taken up by trypanosomatids is believed to play a central role in the production of other amino acids (Chappell *et al.* 1972). Also a variety of transaminases have been found in trypanosomatids (Von Brand 1979)

which synthesize amino acids by transferring amine groups from amino acids to keto acids. Evidence for proline synthesis exists for a variety of trypanosomatids. *Leishmania donovani* and *L. tarentolae* promastigotes synthesise proline using arginine, ornithine and citrulline as precursors (Krassner and Flory 1972, Camargo *et al.* 1978) and it is interesting to note that *L. m. mexicana* promastigotes were found to take up arginine and ornithine (Hart and Coombs 1982). It is believed that a similar system may operate in the genus *Leptomonas* (Camargo *et al.* 1978), but there is no evidence for proline synthesis in the *Trypanosoma* using this pathway (Camargo *et al.* 1978). The same pathway is also used to synthesise arginine from ornithine in *Leptomonas samueli, Herpetomonas megaseliae, H. samuelpessoai* and *H. m. muscarum* but not in *H. m. ingenoplastis, Crithidia, Leishmania, Trypanosoma* or in leptomonads, other than the one mentioned above (Camargo *et al.* 1978, Figueirido *et al.* 1978, Yoshida *et al.* 1978).

The amount of knowledge on the mechanism of protein synthesis in trypanosomatids is small. The mRNA formed by transcription of DNA is then translated on ribosomes of the RER. It is believed that control of protein synthesis operates at the translation level. During the differentiation of *L. m. amazonensis* promastigotes to amastigote-like forms due to temperature elevation a variety of proteins which are known to be stage-specific were examined. It was found that even though the proteins were absent in the amastigote-like forms the mRNA was still present and was not different in abundance, size or translatability compared to the mRNA in the promastigote form (Miller 1988). Similar results were found for the bloodstream and culture forms of *T. brucei* (Parsons and Hill 1989).

1.3.1.3 Lipids

Fatty acid molecules and sterols are important constituents of membranes, the composition of which can affect the membrane properties e.g. fluidity. Lipid metabolism in *Leishmania* has been reviewed by Glew and coworkers (1988) and sterol metabolism in parasitic protozoa has been reviewed by Furlong (1989). A wide variety of lipid and fatty acid compositions has been found in trypanosomatids (Godfrey 1967, Dixon and Williamson 1970). Of the dry weight of leishmanias, 2-15% is lipid (Beach *et al.* 1979). Plasmalogens have been found in a number of trypanosomes (Hack *et al.* 1972) and in *Leishmania* (Beach *et al.* 1979) and the plasmalogen, plasmenylethanolamine, forms the major lipid component of *L. donovani* promastigotes grown on lipid free medium (Hermann and Gercken 1980). This lipid molecule seems common in *Leishmania* grown in culture, whereas lipids of the other 2 main groups, choline and inositol derivatives, are virtually absent (Hermann and Gercken 1980). In contrast *Trypanosoma* and *Crithidia* were found to contain inositol and choline derivatives as the major lipid types (Palmer 1973, Oliveira *et al.* 1977).

Leishmania donovani promastigotes synthesise lipid molecules *de novo* (Steiger and Steiger 1977, Jacobs *et al.* 1982) as do *C. fasciculata* and *T. rhodesiense* (Dixon *et al.* 1971, Nes and Nes 1980). As well as being able to synthesise fatty acid molecules, trypanosomatids are also able to synthesise polyunsaturated fatty acids by chain elongation and progressive desaturation of monoenoic acids (Meyr and Holz 1966). Biosynthesis of ether lipids, another lipid type that is abundant in *Leishmania*, in *L. donovani* promastigotes is the same as in other eukaryotic cells (Hermann and Gerken 1980, Hermann *et al.* 1981) involving the incorporation of fatty alcohols and long chain *o*-alkylglycerols. It has been found that one of the key enzymes in this pathway is

associated with the glycosomes of *Leishmania* species and of *T. brucei* (Hart and Opperdoes 1987) and that high concentrations of *o*-alkylglycerols cause inhibition of lipid metabolism and it has been suggested that these molecules may be useful as antileishmanial agents (Hermann and Gercken 1982).

Another possible site of chemotherapeutic attack is in the synthesis of sterols in the Trypanosomatidae. It has been found that this pathway is similar to that of fungi (Docampo *et al.* 1981, Goad *et al.* 1984, Gomez-Eichelmain *et al.* 1988) and that antimycotic agents e.g. ketoconazole and other imidazoles, which inhibit sterol synthesis in fungi also inhibit sterol synthesis in *Leishmania* and *Trypanosoma* (Docampo *et al.* 1981,Berman *et al.* 1984, Goad *et al.* 1985, Berman *et al.* 1986).

Many members of the Trypanosoma must also import fatty acids to meet requirements for membranous organelles (Dixon *et al.* 1971). Culture forms of *T. brucei* take up lipids 10 times faster than bloodstream forms and 3 times faster than *Crithidia* (Voorheis 1980) and their uptake is associated with serum lipoproteins known as high and low density lipoproteins, HDL and LDL respectively, (Coppens et al.1988, Black and Vandeweer 1989). The exact mechanism of uptake has not been elucidated although evidence suggests that in *T. b. brucei* lipoprotein particles may be destabilised in the flagellar pocket, releasing the lipid which enters the cell by fluid phase endocytosis and/or receptor-mediated endocytosis (Vandeweer and Black 1990).

1.3.1.3.1 Lipid anchors

Much interest has been stimulated, in recent years, in the structure and the formation of the glycosyl-*sn*-1,2-dimyristylphosphatidylinositols (GPI) and

their role in anchoring proteins, and other structures, to the membrane. These structures have been found in *T. congolense, T. equiperdum* (Lamont *et al.*,1987), *T. brucei* (Figure 5) (Ferguson *et al.* 1985), *T. cruzi* (Ferguson and Homans 1989) and *Leishmania* (McConville *et al.* 1987, McConville and Bacic 1990), as well as many other cell types, including *Plasmodium* and mammalian cells (Ferguson and Williams 1988).

African trypanosomes evade the mammalian host's immune response by antigenic variation, which involves the sequential expression of genes which code immunologically distinct variant surface glycoproteins (VSGs) (reviewed by Boothroyd 1985). The VSG forms a dense coat on the surface of the cell and appears to act as a permeability barrier, protecting the trypanosome from the host's immune response.

While primary VSG mRNA translation products suggested a molecule with a hydrophobic C-terminal extension (Boothroyd *et al.* 1980), the mature molecule was found to have this extension replaced with a GPI, which acted as the membrane anchor (Ferguson *et al.* 1985).

In *Leishmania* and *T. cruzi*, GPI anchors also anchor proteins to the surface membrane, but these organisms also have many structurally related GPI molecules which have no analogue in higher eukaryotes (Ferguson and Williams 1988). These structures are the lipophosphoglycans (LPGs) and the glycoinositolphospholipids (GIPLs) of *Leishmania* (Figure 6), and the lipopeptidophosphoglycans of *T. cruzi*, which share the substructure Manα1–4GlcNH$_2$$\alpha$1-6*myo*-inositol-1-phosphate, and,therefore, may also share biosynthetic enzymes for their formation (Ferguson and Homans 1989). The LPG, on the surface of *Leishmania* promastigotes, may be involved in the modulation of protein kinase C activity, and therefore the oxidative burst

Figure 5. Complete primary structure of the GPI anchor of the VSG of *Trypanosoma brucei* (Ferguson and Homans 1989).

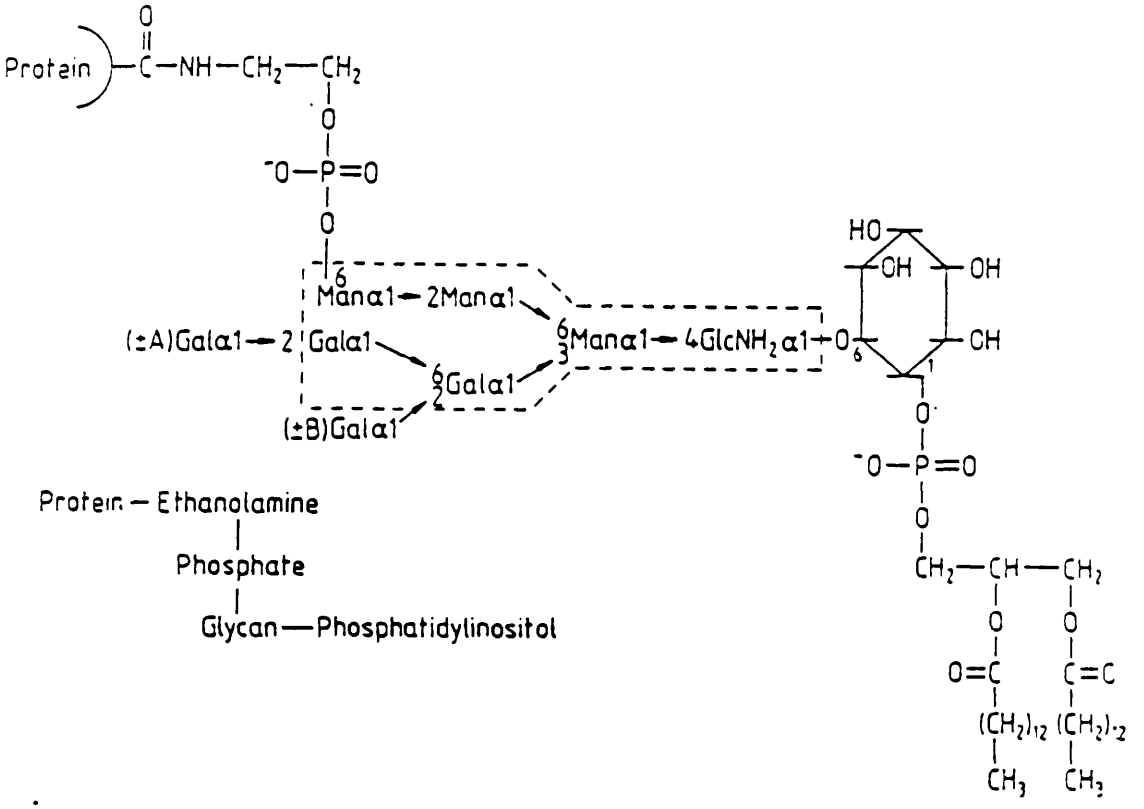


Figure 6. The strucure of the GIPL's of *Leishmania major* (Rosen *et al* 1989).

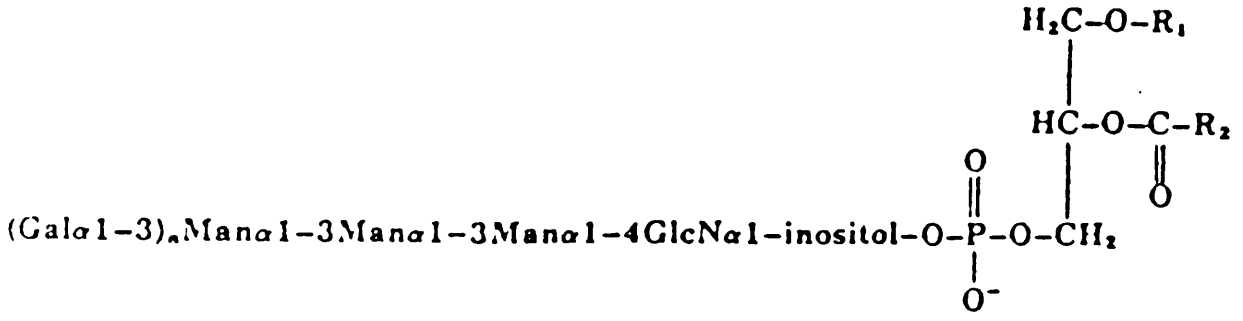


Figure 7. Model of GPI addition to protein. A cleavable N-terminal sequence directs the nascent polypeptide across the rough endoplasmic reticulum. A C-terminal GPI addition signal peptide is recognised by an enzyme or enzyme complex (T) which cleaves off the peptide and adds the GPI precursor (Ferguson and Homans 1989).

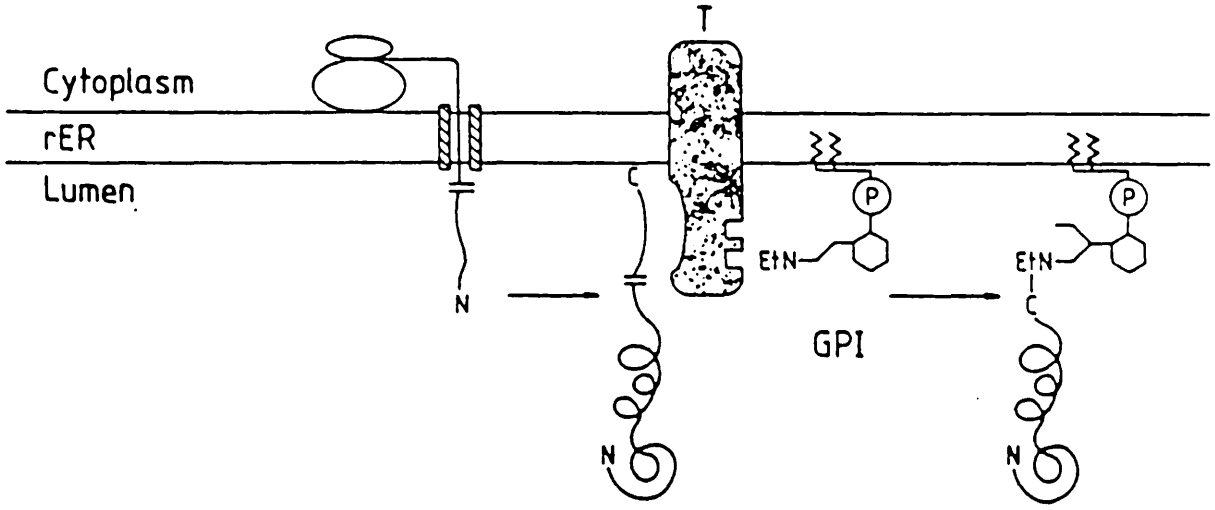
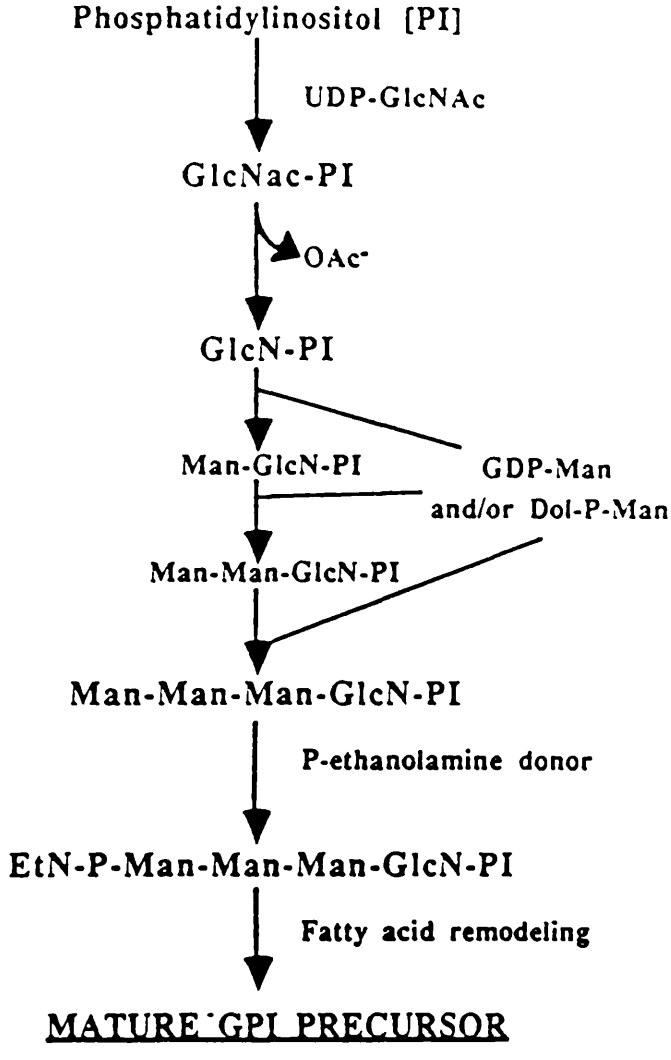


Figure 8. The biosynthetic pathway of GPI precursor formation (Ferguson and Homans 1989).



(McNeely and Turco 1987) due to the presence of *lyso*-alkylglycerol in the structure (McNeely and Turco 1987).

As stated above the VSG of the African trypanosome protects the protozoon against the host's immune response by acting as a permeability barrier. But the VSG is believed to act against the immune response in another way. An endogenous phosphatidylinositol-phospholipase C was found to convert the membrane bound VSG into a soluble form (Lamont *et al* 1987). This GPI-PLC was found at high activities in these cells and is believed to release soluble VSG into the environment , which titrates the host's antibodies and reduces the rate of trypanosome elimination. There is little evidence for the GPI-PLC being involved in antigen switching (Lamont *et al.* 1987).

Figure 7 summarises the process by which the GPI anchor is believed to be attached to the VSG protein. Firstly, the primary translation product passes into the lumen of the RER, due to the presence of an N-terminal signal sequence. An enzyme, or enzyme complex, then recognises and removes a C-terminal signal sequence and replaces it with the GPI precursor. This VSG molecule is then transported to the plasma membrane, *via* the Golgi apparatus and the flagellar pocket (Duszenko *et al.* 1988), with the addition of a galactose to the GPI in the Golgi apparatus (Backs *et al.*1988).

The biosynthetic pathway for the mature GPI of the VSG of African trypanosomes has been elucidated (Figure 8). GlcNAc is added to phosphotidylinositol from UDP-GlcNAc, forming GlcNAc-PI, which is then deacetylated to form $GlcNH_2$-PI. Three mannose residues are then added from GDP-Man forming Man-Man-Man-$GlcNH_2$-PI. Ethanolamine phosphate is added and then the fatty acids of the PI are remodelled forming the dimyristyl-PI structure of the mature GPI precursor (Ferguson and Homans 1989, Masterson *et al.* 1989).

The relationship between the GPI anchor and important surface molecules of *Trypanosoma* and *Leishmania* makes the pathway by which the GPI is produced an attractive target for chemotherapy, and, therefore, much study is being carried out at present on this pathway.

1.3.2 Catabolic processes

Trypanosomatids produce energy *via* glycolysis, the tricarboxylic (TCA) cycle, amino acid catabolism, ß-oxidation of fatty acids and the respiratory chain. Studies on the enzyme levels and characterisation of *in vivo* and *in vitro* metabolic processes have revealed many differences between genera, species and even stages of the Trypanosomatidae.

1.3.2.1 Substrate utilization and end products

Leishmania promastigotes produce succinate, CO_2, pyruvate, alanine and D-lactate as the major end products of energy metabolism (Steiger and Meshnick 1977, Hart and Coombs 1982, Darling *et al.* 1987) although the ways in which the different pathways of catabolism contribute to overall metabolism have not been characterised fully.

Leishmania braziliensis, L. tropica and *L. donovani* promastigotes were found to be able to grow in media without added glucose (Mukkada *et al.* 1974, Marr and Berens 1977) and even when glucose was added maximum utilization was not until late-log and stationary phases of growth. Later studies on the promastigotes of *L.donovani, L. braziliensis* and *L. m. mexicana*, however, showed that high rates of glucose utilization occurred throughout cell growth (Steiger and Meshnick 1977, Hart and Coombs 1982).

Studies on the transport of glucose in *L. donovani* and *L. tropica* promastigotes (Zilberstein and Dwyer 1984, Schaefer and Mukkada 1986)

revealed a carrier-mediated, energy dependent transport system with a high affinity and specificity for D-glucose. It has also been found that the glucose transporter, and also the proline transporter, of *L. donovani* promastigotes are symports, importing protons and glucose into the cells at the same time (Zilberstein and Dwyer 1985).

The uptake mechanisms for proline and other amino acids has been discussed in a previous section (1.3.1.2). There is contradictory evidence on how important amino acids are to the energy metabolism of leishmanias. While Krassner and Flory (1972) and Mukkada and coworkers (1974) give evidence that proline plays an important role in the metabolism of established *L. tropica* promastigote cultures, *L. m. mexicana* promastigotes are found to catabolise proline to CO_2 at a slow rate (Hart *et al.* 1981, Hart and Coombs 1982) and it was suggested that a possible reason for the different results was because the latter studies involved freshly transformed promastigotes, as opposed to long established laboratory cultures (Hart *et al.* 1981).

A little work has been done on the substrate utilization of *L. m. mexicana* amastigotes (Hart *et al.* 1981, Hart and Coombs 1982). The major end products were CO_2 and succinate, but substrate utilization was very different from that found for promastigotes. Glucose was found to be catabolised at a 10-fold lower rate by amastigotes, compared to promastigotes. Non-esterified fatty acids were utilised at a 10-fold higher rate by amastigotes, however, suggesting that macrophage fatty acids may be an important energy substrate for the intracellular parasite. *Leishmania b. panamensis* also increase fatty acid utilization when they are transformed to amastigote-like forms under increased temperature (Blum 1987). The amastigotes take up fatty acids in an albumen complex (Berman *et al.*1987) and as macrophages regulate the amounts of lipoproteins in the serum (Brown

*et al.*1979) they may provide a supply of energy substrates for the amastigote. The high rate of fatty acid catabolism in *Leishmania* is at variance with results for other trypanosomes where fatty acids are utilized only slowly in both insect and mammalian forms (Bowman and Flynn 1976).

As in *Leishmania*, metabolism alters between stages of the Trypanosoma. Procyclic forms of the *T. brucei* subgroup utilise proline and very little glucose (Evans and Brown 1972, Fairlamb and Opperdoes 1986) and produce CO_2 as the major end product (Fairlamb and Opperdoes 1986). They also catabolise threonine to glycine and acetate (Cross *et al.* 1975). The bloodstream forms utilise glucose producing pyruvate and glycerol. *Trypanosoma cruzi* amastigotes consume carbohydrate and produce succinate at a 2-fold higher rate than epimastigotes, while ammonia production only begins on transformation to epimastigotes (Engel *et al.* 1987).

Trypanosoma brucei also alter the mechanisms of glucose uptake in a stage-dependent manner. In the procyclic form, the glucose transporter is similar in many respects to that of *Leishmania* promastigotes in that it is an energy-dependent, high affinity receptor which possibly acts as a glucose/H^+ symporter (Parsons and Nielsen 1990). Glucose enters the long slender bloodstream form, however, by permeation driven by a positive concentration gradient, which is maintained by the high rate of flux through the glycolytic pathway (Gruenberg *et al.* 1978).

The other trypanosomatids produce a wide variety of metabolic end products including succinate (all trypanosomatids (Von Brand 1973)), pyruvate (*C. fasciculata, C. oncopelti* (Ryley 1955, Cazzulo *et al.* 1985), acetate (*T. brucei* sub group, *T. congolense, T. vivax, T. equiperdum, T. cruzi, C. fasciculata* (Ryley1962, 1965, Cazzulo *et al.* 1985, Fairlamb and Opperdoes

1986)), ethanol (*C. fasciculata, C. oncopelti* (Ryley 1955, Cazzulo *et al.* 1985)), glycerol (*C. oncopelti, T. brucei* subgroup, *T. cruzi, T. vivax* (Ryley 1955, 1956, 1962, Fairlamb and Opperdoes 1986)), L-lactate (*T. congolense, T. lewisi* (Ryley 1956, Darling *et al.* 1988)), D-lactate (*T. lewisi, Leishmania* (Blum *et al.* 1987, Darling and Blum 1988, Darling *et al.* 1988)), ammonia (*C. fasciculata, T. cruzi* (Cazzulo *et al.* 1985)) and alanine (*T. cruzi* (Engel *et al.* 1987)).

Little is known about the substrate utilisation and end products of *Herpetomonas*. In common with *Crithidia* sp., they are able to catabolise exogenous citrulline and arginine to CO_2, ornithine and ammonia (Figuerido *et al.* 1978, Yoshida *et al.* 1978) and have no requirement for proline (Yoshida *et al.*1978).

1.3.2.2 Effects of gaseous conditions

Oxygen is an essential requirement for the growth of *L. m. mexicana* promastigotes *in vitro* and there was shown to be a positive correlation between O_2 concentration and growth; the maximum growth was with 20% O_2 (v/v) (Hart and Coombs 1981). The concentration of CO_2 has little effect on growth. Amastigotes required some O_2 to transform to promastigotes but substantially increasing the concentration of O_2 had little effect on the rate of transformation. Carbon dioxide, however, had a positive effect on amastigote transformation, in that the rate was much faster at 5% (v/v) CO_2 compared to 0.1% CO_2. These results suggested that amastigotes are adapted for low O_2 environments and that CO_2 could act as a trigger for amastigote transformation in the sandfly (Hart and Coombs 1981). There is no evidence for CO_2 concentrations being higher in the gut of the sandfly than in the parasitophorous vacuole, however, and it may be that the amastigotes themselves are adapted for growth in high CO_2 concentrations. All other trypanosomatids, including *H. muscarum*, were

considered to require O_2 for growth, although *H. ingenoplastis* may be an exception for it grows equally well under both aerobic and anaerobic conditions (Coombs 1990).

Glucose metabolism can be affected by gaseous conditions. Louis Pasteur discovered that when yeast cells were incubated under aerobic conditions glucose consumption decreased dramatically compared with cells incubated anaerobically. This phenomenon was named the Pasteur effect and it occurs in mammalian cells also. In aerobic conditions the mammalian electron transport chain functions normally, reducing oxygen to water and forming ATP molecules. Excess ATP molecules inhibit the third enzyme of glycolysis, phosphofructokinase, as does citrate formed in the tricarboxylic acid cycle, resulting in a build up of glucose-6-phosphate, which in turn inhibits hexokinase, the first enzyme of glucose catabolism. Under anaerobic conditions, however, ATP is rapidly used up, as the electron transport chain is non-functional, relieving inhibition of phosphofructokinase and hexokinase and causing an increase in glucose catabolism (Stryer 1981).

Some trypanosomatids have been studied in a similar way. Using glucose as the only carbon source, *L. braziliensis, L. major* and *L. m. amazonensis* promastigotes decreased glucose utilisation dramatically under anaerobic conditions compared to aerobic conditions (Darling *et al.* 1987, 1988, 1989) and increased the consumption of an intracellular pool of alanine (Darling *et al.* 1989). This is referred to as the 'reverse' Pasteur effect. The reverse Pasteur effect in Leishmania does not occur under anaerobic conditions in the presence of 5% CO_2. Under these conditions glucose consumption continues as under aerobic conditions suggesting that CO_2 is being fixed. It should be noted Cazzulo and coworkers (1986) were unable to find evidence for a reverse Pasteur effect in *L. mexicana*

promastigotes, with glucose as the only carbon source. Evidence for a reverse Pasteur effect has also been found for *T. lewisi* bloodstream forms and procyclic forms of *T. brucei gambiense* (Darling *et al.* 1988) and in the plant trypanosomatid *C. oncopelti* (Ryley 1955) but not for culture forms of *T. cruzi* or *C. fasciculata* (Cazzulo *et al.* 1988).

Glucose catabolism by *Leishmania* is also affected by gaseous conditions. Under aerobic conditions the products are CO_2, succinate, alanine, acetate, pyruvate and D-lactate (Darling *et al.* 1987). Anoxic incubation conditions cause an increase in D-lactate production and the addition of glycerol as a major end product (Darling *et al.* 1987, 1988) while the addition of 5% CO_2 under anaerobic conditions causes a decrease in glycerol production and an increase in succinate production again suggesting the presence of a CO_2 fixing pathway (Darling *et al.* 1989).

Anaerobic conditions caused a substantial increase in the production of glycerol by procyclic forms of *T. b. gambiense*. A smaller but similar effect occurred with *T. lewisi* bloodstream-forms, where 50% of the glucose carbons end up in the two isomers of lactate. The relative amounts of these two isomers alters with incubation conditions. Under anaerobic conditions there is an increase in L-lactate and a decrease in D-lactate compared to aerobic conditions (Darling *et al.* 1988). It is believed that glucose is the only source of carbon used by the bloodstream forms of the *T. brucei* subgroup (Fairlamb and Opperdoes 1986) and under aerobic conditions all the carbons are converted into pyruvate which is excreted as the only end product. Under anaerobic conditions, however, there is an equal distribution of glucose carbons to glycerol and pyruvate. Although the products change with oxygen tensions, there is no evidence for either a Pasteur or reverse Pasteur effect in these cells (Ryley 1956, Opperdoes *et al.* 1976, Fairlamb *et al.* 1977, Fairlamb

and Opperdoes 1986).

1.3.2.3 Catabolic pathways

The three major pathways of carbohydrate catabolism in higher eukaryotes - glycolysis, the TCA cycle and the pentose phosphate shunt - have all been shown to be present in the Trypanosomatidae, although in some cases they are incomplete or operate in the reverse direction. While the major entry point for carbohydrate catabolism is at glycolysis, the products of the oxidation pathways of amino acids and fatty acids enter at the TCA cycle. Much of the work in these respects has been done on those trypanosomatids that are pathogenic to animals and on *C. fasciculata*.

1.3.2.3.1 Glycolysis and the pentose phosphate shunt and their regulation

Both glycolysis and the pentose phosphate shunt are pathways of glucose catabolism. But where glycolysis produces pyruvate and ATP as the end products, the pentose phophate shunt produces reducing power in the form of NADPH as well as five-carbon sugars. Enzymes of these two pathways have been found in *L. donovani, L. tropica, L. mexicana,* and *L. braziliensis* promastigotes, as well as in the amastigote forms of *L. mexicana* and *L. donovani* (Ghosh and Datta 1971, Martin *et al.* 1976, Coombs *et al.* 1982, Meade *et al.* 1984). The activities for the enzymes of both pathways are higher in promastigotes than in amastigotes (Coombs *et al.* 1982, Meade *et al.* 1984). The activities of these pathways have also been confirmed by the use of radiolabelled glucose (Chatterjee and Datta 1973, Marr 1980, Keegan *et al.* 1987).

Glycolysis and the pentose phosphate pathway have also been found in other trypanosomatids, incuding *Trypanosoma* and *Crithidia* sp. (Von Brand

1973, Bowman and Flynn 1976, Von Brand 1979). Most research has been done on the remarkable change in glucose catabolism that members of the *T. brucei* subgroup undergo during the transformation from long slender bloodstream form through to the procyclic form. The main catabolic pathway in bloodstream forms of *T. brucei* is glycolysis, producing pyruvate. The high specific activities for the glycolytic enzymes and the high rate of glycolytic flux through the pathway, 0.08 μmol of glucose consumed per minute per mg of protein, suggest that glucose is probably the only energy source this form uses (Fairlamb and Opperdoes 1986, Opperdoes 1987). In the procyclic form the activities of the majority of the glycolytic enzymes are less, especially hexokinase (HK) which is at 15-fold lower activity than in the long slender bloodstream form, and the rate of glucose consumption is markedly reduced (Ryley 1962, Opperdoes 1986). The pentose phosphate shunt is present in bloodstream forms of *T. brucei* but does not contribute a great deal to glucose catabolism. It seems mainly to be involved in forming pentose rings for RNA synthesis (Constantinides *et al.* 1990).

The lack of a Pasteur effect in *Leishmania* may be explained by the finding that the HK of the these organisms is unaffected by glycolytic and phosphorylated intermediates (Berens *et al.*1980) while phosphofructokinase (PKF), although allosterically activated by its substrate, fructose-6-phosphate, and also AMP, is unaffected by other intermediates which regulate this enzyme, and therefore glycolysis, in mammalian cells (Berens and Marr 1977a). The first enzyme of the pentose phosphate shunt, glucose-6-phosphate dehydrogenase, was also found to be not subject to metabolic regulation (Berens *et al.* 1980). Similarly, *T. brucei* bloodstream form PFK and HK were also found to be non-regulated (Nwagwu and Opperdoes 1981). The rate-limiting step of glycolysis in these forms is

believed to be at the site of glucose uptake, which proceeds at a slower rate than glucose metabolism (Gruenberg *et al.* 1978).

Another enzyme that is involved in regulation of glycolysis in mammalian cells is pyruvate kinase (PK). This enzyme converts phosphoenolpyruvate to pyruvate, which can then be used in the TCA cycle, CO_2 fixation and amino acid metabolism, and is allosterically inhibited by ATP. The isofunctional enzyme is also regulated in many trypanosomatids. It is present at high activities in the procyclic form of *T. brucei,* although inactive in the bloodstream form (Misset and Opperdoes 1987). In crude extracts the activity is greatly increased by μM concentrations of fructose-2,6-bisphosphate, a metabolic regulator of PFK activity in mammalian cells, and mM concentrations of fructose-1,6-bisphosphate. It is also allosterically activated by phosphoenolpyruvate and inhibited by ATP and inorganic phosphate. The PK of *L. major* promastigotes and *C. lucilliae* are similarly regulated (Van Schaftingen *et al.* 1985). PK activity is cytosolic and is active in bloodstream forms due to the presence of fructose-2,6-bisphosphate in the cell (Van Schaftingen *et al.* 1985). The level of this metabolite in the cell is controlled by the relative activities of the two enzymes 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase, which also occur in the cytosol of trypansomatids as two separate enzymes, as opposed to the isofunctional enzymes in plants and animal tissue which are bifunctional (Van Schaftingen *et al.* 1987). The variation between stages and between species makes the study of regulation both complex and fascinating.

While other workers have found evidence for a metabolically-regulated PK in *L. tropica* and *L. major* promastigotes (Mukkada et al 1974, Etges and Mukkada 1988), *T. cruzi* epimastigotes (Cazzulo *et al.* 1989) and in the bodonid flagellate *Trypanoplasma borelli* (Opperdoes *et al.* 1988)

similar regulation was not found for *L. donovani*, *L. braziliensis* or *C. fasciculata* (Berens and Marr 1977, Cazzulo *et al.* 1989). Where the results of some of the earlier findings require confirmation, some of the more recent findings do raise doubts about the possible importance of PK in the very active glucose metabolism in *C. fasciculata* (Cazzulo *et al.*1989). Currently there is increased interest in the presence and function of high molecular weight polyphosphates in many trypanosomatids, including *C. luciliae, C. fasciculata*, and *L. major* promastigotes (Janekidevi et al. 1965, Grote *et al.* 1990, LeFurgey *et al.* 1990). These may serve as important regulators of the concentrations of important metabolites, e.g. ATP and ADP, inside the cell and therefore may be involved in the regulation of a variety of processes, including glycolysis (Grote *et al.* 1990).

Another site of regulation of glycolysis may be in the structure of the glycosome. This unique organelle is discussed in section 1.3.3.

1.3.2.3.2 Carbon dioxide fixation pathways

Succinate is excreted by a variety of parasites, and in many cases it is a product of the TCA cycle operating in both the forward and reverse directions (Von Brand 1979). Because the succinate generated in the forward TCA cycle is excreted, oxaloacetate has to be regenerated if the cycle is to continue. A variety of CO_2-fixation reactions have been implicated in this. The following have all been reported to occur in various trypanosomatids:

i) Pyruvate carboxylase (PC)

Pyruvate + HCO_3^- + ATP → oxaloacetate + ADP + P_i

ii) Phosphoenolpyruvate carboxykinase (PEPCK)

$PEP + HCO_3^- + ADP \rightarrow$ oxaloacetate + ATP

iii) 'Malic' enzyme (ME)

$Pyruvate + HCO_3^- + NADPH \rightarrow$ malate + $NADP^+$

In the case of ME, oxaloacetate can be regenerated from malate when it enters the TCA cycle (See section 1.3.2.3.3).

Not all CO_2-fixation pathways are found in all trypanosomatids. *Leishmania donovani* promastigotes were shown to incorporate radioactively-labelled CO_2 forming succinate as a major end product under anaerobic conditions during glucose catabolism (Chatterjee and Datta 1973). Pyruvate was the first acceptor of the CO_2 and malate was found to be the first product labelled. The authors suggested that the CO_2-fixing enzyme was ME which was shown to be present using a spectrophotometric assay (Chatterjee and Datta 1973). Further studies on *L. donovani* promastigote cell free extracts using radioactive isotopes suggested that probably all three CO_2-fixing enzymes, described above, were present (Chatterjee and Datta 1974). It was noted however, that although the radioactive assays suggested the presence of PEPCK and PC, neither could be detected using a spectrophotometric assay with MDH as a linking enzyme. Also, the reaction catalysing the carboxylation of PEP to oxaloacetate was found to be GDP-specific, while the reaction catalysing the carboxylation of pyruvate to oxaloacetate was found to prefer GTP to ATP. In all other trypanosomatids studied, these reactions have been shown to be ADP-specific (Klein *et al.* 1975). Berens and Marr (1977a) however, were unable to detect PEPCK in *L. donovani* promastigotes and they suggested that PC is probably the enzyme that

catalyses CO_2-fixation. PC was detected at low activities in promastigotes of *L. braziliensis, L. tropica* and *L. mexicana* (Martin *et al.* 1976, Berens and Marr 1977a) and found at higher activities in amastigotes of *L. donovani* (Meade *et al.* 1984). Neither Martin and coworkers (1976) or Meade and coworkers (1984) looked for the presence of PEPCK which was, however reported to be present in *L. m. mexicana,* the higher activities being present in amastigotes (Coombs *et al.* 1982, Mottram 1984, Cazzulo *et al.* 1985, Mottram and Coombs 1985).

Epimastigotes of *T. cruzi*, under anaerobic conditions, produce succinate in a pathway that is completely dependent on CO_2, while CO_2 enhances the production of succinate under aerobic conditions (Bowman *et al.* 1963). It was concluded that succinate production was due mostly to the forward TCA cycle under aerobic conditions, and due to the reverse TCA cycle under anaerobic conditions. The enzymes of CO_2-fixation found in *T. cruzi* epimastigotes are PEPCK and ME, (PC activity was not detectable), with PEPCK being six-fold more active than ME at their respective pH optima, suggesting that PEPCK is the main CO_2-fixing enzyme (Cataldi de Flombaum et al. 1977). *Crithidia fasciculata* similarly fixes CO_2 during glucose catabolism (Bowman 1974). PEPCK is believed to be the main enzyme responsible for CO_2-fixation (Bacchi *et al.* 1970. Klein *et al.* 1975, de los Santos *et al.* 1985) although this organism does contain a low PC activity (Bacchi *et al.* 1970, Klein *et al.* 1975) and ME is also present (Cazzulo *et al.* 1980). *Trypanosoma brucei* long slender bloodstream forms do not fix CO_2 (Opperdoes and Gottem 1982) but the procyclic forms do, and like *T. cruzi* and *C. fasciculata*, although both ME and PEPCK are present in these forms, it is the latter enzyme which is believed to be mainly responsible for CO_2-fixation (Opperdoes and Cottem 1982). The presence of both PEPCK and ME in some

trypanosomatids could present a problem unless there is a method by which the wasteful recycling of C_4-dicarboxylic acid produced by PEPCK to C_3-monocarboxylic acid, due to the reaction of ME, is prevented. This is achieved through the different subcellular locations of the two enzymes. 'Malic' enzyme is totally cytosolic in the case of *C. fasciculata, T. brucei* procyclic forms and *L. m. mexicana* promastigotes (Cazzulo *et al.* 1980, Opperdoes and Cottem 1982, Mottram and Coombs 1985) while in *T. cruzi* epimastigotes there are two forms of ME; ME1, which is mitochondrial and ME2, which is cytosolic (Cazzulo *et al.* 1980, Cannata *et al.* 1982). In all the trypanosomatids studied PEPCK appears to occur exclusively in the glycosomes (Cazzulo *et al.* 1980, Cannata *et al.* 1982, Opperdoes and Cottem 1982, Mottram and Coombs 1985). Therefore the substrates and products of the two enzymes are prevented from mixing and a futile cycle is avoided.

Recently an 'internal' CO_2-fixation pathway has been detected in *T. cruzi* epimastigotes. In the presence of glucose, CO_2 is fixed forming phosphorylcholine as an end product. It is believed that the CO_2 used in this case would be that liberated endogenously from the reaction of glucose-6-phosphate dehydrogenase (Frydman *et al.* 1990).

1.3.2.3.3 The tricarboxylic acid cycle

The tricarboxylic acid cycle, also known as the Krebs or citric acid cycle, plays a central role in the catabolism of many molecules, including fatty acids and amino acids, in many organisms, including mammals. In this cycle the four-carbon compound, oxaloacetate, condenses with the two-carbon compound, acetyl CoA, produced by pyruvate dehydrogenase. The resulting six-carbon compund is then decarboxylated and oxidised through a series of reactions to regenerate oxaloacetate, and forming two molecules of CO_2, one

molecule of GTP, three molecules of NADH and one molecule of $FADH_2$ per cycle. The latter two molecules are then reoxidised by the electron transport chain, forming three and two molecules of ATP, respectively (see below). In many plants and microorganisms, there is a bypass of the CO_2-evolving steps, called the glyoxylate bypass. This enables the organisms to oxidise fatty acids or acetate as the sole carbon sources in this bypass. Isocitrate is cleaved to form succinate and glyoxylate by the enzyme isocitrate lyase. The glyoxylate formed is then condensed with acetyl CoA by malate synthase forming malate, which is subsequently oxidised to oxaloacetate. The product of the glyoxylate bypass is succinate which can be released from catabolism and used for biosynthetic purposes, especially in the formation of carbohydrate molecules. The TCA cycle and the glyoxylate bypass can operate at the same time, allowing energy requirements to be maintained via oxidative phosphorylation and carbohydrates to be formed from fatty acids. In higher plants and some protozoa the enzymes of the glyoxylate bypass are contained within microbodies called glyoxysomes.

In mammalian *Leishmania* sp. most of enzymes of the forward TCA cycle have been found in both promastigotes and amastigotes. The exception is NAD-linked isocitrate dehydrogenase (NAD-ICDH), although an NADP-ICDH is present (Martin *et al.* 1976, Meade *et al.* 1984). With *L. donovani* the activities of NADP-ICDH, α-ketoglutarate dehydrogenase and fumarase are all higher in promastigotes than amastigotes (Meade *et al.* 1984). With *L. m. mexicana,* however, promastigotes have higher activities than amastigotes of α-ketoglutarate dehydrogenase, citrate synthase and succinate dehydrogenase (Coombs et al. 1982) although the activities of these three enzymes, especially citrate synthase, was low in both forms (Coombs *et al.* 1982, Von

Brand 1979) suggesting that the forward TCA cycle may be very slow compared with the formation of oxaloacetate by CO_2 fixation, especially in amastigotes (Marr 1980). This has been confirmed using radiolabelled compounds. However it was found that the activity of citrate synthase was adequate for the generation of citrate for both the TCA cycle and the glyoxylate bypass (Simon *et al.* 1978). The enzymes of the glyoxylate bypass have also been found in *L. mexicana, L. donovani, L. tropica* and *L. braziliensis* promastigotes (Mukkada 1977) and an active bypass has been shown to be present using radiolabelled compounds (Simon *et al.* 1978). However Mottram (1984) could find no evidence for the presence of isocitrate lyase in either the amastigote or promastigote forms of *L. m. mexicana.*

The TCA cycle is also present and active in *T. cruzi*, although the activity differs between strains (Von Brand 1979). It operates, in the main, in a forward direction under aerobic conditions, forming succinate and CO_2 as major end products (Bowman *et al.* 1963). Like *Leishmania* there is no evidence of an NAD-ICDH activity in any form of *T. cruzi* (Adroher *et al.* 1988). The metacyclic forms of *T. cruzi* have higher activities of NADP-ICDH, citrate synthase, and succinate dehydrogenase than epimastigote forms. Also the K_ms of citrate synthase and NADP-ICDH for their substrates decrease in the metacyclic forms. It has been suggested that the increase in activity of the TCA cycle in the metacyclic forms probably correlates with the increased use of proline by these forms (Adroher *et al.* 1988). In contrast, cultured amastigote-like forms of *T. cruzi* have a repressed TCA cycle which is derepressed in epimastigotes, perhaps indicating a shift away from the use of amino acids to the use of glucose as a carbon source in the amastigote-like form (Engel *et al.* 1987).

In the long, slender bloodstream forms of *T. brucei* the TCA cycle

enzymes are completely absent. While in the short, stumpy bloodstream form, although the TCA cycle is present, it is not fully active due to the limiting activities of citrate synthase and succinate dehydrogenase (Bowman and Flynn 1970). On transformation to the procyclic form, an active TCA cycle appears. This may be involved in proline catabolism. Whether it is fully active is unknown as both citrate synthase and NAD-ICDH have not been detected and neither has citrate itself been shown to be produced, although succinate, malate and fumarate are (Fairlamb and Opperdoes 1986). As in other trypanosomatids, NADP-ICDH is present and is found totally in the mitochondrion (Opperdoes *et al.* 1977). This is in contrast to many higher eukaryotes, where this enzyme has a dual location in the cytosol and in the mitochondrion. While the action of the TCA cycle in the forward direction is uncertain in many trypanosomatids, it has been found to be very active in the reverse direction under certain gaseous conditions and is believed to be involved in the formation of succinate via the reverse reactions of MDH and fumarase and the activity of the enzyme NADH-fumarate reductase (FR) which forms NAD and succinate from NADH and fumarate. FR could not be detected in promastigotes of *L. m. mexicana,* but was detected in the amastigote form (Mottram 1984). The appearance of FR in amastigotes was concomitant with the increase in the activities of PEPCK and MDH, the enzymes involved in the CO_2-fixation pathway (Mottram 1984). Using radioactively labelled CO_2, Chatterjee and Datta (1973, 1974) demonstrated that *L. donovani* promastigotes fixed CO_2 and that the label was found in fumarate, malate and succinate, indicating an active reverse TCA cycle. Using similar techniques, this pathway has also been demonstrated in *T. cruzi*, T. *brucei* and *C. fasciculata* (Baernstein 1953, Ryley 1962, Bacchi *et al.* 1970). In the case of *T. cruzi* epimastigotes incubated under anaerobic

conditions in the presence of CO_2, the resulting succinate produced is completely due to the reverse TCA cycle via MDH, fumarase and FR. In *T. cruzi* epimastigotes 96% of the FR activity is found in the mitochondrion and 3% in the cytosol, with the mitochondrial activity being associated with the mitochondrial membrane, along with succinate dehydrogenase, NADH dehydrogenase and the cytochromes (Boveris *et al.* 1986). The mitochondrial activity is inhibited by malonate, succinate and cyanide and the succinate dehydrogenase/fumarate reductase ratio of 0.28 indicates that FR has an important physiological role. This ratio is often used as an indication of the role of the TCA cycle in metabolism, for example in the obligate anaerobe *Micrococcus lactideus* the ratio is 0.03 (Warringa *et al.* 1958), while in the aerobic tissue of beef heart the ratio is 60 (Kimura *et al.* 1967), and in the facultative anaerobe *Fasciola hepatica* the ratio is 2.3 (Lara 1976). It is believed that in *T. cruzi*, oxaloacetate, formed by the carboxylation of PEP (See section 1.3.2.3.2) may inhibit succinate dehydrogenase and thereby control the direction of flux through the TCA cycle under different gaseous conditions (Boveris *et al.* 1986).

Amino acid catabolising enzymes have been demonstrated in a variety of *Leishmania* species (Martin *et al.* 1976, Meade *et al.* 1984, Mottram 1984) and *L. b. panamensis* has been shown to catabolise alanine and glutamate *via* a functioning forward TCA cycle, forming CO_2 as a major end product, at comparable rates to glucose (Keegan *et al.* 1987). Activities of the amino acid catabolising enzymes vary with the stage of the life cycle in *Leishmania*. While *L. major* metacyclic and promastigote forms have similar levels of alanine aminotransferase and aspartate aminotransferase, the level of NAD-linked glutamate dehydrogenase in the promastigote forms is higher than in the metacyclic forms (Mallinson and Coombs 1986). *Leishmania m. mexicana*

promastigotes, however, have higher activities of alanine aminotransferase and aspartate aminotransferase than amastigote forms (Mallinson and Coombs 1986). The activity of NAD-glutamate dehydrogenase also decreases in amastigotes of *L. donovani* and *L. m. mexicana* (Meade *et al.* 1984,Mallinson and Coombs 1986) but this activity is replaced by an NADP-linked glutamate dehydrogenase in *L. m. mexicana* amastigotes, this activity being absent in the promastigote forms (Mottram 1984). The APT of Leishmania has a broad specificity and can form α-ketoglutarate for the TCA cycle using aspartate, alanine, tryptophan and tyrosine (Le Blanq and Lanham 1984). *Leishmania* can also catabolise amino acids for the glyoxylate bypass via L-alanine-glyoxylate aminotransferase and glyoxylate-aspartate aminotransferase (Simon *et al.* 1978).

1.3.2.3.4 The respiratory chain

In mammals, NADH and $FADH_2$ from glycolysis and the TCA cycle are reoxidised by a respiratory chain containining a number of electron carriers, among which are the cytochromes, the final electron acceptor being O_2 which is reduced to form H_2O. The structure of this respiratory chain in the inner mitochondrial membrane is such that during electron transport protons are moved from the matrix to the intermembrane space, forming a proton gradient over the inner membrane. In the inner membrane there is a vectorially arranged ATPase which utilises the proton gradient to form an ATP molecule. The only way for the protons to equlibrate over the membrane is for them to pass through the proton channel provided by the F_0 subunit of the ATPase, which in turn causes the F_1 subunit to form ATP molecules from ADP and P_i in the matrix of the mitochondrion. Oligomycin renders this

Figure 9.

The electron transport pathways of *H. muscarum* (A) and *H. ingenoplastis* (B) (From Hajduk 1980).

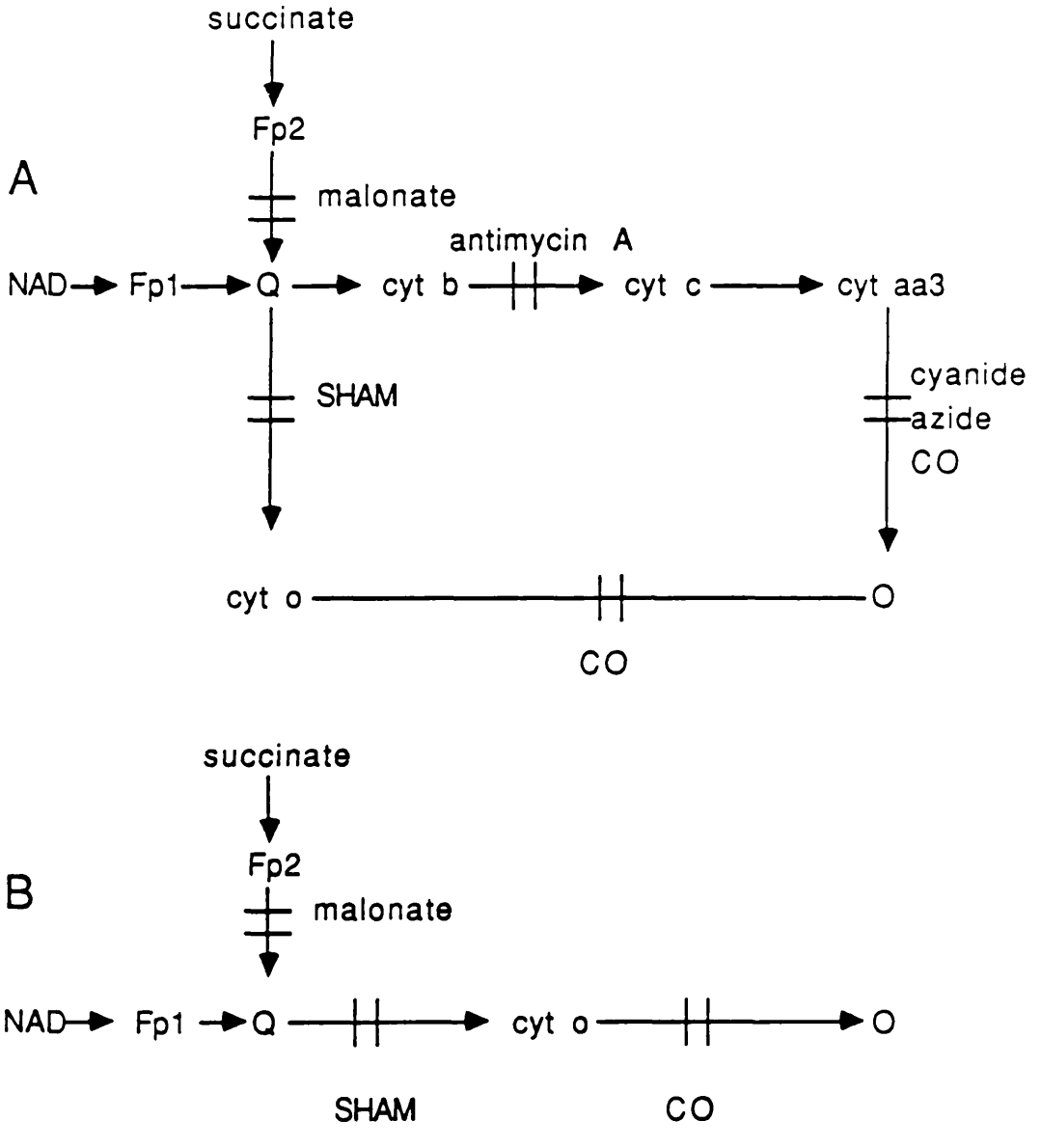


Figure 10.

Compartmentation of glycolysis in *T. brucei* long slender bloodstream trypomastigotes (From Fairlamb and Opperdoes 1986).

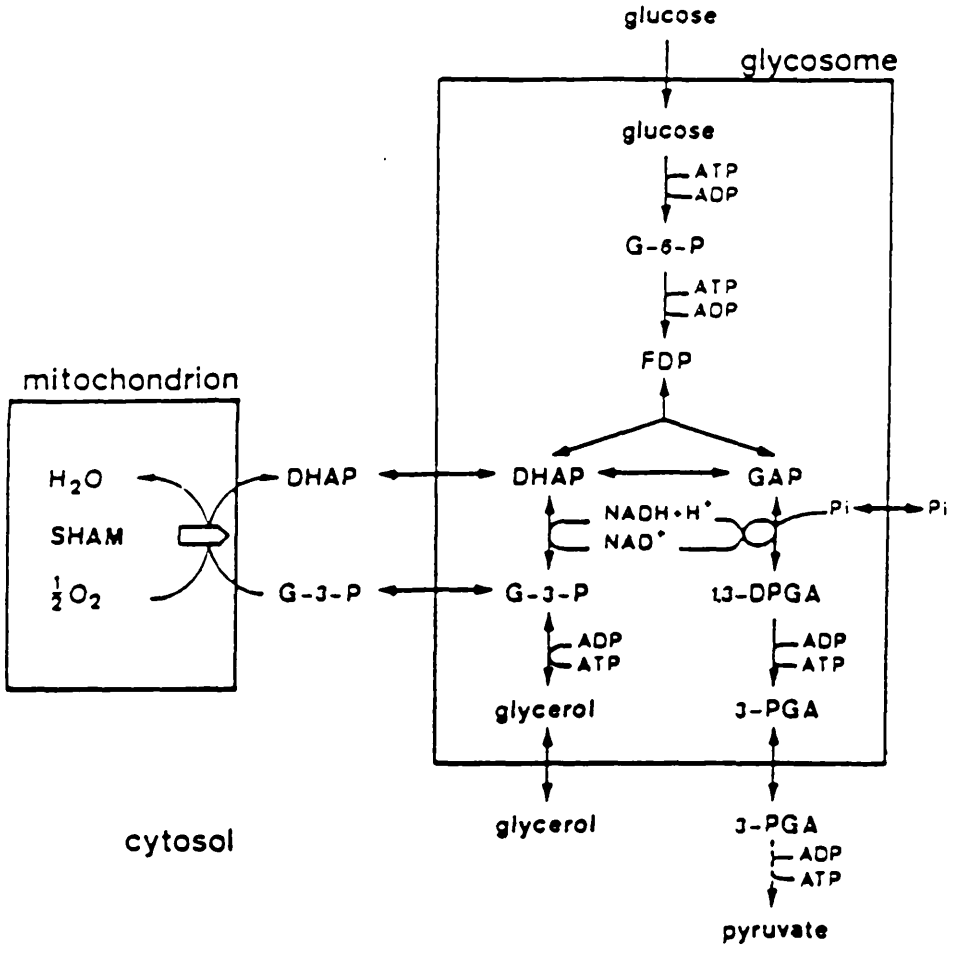
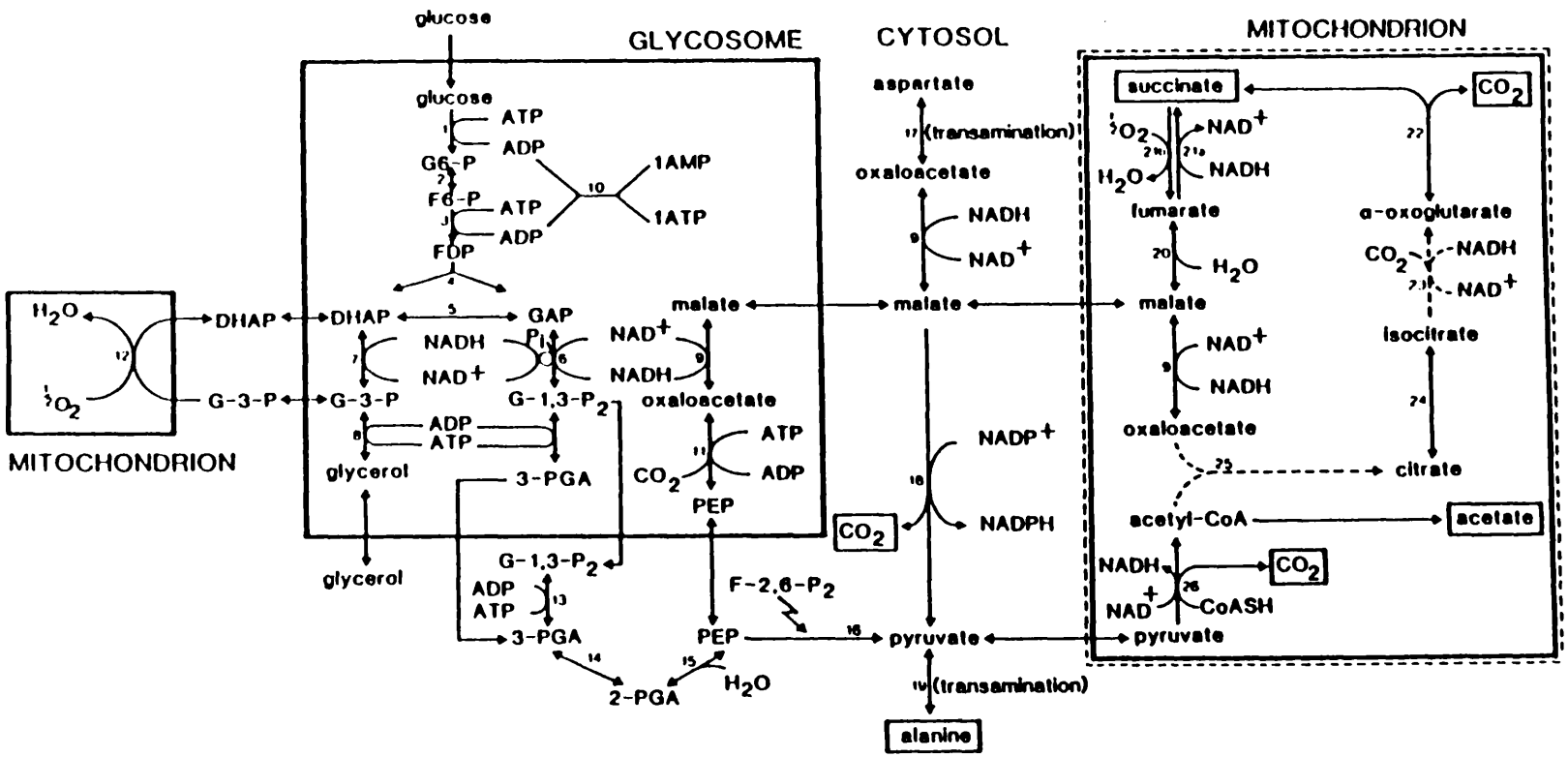


Figure 11.

Pathways of glucose metabolism in *T. brucei* procyclic trypomastigotes. The enzyme locations in the glycosome and cytosol have been established, but the location of the mitochondrial enzymes (except for glycerophosphate oxidase and malate dehydrogenase) have not been clearly demonstrated. End products of aerobic or anaerobic metabolism are in *boxes*. The *dashed lines* indicate enzymes whose presence remains uncertain. 1, hexokinase; 2, phosphoglucoseisomerase; 3, phosphofructokinase; 4, aldolase; 5, triosephosphate isomerase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, glycerol-3-phosphate dehydrogenase; 8, glycerol kinase; 9, malate dehydrogenase; 10, adenylate kinase; 11, phosphoenolpyruvate carboxykinase; 12, glycerol-3-phosphate oxidase; 13, phosphoglycerate kinase; 14, phosphoglycerate mutase; 15, enolase; 16, pyruvate kinase; 17, aspartate aminotransferase; 18, malic enzyme; 19, alanine aminotransferase; 20, fumarate hydratase; 21a, fumarate reductase; 21b, succinate dehydrogenase; 22, α-oxoglutarate decarboxylase; 23, isocitrate dehydrogenase; 24, aconitase; 25, cirate synthase; 26, pyruvate dehydrogenase (From Opperdoes 1987).


glucose
GLYCOSOME
CYTOSOL
MITOCHONDRION
glucose
ATP
ADP
G6-P
F6-P
ATP
ADP
1AMP
1ATP
FDP
H2O
½O2
MITOCHONDRION
DHAP
G-3-P
DHAP
GAP
NADH
NAD+
Pi
G-3-P
G-1,3-P2
NAD+
NADH
malate
oxaloacetate
ADP
ATP
glycerol
3-PGA
CO2
ATP
ADP
PEP
glycerol
G-1,3-P2
ADP
ATP
3-PGA
F-2,6-P2
PEP
2-PGA
H2O
aspartate
(transamination)
oxaloacetate
NADH
NAD+
malate
NADP+
CO2
NADPH
pyruvate
(transamination)
alanine
MITOCHONDRION
succinate
CO2
½O2
NAD+
H2O
NADH
fumarate
α-oxoglutarate
H2O
CO2
NADH
NAD+
malate
isocitrate
NAD+
NADH
oxaloacetate
citrate
acetyl-CoA
acetate
NADH
CO2
NAD+
CoASH
pyruvate

ATPase inactive and therefore this oligomycin-sensitive ATPase is often used as a mitochondrial marker in cell fractionation experiments. The protons are pumped over the membrane yielding three ATP molecules, due to the oxidation of NADH, and two ATP molecules from the oxidation of $FADH_2$. In aerobic organisms the final electron acceptor, O_2, is therefore crucial in meeting their ATP, and therefore their energy, requirements.

The respiratory chain of *Leishmania* is similar to that of mammals in that it contains cytochromes b, c and aa_3 (Hill 1976, Martin and Mukkada 1979, Hart *et al.* 1981b) as well as flavoproteins (Martin and Mukkada 1979). Differences do exist, however. Both the promastigote and amastigote forms of *L. m. mexicana* require high concentrations of cyanide and azide for greater than 70% inhibition of respiration, and rotenone, which inhibits the transfer of electrons between NADH-Q reductase and ubiquinone in mammals, has no effect on respiration (Hart *et al.* 1981b). Martin and Mukkada (1979) suggest a scheme for the respiratory chain of *L. tropica* promastigotes that is similar to that of aerobic eukaryotes and find no evidence for the presence of cytochrome o, or of a branched respiratory chain, as is found in some facultative anaerobes (Kohler 1985). While it has been shown that on transformation of amastigotes to the promastigotes both O_2 consumption and mitochondrial mass increase in *Leishmania* (Rudzinska *et al.* 1964, Simpson 1968) there is no evidence for any difference in the respiratory chain between the two forms (Hart *et al.* 1981b) and changes in mitchondrial mass could be due to another function e.g. the TCA cycle.

Epimastigotes of T. cruzi contain cytochromes c, aa_3, and a b-like cytochrome and the fact that the respiration is only 5% insensitive to cyanide inhibition implies little or no alternative oxidase activity (Docampo *et al.* 1978). However, recent evidence shows that while the cytochromes of axenic

amastigotes are repressed, with little cytochrome b-like molecule and no evidence for cytochrome o at all, there is an increase in cytochrome b-like molecule and cytochrome o appears, along with the TCA cycle enzymes, upon transformation to epimastigotes (Engel *et al.* 1987). As with the TCA cycle enzymes, cytochrome content varies with the strain of *T. cruzi* (Rogerson and Gutteridge 1979, Engel *et al.* 1987). The cytochrome c of *T. cruzi* has a different absorption maximum (558 nm) from the mammalian form (550 nm) (Bowman and Flynn 1976). The respiratory chain for this species was elucidated by Boveris and coworkers (1987). They demonstrated the presence of NADH dehydrogenase and succinate dehydrogenase, with cytochrome a_{611} being the main final electron donor (Docampo *et al.* 1978, Boveris *et al.* 1987). The presence of ubiquinone was inferred from the results for *C. fasciculata* (Kusel and Storey 1972).

Much more is known about the respiratory chain of *T. brucei* (Engel *et al.* 1987, Turrens 1989). In the bloodstream form, cytochromes are absent and respiration is cyanide, azide and antimycin A insensitive. Reoxidation of NADH is carried out by the glycerol-3-phosphate oxidase system in the mitochondrion (Hill 1976, Opperdoes *et al.* 1977). In the mitochondrion the glycerol-3-phosphate oxidase is made up of two components:

i) glycerol-3-phosphate dehydrogenase, which converts glycerol-3-phosphate, which enters from the glycosome, into dihydroxyacetone phosphate(DHAP), which is transported back into the glycosome. The electrons from the glycerol-3-phosphate are passed, probably *via* ubiquinone, to,

ii) a salicylhydroxamic acid (SHAM)-sensitive terminal oxidase which is the final electron donor.

The glycerol-3-phosphate oxidase of the bloodstream forms has a lower

affinity for O_2 (K_m 2.1 uM) than the terminal oxidase of the cytochrome system of the procyclic forms (K_m 0.1 uM) (Opperdoes and Fairlamb 1986). Despite the fact that this system does not involve oxidative phosphorylation, the bloodstream form does contain an oligomycin-sensitive ATPase with an F_0 and an F_1 subunit, as well as an active proton pump. However, the reaction of the ATPase is in the direction of ATP hydrolysis and does not involve respiration, suggesting that the promitochondria of the boodstream forms have a function which may be a transport process of some type (Nolan and Voorheis 1990).

On transformation to the procyclic form cytochromes b,c and aa_3 appear in the mitochondria, as well as a second terminal oxidase, as shown by the respiration being inhibited 60% by cyanide and 30% by SHAM (Fairlamb and Opperdoes 1976, Hill 1976). That the second oxidase is cytochrome has not been confirmed (Bowman and Flynn 1976, Fairlamb and Opperdoes 1986). As with *T. cruzi,* the cytochrome c of *T. brucei* is slightly different from the mammalian form (Bowman and Flynn 1976). Glycerol-3-phosphate is also oxidised by the procyclic forms of *T. brucei,* not by a glycerol-3-phosphate oxidase but by a glycerol-3-phosphate reductase, which involves FAD as opposed to NAD (Fairlamb and Bowman 1977a, b) and flavoproteins (Hill 1976).

One recent discovery concerning respiration in the procyclic form is the contribution of FR to the electron transport chain. Turrens (1987) showed that the FR is involved in free radical production in a similar way to the facultative anaerobe *Monieza expansa* where cytochrome o is also involved, and suggested that other trypanosomes, e.g. *T. cruzi,* may have this system. Further work demonstrates that FR reoxidises NADH producing succinate which is then oxidised by the respiratory chain (Boveris et al. 1986, Turrens 1989). It

is suggested that FR may bypass complex I, which is why several enzymes of the TCA cycle are missing (Turrens 1989). The NADH generated by other reactions could be transported into the mitochondrion, utilising the malate shuttle, allowing the production of NADH or fumarate, depending upon the energetic requirements of the cell. Furthermore, it has been postulated that the NADH-ubiquinone segment may be replaced by FR, as respiration is inhibited by 3-methoxyphenylacetic acid, an inhibitor of FR (Turrens 1989).

Differences in the mitochondrial structure between *H. muscarum* and *H. ingenoplastis* are reflected in differences in the cytochrome system (Hajduk 1980). Respiration of *H. muscarum* is sensitive to cyanide, azide and antimycin A and the respiratory chain contains cytochromes b, c and aa_3. In contrast, the respiration of *H. ingenoplastis* is senstive to SHAM but insensitive to cyanide, azide and antimycin A and the respiratory chain contains cytochrome c and a b-like cytochrome, cytochrome aa_3 being absent (Hajduk 1980). Carbon monoxide-binding data suggested the presence of a cytochrome o as a terminal oxidase in both *H. muscarum* and *H. ingenoplastis*, although the fact that *H. muscarum* respiration is 90% cyanide sensitive suggests that cytochrome aa_3 is the main terminal oxidase. Taking this into account , Hajduk (1980) proposed the respiratory chain schemes outlined in figure 9.

Branched electron transport chains have been suggested for *T. mega, C. fasciculata, C. oncopelti, Leptomonas* sp. and *Blastocrithidia culicia* (Bowman and Flynn 1976, Hill 1976). Little work has been reported on electron transport-linked phosphorylation in the Trypanosomatidae. Data on *C. fasciculata* suggest that there are two energy coupling sites between succinate and O_2 corresponding to sites I and II in mammals, i.e. the NADH-Q reductase complex and the QH_2-cytochrome c reductase complex, respectively.

They yield 0.5-0.6 ATP molecules/2 electrons/site, the low efficiency perhaps being due to competition with cytochrome o for O_2 (Kusel and Storey 1972) The rate of oxidative phosphorylation can be controlled by ADP levels, as in other organisms (Toner and Weber 1972).

1.3.2.3.5 Proteinases

Proteinases are enzymes that carry out protein degradation, forming small peptides and amino acids, and are involved in nutrition and intracellular protein degradation (North 1982). Proteinases also activate other proteinases, and hormones, and, therefore, can have a regulatory function (North 1982). Four classes of proteinases are generally recognised, which are serine, aspartate, cysteine and metalloproteinases. These classifications are made on the basis of catalytic function, distinguished by the use of a variety of specific and non-specific inhibitors (North 1982). They also tend to differ with respect to pH optima (North 1982), although this can vary according to the substrate, e.g. the pH optimum of casein hydrolysis is generally higher than that of haemoglobin hydrolysis (North 1982).

Proteinases have been found in a wide variety of Trypanosomatidae. Data suggests that both qualitative and quantitative differences exist between monogenetic and digenetic species, with proteinase activity being generally more active in the latter group, and between mammalian and insect stages in the life cycles of digenetic species, the proteinases being more active in mammalian forms (Camargo *et al.* 1978, Campetella *et al.* 1990, North *et al.* 1990). Proteinases have been found in *L. donovani, L. braziliensis, L. major* and *L. mexicana* (Camargo *et al.* 1978, Steiger *et al.* 1979) but it is only with the latter two species that there has been any extensive characterisation of the enzyme. In 1981, North and Coombs analysed the proteinases of *L. m.*

mexicana amastigotes using polyacrylamide gel electrophoresis (PAGE) with haemoglobin incorporated into the gel. They showed multiple bands of proteinase activity with a pH optimum of 4.0 and suggested that these bands may represent cysteine proteinases. That *L. m. mexicana* amastigotes may have high activities of cysteine proteinases was confirmed when it was shown that leupeptin, antipain and two epoxysuccinate compounds, all cysteine proteinase inhibitors, inhibited proteinase activity in lysates (Coombs *et al.*1982). These cysteine proteinase activities are believed to be important in survival and growth of amastigotes in macrophages because:

i) cysteine proteinases are twenty fold more active in amastigotes of *L. m. mexicana* compared to promastigotes, and 200-800 fold more active than the monogenetic parasites *H. ingenoplastis, H. muscarum, C. fasciculata* and *Leptomonas ctenocephali* (Coombs 1982),

ii) inhibitors of cysteine proteinases inhibit the growth of *L. m. mexicana* amastigotes in macrophages, and also dramatically inhibit amastigote transformation *in vitro* (Coombs *et al.* 1982, Coombs and Baxter 1984),

Amastigotes of *L. m. mexicana* contain several low molecular weight (16-36 kDa), high activity enzymes, which also occur at lower activity in metacyclic forms (North and Coombs 1981, Lockwood *et al.* 1987, Alfieri *et al.* 1989) but are absent from all forms of *L. major* and *L. donovani.* These proteinases appear to be located in lysosome-like organelles which are found only in *L. m. mexicana* and *L. m. amazonensis* amastigotes (Coombs and Pupkis 1986, Pupkis *et al.* 1986) and constitute 14% of the total cellular volume. The cysteine proteinases in these organelles may produce amines which are released into the parasitophorous vacuole, perhaps regulating the pH of this harsh environment (Coombs and Sanderson 1986).

Intracellular cysteine proteinases have also been found in *T. cruzi* and

T. brucei. Epimastigotes of *T. cruzi* were found to contain a 60 kDa proteinase with an acidic pH optimum, suggesting a cysteine proteinase (Tangel *et al.* 1981). This has since been supported using inhibitors, and by the fact that sequence analysis of the proteinase shows considerable homology to the cysteine proteinases papain, cathepsin B and cathepsin L (Cazzulo *et al.* 1989b, Cazzulo *et al.* 1990a, Eakin *et al.* 1990). This proteinase, termed 'cruzipain', is a dimer of subunits of 30-35 kDa molecular weight and has oligosaccarides attached that make up 10% of the total molecular weight (Cazzulo *et al.* 1990b). In spite of the fact that this proteinase activity seems to be contained within lysosomes (Bontempi *et al.* 1989, Cazzulo *et al.* 1990a) the oligosaccharide attatched does not contain mannose-6-phosphate residues, suggesting that *T. cruzi* and other trypanosomatids do not target lysosomal enzymes to organelles by the mannose-6-phosphate pathway. The cysteine proteinases of *T. brucei*, of which there are believed to be four (Robertson *et al.* 1990), also demonstrate homology to the cysteine proteinases from other organisms. Robertson and coworkers (1990) showed that the cysteine proteinases were similar to cathepsin L with respect to specificity for the artificial proteinase substrates, the peptidylaminomethylcoumarins. Analysis of the cDNA sequence of one cysteine proteinase of *T. brucei* demonstrated that the sequences for the pre, pro and central regions are very similar to cathepsin L, but there is a 108 amino acid extension which distinguishes it from mammalian cysteine proteinases, and may be involved in targetting the enzyme within the cell (Mottram *et al.* 1989). One 28 kDa activity was found to be more active in short stumpy forms, compared to long slender or procyclic forms in a pleomorphic strain, suggesting developmental regulation (Pamer *et al.* 1989). As in other trypanosomatids, these low molecular weight cysteine proteinases are

probably contained within lysosomes (Lonsdale-Eccles and Grab 1987).

As well as major intracellular proteinase activities, trypanosomatids also have surface activities. On the surface of *L. major* promastigotes is a protein known as gp63, which is anchored to the plasmalemma by a glycolipid anchor (McKerrow 1989). This protein is the major surface protein of *L. major* promastigotes with approximately 500000 molecules reported on the surface of a cell (McKerrow 1989). Etges and coworkers (1986) reported that gp63 has a proteinase activity, and have since characterised the protein as a zinc metalloproteinase (Bouvier *et al.* 1989). It retains its activity in the soluble form, suggesting that the enzyme may be involved in amastigote survival or, when bound to the promastigote, may cleave the host complement factor C3b to C3bi, inducing binding of the parasite to the macrophage surface (Etges *et al.* 1986, Bordier 1987). The former suggestion has since been disproved by Heumann and coworkers (1989), who found that while both the soluble and the membrane-bound enzyme interacted with α–2 macroglobulin, a natural proteinase inhibitor found in the plasma, the soluble form was rendered inactive, whereas the membrane-bound form was unaffected, demonstrating that only the membrane-bound enzyme could be active in the host.

A similar surface proteinase activity occurs in 15 species of *Leishmania,* including *L. donovani* and *L. mexicana*, as well as in *Crithidia* sp. (Coombs *et al.* 1987, Lockwood *et al.* 1987, Bouvier *et al.* 1989) and in *T. cruzi* (Piras *et al.* 1985, Greig and Ashall 1990). In the latter organism, this is a 60 kDa metallopeptidase activity associated with the membrane which is conserved between strains (Greig and Ashall 1990) and like the PSP of *L. major* may be involved in the adhesion to and infectivity of *T. cruzi* in host cells (Piras *et al.* 1985, Russell and Wilhelm 1986).

1.3.3 Glycosomes

These organelles were first discovered in *T. brucei* (Opperdoes and Borst 1977) and have since been found in *Crithidia* (Taylor *et al.* 1980, Michels 1989), *Leishmania* (Coombs *et al.* 1982, Hart and Opperdoes 1984, Mottram 1984), *Phytomonas* (Michels 1989), *T. cruzi* (Taylor *et al.* 1980, Cannata *et al.* 1982) and the bodonid flagellate *Trypanoplasma borelli* (Opperdoes *et al.* 1988, Michels 1989), suggesting that the glycosome is another feature unique to the Kinetoplastida. These organelles have been separated, after cell lysis using silicon carbide (Opperdoes and Borst 1977) or alumina (Mottram 1984), using a combination of differential centrifugation and isopycnic centrifugation (Mottram 1984, Opperdoes and Borst 1977) and have been purified to near homogeneity (Opperdoes *et al.* 1984) (See section 1.1 for details). Investigations have led them to be classified as microbodies along with peroxisomes and glyoxysomes, due to:

i) their morphology (Fairlamb and Opperdoes 1986),

ii) the presence of catalase in the glycosomes of *C. luciliae* and *T. borelli* (Michels 1989. Opperdoes *et al.* 1988),

iii) the presence of a β-oxidation pathway (Opperdoes 1984),

iv) the fact that the enzymes are synthesised on free ribosomes (Clayton 1987, Hart *et al.* 1987).

In the first investigation using sucrose gradients to separate cell organelles of bloodstream forms of *T. brucei* (Opperdoes and Borst 1977), it was found that the first seven enzymes of glycolysis [HK, PGI, PFK, aldolase (ALDO), triosephosphate isomerase (TIM), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK)] and two enzymes

of glycerol metabolism [glycerol kinase (GK), glycerol-3-phosphate dehydrogenase)] were associated with a particulate fraction. Similar results have subsequently been reported for other trypanosomatids (Taylor *et al.* 1980, Cannata *et al.* 1982, Hart and Opperdoes 1984, Opperdoes 1987, Michels 1989).

Another pathway associated with the glycosomes is CO_2 fixation. Glycosomal PEPCK forms ATP and oxaloacetate from PEP, ADP and CO_2 and the NADH generated in glycolysis is reoxidised by the glycosomal MDH in the procyclic forms of *T. brucei* (Opperdoes and Cottem 1982, Broman *et al.* 1983). PEPCK has also been found to be associated with the glycosomes of *T. cruzi* epimastigotes (Cannata *et al.* 1982), *Leishmania* promastigotes (Hart and Opperdoes 1984, Mottram 1984) and *Crithidia* (Cannata *et al.* 1982). Of these, only *T. cruzi* has been shown to have MDH associated with the glycosomes (Cannata *et al.* 1982) although evidence suggests that the case is similar for *Leishmania* (Mottram 1984). Two key enzymes of ether-lipid biosynthesis, DHAP acyltransferase and acylDHAP reductase, are also present in the glycosomes of procyclic forms of *T. brucei* (Opperdoes 1984) and in *Leishmania* promastigotes (Hart and Opperdoes 1984). Also present are acyl CoA synthetase (Opperdoes 1987) and acyl CoA reductase (Opperdoes 1984). These are involved in converting long chain fatty acids into the corresponding alcohols which are believed to be substrates for ether-lipid synthesis (Opperdoes 1984, 1987). The presence of GK, GPDH, TIM and acyl CoA synthetase also suggest that the glycosomes of *T. brucei* procyclic forms contain the complete pathway for the glycerol-ether lipid synthesis.

Adenylate kinase is present as an integral membrane protein of the glycosomes of *T. brucei* (McLaughlin 1985, Opperdoes 1987) and is also present in *Leishmania* (Hart and Opperdoes 1984). This enzyme is believed

to function as a regulator of ATP/ADP levels in the glycosome to prevent glycolysis running down due to the lack of ATP.

Also present in in the glycosome of *T. cruzi, Leishmania, Crithidia* and *T. brucei* are orotate phosphoribosyltransferase and orotidine-5'-phosphate decarboxylase, the last two enzymes of the pyrimidine biosynthetic pathway (Hammond and Gutteridge 1980, Hammond *et al.* 1981, Hammond and Gutteridge 1982) and hypoxanthine-guanine phosphoribosyltransferase, in *L. mexicana, T. cruzi* and *T. brucei,* which is involved in the salvage of purine bases (Gutteridge and Davies 1982, Hammond *et al.* 1985, Hassan *et al.* 1985).

Glycosomal contents alter with the stage of life cycle of *T. brucei* (Figures 10 and 11). In the long slender bloodstream form glucose is converted to 3-phosphoglycerate (3-PGA) and glycerol-3-phosphate (G-3-P) in the glycosome. Under aerobic conditions the following reactions are catalysed:

Glucose + 2DHAP + $2P_i$ ----> 2(G-3-P + 3-PGA)

Glycerol + 2DHAP + $2P_i$ ---> 2G-3-P + 3-PGA

where the consumption and production of NADH and ATP are balanced (Opperdoes and Borst 1977). Net ATP synthesis occurs in the cytosol where the 3-PGA is converted to pyruvate, which is excreted (See section 1.3.2.1). The G-3-P is oxidised to DHAP by a mitochondrial *sn*-glycerol-3-phosphate oxidase (Opperdoes et al. 1977) (See section 1.3.2.3.4). Under anaerobic conditions glucose is catabolised to equimolar amounts of pyruvate and glycerol (Hammond and Bowman 1980, Opperdoes 1987). There is a net ATP synthesis as the glycerol and ATP are formed from G-3-P and ADP by GK in the glycosome. It is envisaged that the high activity of GK in bloodstream

forms, 10-fold higher than in procyclic forms, (Broman *et al.* 1983, Krakow and Wang 1990) together with the high activities of ATP utilising enzymes in the glycosomes allow efficient production of ATP and glycerol, although there is no evidence of channelling of ATP between GK and HK or PFK (Hammond *et al.* 1985).

In the procyclic forms there is a general decrease of the glycolytic pathway within the glycosome (Opperdoes *et al.* 1981) and PGK is found almost exclusively in the cytosol (Misset and Opperdoes 1987). A decrease in the activity of the DHAP:G-3-P shuttle is linked with the appearances of MDH and PEPCK in the glycosomes i.e. the CO_2 fixation pathway (Opperdoes *et al.* 1981, Opperdoes and Cottem 1982). As explained above MDH in the glycosome regenerates NAD produced by glycolysis (Broman *et al.* 1983) while PEPCK regenerates ATP in the glycosome. Thus in the bloodstream forms these enzymes serve the same functions as GPDH and PGK, respectively(Opperdoes and Cottem 1982), so together they help maintain both the redox and the energy balances.

The exact nature of the transportation of the various glycolytic intermediates over the glycosomal membrane has yet to be fully elucidated. Evidence, to date, suggests that in the bloodstream form of *T. brucei* the glycosomal membrane is relatively impermeable to substrates for enzyme activity (Visser and Opperdoes 1980) and that there is no equilibration between the cytosol and the glycosomes, except for G-3-P, the substrate for the mitochondrial glycerol-3-phosphate oxidase (Visser *et al.* 1981). It is believed that there are specific translocators for G-3-P and hexose monophosphates (Nwagwu and Opperdoes 1980, Visser *et al.* 1981) which allow interchange of intermediates between the glycosomes and the mitochondrion or pentose phosphate shunt, respectively. The glycosomal

membrane of the procyclic forms is permeable to small molecules regardless of charge e.g. ATP, PEP, NAD, (Patthey and Deshusses 1987) suggesting that not only do the contents of the glycosomes alter with cell type but that the properties of the membrane also alter, suggesting that the membrane may play a role in the regulation of glycolysis.

When glycosomes are lysed, the glycolytic enzymes, apart from PGI, appear as a multienzyme complex in *T. brucei* and *L. m. mexicana* (Oduro *et al.* 1980, Mottram 1984), which can be dissociated into its constituent enzymes by 0.15M sodium chloride (Oduro *et al.* 1980, Mottram 1984). There is, however, no evidence for substrate channelling (Aman and Wang 1985, Aman *et al.* 1986), but rather the high local concentrations of substrate (mM) and enzymes in the glycosome allow an efficient glycolytic flux (Misset *et al.* 1987).

Great interest has been shown in how the glycosomal proteins are translocated across the membrane. In *T. brucei*, enzymes are synthesised on free polysomes in the cytosol (Clayton 1987, Hart *et al.* 1987) and imported into the glycosomes within 5 minutes without any post-translational processing. The results of Hart and coworkers (1987) suggest that ALDO, GAPDH and GPDH have half-lives in the glycosomes of only 30 minutes, while in the bloodstream forms the half-life of ALDO was at least 3 hours (Clayton 1987). However, Clayton (1988) has since questioned this difference in protein stability between forms, finding that proteins of the bloodstream and procyclic forms are equally stable.

Studies on the glycosomal proteins and their genes have revealed differences between them and their iosofunctional enzymes in other organisms. These differences may be related to glycosomal import and/or the multienzyme complex. Misset and coworkers found that the isoelectric

points of 8 of the 9 glycolytic enzymes found in bloodstream forms of *T. brucei,* PGI excepted, were greater than those for corresponding enzymes from other organisms. The higher isoelectric point is probably due to the presence of a relatively greater number of positively charged amino acids in the primary structure. In the case of GAPDH, the glycosomal form (GAPDHg) is 4-5 kDa larger than the cytosolic form (GAPDHc) (MIchels *et al.* 1986, Misset *et al.* 1987, Opperdoes 1987) due to a C-terminus extension and short amino acid insertions, containing positive amino acids. The cytosolic and glycosomal isoenzymes of PGK also differ in that PGKg is 2 kDa larger, due to a C-terminus extension, and that the PGKg has several positive amino acid substitutions compared to PGKc (Osinga *et al.* 1985). ALDO, on the other hand, has an N-terminus extension (Clayton 1985) and TIM does not differ in size at all compared to isofunctional enzymes from other organisms but, like GAPDHg and PGKg, does have two clusters of positive amino acids, termed 'hotspots' (Misset *et al.* 1986, Swinkels *et al.* 1986).

Wierenga and coworkers (1987a) modelled the primary structure of TIM, PGKg, and GAPDHg onto the three-dimensional structure of the corresponding enzymes from other organisms revealing hotspots, clusters of positive amino acids, 40Å apart. This has been confirmed for TIM where the three-dimensional structure was determined at 2.4Å revealing two hotspots 39Å apart on the same subunit, while it was observed that the distance between any other two hotspots was no greater than 45Å (Wierenga et al. 1987b). It was proposed that these hotspots may be important in the import of the enzymes into the glycosomes. However evidence recently put forward would suggest that while the hotspots may have a role in the formation of the glycosomes, they are certainly not essential to import. The GAPDHg in epimastigotes of *T. cruzi* lacks one

hotspot and three-dimensional modelling confirms that the 40Å separation of hotspots is not essential (Kendall *et al.* 1990). Alexander and coworkers (1990) found that a juxtaposition of an acidic residue with hotspot 1 did not prevent import of PGKg in to the glycosomes of *T. brucei,* while the presence of hotspot 2 on PGKc did not cause import. On the other hand, the C-terminus was found to be very well conserved between alleles of PGK. These data suggest that import is much more complicated than a case where only hotspots are essential for uptake. They may well be only involved in the assembly of the multienzyme complex. With respect to import, Aman and Wang (1987) suggested that two integral proteins of glycosomal membranes in *T. brucei* procyclic forms may be involved in protein translocation.

As stated above, glycosomes are believed to be related to other microbodies. Therefore, elucidating the mechanism of glycosome assembly may be aided by looking at how other microbodies are formed. For import of firefly luciferase into peroxisomes, the targeting signal is a 12 amino acid section at the extreme C-terminus (Gould *et al.* 1987). Perhaps, therefore, it is the C-terminus extensions of GAPDHg and PGKg that are important for glycosomal import. It has been found that the import of proteins into the peroxisome is perhaps facilitated by ATP hydrolysis (Imanaka *et al.* 1987), while other evidence suggests that a membrane potential is important (Belliona and Goodman 1987, Borst 1989). This may be formed by a protein translocating ATPase in the peroxisomal membrane (Douma *et al.* 1989). In the case of glycosomes, while there is no external requirement for ATP in order to import PGKg into the glycosomes of *T. brucei* bloodstream forms, it has been suggested that a source of ATP, which may be hydrolysed in order to provide energy, may be supplied by the high concentrations (20-30 μM) inside the glycosomes (Sommer *et al.* 1990).

As can be seen, much has still to be learned about the subject of the glycosomal biochemistry and molecular biology, but they have many unique properties which could make them prime targets for chemotherapeutic attack.

1.4 Aims of the project

The overall aim of this project was to investigate various aspects of the energy metabolism of *H. muscarum* and *H. ingenoplastis* in order to explain the many differences between the two organisms with respect to cell biology, biochemistry and growth and how these may relate to the organisms' natural environment. One aim was to discover whether *H. muscarum* and *H. ingenoplastis* were able to utilise glucose from their surroundings and, if so, how the glucose was catabolised and whether any differences existed between the pathways operating in the two species. A second aim was to find out how gaseous conditions affected glucose catabolism and a third aim was to determine whether pyruvate kinase played an important role in controlling glucose catabolism in *H. ingenoplastis.* As an aside, preliminary work was done on the excretion of proteinases into the environment, with the aim of finding if alternative substrates to glucose may also be utilised.

2.0 MATERIALS AND METHODS

2.1 Cells

2.1.1 Maintenance and cultivation

Herpetomonas muscarum (ATCC 30260) was routinely grown in 5 ml LIT (complete) medium (Carmargo *et al.* 1964) supplemented with 5 % (v/v) heat inactivated foetal calf serum (HIFCS) and 25 μg ml^{-1} gentamycin sulphate at 25°C in sealed 30 ml universals using air as the gas phase. *Herpetomonas ingenoplastis* (ATCC 30269) was routinely grown in LIT medium supplemented with HIFCS and gentamycin sulphate as above at 25°C in sealed 30 ml universals with a minimum gas (air) phase. Both cultures were sub-passaged every 3-4 days when at late-log/early-stationary phase of growth.

2.1.2 Harvesting of the cells

For experimental work *H. muscarum* and *H. ingenoplastis* were grown in 500 ml medi-flats containing 250 ml and 500 ml, respectively, LIT medium supplemented with 5 % (v/v) HIFCS and 25 μg ml^{-1} gentamycin sulphate. The cultures were inoculated with cells to give a starting density of $1x10^5$ cells ml^{-1} and were harvested 3 days later when *H. muscarum* had reached a density of $2\text{-}2.5x10^5$ cells ml^{-1} and *H. ingenoplastis*, $8\text{-}9.5x10^6$ cells ml^{-1}. The cells were harvested and washed by centrifugation at 2100g for 10 minutes at 4°C in an MSE Chilspin, unless otherwise indicated. The number of washes and the medium used depended upon the study undertaken, details of which will be given in the appropriate sections.

2.2 Enzyme analysis

2.2.1 Preparation of cell homogenates for enzyme assays

Cells used in the enzyme assays were washed twice in 0.25 M sucrose, pelleted and used immediately or stored at -70^{o}C until required. Activities were measured using homogenates, prepared by resuspending the pellets in 0.25 M sucrose containing 0.1 % Triton X-100 on ice and leaving for 10 minutes before use, or soluble extracts, prepared by centrifuging the homogenates for 2 minutes at 10000g in an Eppendorf microfuge, the resulting supernatant being used in the assays.

2.2.2 Enzyme assays

All the enzyme assays were performed on a Phillips PU8700 UV/visible spectrophotometer incorporating a temperature controlled cell holder. All the assays were carried out in a total volume of 1 ml, at 25^{o}C and 340 nm, unless specified otherwise. The following assays based on the methods quoted were used:

Hexokinase was assayed according to the method of Martin and coworkers (1976) in a reaction mixture containing 100 mM Tris-HCl pH 7.3, 6.6mM $MgCl_2$, 0.66 mM EDTA, 6.6 mM glucose, 0.33 mM NADP, 0.7 units yeast glucose-6-phosphate dehydrogenase and homogenate. The reaction was initiated with 1 mM ATP.

Phosphoglucoseisomerase (Martin *et al.* 1976) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 0.66 mM NADP, 3.3 mM fructose-6-phosphate and 0.7 units yeast glucose-6-phosphate dehydrogenase. The reaction was initiated with homogenate.

Phosphofructokinase (Mukkada *et al.* 1974) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 7.3, 6.6 mM $MgCl_2$, 0.1 mM NADH, 3.3 mM fructose-6-phosphate, 0.2 units rabbit muscle aldolase, 2 units triose-phosphate isomerase/glycerophosphate dehydrogenase and homogenate. The reaction was initiated with 0.4 mM ATP.

Phosphoglycerate kinase (Bergmeyer 1974) was assayed in a reaction mixture containing 100 mM triethanolamine (TEA) buffer pH 7.5, 2 mM $MgSO_4$, 0.6 mM ATP, 2.6 mM glycerol-3-phosphate, 0.1 mM NADH and 2.5 units yeast glyceraldehyde-3-phosphate dehydrogenase. The reaction was initiated with homogenate.

Pyruvate kinase (Mottram 1984) was assayed in a reaction mixture containing 100 mM TEA buffer pH 7.5, 4 mM $MgCl_2$, 0.5 mM NADH, 75 mM KCl, 4 mM phosphoenolpyruvate, 18 units rabbit muscle L-lactate dehydrogenase and homogenate. The reaction was initiated with 3 mM ADP.

Glucose-6-phosphate dehydrogenase (Mottram 1984) was assayed in a reaction mixture containing 100 mM TEA buffer pH 7.5, 0.33 mM NADP, 13.3 mM EDTA and homogenate. The reaction was initiated with 2 mM glucose-6-phosphate.

6-Phosphogluconate dehydrogenase (Martin *et al.* 1976) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 7.3, 2 mM 6-phosphogluconate and 0.33 mM NADP. The reaction was initiated with homogenate.

Pyruvate decarboxylase (Cazzulo and De Cazzulo 1985) was assayed in a reaction mixture containing 100 mM potassium phosphate buffer pH 7.0, 3.3 mM $MgCl_2$, 6.7 mM pyruvate, 0.33 mM NADH, 0.5 mM thiamine pyrophosphate and 10 units alcohol dehydrogenase. The reaction was initiated with homogenate.

Alcohol dehydrogenase was assayed in both directions. Ethanol oxidation (Wornick 1975) was assayed in a reaction mixture containg 50 mM glycine-NaOH pH 10, 0.67 mM NAD and homogenate. The reaction was initiated with 1.2 % (v/v) ethanol. Aldehyde reduction (Cazzulo and De Cazzulo 1985) was assayed in a reaction mixture containing 100 mM potassium phosphate buffer pH 7.0, 0.3 mM NADH and homogenate. The reaction was initiated with 0.67 mM acetaldehyde. Before adding the homogenate to the reaction mixture, it was preincubated with 1 mM $MnCl_2$ for 10 minutes at 25^{o}C to stimulate the alcohol dehydrogenase activity.

Pyruvate dehydrogenase (Martin *et al.* 1976) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 3.5 mM NAD, 0.35 mM thiamine pyrophosphate, 1.1 mM coenzyme A, 3.5 mM L-cysteine and homogenate. The reaction was initiated with 7 mM pyruvate.

α-Ketoglutarate dehydrogenase (Martin *et al.* 1976) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 3.5 mM NAD, 0.35 mM thiamine pyrophosphate, 1.1 mM coenzyme A, 3.5 mM L-cysteine and homogenate. The reaction was initiated with 7 mM α–ketoglutarate.

α-Ketobutyrate dehydrogenase was assayed by modifying the assay for α-ketoglutarate dehydrogenase (Martin *et al.* 1976). The reaction mixture contained 100 mM Tris-HCl pH 8.0, 1.5 mM $MgCl_2$, 3.5 mM NAD, 0.35 mM thiamine pyrophosphate, 1.1 mM coenzyme A, 3.5 mM L-cysteine and homogenate. The reaction was initiated with 7 mM α-ketobutyrate.

Phosphoenolpyruvate carboxykinase (Bentle and Lardy 1976) was assayed in a reaction mixture containing 100 mM imidazole buffer pH 6.6, 4 mM

$CoCl_2$, 0.15 mM NADH, 4 mM phosphoenolpyruvate, 40 mM $NaHCO_3$, 15 units bovine malate dehydrogenase and homogenate. The reaction was initiated with 1 mM ATP.

'Malic' enzyme (Mottram 1984) was assayed in a reaction mixture containing 100 mM TEA pH 7.5, 3.6 mM $MnSO_4$, 0.91 mM NADP and homogenate. The reaction was initiated with 3.6 mM malate.

Citrate synthase (Adroher *et al.* 1988) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 0.1 mM 5-5'-dithiobis-(2-nitrobenzoate), 0.3 mM acetyl CoA and homogenate. The reaction was initated with 0.5 mM fresh oxaloacetate. The formation of the mercapton ion was followed at 412 nm.

Aconitase (Racker 1950) was assayed in a reaction mixture containing 50 mM potassium phosphate/30 mM citrate buffer pH 7.0. The reaction was initiated with cell-free lysate and the formation of cis-aconitate was followed at 210 nm.

Isocitrate dehydrogenase (Martin *et al.* 1976) was assayed for both NAD- and NADP-dependent activity in a reaction mixture containing 100 mM TEA pH 7.5, 4 mM $MnSO_4$, 1.6 mM NAD or 0.3 mM NADP and homogenate. The reaction was initiated with 6.7 mM isocitrate.

Succinate thiokinase (Cha 1972) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 50 mM succinate, 10 mM $MgCl_2$, 0.2 mM GTP amd 0.1 mM coenzyme A. The reaction was initiated with soluble extract and was followed at 235 nm.

Succinyl CoA synthase (Bridger *et al.* 1972) was assayed in a reaction mixture containing 100 mM Tris-HCl pH 8.0, 50 mM succinate, 10 mM $MgCl_2$, 100 mM KCl, 0.4 mM ATP and 0.1 mM coenzyme A. The reaction was initiated with soluble extract and was followed at 235 nm.

Succinate dehydrogenase (Martin *et al.* 1976) was assayed in a reaction

mixture containing 100 mM sodium phosphate buffer pH 7.3, 10 mM KCN, 15 mM succinate and 1 mM potassium ferricyanide. The reaction was initiated with homogenate and was followed at 420 nm.

Fumarate reductase(Mottram 1984) was assayed in a reaction mixture containing 100 mM sodium phosphate buffer pH 7.3, 2.5 mM fumarate and 0.45 mM NADH. The reaction was initiated with homogenate.

Fumarase (Racker 1950) was assayed in a reaction mixture containing 100 mM potassium phosphate buffer pH 7.0 and 20 mM malate. The reaction was initiated with soluble extract and was followed at 210 nm.

Malate dehydrogenase (Martin *et al.* 1976) was assayed in a reaction mixture containing 100 mM TEA pH 7.5, 0.2 mM NADH, and homogenate. The reaction was initiated with 2 mM malate.

Isocitrate lyase (Mottram 1984) was assayed in a reaction mixture containing 100 mM imidazole buffer pH 6.6, 6.7 mM $MgSO_4$, 0.67 mM EDTA, 3.3 mM isocitrate and 3.3 mM phenylhydrazine hydrochloride. The reaction was initiated with soluble extract and was followed at 324 nm.

Glutamate dehydrogenase (Mottram 1984) was assayed in a reaction mixture containing 100 mM TEA pH 8.0, 0.5 mM NADH, 320 mM $(NH_4)_2SO_4$ and homogenate. The reaction was initiated with 6.6 mM α-ketoglutarate.

Alanine aminotransferase (Mottram 1984) was assayed in a reaction mixture containing 100 mM potassium phosphate buffer pH 7.0, 0.5 mM NADH, 15 mM alanine, 3.6 units L-lactate dehydrogenase and homogenate. The reaction was initiated with 6.6 mM α-ketoglutarate.

NADH: methyl viologen oxidoreductase and pyruvate:methyl viologen oxidoreductase (Thong and Coombs 1987) were assayed at 37^{o}C and the reactions were followed at 600 nm, under anaerobic conditions - achieved by

bubbling argon through the reaction mixture, which was contained within a 2 mm path-length cell sealed with a subaseal, for 2-3 minutes. The reaction mixture for NADH: methyl viologen oxidoreductase, in a final volume of 0.53 ml, contained 100 mM sodium phosphate buffer pH 6.5, 9.4 mM NADH, 250 mM 2-mercaptoethanol and 20 mM methyl viologen. The reaction mixture for pyruvate: methyl viologen oxidoreductase, also in a final volume of 0.53 ml, contained 100 mM sodium phosphate pH 6.5, 2.5 mM pyruvate, 0.25 mM coenzyme A, 20 mM methyl viologen and 250 mM 2-mercaptoethanol. The reaction mixtures were initiated with homogenate.

Proteinase (Hunter 1989) was assayed using benzoyl-prolyl-phenylalanyl-*p*-nitroanilide (BzPFR) as the proteinase substrate and monitoring the release of *p*-nitroaniline at 405 nm in a reaction mixture containing 100 mM sodium phosphate buffer pH 6.0, 0.08 mM BzPFR and 8.3 mM dithiothreitol. The reaction was initiated with homogenate.

2.2.3 Limits of enzyme assays

All the assays used were tested to determine their range of reliability and to check that substrate concentrations were not limiting. In some cases changes were made to the original methods if these were found to improve the detectable activities of the enzymes.

2.3 Protein determinations

Protein concentrations were determined using the method of Sedmak and Grossberg (1977). A 0.06% solution of Coomassie Brilliant Blue G250 was prepared in 0.3 M perchloric acid. The solution was stirred for over 60 minutes and filtered through a Whatman No. 1 filter paper. Stock solutions required dilution with 0.3 M perchloric acid to give absorbances of 1.3-1.5 absorbance

units at 465 nm. A 1 ml aliquot of the reaction mixture was added to an equal volume of the protein sample (diluted with distilled, deionised water), mixed and the absorbances determined at 620 and 465 nm. Absorbances for a blank of 1 ml of reagent plus 1 ml of distilled, deionised water were determined at the same time. Calculations were as follows:

$$(A_{620\ nm}/A_{465\ nm})_{sample} - (A_{620\ nm}/A_{465\ nm})_{blank}$$

and the results were compared with a standard curve prepared with 0-50 μg of bovine serum albumin.

2.4 Metabolite analysis

2.4.1 Long-term incubations

Cultures comprised 10 ml LIT medium supplemented with HIFCS and gentamycin sulphate (see section 2.1.2) in 30 ml universals fitted with subaseals. Prior to inoculation of the medium with the cells, to a starting density of $1x10^5$cells ml^{-1}, the medium was vigorously bubbled with argon for 5 minutes in order to obtain anaerobic conditions. Aerobic conditions were obtained by leaving the cultures with a normal atmospheric gas phase. After inoculation, the gas phase of the inoculated culture was gassed for a further 5 minutes. Whenever samples were removed for analysis (using a syringe and needle to pierce the subaseal) the cell densities were determined and the gas phase was gassed for a further 5 minutes in order to maintain anaerobic conditions. Before analysis, cells were removed from the medium samples by centrifugation at 10000g for 5 minutes, in an Eppendorf microfuge, and the

supernatent was stored at -70^{o}C or analysed immediately.

2.4.2 Short-term incubations

2.4.2.1 Harvesting of cells

The cells, which had been grown in LIT medium supplemented with HIFCS and gentamycin sulphate (see section 2.1.2), were harvested at late-log/early-stationary phase and were washed by centrifugation at 2100g for 10 minutes at 18^{o}C in an MSE Chilspin. The cells were washed three times in Hanks balanced salts solution (HBSS) containing 10 mM HEPES pH 7.2 and glucose to a final concentration of 10 mM (HB7.2).

2.4.2.2 Incubation conditions

The cells were incubated in 30 ml glass universals, in 10 ml of medium, under four different gaseous conditions which were aerobic, anaerobic, aerobic plus CO_2, and anaerobic plus CO_2. The former two cultures were carried out using HB7.2, and the latter two being carried out with HBSS containing 10 mM Hepes, 10 mM sodium bicarbonate, pH 7.2, and glucose to a final concentration of 10 mM ($HBCO_2$). Aerobic conditions were maintained by stoppering the universal with cotton wool to allow free gaseous exchange between the incubation vessel and the atmosphere. Anaerobic, anaerobic plus CO_2, and aerobic plus CO_2 conditions were obtained in universals sealed with suba-seals by bubbling the relevant medium vigorously for 5 minutes with argon, a 95% air/5% CO_2 mixture or a 95% nitrogen/5% CO_2 mixture, respectively. Prior

to addition of the cells, to a density of $2x10^8$ cells ml^{-1}, the media were equilibrated to 25°C. The incubations were carried out in a shaking water bath at 25°C, the vessels were gently agitated and the gas phases of the incubation vessels were gently purged with the relevant gases (see above). Samples were taken at 20 minute intervals, the cells removed by centrifugation at 10000g for 5 minutes at 18°C in an Eppendorf microfuge, and the supernatants removed for analysis. During the incubations, cells were frequently checked for motility and integrity.

2.4.3 Analysis using enzyme-based assays

2.4.3.1 Preparation of extracts

In order to assay metabolites produced and consumed during growth of cells in LIT medium, protein, which may have interfered with the assays, was removed by the following method:

i) equal volumes of the cell-free LIT medium and 1 M ice-cold perchloric acid were mixed and left for 10 minutes on ice,

ii) the mixture was centrifuged at 10000g for 10 minutes in an Eppendorf microfuge,

iii) the resulting supernateant was mixed (per ml) with 110 µl of 5 M potassium hydroxide, and left on ice for 15 minutes, before being centrifuged for 5 minutes at 10000g to remove the potassium chlorate precipitate formed.

Aliquots of the final supernatants were used in the enzyme-based metabolite assays.

2.4.3.2 Enzyme-based assays

All the assays were performed in a total of 1.5 ml, and the incubations were carried out at 37^{o}C, until the reaction was complete, whereupon absorbances were read at 340 nm on a Phillips PU 8700 UV/visible light spectrophotometer. Concentrations were calculated by comparing with controls, taking the molar extinction coefficient of NAD(P)H to be 6220 $M^{-1}cm^{-1}$.

Glucose (Kunst *et al.* 1984) was assayed in a reaction mixture containing 66 mM TEA pH 7.5, 3.3 mM $MgCl_2$, 2.3 mM ATP, 2.3 mM NADP and sample. The reaction was initiated with a mixture of hexokinase (5 units) and glucose-6-phosphate dehydrogenase (10 units). Absorbances were taken 90 minutes later.

Pyruvate (Lamprecht and Heinz 1984) was assayed in a reaction mixture containing 100 mM TEA pH 7.5, 1.2 mM EDTA, 0.33 mM NADH and sample. The reaction was initiated wirth 1.5 units of L-lactate dehydrogenase. Absorbances were taken 60 minutes later.

D-Lactate (Gawehn 1984) was assayed in a reaction mixture containing 30 mM L-glutamate/225 mM glycylglycine buffer pH 10, 3.3 mM NAD, 10 units alanine aminotransferase and sample. The reaction was initiated with 11 units of D-lactate dehydrogenase. Absorbances were taken 60 minutes later.

L-Lactate (Noll 1984) was assayed in a reaction mixture containing 160 mM L-glutamate buffer pH 8.9, 1 mM NAD, 9 units alanine aminotransferase and sample. The reaction was initiated with 7 units of L-lactate dehydrogenase. Absorbances were taken 90 minutes later.

Ammonia (Tabor 1970) was assayed in a reaction containing 100 mM potassium phosphate pH 7.0, 16.7 mM α-ketoglutarate, 0.33 mM NADH and 2 units of glutamate dehydrogenase. The reaction was initiated with sample.

Absorbances were taken 30 minutes later.

Ethanol (Cornell 1983) was assayed in a reaction mixture containing 300 mM Tris/200 mM L-lysine-KOH buffer pH 9.7, 1.6 mM NAD and sample. The reaction was initiated with 10 units of alcohol dehydrogenase. Absorbances were taken 15 minutes later.

Glycerol (Wieland 1984) was assayed in a reaction mixture containing 130 mM glycine/660 mM hydrazine buffer pH 9.8, 1.6 mM $MgCl_2$, 1.4 mM ATP 0.66 mM NAD, 10.5 units α-glycerophospate dehydrogenase and sample. The reaction was intiated with 1 unit of glycerol kinase. The absorbances were taken 90 minutes later.

2.4.4 High Performance Liquid Chromatography (HPLC)

2.4.4.1 Preparation of extracts

Samples from both short-term incubations and long-term growth experiments were treated in the following manner for analysis of short-chain fatty acids (SCFAs) using isocratic HPLC. All the solvents and samples used were either filtered through 0.22 μm filters (Millipore) or were of Aristar, or equivalent grade. SCFAs were extracted from media into 0.1 N NaOH, using a modification of the method of Guerrant and coworkers (1982).

i) 0.2 ml of the sample was mixed with 40 μl 18 N H_2SO_4, 1 ml of diethyl ether and 0.12 g of NaCl for 1 minute on a vortex mixer;

ii) the mixture was centrifuged for 1 minute at 10000g in an Eppendorf microcentrifuge to partition the aqueous and organic phases; 0.85 ml of the organic phase was removed;

iii) 0.1 ml of 0.1 N NaOH was added to the organic phase and mixed on a vortex

mixer for 1 minute;

iv) the mixture was again centrifuged for 1 minute at 10000g, and the organic supernatant discarded;

v) the extract was left to stand for 10 minutes in a fume cupboard, to allow any residual organic residue to evaporate.

2.4.4.2 Analysis using the Polypore H column

Aliquots,20 µl, of the extracted samples were injected onto a Polypore H column , which was maintained at 70°C, with 0.01 M H_2SO_4 as the mobile phase. The flow rate was 0.3 ml min^{-1} and the elution profile was monitored for 23 minutes at 210 nm, by which time all the SCFAs were found to be eluted. The SCFAs were identified by comparing the retention times of peaks with those of known standards.

2.5 Studies on the subcellular location of enzymes

2.5.1 Particulate and soluble activities

The distribution of succinate dehydrogenase, fumarate reductase and glucose-6-phosphate dehydrogenase between soluble and particulate fractions was determined using a modification of the method of Boveris and coworkers (1986). Cells, suspended in ice-cold 0.25 M sucrose, were lysed by three cycles of freezing, at -70°C, followed by thawing, at 25°C, and passing the suspension three times through a 0.26G needle after each freeze-thaw cycle. An aliquot of the lysate was removed for later analysis, and the remaining lysate was centrifuged at 10000g for 5 minutes in an Eppendorf microfuge. The

supernatant was removed and the pellet resuspended in ice-cold 0.25 M sucrose, before use in enzyme analysis.

2.5.2 Fractionation by differential centrifugation

2.5.2.1 Cell lysis

Cell lysis was performed on ice. Cells were harvested, as described in section 2.1.2, and were washed twice in 0.25 M sucrose. Cells were resuspended in a minimum amount of ice-cold breakage buffer (0.25 M sucrose, 25 mM Tris-HCl pH 7.5, 1 mM EDTA) in a porcelain mortar. Acid washed and defined alumina was gradually added and stirred in using a glass rod, forming a creamy paste. When over 95% of the cells were lysed (as adjudged by microscopic observation), the paste was resuspended in 4-5 volumes of breakage buffer. The alumina was removed from the lysate by centrifugation at 180g for 5 minutes in an MSE Chilspin, and the supernatant taken as the homogenate.

2.5.2.2 Differential centrifugation

All centrifugation steps were performed at $4^{o}C$. The homogenate was centrifuged at 2100g for 10 minutes, to give pellet 1 (P1). The resulting supernatant was centrifuged at 16000g for 10 minutes to give pellet 2 (P2). The supernatant removed from P2 was centrifuged at 240000g for 60 minutes to give pellet 3 (P3) and the supernatant (S). All the pellets were resuspended in 1 ml of breakage buffer. The fractions were used immediately and were assayed in the presence of 0.1% Triton X-100, to ensure organelle lysis.

2.6 Proteinases

2.6.1 pH profile of total cellular proteinase activity

Proteinase activity was determined using the artificial substrate, BzPFR. The pH profile was determined using homogenates, prepared by the method outlined in section 2.2.1. The buffers used were 100 mM sodium acetate buffer pH 5.0, 100 mM sodium phosphate buffer pH 6.0-7.0 and 100 mM Tris-HCl pH 8.0-8.9.

2.6.2 Gelatin gel analysis of extracellular activities

2.6.2.1 Gelatin-SDS-PAGE preparation

Samples of the medium were collected and cells removed by centrifugation at 2100g for 10 minutes in an MSE Chilspin. The supernatants were filtered through a nitrocellulose filter (Millipore) with a 0.45 μm pore size to remove any remaining cells. The lack of cells was confirmed by microscopic observation.

Samples were then diluted 1:1 with sample buffer (0.125 M Tris-HCl pH 6.8, 4 mg ml^{-1} bromophenol blue, 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 20% (v/v) glycerol) before being loaded onto a SDS discontinuous buffer system (Lockwood 1987) using a Bio-Rad Mini-protean II system. The stacking gel contained 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 3.75% (w/v) acrylamide, 0.1% (w/v) bis-acrylamide, 0.075% (w/v) ammonium persulphate and TEMED at 5 μl/10 ml of stacking gel mix. The resolving gel contained 375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.2% (w/v) gelatin, 7.5% (w/v) acrylamide, 0.2% (w/v) bis-acrylamide, 0.075% (w/v) ammonium persulphate and TEMED,

at 5 µl/10 ml of resolving gel mix. Electrophoresis was carried out over 2 hours at 15 mA/gel.

2.6.2.2 Proteinase staining

After electrophoresis, the gels were incubated for 20 minutes in 500 ml of 2.5% (v/v) Triton X-100 to remove SDS, allowing the activation of proteinase activity. The proteinase bands were developed in buffer for 18 hours, at 25°C. The standard incubation buffer used, unless otherwise stated, was 100 mM sodium acetate buffer pH 5.5, containing 1mM DTT. The bands were visualised in a stain containing 0.05% (w/v) PAGE blue 83, 25% (v/v) isopropanol and 10% (v/v) acetic acid for 1 hour, after which the gel was destained in 10% (v/v) acetic acid. Molecular weights of individual proteinases were determined by comparing their relative mobilities with the relative mobilities of protein standards (high molecular weight standard mixture SDS-6H, Sigma Chemical Co., Ltd.).

2.6.2.3 pH profile of extracellular activities

In order to obtain a pH profile of the extracellular activities, slices of SDS-PAGE gels were incubated in a range of buffers (see section 2.6.1), after incubation in 0.1% (v/v) Triton X-100 for 20 minutes to restore proteinase activity.

2.7 Purification and characterisation of pyruvate kinase of *H. ingenoplastis*

2.7.1 Modifying the pyruvate kinase assay

The assay outlined in section 2.2.2 was modified in order to make the elution profiles of pyruvate kinase from FPLC easier to follow. The activity of pyruvate kinase was measured with various concentrations of the activator fructose-1.6-bisphosphate added to the enzyme assay, in order to find the optimal concentration of the activator to use routinely in the assay during pyruvate kinase purification. This modified assay was not used during the characterisation of the purified enzyme, in which the assay used was that outlined in section 2.2.2.

2.7.2 Solubilization of the particulate enzymes

Approximately $7x10^8$ *H. ingenoplastis* cells were lysed in 20 mM TEA pH 7.3, containing 0.1% Triton X-100, at 4^oC, and were then centrifuged at 10000g for 5 minutes in an Eppendorf microfuge. The resulting pellet was resuspended in 20mM TEA pH7.3 and aliquots exposed to several concentrations of NaCl, and left on ice for 10 minutes. The lysates were then centrifuged at 10000g for 5 minutes and the supernatants removed. The pellets were resuspended in 20 mM TEA pH 7.3, and both the pellets and the supernatants assayed for various enzyme activities.

2.7.3 Enzyme purification

Pyruvate kinase purification was carried out using FPLC (Fast Protein Liquid Chromatography) (Pharmacia, Sweden) with the advantages over

normal column chromatography of fast elution times and operation at room temperature.

2.7.3.1 Cell lysis and preparation

Approximately $4x10^9$ cells were lysed by incubation in 20 mM TEA pH 7.3, containing 0.1% Triton X-100, at 4^oC. The homogenate (H) formed was centrifuged at 10000g for 5 minutes in an Eppendorf microfuge, and the resulting supernatant (S1) was filtered through a 0.22 μm nitrocellulose filter (Millipore) before applying to an anion exchange column.

2.7.3.2 Anion exchange chromatography

The filtered S1 was loaded onto a MonoQ anion exchange column (Pharmacia, bed volume 1 ml) which had been pre-equilibrated with buffer A (20 mM TEA pH 7.3). Unbound protein was eluted at a flow rate of 1.0 ml min^{-1}, with 2 ml of buffer A, and the bound protein was then eluted with the following gradient of NaCl, in buffer A:

0.0-2.0 minutes, 0 mM NaCl;

2.0-8.5 minutes, 0-120 mM NaCl;

8.5-23.5 minutes, 120-140 mM NaCl;

23.5-23.6 minutes, 140-1000 mM NaCl;

23.6-27.0 minutes, 1000 mM NaCl;

27.0-27.1 minutes, 1000-0 mM NaCl;

27.1-31.0 minutes, 0 mM NaCl.

The eluant was collected in 1 ml fractions, and those containing pyruvate kinase activity were pooled to form supernatant 2 (S2), which was applied to a gel

filtration column.

2.7.3.3 Gel filtration chromatography

The S2 fraction was applied to a Superose 6 gel filtration column (Pharmacia, bed volume 25 ml) which had been pre-equlibrated with buffer B (20m mM TEA pH 7.3, 1 mM EDTA, 5 mM 2-mercaptoethanol). The column was eluted with buffer B at a flow rate of 0.5 ml min^{-1}, and 1 ml fractions were collected. The pooled pyruvate kinase fractions from the gel filtration step, supernatent 3 (S3), were used in all subsequent characterisation experiments.

2.7.4 Polyacrylamide gel electrophoresis

Samples were diluted with water and mixed with an equal volume of sample buffer (see section 2.6.2.1) and boiled for 1 minute before being run on an SDS-discontinuous polyacrylamide gel system (Haimes 1981) using a Bio-Rad Mini-Protean II system. The stacking gel was formed as in section 2.6.2.1, while the resolving gel contained 375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 7.5% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 0.075% (w/v) ammonium persulphate, and TEMED at 5 µl per 10 ml of resolving gel mix. Electrophoresis was carried out at 15 mA per gel, for 2 hours.

2.7.4.1 Staining for protein

Protein bands were resolved by immersing the gel in stain (see section 2.6.2.2) for 30 minutes, before destaining with 10% acetic acid. Molecular weights were determined by comparing their relative mobilities to those of protein standards (high molecular weight standard mixture SDS-6H, Sigma Chemical Co., Ltd.)

2.7.5 pH profile of pyruvate kinase activity

The activity of the partially-purified pyruvate kinase, in S3, was determined in the pH range 6.0-9.0. The buffers used were 100 mM imidazole buffer pH 6.0-7.5 and 100 mM TEA pH 7.0-9.0.

2.7.6 Enzyme kinetics

Partially-purified pyruvate kinase was assayed as outlined in section 2.2.2. Activity was measured at a variety of KCl, $MgCl_2$, ADP and PEP concentrations. The K_m and V_{max} values for ADP were calculated using an Eadie-Hoftsee plot (Engel 1981) and determined using linear regression analysis of plots of velocity versus velocity/ADP concentration, for 5 concentrations of ADP.

2.8 Materials

All chemicals and enzymes, unless otherwise stated, were obtained form Sigma Chemical Co.,Ltd., Poole, Dorset, England.

Foetal calf serum was obtained from Gibco-Biocult, Paisley, Scotland.

Tryptose was obtained from Oxoid Ltd., Basingstoke, Hampshire, England.

Liver infusion broth was purchased from Difco Laboratories, Michigan, U.S.A.

Sodium acetate and NaCl were obtained from Formachem (Research International), Strathaven, Scotland.

Aristar grade H_2SO_4, SDS, NaOH, $MgCl_2$, $NaHCO_3$, NaH_2PO_4 and Na_2HPO_4 were otained from BDH Chemicals Ltd.,Poole, Dorset, England.

Glacial acetic acid was obtained from May and Baker Ltd., Dagenham,

England.

Triethanolamine was purchased from The Boehringer Corporation (London) Ltd., Lewes, East Sussex, England.

HPLC grade water was obtained from FSA Laboratory Supplies, Loughborough, England.

MonoQ and Superose 6 columns were obtained from Pharmacia Fine Chemicals Ltd., Milton Keynes, England.

Gilson HPLC equipment was obtained from Anachem (Scotland).

3.0 RESULTS

3.1 Glucose catabolism

3.1.1 Glycolysis and the pentose phosphate shunt

The specific activities of selected enzymes of glycolysis and the pentose phosphate shunt, in *H. muscarum* and *H. ingenoplastis,* are given in Table 3. The first three enzymes of glycolysis, hexokinase, phosphoglucoseisomerase and phosphofructokinase were found to be at very high specific activities in both organisms. At much lower activities were the enzymes 3-phosphoglycerate kinase, pyruvate kinase and the first two enzymes of the pentose phosphate shunt, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The activies of all the enzymes were similar in both *H. muscarum* and *H. ingenoplastis.*

3.1.2 Glucose consumption

The results in Tables 4 and 5 show that glucose was consumed by both *H. muscarum* and *H. ingenoplastis* during growth in complex medium under both aerobic and anaerobic conditions. After 72 hours in culture, approximately 90% of the glucose was consumed by *H. muscarum* and approximately 70% in the case of *H. ingenoplastis. Herpetomonas muscarum* produced ethanol as a major end product under both aerobic and anaerobic conditions, and also seemed to consume the small amount of glycerol present in the medium. *H. ingenoplastis* also produced ethanol as a major end product (Table 5), but in this case small amounts of glycerol were excreted. Neither *H. ingenoplastis* nor *H. muscarum* produced detectable levels of D-lactate, L-lactate or pyruvate. Enzymatic assays also reavealed that ammonia was not excreted by either organism (limit of detection 50 μM).

Table 3.

Comparison of the activities of selected enzymes of glycolysis and the pentose phosphate shunt in *H. muscarum* and *H. ingenoplastis*. The results are in nmoles min^{-1} (mg protein)$^{-1}$ and are the means (±SD) from the number of experiments in parentheses.

	H. muscarum	*H. ingenoplastis*
Hexokinase	782 ± 211 (7)	906 ± 305 (7)
Phosphoglucoseisomerase	1226 ± 288 (6)	1618 ± 405 (6)
Phosphofructokinase	887 ± 364 (5)	1038 ± 431 (5)
3-Phosphoglycerate kinase	47 ± 18 (5)	92 ± 67 (5)
Pyruvate kinase	43 ± 12 (3)	150 ± 55 (3)
Glucose-6-phosphate dehydrogenase	183 ± 84 (7)	203 ± 95 (7)
6-Phosphogluconate dehydrogenase	73 ± 35 (4)	69 ± 23 (4)

Table 4.

Growth, glucose consumption and the excretion of end products by *H. muscarum* incubated under different conditions. Concentrations of metabolites are given in mM, and the cell densities as the number of cells $x10^6$(ml culture)$^{-1}$. The concentrations of the metabolites were obtained using the enzyme-based assays. Figures given are the means (±SD) from the number of experiments in parentheses, or from single experiments.

	0 hours	72 hours	
		Aerobic	Anaerobic
Glucose	4.4 ± 0.2 (3)	0.6 ± 0.2 (2)	0.4 ± 0.0 (2)
D-Lactate	0.23	0.23	0.23
L-Lactate	0.45	0.47	0.25
Pyruvate	0.13	0.15	0.14
Ethanol	0.8 ± 0.4 (2)	2.6 ± 0.1 (2)	2.8 ± 0.0 (2)
Glycerol	0.3 ± 0.0 (2)	<0.2	<0.2
Cell No.	0.1	36 ± 10 (3)	27 ± 8.0 (3)

Table 5.

Growth, glucose consumption and the excretion of end products by *H. ingenoplastis* incubated under different conditions. Concentrations of metabolites are given in mM, and the cell densities as the number of cells $x10^6$(ml culture)$^{-1}$. The concentrations of metabolites were obtained using enzyme-based assays. Figures given are the means(±SD) from the number of experiments in parentheses, or from single experiments.

	0 hours	72 hours	
		Aerobic	Anaerobic
Glucose	3.9 ± 0.4 (3)	1.2 ± 0.4 (3)	1.1 ± 0.6 (3)
D-Lactate	0.25	0.21	0.29
L-Lactate	0.41	0.37	0.42
Pyruvate	0.15	0.17	0.14
Ethanol	0.8 ± 0.4 (3)	4.5 ± 1.0 (3)	4.4 ± 0.9 (3)
Glycerol	0.3 ± 0.1 (3)	0.6 ± 0.1 (3)	0.6 ± 0.0 (3)
Cell No.	0.1	3.2 ± 0.4 (3)	2.9 ± 0.9 (3)

3.1.3 Cell growth and catabolism

There was a correlation between the growth of the organisms, glucose consumption and ethanol production (Figures 12a-d). Glucose was consumed and ethanol produced most rapidly during the log phase of growth. The results in figures 12a and 12b show how *H. muscarum* grew faster under aerobic conditions than anaerobic conditions with a doubling time of 11 hours over the first 30 hours. Stationary phase was reached at approximately 60 hours under aerobic conditions and 80 hours under anaerobic conditions (doubling time 16 hours). *Herpetomonas ingenoplastis* grew similarly under both aerobic and anaerobic condition, reaching stationary phase by 80 hours (Figures 12c and 12d), with a doubling time of 18 hours over the first 60 hours. The results in Figures 12a-d confirm those in Tables 4 and 5, which show that ethanol is produced in greater amounts by *H. ingenoplastis* than by *H. muscarum.*

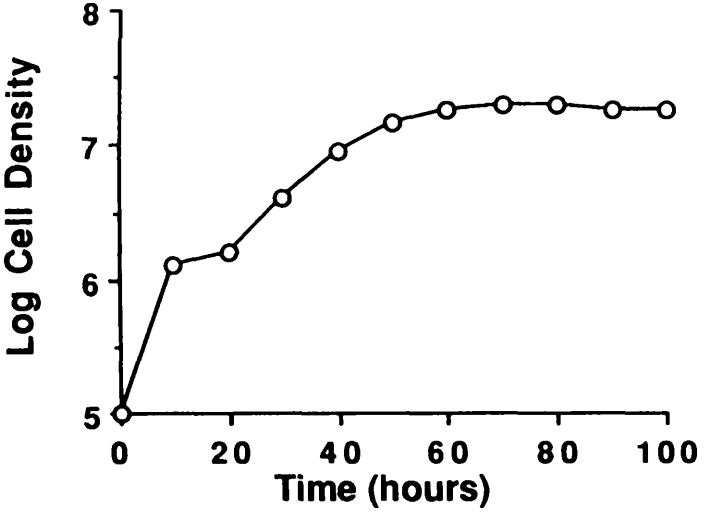
3.1.4 Short-term incubations

It could not be ascertained from the results for growth of the organisms in complex medium whether or not ethanol is produced from glucose. Experiments were therefore carried out using buffered Hanks balanced salts solution containing 10 mM glucose as the only carbon source. Initial results showed that the best cell concentration for these short-term incubations was $2\text{-}3\text{x}10^8$ cells ml^{-1} in that all the cells remained motile and glucose consumption was approximately linear over the 60 minute incubation. Typical results (Figures 13a-d) show that ethanol was produced during incubation of both *H. muscarum* and *H. ingenoplastis*, and that, as during growth in complex medium, ethanol was produced in greater concentrations by *H. ingenoplastis* under aerobic incubation conditions than under anaerobic conditions, 23 nmoles

Figures 12a-d

Growth (○) glucose consumption (●) and ethanol production (□) by *H. muscarum* (a and b) and *H. ingenoplastis* (c and d) under aerobic and anaerobic conditions, respectively. Cells were grown in 10 ml of LIT medium containing 5% (v/v) HIFCS and 25μg ml^{-1} gentamycin sulphate in 30 ml universals. Aerobic cultures were obtained by leaving the cultures with a normal atmospheric gas phase, while anaerobic conditions were obtained by bubbling the medium vigorously with argon for 5 minutes prior to inoculation. The data shown are representative of three experiments in which samples were removed at 10 hour intervals. After centrifugation, to remove cells, and deproteinisation of the samples, they were assayed in duplicate for glucose and ethanol. The cell densities are given as $\log_{10}$ of the cell number (ml culture)$^{-1}$ and the concentrations of the metabolites, ethanol and glucose, are in mM.

Figure 12a



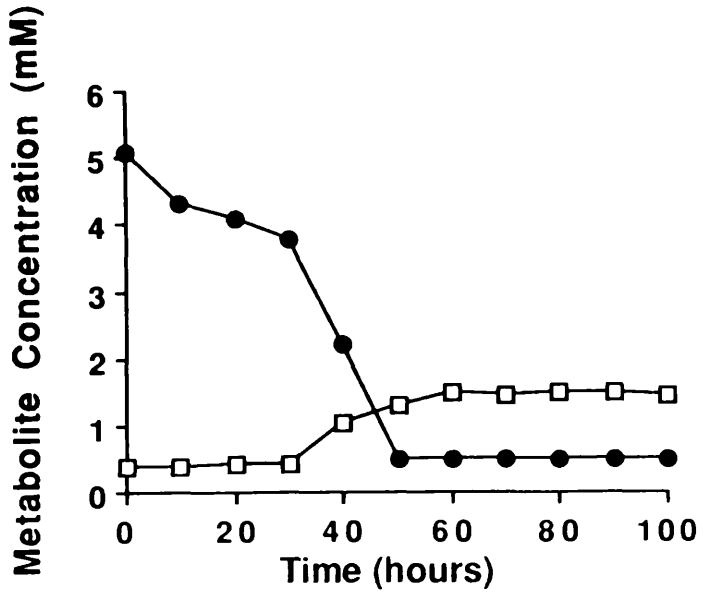
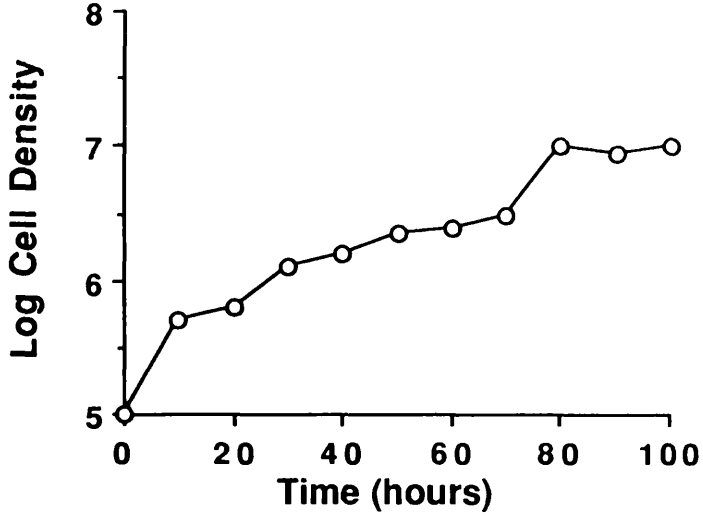


Figure 12b



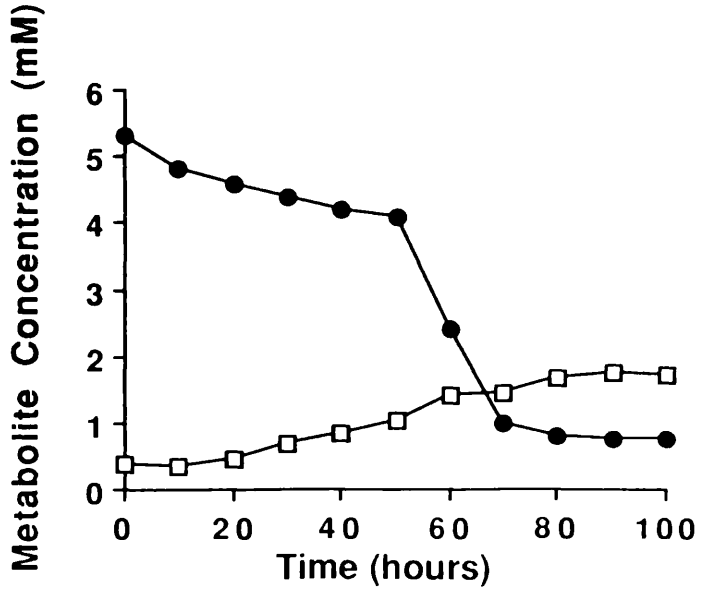
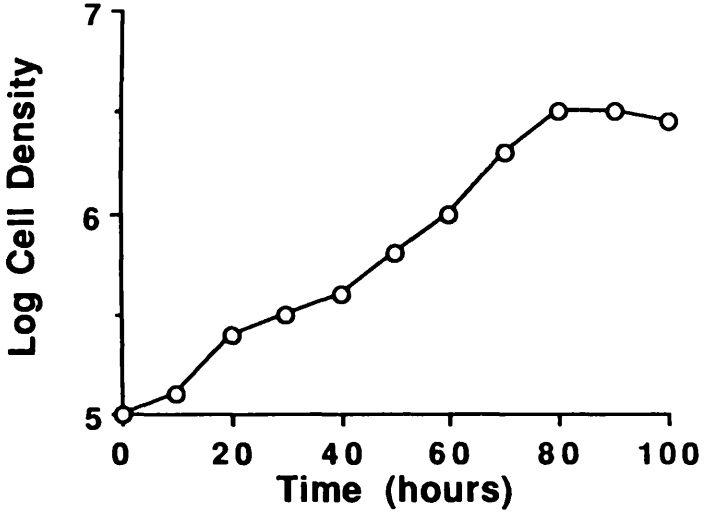


Figure 12c



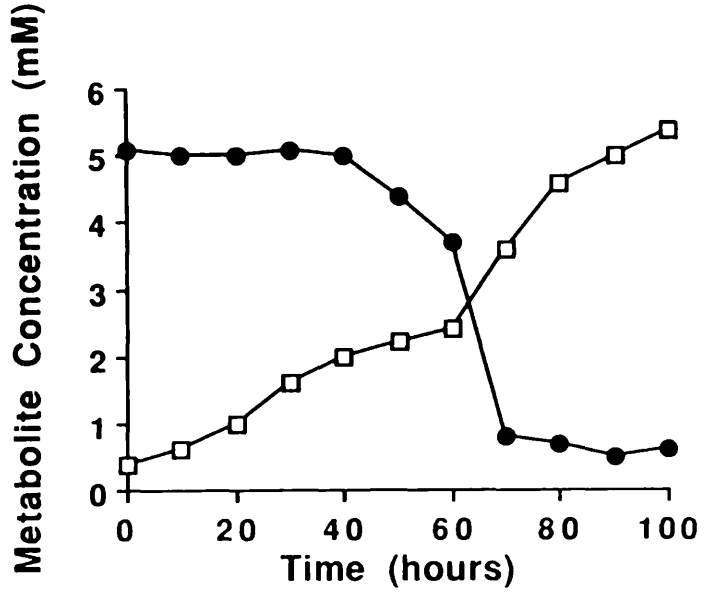
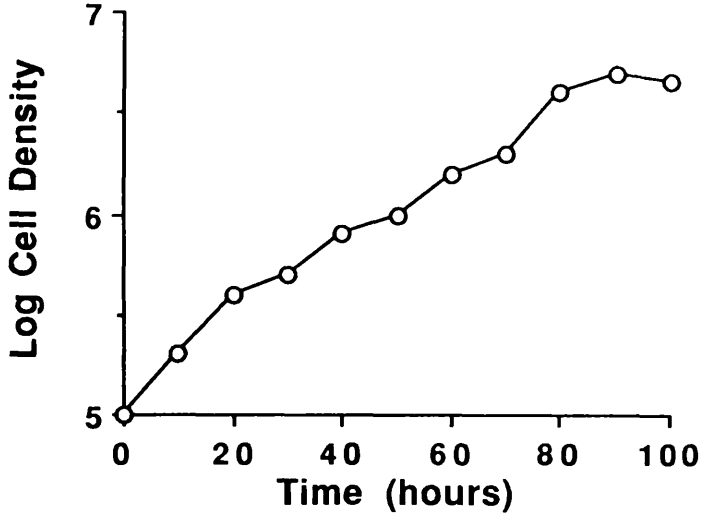
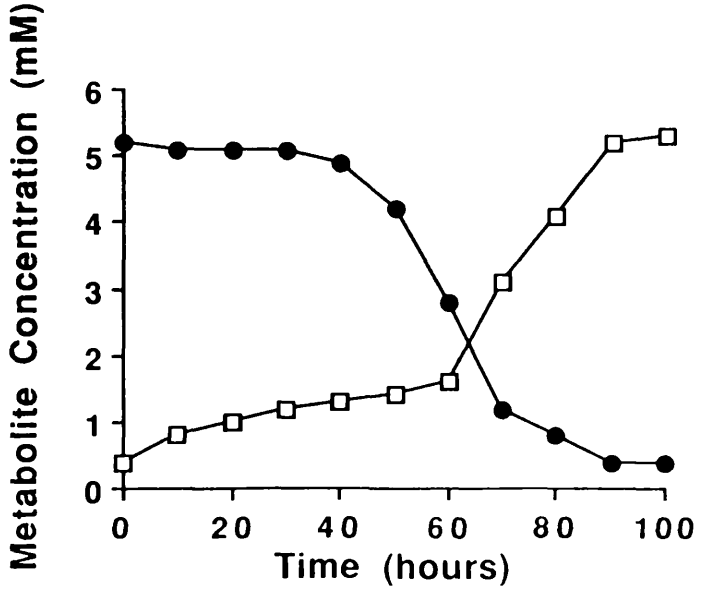


Figure 12d





Figures 13a-d.

Glucose consumption (o) and ethanol production (●) during a 60 minute incubation at 25^{o}C of *H. muscarum* (a and b) and *H. ingenoplastis* (c and d) under aerobic and anaerobic conditions, respectively. Cells were incubated at 2-3x10^{8} cells ml^{-1} in Hanks balanced salts solution containing 10 mM glucose and 10 mM Hepes, pH 7.2. Samples were taken at 20 minute intervals, microscopically examined for cell motility and integrity, and centrifuged to remove cells, before the medium was assayed, using duplicate samples, for the presence of glucose and ethanol. The concentrations are given in mM and the results are representative of five experiments.

Figure 13a

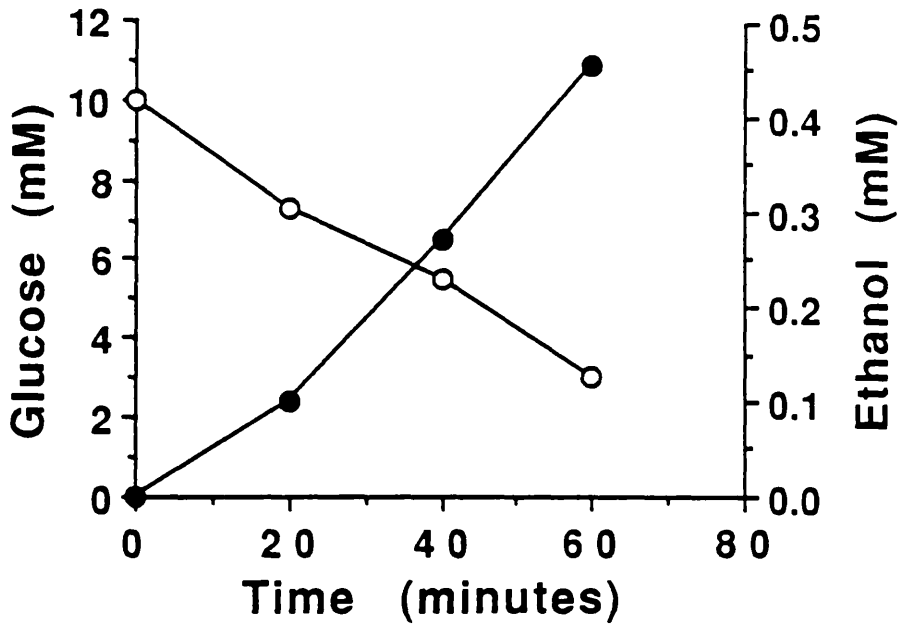


Figure 13b

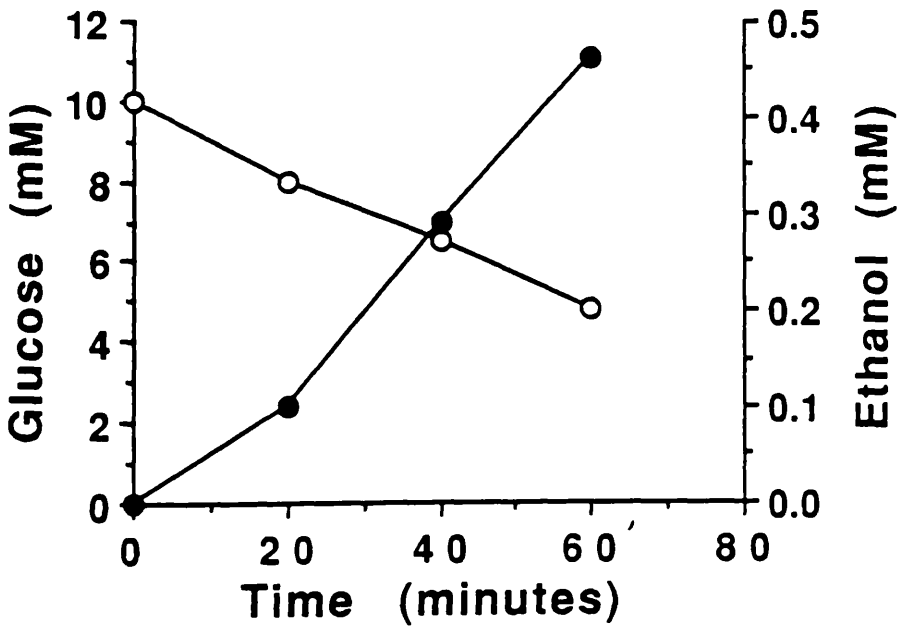


Figure 13c

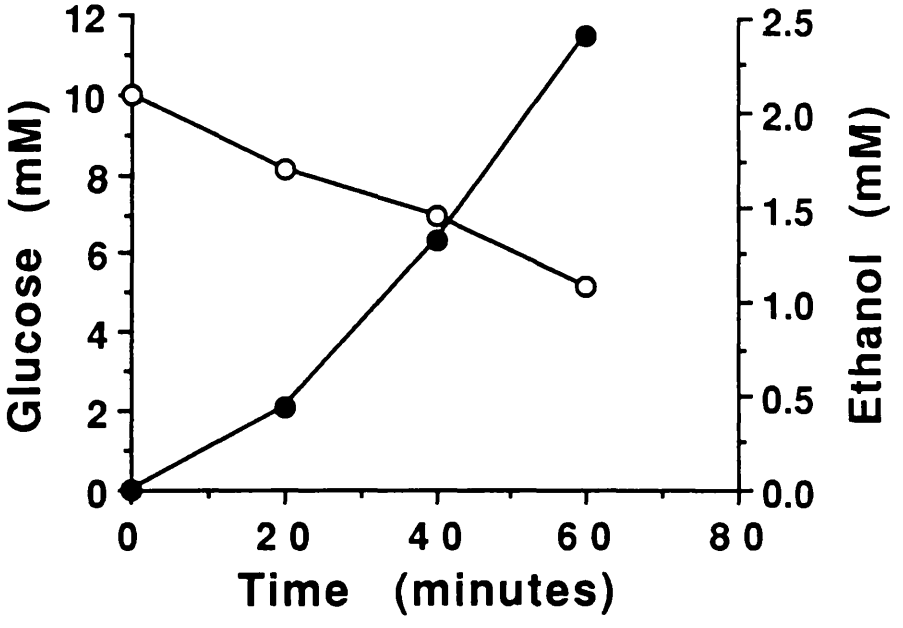
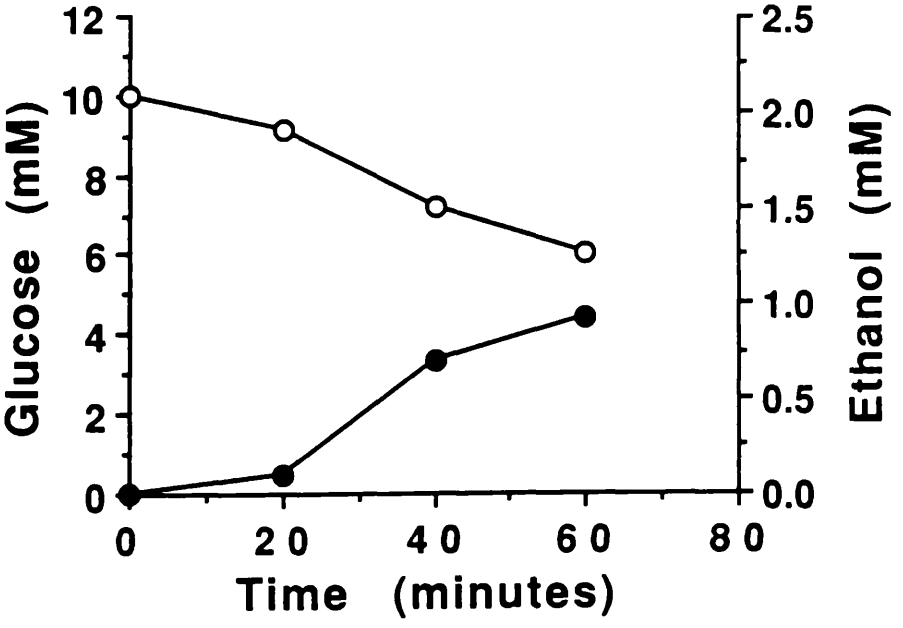


Figure 13d



min^{-1} (mg protein)$^{-1}$ and 9 nmoles min^{-1} (mg protein)$^{-1}$, respectively (Figures 13c and 13d).

3.1.5 HPLC analysis

In order to establish whether organic short-chain fatty acids (SCFA's) were produced by either organism, media samples were analysed using isocratic HPLC with a Polypore H column, and 0.01 M H_2SO_4 as the fluid phase. Table 6 shows the retention times of 20 SCFA's in this procedure. The results show that there was very little coelution of the acids. The only acids that coeluted were the two isomers of lactic acid. All 20 SCFA's were shown to have very reproducible and specific retention times.

In order to quantify organic acid production, a reproducible procedure of extraction of the organic acids was required. The method developed was based upon that described by Guerrant and coworkers (1982) and involved:

a) extraction of SCFA's from the media to chloroform, under high salt, low pH conditions;

b) extraction of SCFA's from the chloroform to sodium hydroxide. In this form it was injected onto the column.

A potential problem that I envisaged was the evaporation of chloroform and consequent reduction in the extraction efficiency. The extraction procedure was compared at 4^{o}C and room temperature. It was found that reducing the temperature did not alter the percentage extraction.

It was found that each acid had a specific a specific extraction percentage, and that this differed considerably from acid to acid (Table 7). For instance, only 21 $\pm$ 3% of the L-lactate was exracted from water, whereas both propionate and α-ketobutyrate were extracted to over 90%. In order to ensure

Table 6.

Retention times of organic acids used to calibrate the polypore H column. Results are in minutes and are the means (±SD) of the number of experiments shown in parentheses.

Organic acid	Retention time
L-Tartaric acid	5.51 ± 0.01 (9)
Malic acid	5.59 ± 0.03 (9)
Malonic acid	6.23 ± 0.04 (9)
Pyruvic acid	6.40 ± 0.05 (10)
Succinic acid	6.81 ± 0.02 (10)
α-Ketobutyric acid	7.04 ± 0.07 (9)
D-Lactic acid	7.59 ± 0.02 (10)
L-Lactic acid	7.60 ± 0.02 (10)
Fumaric acid	7.79 ± 0.02 (10)
Formic acid	8.25 ± 0.03 (9)
α–Ketoisocaproic acid	8.54 ± 0.02 (9)
Acetic acid	8.79 ± 0.01 (9)
Propionic acid	10.00 ± 0.02 (9)
Isobutyric acid	10.96 ± 0.01 (9)
n-Butyric acid	11.69 ± 0.03 (9)
Isovaleric acid	13.00 ± 0.04 (9)
p-Hydroxyphenylacetic acid	13.79 ± 0.05 (9)
n-Valeric acid	15.05 ± 0.05 (9)
Methylvaleric acid	17.90 ± 0.08 (9)
n-Caproic acid	20.05 ± 0.11 (9)

Table 7.

The extraction efficiencies of some organic acids. The results are the means (±SD) from three experiments. Organic acids, dissolved in HPLC grade water, were extracted, firstly into chloroform, and then into 0.1N NaOH. The original and the extracted organic acids were then injected onto a Polypore H column, using 0.01 M H_2SO_4 as the fluid phase, and the peak heights used to calculate the extraction efficiencies.

Organic acids	Percentage extraction
Pyruvic acid	64 ± 4
Succinic acid	50 ± 4
α-Ketobutyric acid	92 ± 5
L-Lactic acid	21 ± 3
Acetic acid	74 ± 4
Propionic acid	92 ± 8

Figures 14 a-c

These figures show the relationship between the concentration of short-chain fatty acids and the height of the peak eluted from the HPLC, as measured by absorbance at 210 nm.

Figure 14a

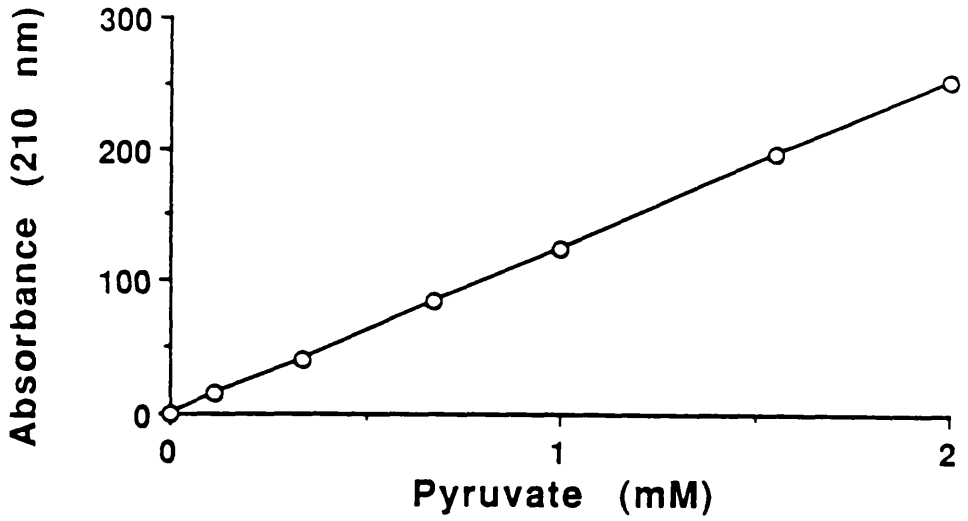


Figure 14b

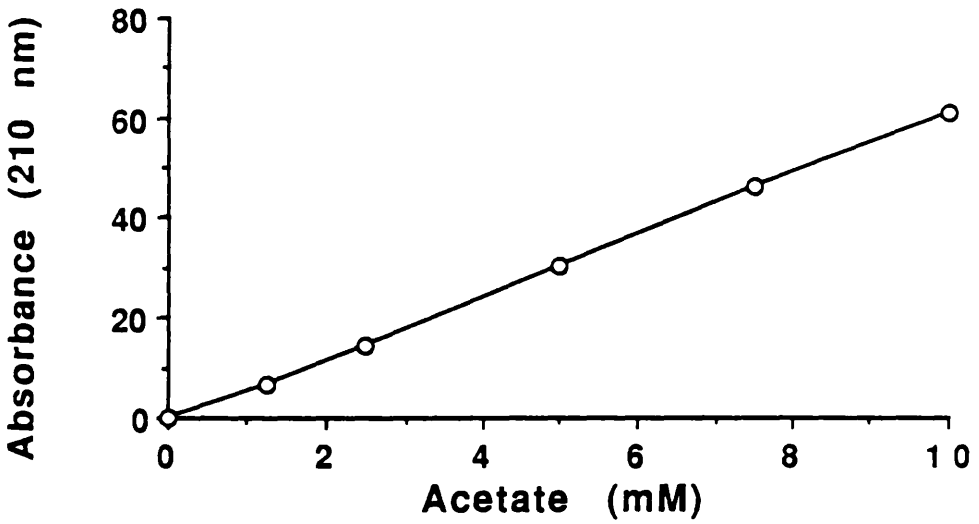
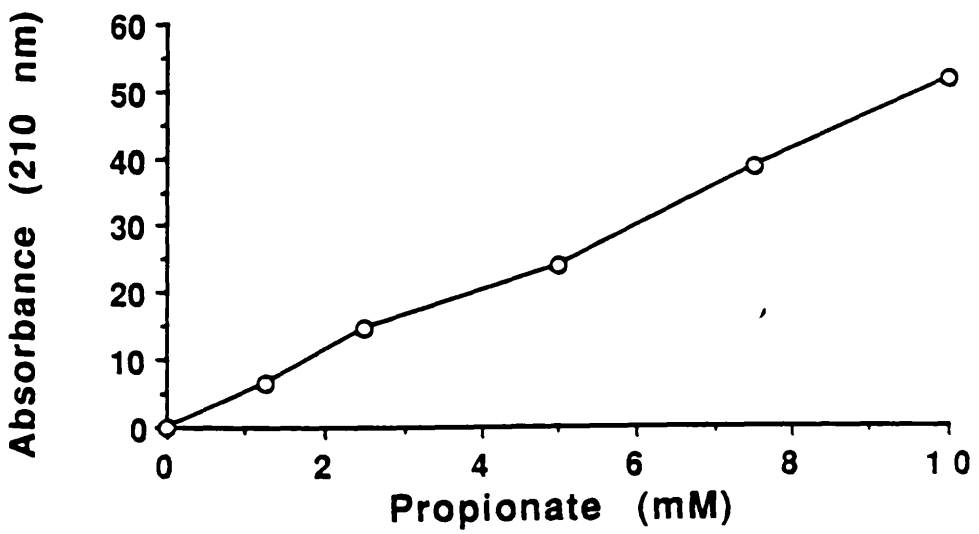


Figure 14c



accuracy during the quantification of SCFA production, two sets of standards containing succinate, acetate and propionate at 10 and 5 mM were routinely extracted in duplicate at the same time as the samples and the concentrations of the acids in the experimental samples calculated using the absorbances of the extracted standards for comparison.. As well as having different extraction efficiencies and retention times, the organic acids also absorbed light at 210 nm to different extents (Figures 14a-c). Whereas 20 µl of a 2 mM pyruvate solution has an absorbance of 250 mV (Figure 14a), 20 µl of a 10 mM propionate solution has an absorbance of 54 mV (Figure 14c). Figures 14a-c do show, however, that the peak height absorbances were directly proportional to the SCFA concentrations.

3.1.6 Organic acid production

Analysis of 72 hour cultures using the standard extraction procedure and the HPLC method developed showed that acetate, succinate and propionate were excreted into the medium by both *H. muscarum* and *H. ingenoplastis* (Table 8). Both organisms excreted acetate and propionate during the log phase of growth (Figures 15a-d), whereas succinate excretion was not detectable until stationary phase. While with *H. muscarum*, the production of the three SCFA's was similar under both aerobic and anaerobic conditions (Table 8, Figures 15a and 15b), *H. ingenoplastis* produced more propionate under anaerobic conditions than aerobic conditions (Table 8, Figures 15c and 15d).

Figure 16 shows examples of elution profiles of the organic acids extracted from LIT medium before (Figure16B and D) and after cell growth for 72 hours (Figures 16A and C). These figures showed that accurate quantification of SCFA's in complex medium was not possible using this method as the trailing edges and leading edges of the peaks overlapped. This was

Table 8.

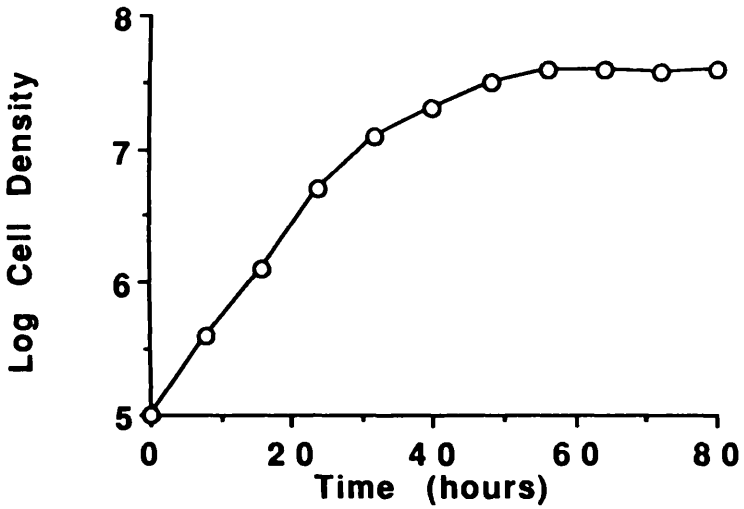
Excretion of organic acids by *H. muscarum* and *H, ingenoplastis* during growth for 72 hours in LIT medium. The products were analysed by isocratic HPLC on a Polypore H column. The figures for metabolite concentrations are in mM and are the means (±SD) of three experiments.

	H. muscarum		*H. ingenoplastis*	
	Aerobic	Anaerobic	Aerobic	Anaerobic
Acetate	5.06 ± 1.21	3.36 ± 1.03	2.90 ± 0.98	2.40 ± 0.46
Propionate	1.24 ± 0.53	0.96 ± 0.21	2.92 ± 0.82	3.92 ± 0.93
Succinate	3.45 ± 0.62	4.52 ± 1.34	2.50 ± 0.52	2.43 ± 0.60

Figures 15a-d

Production of short-chain fatty acids during the growth of *H. muscarum* (a and b) and *H. ingenoplastis* (c and d) under aerobic and anaerobic conditions, respectively. Cells were treated as Figure 12, and samples were removed at 8 hour intervals. Concentrations of succinate (□), propionate (■) and acetate (●) are in mM and the cell densities (○) are given as $\log_{10}$ of the cell number (ml culture)$^{-1}$.

Figure 15a



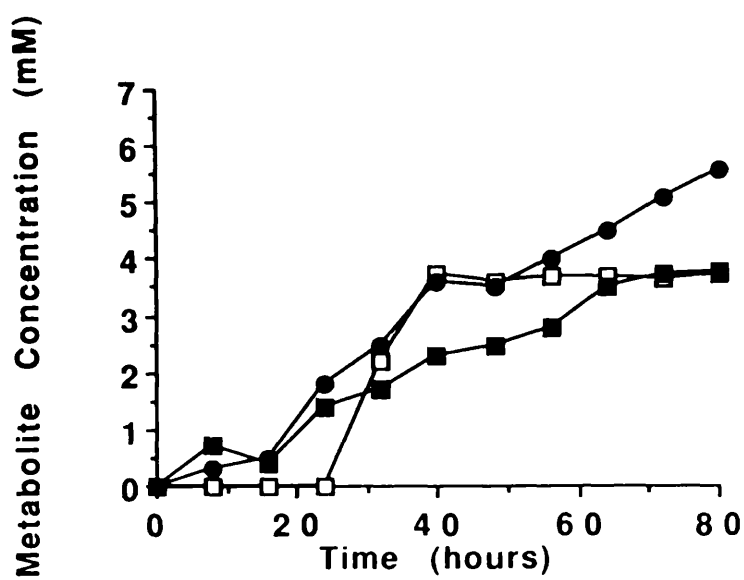
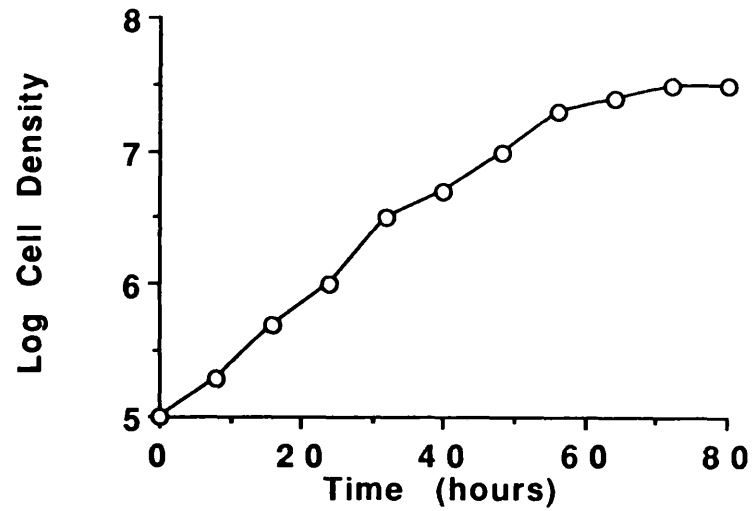


Figure 15b



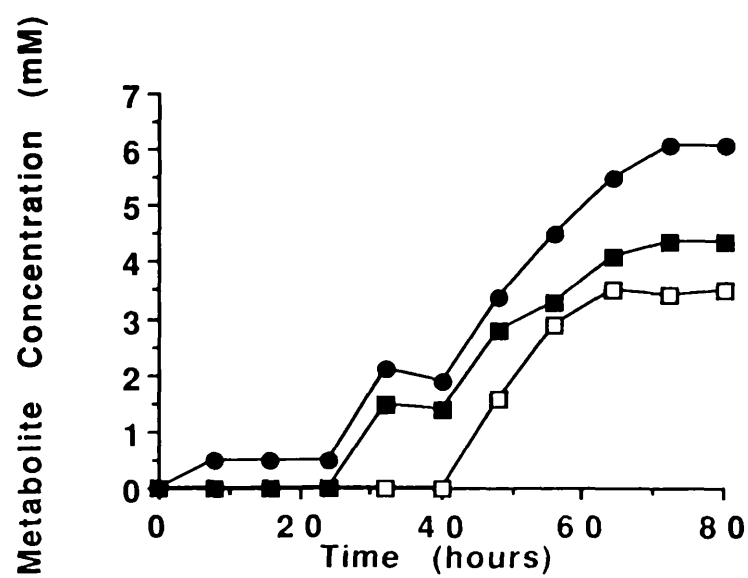
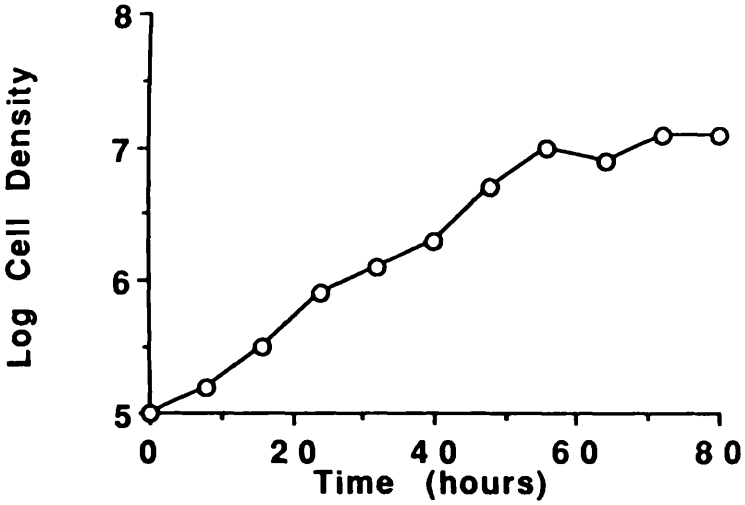


Figure 15c



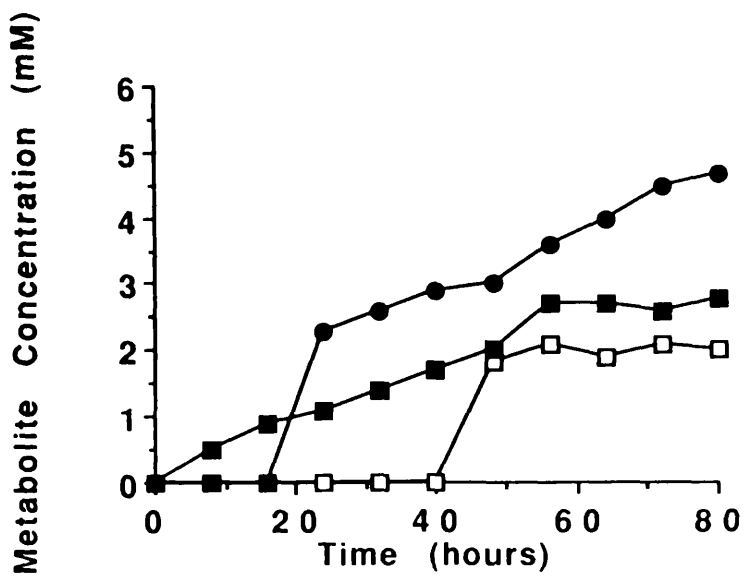
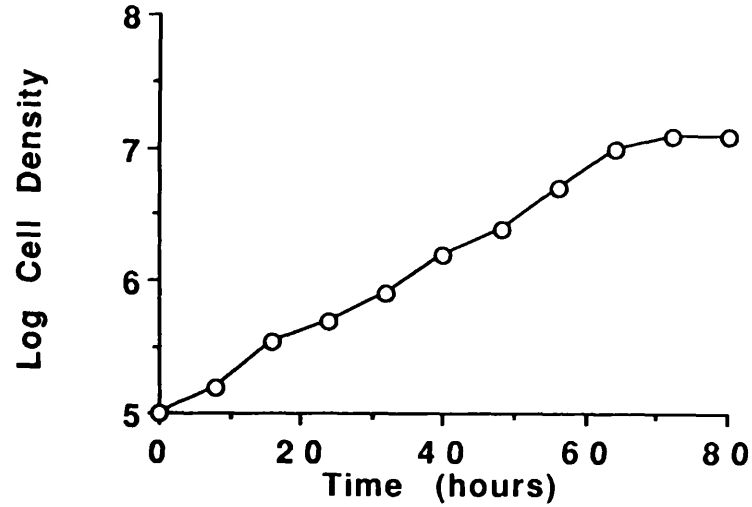
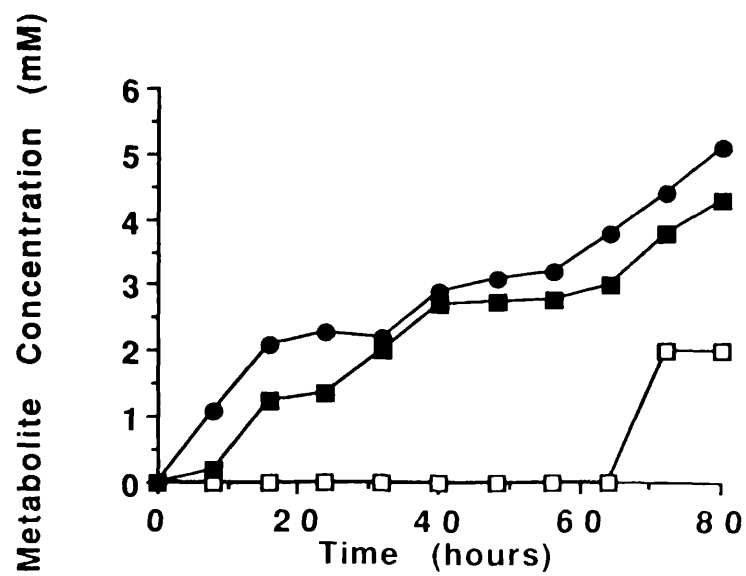


Figure 15d





especially noticable in the case of pyruvate (peak 2) and succinate (peak 3) which overlapped so considerably as to make them difficult to quantify. An example is Figure 16D in which the tip of the succinate peak can be seen on the trailing edge of the pyruvate peak.

Accurate quantification was simpler if cells were incubated in Hanks balanced salts solution containing glucose as the only carbon source. Figure 17a shows that there is very little background when Hanks balanced salts solution is extracted (compare Figure 17a with Figure 16D), consequently it is easier to detect and quantitate succinate, acetate and propionate due to the resolution of the peaks (Figure 17b).

The use of this procedure allowed the more accurate quantitation of acid excretion by the cells. Table 9 shows the excretion products of *H. muscarum* and *H. ingenoplastis* when incubated in Hanks balanced salts solution containing glucose or glucose plus bicarbonate. No evidence was found for the production of ammonia, glycerol, pyruvate or D- or L-lactate using enzyme-based assays, or, in the case of the latter 3 compounds, using isocratic HPLC analysis.

H. muscarum consumed glucose under all four gaseous conditions, and at a lower rate under anaerobic rather than aerobic conditions. The presence of added bicarbonate, however, reversed this situation (Table 9). *Herpetomonas muscarum* excreted succinate and acetate as major end products, and ethanol as a minor end product under all four conditions. Propionate was detected only in anaerobic cultures without added bicarbonate. Production of ethanol, succinate and acetate increased under anaerobic conditions in the presence of bicarbonate, with succinate being excreted at 6-fold greater concentrations compared with aerobic conditions.

Herpetomonas ingenoplastis excreted ethanol, succinate, acetate and

Figure 16

The elution profiles of LIT medium which was extracted into 0.1N NaOH and injected onto a Polypore H column, using HPLC. Figures B and A show the elution profiles of LIT medium prior to inoculation and from an *H. muscarum* culture incubated aerobically for 70 hours, respectively. Figures D and C are elution profiles of LIT medium prior to inoculation and from an *H. ingenoplastis* culture incubated aerobically for 70 hours, respectively. The vertical axis shows the relative absorbance at 210 nm, compared with the largest peak, and the horizontal axis shows the retention time in minutes. The numbered peaks are : 1. solvent front, 2. pyruvate, 3. succinate, 4. fumarate, 5. acetate, 6. propionate.

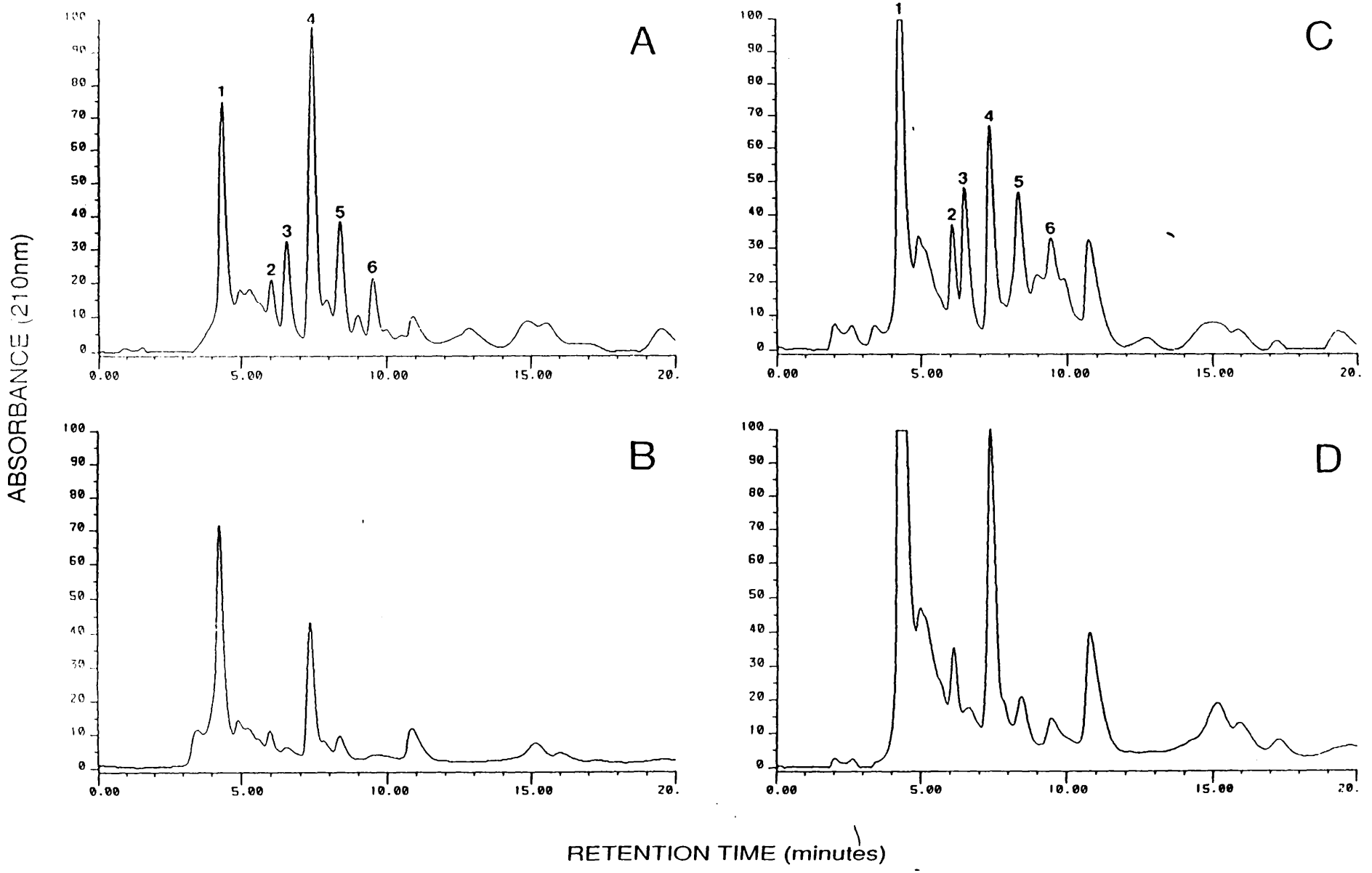
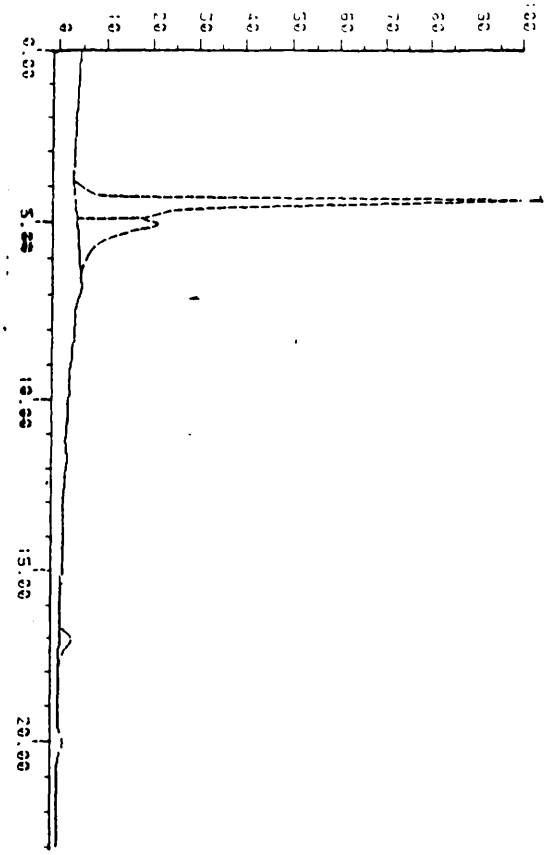

A
B
C
D
1
2
3
4
5
6
ABSORBANCE (210nm)
RETENTION TIME (minutes)
0.00
5.00
10.00
15.00
20.

Figure 17

The elution profiles for extracted Hanks balanced salts solution containing 10 mM glucose and 10 mM Hepes, pH 7.2 before (A) and after (B) a 60 minute incubation of *H. ingenoplastis* at $3x10^8$ cells ml^{-1} under anaerobic conditions. The axes are labelled as per Figure 16. The peaks are: 1. solvent front, 2. succinate, 3. acetate, 4. propionate.

ABSORBANCE (210 nm)



RETENTION TIME (minutes)

Table 9.

Excretion products of herpetomonads during incubations in HBSS containing 10 mM glucose, buffered to pH 7.2. The figures given (in mM) are the concentration in the incubation medium after 40 minute incubations and are the means (±SD) of three experiments.

	Aerobic	Anaerobic	Aerobic plus CO_2	Anaerobic plus CO_2
H. muscarum				
Glucose	4.98 ± 0.33	6.63 ± 0.45	5.21 ± 0.22	4.45 ± 0.35
Ethanol	0.38 ± 0.09	0.33 ± 0.17	0.71 ± 0.14	0.22 ± 0.05
Succinate	1.89 ± 0.96	1.80 ± 0.55	2.96 ± 1.16	8.31 ± 2.66
Acetate	2.13 ± 0.13	3.06 ± 0.80	3.70 ± 1.29	4.18 ± 0.66
Propionate	<0.05	0.21 ± 0.03	<0.05	<0.05
H. ingenoplastis				
Glucose	4.85 ± 0.42	7.93 ± 0.37	5.95 ± 0.68	6.92 ± 0.61
Ethanol	2.67 ± 1.43	0.85 ± 0.37	1.60 ± 0.36	0.89 ± 0.05
Succinate	0.70 ± 0.14	1.25 ± 0.41	1.04 ± 0.59	2.91 ± 0.91
Acetate	2.85 ± 0.64	2.59 ± 1.36	2.35 ± 0.21	2.87 ± 0.86
Propionate	1.07 ± 0.36	2.68 ± 1.31	1.05 ± 0.12	3.15 ± 1.20

propionate as major end products of glucose catabolism. The results suggest that glucose is consumed at higher rates under the two anaerobic conditions, compared with the two aerobic conditions. While the addition of bicarbonate to the anaerobic cultures did not restore the rate of glucose consumption to that of the aerobic conditions, it did increase the rate of glucose consumption. Ethanol production under anaerobic conditions, in the presence of bicarbonate, was approximately half of that found under aerobic conditions. While succinate production by *H. ingenoplastis* was, generally, less than that of *H. muscarum*, the trend was similar, in that succinate production appeared to increase in the presence of added bicarbonate, although the differences were less pronounced than with *H. muscarum.* Acetate production remained similar under the different gaseous conditions, whereas propionate production did alter, being produced in greater amounts under anaerobic conditions. The difference was especially pronounced for anaerobic conditions with bicarbonate. Under these conditions propionate production was approximately 4-fold greater than for aerobic conditions.

Neither of the organisms were analysed for CO_2 production, either during growth in complex medium or during incubations in HBSS.

3.1.7 Enzyme pathways of catabolism

Enzymes potentially responsible for the production of ethanol from pyruvate were detected at high activity in both herpetomonads (Table 10). The specific activity of pyruvate decarboxylase in *H. muscarum* was approximately 2-fold higher than that of *H. ingenoplastis.* In both organisms the activities of alcohol dehydrogenase was over 10-fold higher in the direction of aldehyde reduction than in the direction of ethanol oxidation.

Herpetomonas muscarum was also found to have three

Table 10.

The activities of enzymes putatively involved in ethanol production in *H. muscarum* and *H. ingenoplastis*. The figures given are in nmoles min^{-1} (mg protein)$^{-1}$ and are the means (±SD) of the number of experiments in parentheses.

	H. muscarum	*H. ingenoplastis*
Pyruvate decarboxylase	300 ± 21 (3)	143 ± 32 (3)
Alcohol dehydrogenase (ethanol oxidation)	14 ± 4 (4)	29 ± 21 (4)
Alcohol dehydrogenase (aldehyde reduction)	184 ± 81 (4)	280 ± 80 (4)

Table 11.

The activities of the NAD-linked α-keto acid dehydrogenases in *H. muscarum* and *H. ingenoplastis*. The figures given are in nmoles min^{-1} (mg protein)$^{-1}$ and are the means (±SD) of the number of experiments in parentheses.

	H. muscarum	*H. ingenoplastis*
Pyruvate dehydrogenase	43 ± 25 (5)	57 ± 51 (5)
α-Ketobutyrate dehydrogenase	38 ± 22 (6)	52 ± 38 (6)
α-Ketoglutyrate dehydrogenase	49 ± 31 (5)	<2 (5)

Table 12.

The activities of the enzymes involved in CO_2-fixation in *H. muscarum* and *H. ingenoplastis*. The figures given are in nmoles min^{-1}(mg protein)$^{-1}$and are the means (+SD) the number of experiments in parentheses.

	H. muscarum	*H. ingenoplastis*
Phosphoenolpyruvate carboxykinase	368 ± 139 (6)	417 ± 121 (5)
'Malic' enzyme	23 ± 10 (3)	249 ± 41 (3)

α-keto acid dehydrogenase activities (pyruvate dehydrogenase, α-ketobutyrate dehydrogenase and α-ketoglutarate dehydrogenase) whereas only the former two enzymes were detected in *H. ingenoplastis* (Table 11). Another pyruvate metabolising enzyme, pyruvate: methyl viologen oxidoreductase, was also detected in *H. ingenoplastis* (4 ± 5 nmoles min^{-1} (mg protein)$^{-1}$ (3 experiments)) but not in *H. muscarum* (<0.4 nmoles min^{-1} (mg protein)$^{-1}$). The activity detected in *H. ingenoplastis* varied considerably between experiments, however, and in one experiment was not found. NADH: methyl viologen oxidoreductase activity was also detected in lysates of both *H. muscarum* and *H. ingenoplastis* at 9 ± 3 (3 experiments) and 18 ± 5 (4 experiments) nmoles min^{-1} (mg protein)$^{-1}$, respectively.

Two enzymes that fix CO_2 were also detected in both organisms (Table 12). Phosphoenolpyruvate carboxykinase was found at high activities in both *H. muscarum* and *H. ingenoplastis*. 'Malic' enzyme was at low activity in *H. muscarum* but at relatively high activity in *H. ingenoplastis*.

Several enzymes of the TCA cycle were detected in both herpetomonads (Table 13). *Herpetomonas muscarum* contained all the enzymes of the TCA cycle, at differing activities. NAD-linked isocitrate dehydrogenase (ICDH) was undetecteable in *H. ingenoplastis* but NADP-ICDH was present at high activity in both herpetomonads. *Herpetomonas ingenoplastis*, however, appeared to lack succinyl CoA synthase, succinate dehydrogenase and α-ketoglutarate dehydrogenase (Table 11). Succinate thiokinase activity was undetectable in both *H. muscarum* or *H. ingenoplastis*. Fumarate reductase, an enzyme of the reverse TCA cycle, was present in both organisms but was at approximately 3-fold higher activities in *H. ingenoplastis*. Isocitrate lyase, the first enzyme of the

Table 13.

The activities of the TCA cycle and glyoxylate bypass enzymes, in *H. muscarum* and *H. ingenoplastis*. The figures given are in nmoles min^{-1}(mg protein)$^{-1}$ and are the means (±SD) of the number of experiments in parentheses.

	H. muscarum	*H. ingenoplastis*
Citrate synthase	0.04 ± 0.01 (3)	0.03 ± 0.02 (3)
Aconitase	110 ± 13 (3)	77 ± 64 (3)
NAD-linked isocitrate dehydrogenase	15 ± 6 (3)	<2 (3)
NADP-linked isocitrate dehydrogenase	130 ± 22 (3)	76 ± 16 (3)
Succinate CoA synthase	18 ± 1 (3)	<2 (3)
Succinate thiokinase	<2 (3)	<2 (3)
Succinate dehydrogenase	98 ± 37 (3)	<2 (3)
Fumarate reductase	39 ± 6 (3)	110 ± 29 (3)
Fumarase	8019 ± 2068 (4)	4638 ± 1538 (4)
NAD-linked malate dehydrogenase	989 ± 554 (5)	1362 ± 265 (5)
Isocitrate lyase	<1 (3)	<1 (3)

Table 14.

The activities of two enzymes involved in amino acid metabolism in *H. muscarum* and *H. ingenoplastis*. The figures given are in nmoles min^{-1}(mg protein)$^{-1}$ and are the means (±SD) of the number of experiments in parentheses.

	H. muscarum	*H. ingenoplastis*
NAD-linked glutamate dehydrogenase	610 ± 253 (3)	99 ± 47 (3)
Alanine aminotransferase	120 ± 33 (3)	1023 ± 267 (3)

glyoxylate bypass, was not detectable in either organism.

Two enzymes of amino acid metabolism were detected in both species (Table14). Glutamate dehydrogenase was detected at a very high activity in *H. muscarum*, over 6-fold greater than the activity in *H. ingenoplastis*. Conversely alanine aminotransferase was detected at approximately 9-fold greater activity in *H. ingenoplastis* compared with *H. muscarum*.

3.2 Proteinases

3.2.1 Cellular proteinase activity

The cellular proteinase activity, detected using the synthetic substrate BzPFR, was highest at around pH 8-9 in both *H. muscarum* and *H. ingenoplastis* (Figure 18). At pH 8.0 the proteinase activity of *H. ingenoplastis* was over 2-fold greater than that of *H. muscarum*.

3.2.2 Extracellular proteinase activity

Evidence for extracellular proteinases was obtained for both *H. muscarum* and *H. ingenoplastis* using gelatin-SDS-PAGE (Figures 19 and 20). Figure 19 shows the results obtained when the gel was incubated for 10 hours in 0.1 M acetate buffer pH 5.5, plus 1 mM DTT, at 25^{0}C. Proteinase activity was not detected from short-term incubation medium (lane 1) or spent growth medium (lane 3) that had previously contained *H. ingenoplastis*. Two proteinase bands were detected for *H. muscarum*, in the cases of both short-term incubations in HBSS (lane 2) and LIT (lanes 6-8), and spent growth medium (lane 4).

As the intracellular proteinase activity was optimal at about pH 8, it was

Figure 18

The relationship between pH and proteinase acivity using Bz-PFR as substrate, for homogenates of *H. muscarum* (●) and *H. ingenoplastis* (○). The figures for activity are means (± SD) of three experiments.

Figure 18

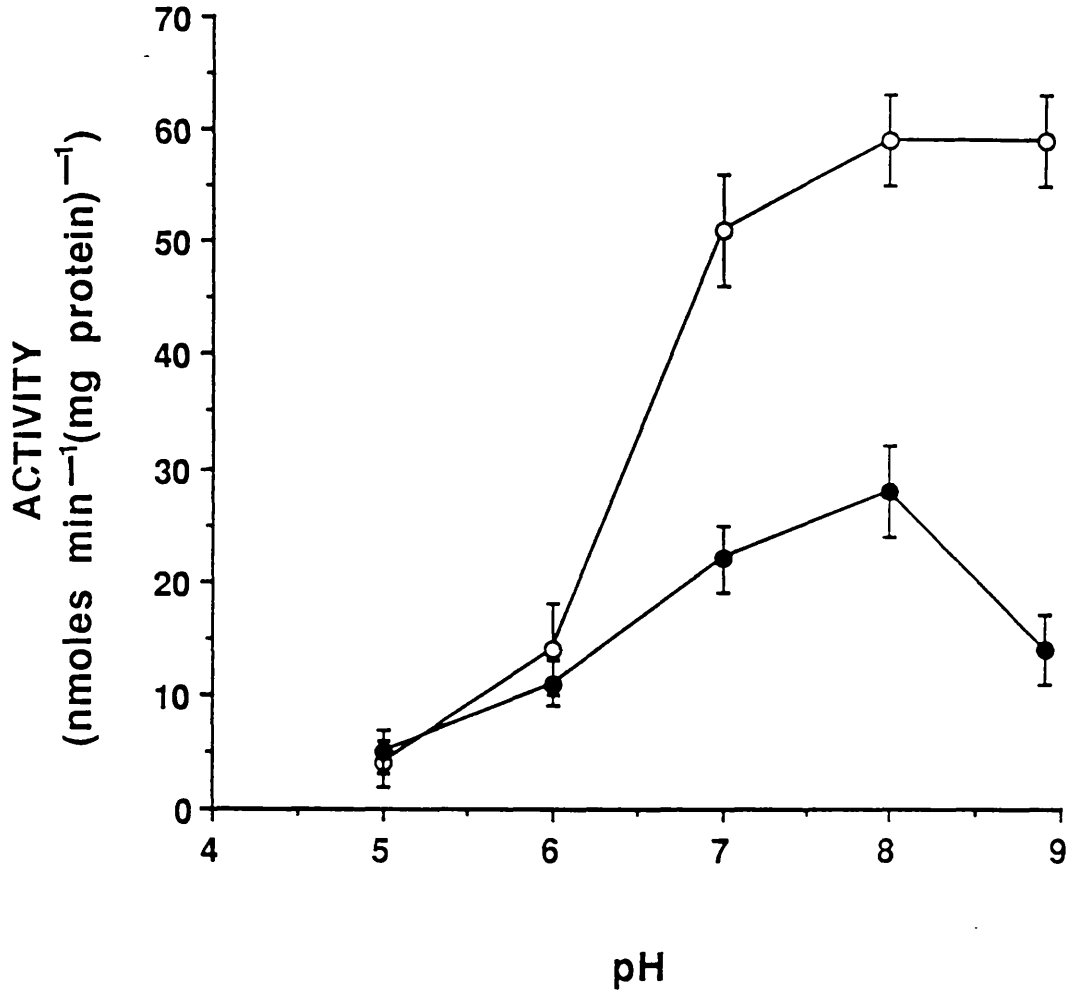

ACTIVITY
(nmoles min^{-1}(mg protein)$^{-1}$)
70
60
50
40
30
20
10
0
4
5
6
7
8
9
pH

Figure 19.

The release of proteinase by herpetomonads during growth and short-term incubations. In the cases of the short-term incubations in HBSS and LIT (minus HIFCS) the cells were incubated at cell densities of approximately $1x10^8$ cells ml^{-1}.

Lane 1, *H. ingenoplastis* short-term incubation (1hour) in HBSS; Lane 2, *H. muscarum* short-term incubation (1 hour) in HBSS; Lane 3, *H. ingenoplastis* after 72 hours growth in LIT medium; Lane 4, *H. muscarum* after 72 hours growth in LIT medium; Lane 5, LIT medium control; Lanes 6-8, *H. muscarum* short-term incubations in LIT medium (minus HIFCS) for 1, 2 and 4 hours respectively. The gel was incubated for 10 hours at 25^oC in 0.1 M acetate buffer pH 5.0 plus 1 mM DTT.

Figure 19

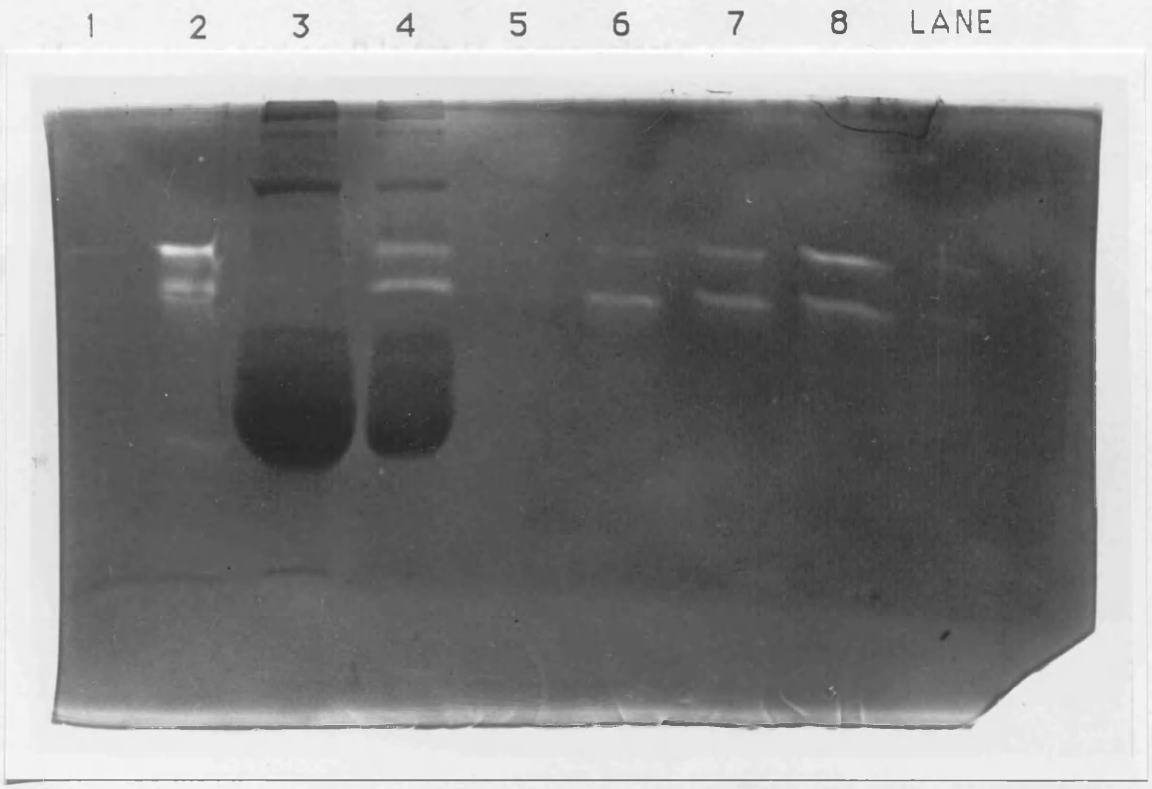


Figure 20.

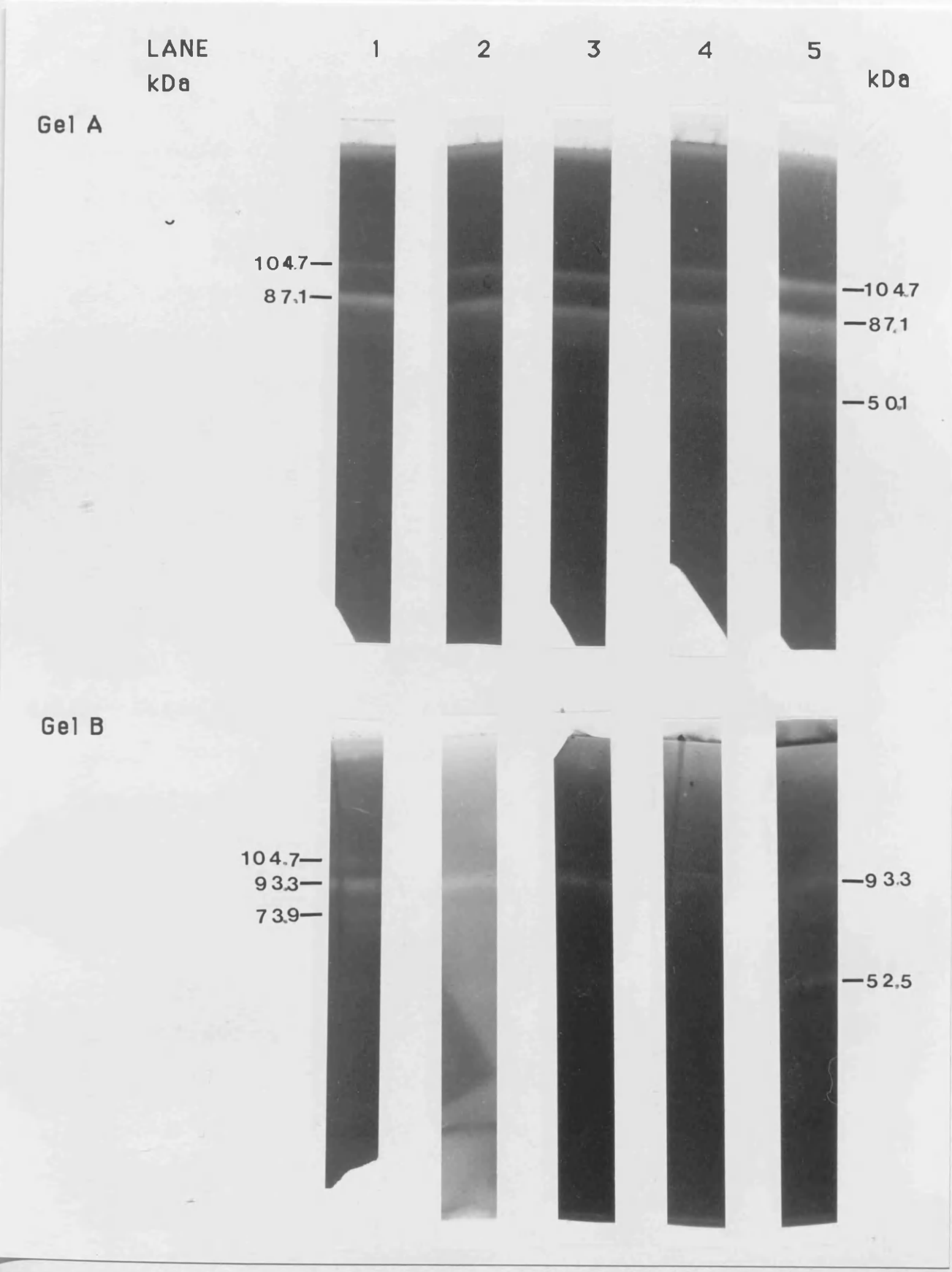
The effect of pH on the released proteinase activity. Medium from short-term incubations in LIT (minus HIFCS) at $2x10^8$ cells ml^{-1} for 2 hours. Gel A is for *H. muscarum* and gel B is for *H. ingenoplastis*.

Lane 1, pH 5.0; Lane 2, pH 6.0; Lane 3, pH 7.0; Lane 4, pH 8.0; Lane 5, pH 8.9.

Gels were incubated for 18 hours at 25^oC in buffer containing 1 mM DTT.

The arrows indicate the positions of the secreted enzymes.

Figure 20


LANE
kDa
1
2
3
4
5
kDa
Gel A
104.7
87.1
104.7
87.1
50.1
Gel B
104.7
93.3
73.9
93.3
52.5

decided to investigate the pH optima of the released enzymes by incubating the gels at different pH's. The results (Figure 20) show that both *H. muscarum* and *H. ingenoplastis* release low proteinase activities into their environment. *Herpetomonas muscarum* (Figure 20, Gel A) shows the presence of three proteinase activities. Two, at 104.7 and 87.1 kDa, are active across a broad pH range (5.0-8.9), but seem to increase in activity with pH. The third activity, 50.1 kDa, is only active at pH 8.9, and then seem to be very low compared to the other two activities. *Herpetomonas ingenoplastis* is similar, with a broad pH band at 93.3 kDa, as well as an activity which appears only at alkaline pH, 52.5 kDa. However, also released by *H. ingenoplastis* are two activities which are only active at pH's below 7.0, which are at 104.4 and 79.3 kDa. The detection of proteinases in medium from *H. ingenoplastis* in this case (Figure 20, Gel B), which do not appear in Figure 19, could be due to the higher cell densities used and the longer gel incubation time. It should be noted that in all cases, both *H. muscarum* and *H. ingenoplastis* cells were very motile at the end of the short-term incubations. In addition, glucose-6-phosphate dehydrogenase, as a cytosolic marker, could not be detected in the medium, again indicating that there had been little cell lysis.

3.3 Subcellular distribution of enzymes

Lysing the cells by freeze-thawing and centrifuging the resulting lysates at 10000g for 5 minutes resulted in the majority of the FR and SDH activities of *H. muscarum* being recovered in the pellet (Table 15). In contrast, the FR activity of *H. ingenoplastis* was partitioned approximately equally between the particulate and soluble fractions. Greater than 90% of the glucose-6-phosphate dehydrogenase was recovered in soluble fraction for both *H. muscarum* and *H. ingenoplastis*, showing that cell lysis had been effected.

Table 15.

Distribution of succinate dehydrogenase, glucose-6-phosphate dehydrogenase and fumarate reductase in particulate and soluble fractions of *H. muscarum* and *H. ingenoplastis* . Cells were resuspended in 0.25 M sucrose and lysed by 3 cycles of freezing at -70°C followed by thawing at 25°C and passage through a 0.26 G needle three times. The resultant lysate was centrifuged at 10000g for 5 minutes. The activities given are expressed as percentages of the activity in the initial lysate, immediately before centrifugation, and are the means (±SD) of 3 experiments.

	Pellet	Supernatent	Total
H. muscarum			
Succinate dehydrogenase	88 ± 6	12 ± 6	100 ± 0
Fumarate reductase	79 ± 6	13 ± 1	91 ± 5
Gucose-6-phosphate dehydrogenase	6 ± 3	93 ± 5	99 ± 3
H. ingenoplastis			
Succinate dehydrogenase	ND[a]	ND[a]	ND[a]
Fumarate reductase	54 ± 2	46 ± 2	100 ± 0
Glucose-6-phosphate dehydrogenase	4 ± 2	94 ± 4	99 ± 2

[a] Not detected - see Table 13.

Table 16.

Distribution of protein and enzymes in the subcellular fractions of *H. muscarum* prepared by differential centrifugation. The figures given are means (±SD) of the number of experiments in parentheses, and are expressed as a percentage of the total activity recovered.

	P1	P2	P3	S	% recovery	No. of experiments
Protein	33 ± 8	21 ± 6	10 ± 3	47 ± 6	108 ± 8	(4)
Hexokinase	18 ± 3	32 ± 5	8 ± 2	35 ± 4	92 ± 9	(4)
Phosphoglucose-isomerase	6 ± 3	19 ± 5	4 ± 2	70 ± 8	86 ± 5	(4)
Glucose-6-phosphate dehydrogenase	5 ± 2	8 ± 3	7 ± 3	94 ± 5	105 ± 3	(4)
Malate dehydrogenase	11	21	14	35	81	(1)
Fumarate reductase	41	37	13	10	91	(1)
NADH oxidase	22	61	22	18	123	(1)
Succinate dehydrogenase	18	31	6	15	70	(1)

Table 17.

Distribution of enzymes in subcelluar fraction of *H. ingenoplastis* prepared by differential centrifugation. The figures given are means (±SD) of the number of experiments in parentheses, and are expressed as a percentage of the total activity recovered.

	P1	P2	P3	S	% recovery	No. of experiments
Hexokinase	19 ± 3	7 ± 1	25 ± 5	38 ± 3	87 ± 6	(3)
Phosphoglucose-isomerase	10 ± 4	3 ± 1	5 ± 1	80 ± 5	103 ± 5	(3)
Glucose-6-phosphate dehydrogenase	10	2	5	82	99	(1)
Malate dehydrogenase	25	12	42	22	101	(1)
NADH oxidase	59	9	22	14	104	(1)
Fumarate reductase	14	42	0	52	108	(1)

The FR activities in the particulate fractions of both *H. muscarum* and *H. ingenoplastis* were not inhibited by succinate (concentrations up to 15 mM), malonate, thenoyltrifluoroacetone (both at concentrations up to 10 mM) and rotenone at 1 mM.

The results from fractionation of *H. muscarum* by differential centrifugation are shown in Table 16. Hexokinase showed a substantial particulate fraction which sedimented in P1 and P2, along with a large part of the PGI. Hexokinase activity recovered in the soluble fraction (S) was about equal to that found in P2, while the PGI activity in S was approximately 4-fold greater than that found in P2. Glucose-6-phosphate dehydrogenase was mostly recovered in the cytosolic fraction (S). Malate dehydrogenase, FR, SDH and NADH oxidase also all showed activity in P2.

The results from differential centrifugation of *H. ingenoplastis* homogenates (Table 17) were very different from those for *H. muscarum*. Only 7% of the HK activity of *H. ingenoplastis* was recovered in P2, whereas 25% ended in P3. Similarly a large percentage, 42%, of MDH was recovered in P3. The amount of FR activity that sedimented in P2 was 42%, with 52% in the cytosolic fraction. Glucose-6-phosphate dehydrogenase was again found mostly in the cytosolic fraction.

3.4.1 Purification of PK from *H. ingenoplastis*

In order to characterise PK, this activity was purified away from PEPCK, which also used PEP and ADP as substrates. To make detection of PK easier during the purification procedures, 5 mM F-1,6-P_2 was used routinely in the assays. In the absence of added F-1,6-P_2, the activity of PK was 150 ± 55 nmoles min^{-1}(mg protein)$^{-1}$(Table 3). Figure 21 shows how the activity of PK in

cellular lysates in direct proportion to concentrations of F-1,6-P_2 up to 5 mM.

When cells were lysed in the presence of 0.1% Triton X-100 in 20 mM TEA, pH 7.5, and then centrifuged for 5 minutes at 10000g, 89 ± 8% (3 experiments) of PEPCK resulted in the pellet, along with 73 ± 10% of HK and 77 ± 8% of MDH. However, the majority of the PK activity, 93 ± 5%, was recovered in the soluble fraction, along with 85 ± 9% of glucose-6-phosphate dehydrogenase. The activities of PEPCK, MDH and HK were released from the pellet by the addition of sodium chloride (Figure 22).

As the activities of PK and PEPCK were largely separated by the simple lysis in Triton X-100 and centrifugation procedure, this was incorporated as the first stage of the enzyme purification. The resultant supernatent was filtered through a 0.22 μm filter, giving Supernatent 1, which was subjected to MonoQ, anion exchange chromatography (Figure 23). Pyruvate kinase eluted after 10 ml, whereas the the remnant MDH and PEPCK eluted in the same fractions, around 8 ml. It is interesting to note that MDH and PEPCK also coeluted, separately from PK, from the Superose 6 gel filtration column and the Alkyl-Superose hydrophobic interactions column in preliminary experiments (data not shown).

The pooled PK fractions from the MonoQ column were then subjected to gel fitration using a Superose 6 column (Figure 24). No MDH or PEPCK was detected in any fractions from this column. The pooled PK fractions were then used in the characterisation studies. Table 18 shows summary data of a typical purification using the above methodology. The yield was consistent between experiments, at about 30%, although the purification factor varied between 51 and 112-fold, with a mean of 70 ± 19-fold (10 experiments). Further steps were found to cause either an unacceptable reduction in yield or a loss in activity. The volume pooled from the Superose 6 column, 3 ml, was too large to apply

directly to either an Alkyl-Superose column or a MonoS, cation exchange column. However, ultrafiltration to reduce the volume resulted in a large loss of activity, greater then 80%. Hence, it was decided to use the material at this stage of the purification in characterisation studies, as the main objective, to remove PEPCK, had been achieved.

Samples from the purification steps were subjected to SDS-PAGE (Figure 25a). The purified sample consistently contained a major band of approximately 63 kDa (Figure 25b) and was believed to be PK. Other minor protein bands were also present.

3.4.2 Analysis of PK purified from H. ingenoplastis

3.4.2.1 pH profile

The purified PK activity was assayed over the pH range 6.0-9.0 (Figure 26). The pH optimum was 7.5, with both TEA and imidazole as buffers. The activity was about 7% higher using imidazole.

3.4.2.2 The effects of divalent cations

In the absence of $MgCl_2$, the purified PK of *H. ingenoplastis* showed no detectable activity (Figure 27). The curve was found to be hyperbolic, with the enzyme saturating at approximately 8 mM $MgCl_2$ and above. The enzyme was also active in the presence of 8 mM $MnCl_2$, although 17 $\pm$ 6% (3 experiments) less than with $MgCl_2$.

3.4.2.3 The effects of KCl

Pyruvate kinase purified from *H. ingenoplastis* was found to be active in the absence of KCl (Figure 28). Nevertheless, the addition of KCl resulted in

Figure 21

The activation of pyruvate kinase activity in lysates of *H. ingenoplastis* by fructose-1,6-bisphosphate

Figure 21

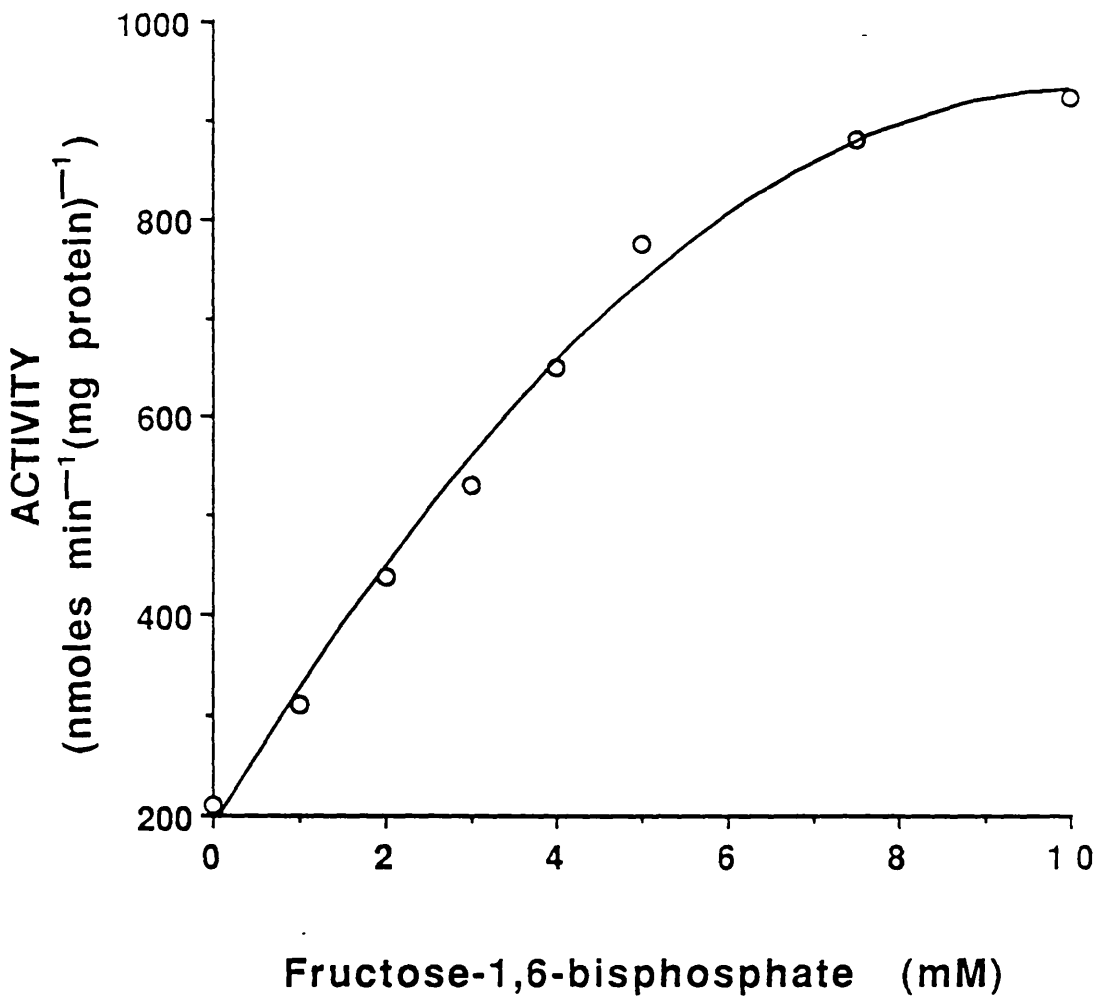

1000
800
600
400
200
0
2
4
6
8
10
ACTIVITY
(nmoles min^{-1}(mg protein)$^{-1}$)
Fructose-1,6-bisphosphate (mM)

Figure 22

The solubilisation of enzymes of *H. ingenoplastis*. The cells were lysed in 20 mM TEA, pH 7.3, containing 0.1% (v/v) Triton X-100 and the lysates centrifuged at 10000 g for 5 minutes at 18^{o}C. The pellet was resuspended and incubated for 10 minutes in a range of NaCl concentrations. The supernatents and resuspended pellets from a subsequent 5 minute, 10000 g centrifugation were assayed for protein (○), PEPCK (■), PK (●), HK (□) and MDH (△).

Figure 22

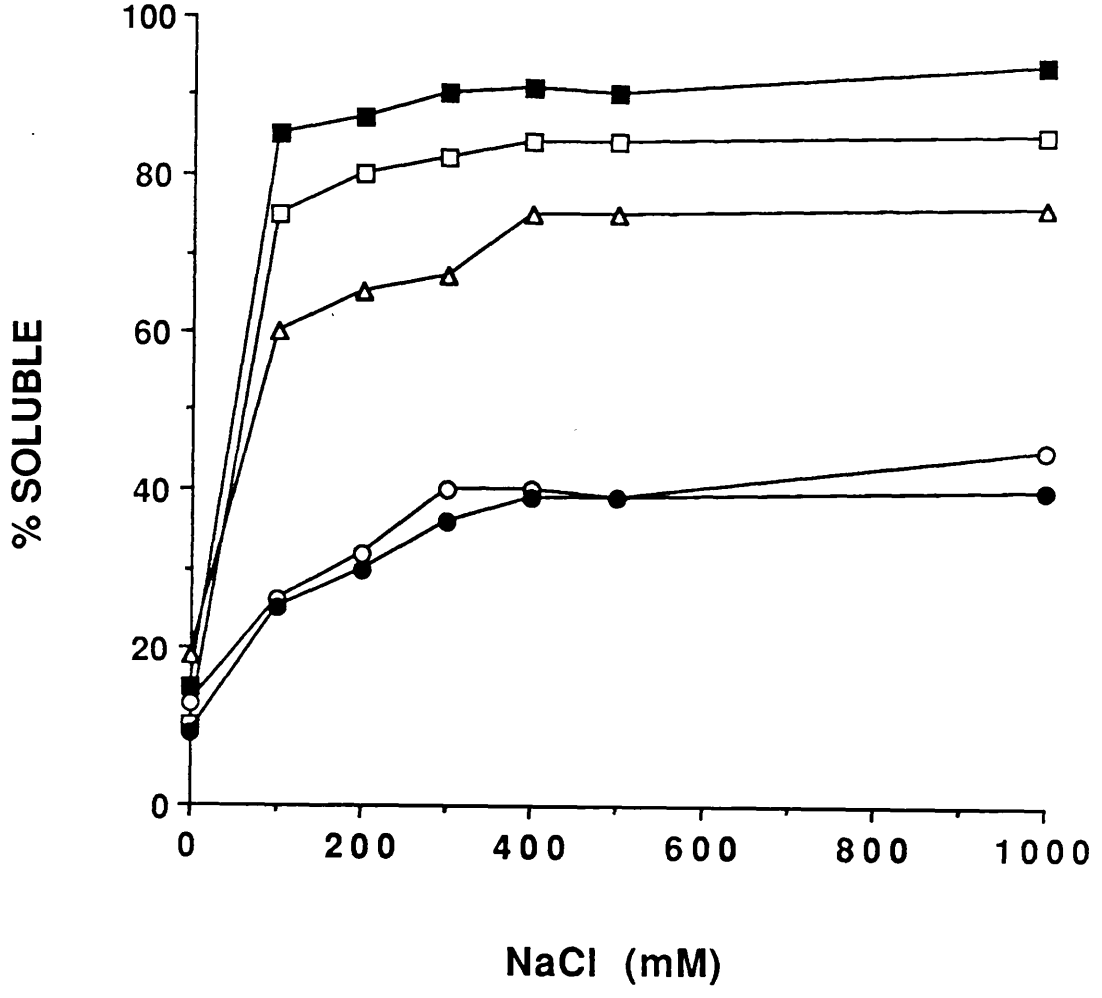


Figure 23

The elution of MDH (□), PEPCK (■), protein (—) and PK (○) from a MonoQ anion exchange column using a gradient of NaCl (– – –).

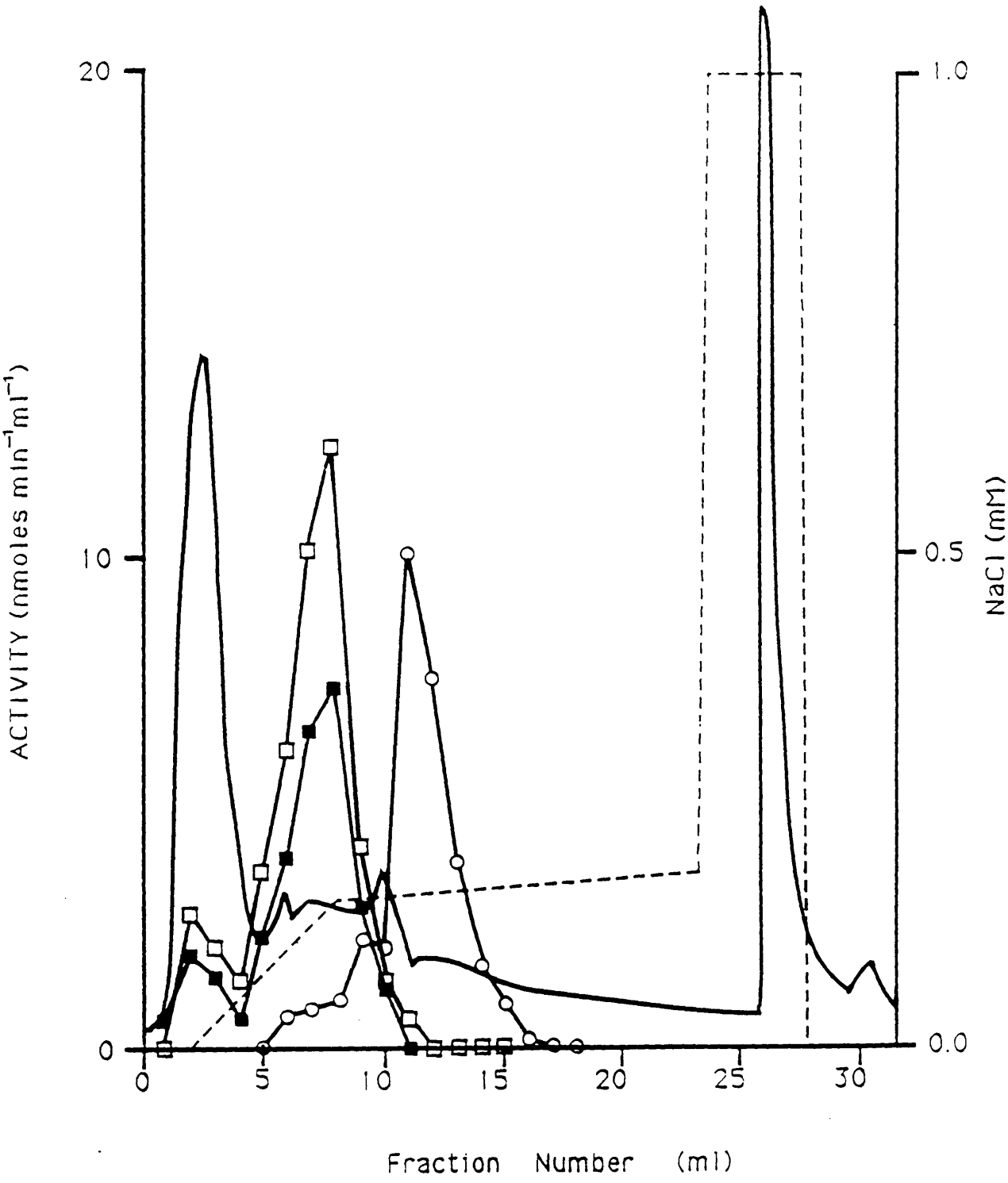

20
10
0
1.0
0.5
0.0
0
5
10
15
20
25
30
ACTIVITY (nmoles min⁻¹ml⁻¹)
NaCl (mM)
Fraction Number (ml)

Figure 24

The elution of PK (o) and protein (—) from a Superose 6 gel filtration column.

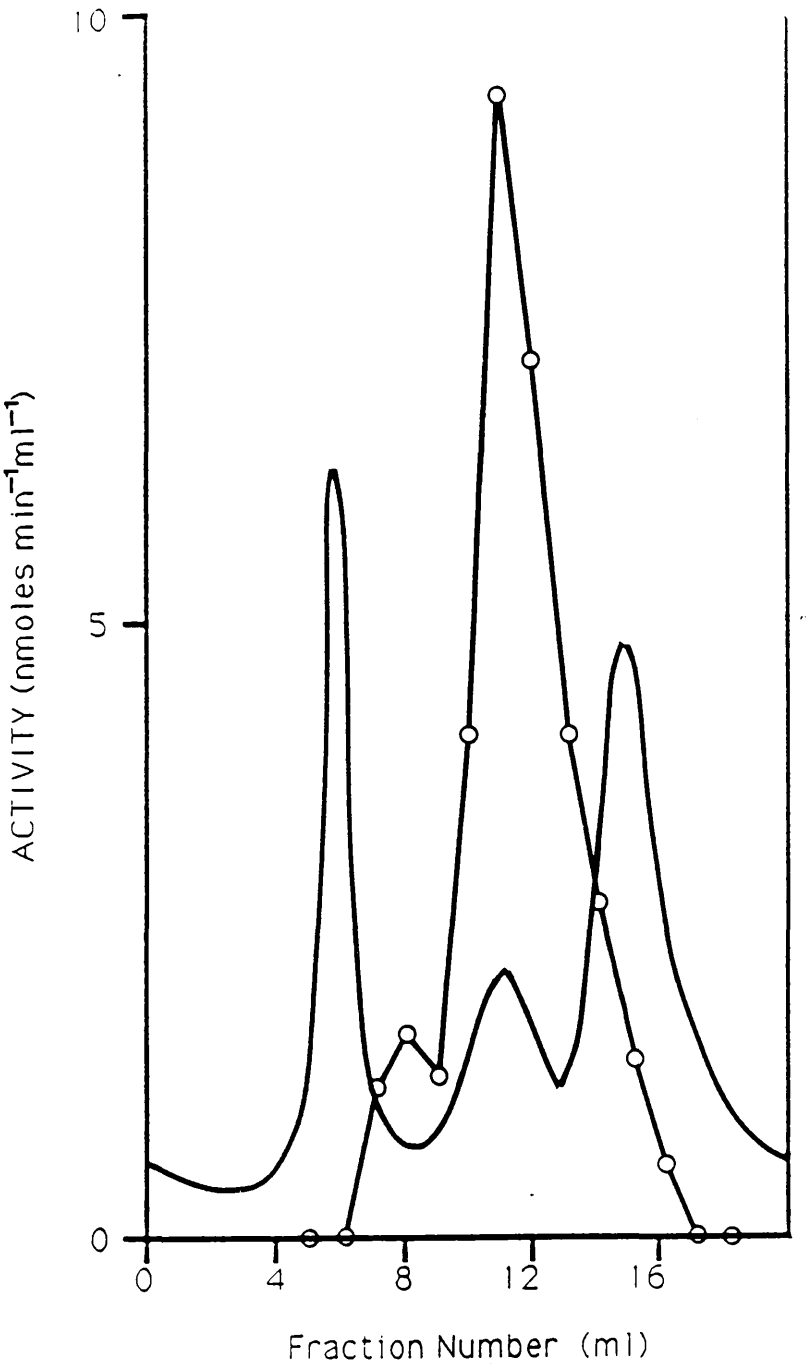

10
5
0
0
4
8
12
16
ACTIVITY (nmoles min^{-1}ml^{-1})
Fraction Number (ml)

Table 18.

Summary of the purification of pyruvate kinase from *H. ingenoplastis*.

Purification step	Volume (ml)	Total protein[a]	Total activity[b]	Specific activity[c]	Yield (%)	Purification factor
Homogenate (H)	1.5	6.63	2664	408	100	1
Supernatent 1 (S1)	1.0	2.78	1403	505	53	1.2
MonoQ (S2)	2.0	0.12	1118	9317	42	22.8
Superose 6 (S3)	3.0	0.03	819	27300	30	66.9

[a] the units for protein content are milligrams.

[b] the units for total activity are nmole min^{-1}, where the assays were performed in the presence of 5 mM fructose-1,6-bisphosphate.

[c] the units for specific activities are nmoles min^{-1} $(mg\ protein)^{-1}$.

increased activity up to 50 mM. Higher concentrations caused apparent slight inhibition.

3.4.2.4 The effects of ADP

At sub-saturating concentrations of PEP, 4 mM, ADP was found to saturate the purified PK at approximately 3 mM (Figure 29a). The curve for velocity versus ADP concentration was hyperbolic resulting in a linear plot of velocity versus velocity/ADP concentration (Figure 29b). Regression analysis of this figure estimates a K_m for ADP of 1.41 mM, and a V_{max} of 14 nmoles min^{1-} $(mg\ protein)^{-1}$

3.4.2.5 The effects of PEP

At saturating ADP concentrations, 3 mM, purified PK was found to be saturated by PEP at 6 mM and above (Figure 30) and the curve of velocity versus PEP concentrations was sigmoidal. This resulted in calculations for K_m and V_{max} being inaccurate.

3.4.2.6 Inhibition by ATP

The addition of 5 mM ATP was found to cause the curve of velocity of pyruvate kinase activity versus PEP concentration to become more sigmoid, than in the absence of ATP (Figure 30). The K_m was again not calculable by linear regression anaysis due to the shape of the curve, but since K_m is defined as the the substrate concentration at $V_{max}/2$, then the graph suggests that there is an increase in the K_m, in the presence of ATP. The fact that there is an increases in K_m and no change to the V_{max} suggests that ATP inhibits PK

Figure 25

a) SDS-PAGE of samples from the stages during the purification of pyruvate kinase.

Key to tracks with the amount of protein applied in parenthesis: 1. Homogenate (8.7 µg), 2. Supernatent (11.1 µg), 3. MonoQ eluant (4.8 µg), 4. Superose 6 eluant (1.2 µg), 5. Low molecular weight markers (10 µg).

b) Molecular eight determination using a calibration curve of low molecular weight markers. The results presented are from Figure 25a.

Figure 25a

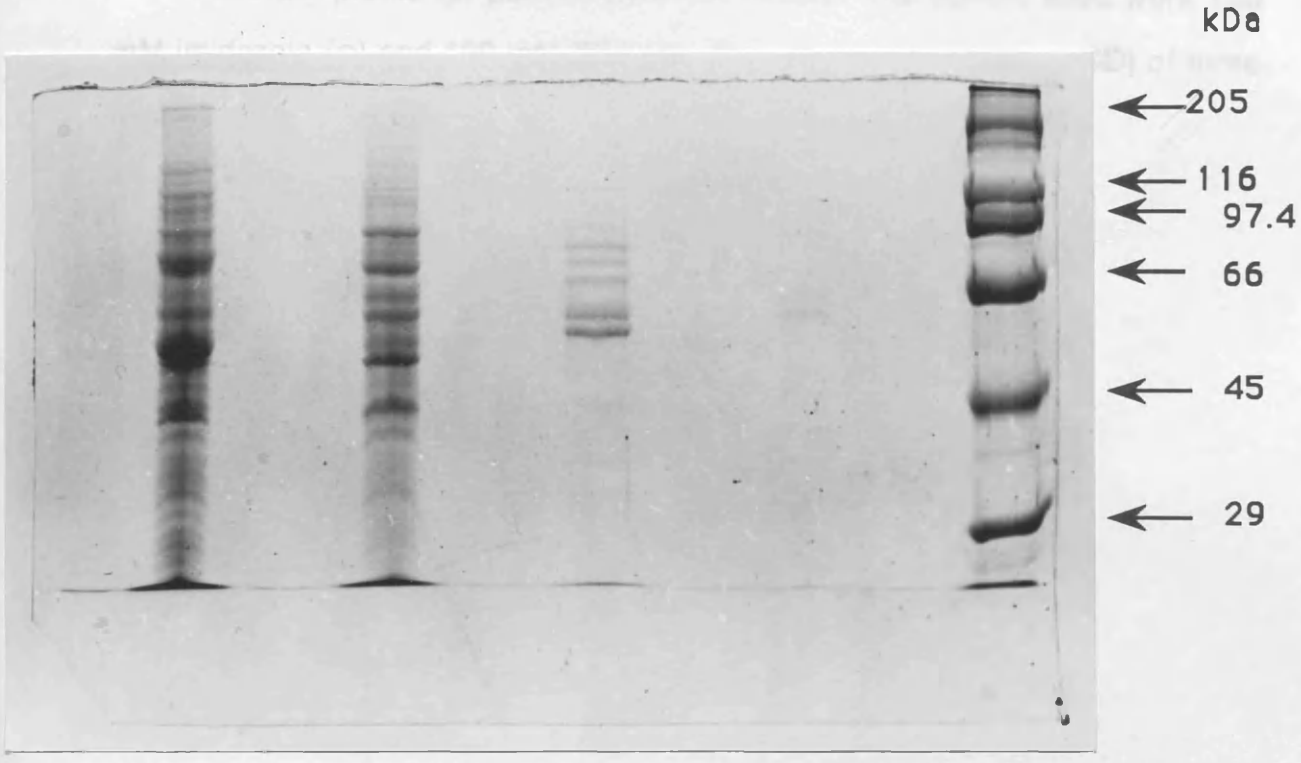


Figure 25b

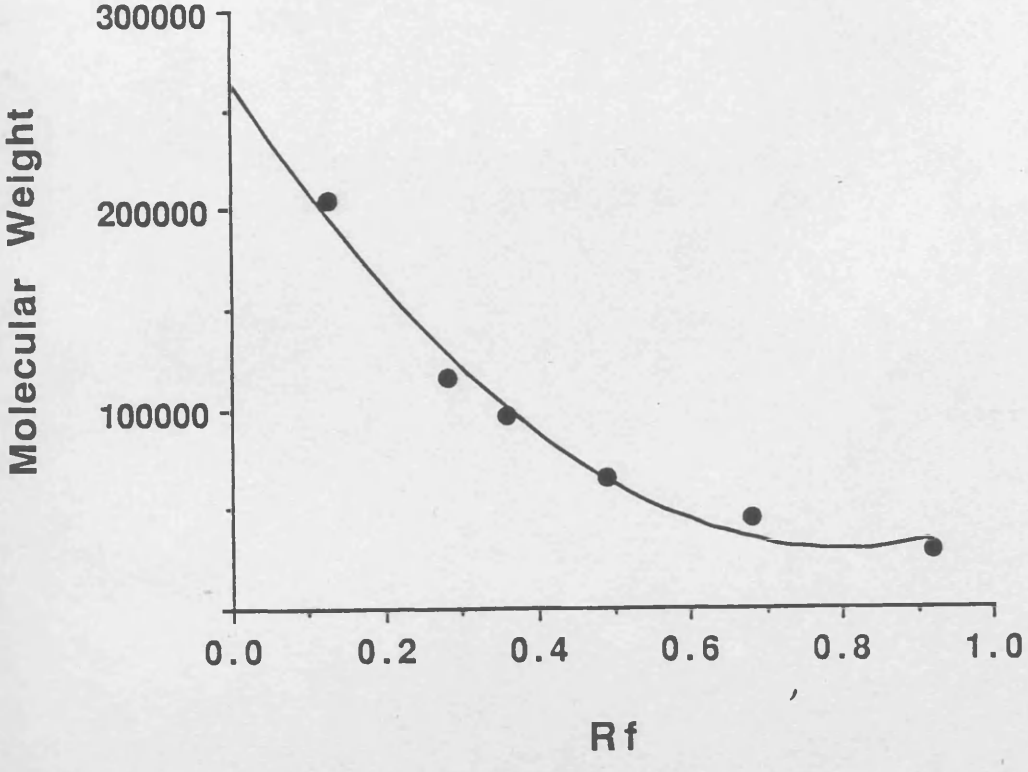


Figure 26

The pH profile for purified pyruvate kinase. The buffers used were 100 mM imidazole (○) and 100 mM TEA (●). The results are means (± SD) of three experiments.

Figure 26

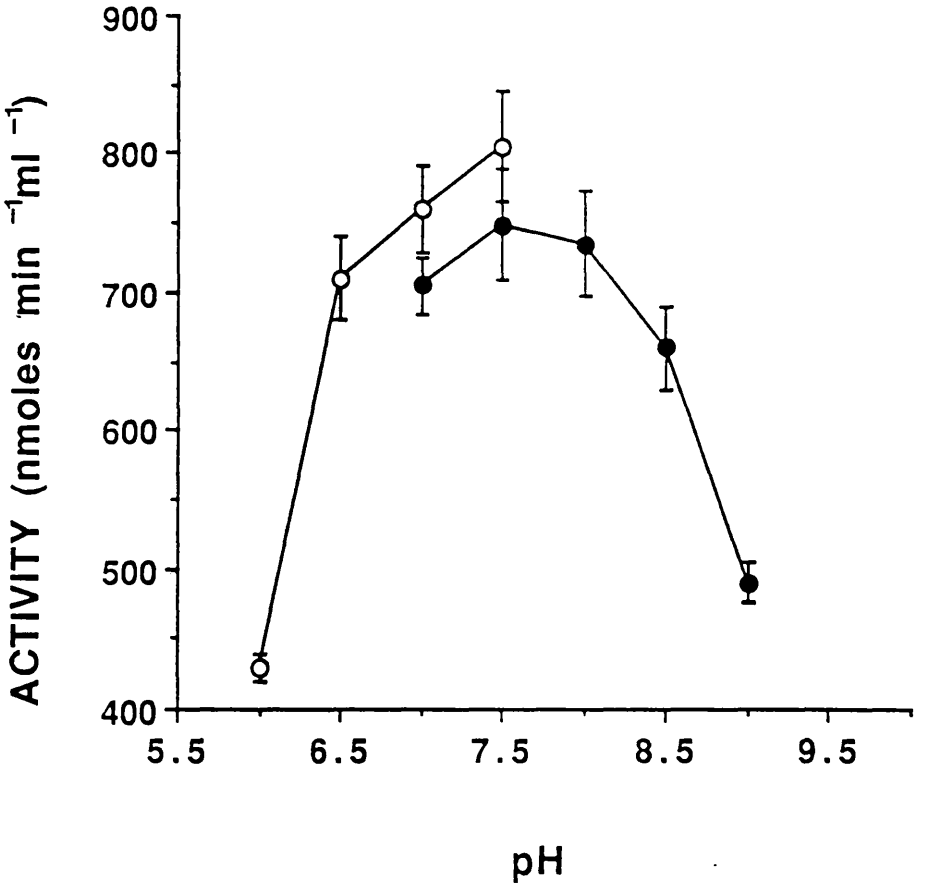

900
800
700
600
500
400
ACTIVITY (nmoles min $^{-1}$ml $^{-1}$)
5.5
6.5
7.5
8.5
9.5
pH

Figure 27

The relationship between activity for purified pyruvate kinase from *H. ingenoplastis* and concentration of $MgCl_2$. Other components in the assay mixture were 4 mM PEP, 3 mM ADP and 75 mM KCl and 1.5 µg of purified pyruvate kinase. The figures given are means (± SD) from 3 experiments.

Figure 27

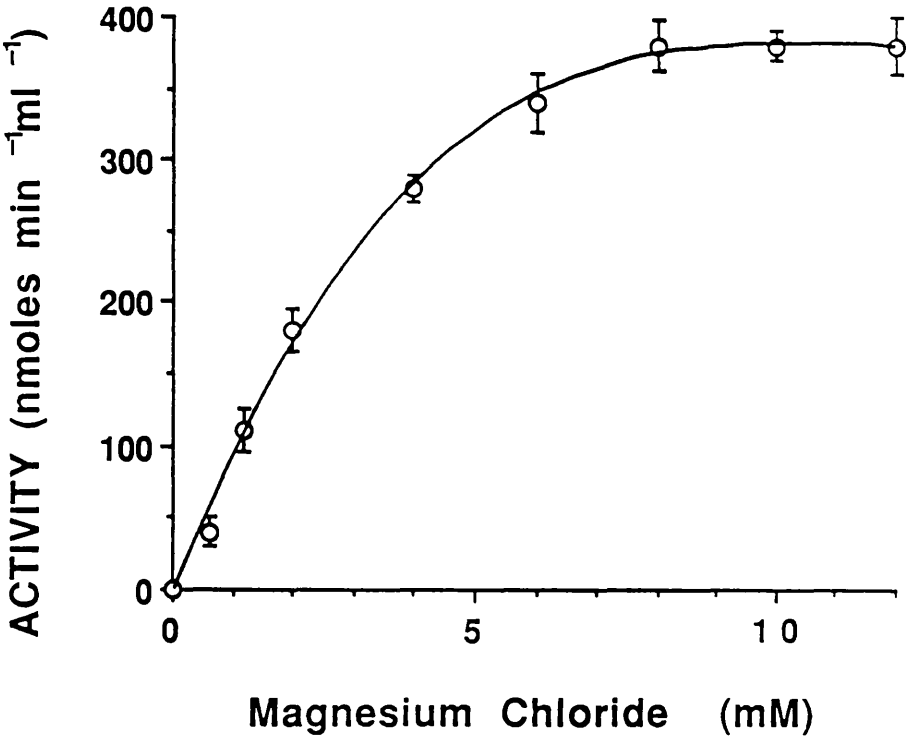

ACTIVITY (nmoles min $^{-1}$ml $^{-1}$)
400
300
200
100
0
0
5
10
Magnesium Chloride (mM)

Figure 28

The relationship between the activity of purified pyruvate kinase from *H. ingenoplastis* and the concentration of KCl. Other components in the assay mixture were 4 mM PEP, 3 mM ADP and 6 mM $MgCl_2$ and 1.5 µg of purified pyruvate kinase. The figures given are means (± SD) from 3 experiments.

Figure 28

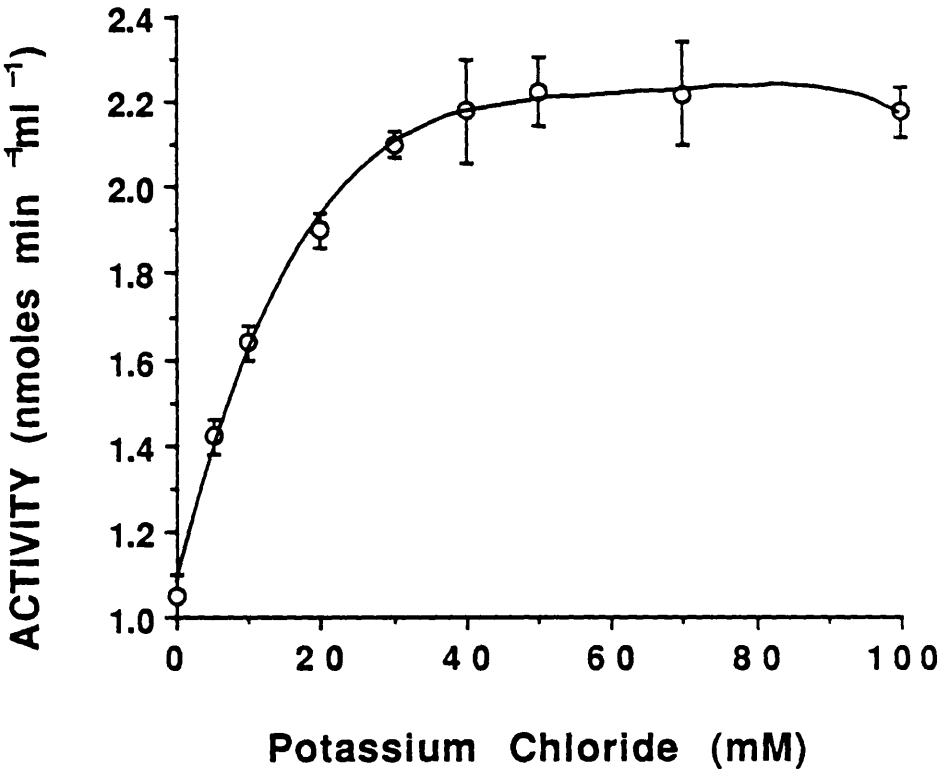
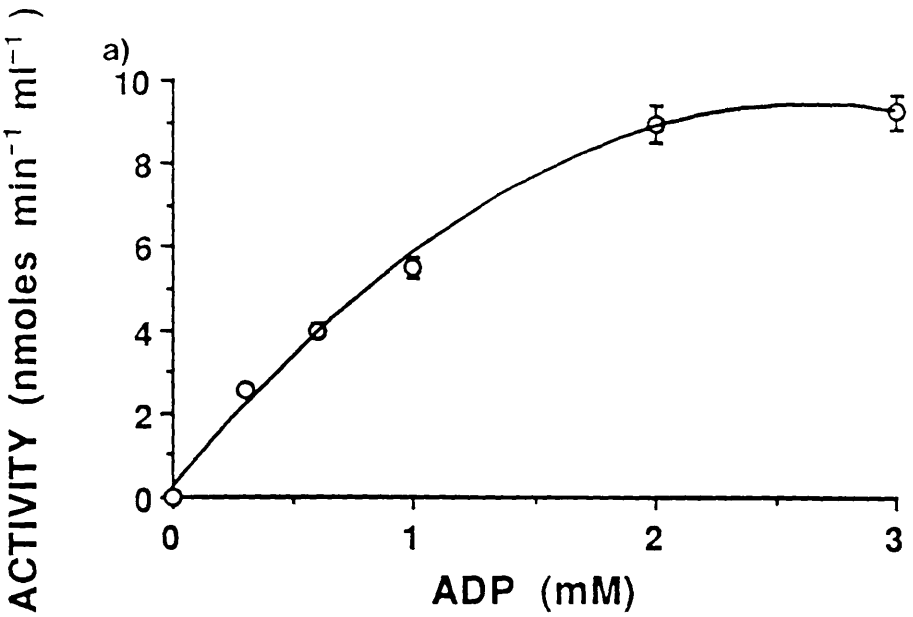

ACTIVITY (nmoles min $^{-1}$ml $^{-1}$)
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0
20
40
60
80
100
Potassium Chloride (mM)

Figure 29

a) The relationship between activity of purified pyruvate kinase from *H. ingenoplastis* and ADP concentrations at 4mM PEP. The figures given are means (± SD) from 3 experiments.

b) An Eadie-Hofstee plot of the data presented in Figure 29a. The vertical axis is enzyme velocity (nmoles min^{-1} ml^{-1}) and the horizontal axis is velocity/ADP concentration.


a)
ACTIVITY (nmoles min^{-1} ml^{-1})
10
8
6
4
2
0
0
1
2
3
ADP (mM)

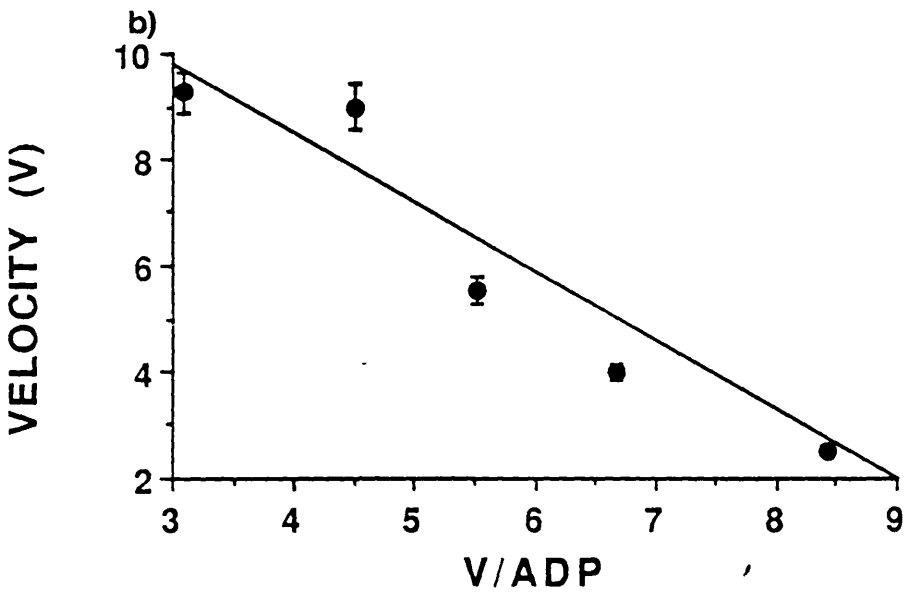

b)
VELOCITY (V)
10
8
6
4
2
3
4
5
6
7
8
9
V/ADP

Figure 30

The relationship between the activity of pyruvate kinase purified from *H. ingenoplastis* and PEP concentration with ADP at 3 mM, in the presence of 30 mM $MgCl_2$ and 75 mM KCl. The effects of fructose-2,6-bisphosphate and ATP on the activity of PK are all shown.

No addition (○), 5 mM ATP (●), 1.5 μM F-2,6-P_2 (□) and 5 mM ATP + 1.5 μM F-2,6-P_2 (■). The data presented are representitive of three experiments.

Figure 30

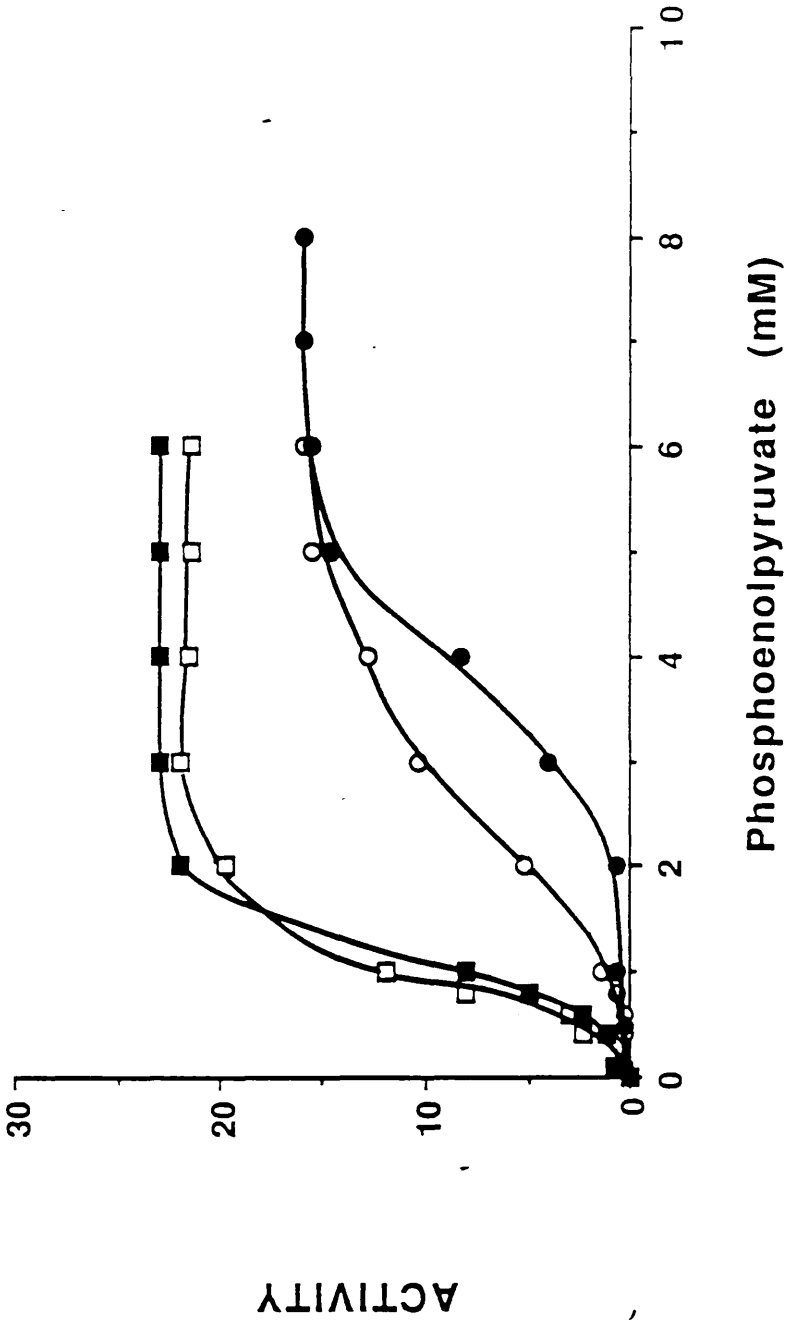


Figure 31

The effect of different ATP concentrations on the activation of purified pyruvate kinase from *H. ingenoplastis* by 1.5 μM F-2,6-P_2, in the presence of 4 mM PEP and 3 mM ADP. The data presented is representitive of 2 experiments. Minus F-2,6-P_2 (●), plus F-2,6-P_2 (○).

Figure 31

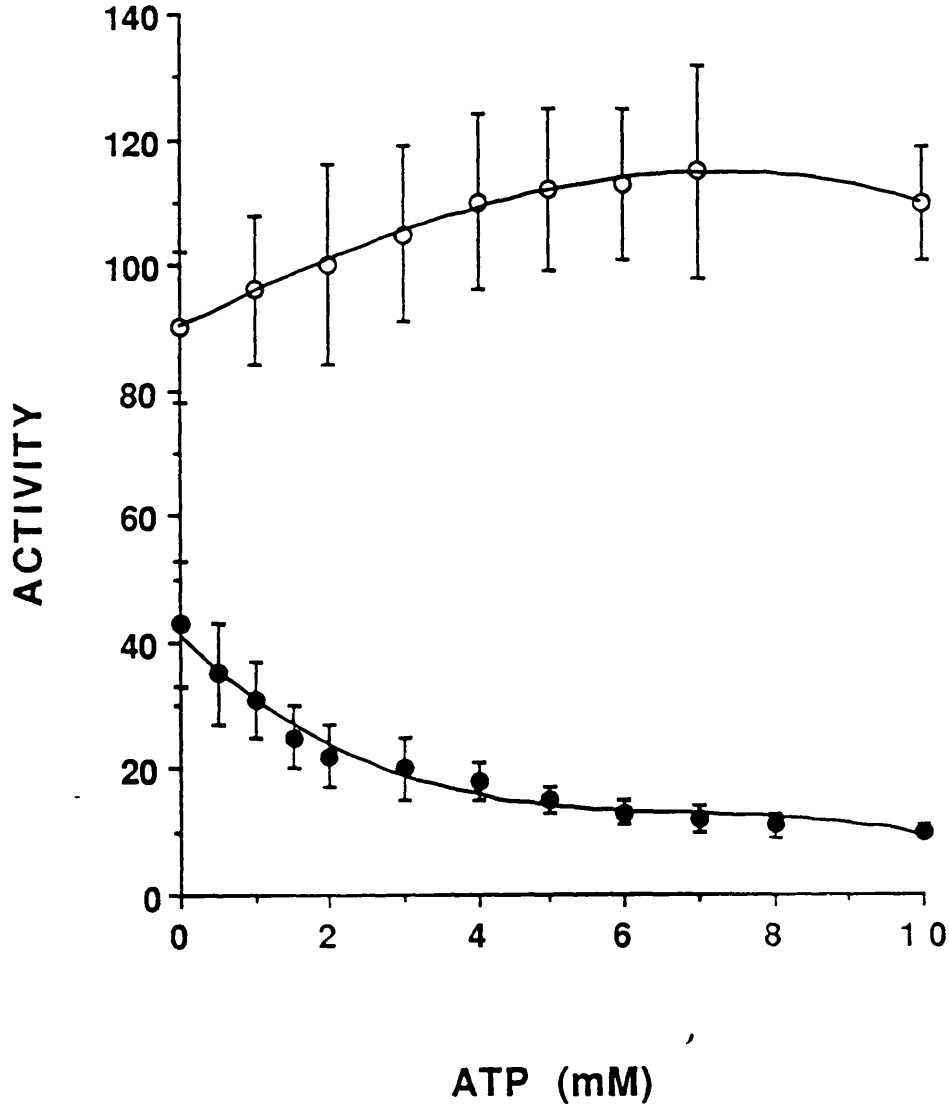


Table 19.

Effects of several compounds on the activity of partially purified pyruvate kinase of *H. ingenoplastis*. The reactions were performed in duplicate in the presence of 4 mM PEP and 3 mM ADP. The results are means (±SD) of 3 experiments.

Compound	Percent of control specific activity
None	100
5 mM ATP	68 ± 6
5 mM GTP	7 ± 5
5 mM 5'-AMP	100 ±2
5 mM Fructose-6-phosphate	100 ± 3
5 mM Fructose-1,6-bisphosphate	332 ± 50
5 mM Glucose-6-phosphate	100 ± 1
5 mM Citrate	104 ± 8
5 mM Malate	50 ± 6
5 mM Fumarate	99 ± 2
10 mM Propionate	103 ± 4
10 mM Succinate	98 ± 2
10 mM $NaHCO_3$	101 ± 1

competitively.

3.4.2.7 Activation by F-2,6-P_2

The addition of 1.5 μM F-2,6-P_2 caused the sigmoid curve of velocity versus PEP concentration, in the absence of other modifiers to become less sigmoidal (Figure 30). Figure 30 also shows that F-2,6-P_2 abolished the allosteric effect of ATP, resulting in a similar, almost hyperbolic, curve to that of PK activity in the presence of F-2,6-P_2 only. While K_m's and V_{max}'s were again incalculable, the results do suggest that F-2,6-P_2 decreases the K_m and increases the V_{max}.

Inhibition of PK was hyperbolic with respect to ATP concentration (Figure 31). However the results suggest that the abolition of the inhibitory effect of ATP by F-2,6-P_2, and subsequent activation, is related to ATP concentrations. In the absence of ATP, 1.5 μM F-2,6-P_2 caused a 2-fold increase in activity under the conditions used. At 5 mM ATP, the presence of 1.5 M F-2,6-P_2 caused a 7.5-fold increase in activity compared with 5 mM ATP alone and a 2.6-fold increase with respect to no modifier at all. The activation of PK by 1.5 μM F-2,6-P_2 seemed to be greatest between 5 and 7 mM ATP, after which there was a subsequent decrease in activation.

3.4.2.8 Other effectors of PK

Several compounds were tested to find if they effected the purified PK activity. The most effective was GTP, reducing the activity by 93%, at a concentration of 5 mM (Table 19). The next most effective was ATP. The only other compound tested that inhibited the activity was 5 mM malate. Fructose-1,6-bisphosphate increased the activity by over 3-fold at a concentration of 5 mM.

4.0 DISCUSSION

4.1 Glucose catabolism in Herpetomonads

The major pathways of glucose catabolism in *H. ingenoplastis* and *H. muscarum* are summarised in schemes A and B, respectively. These schemes have been drawn up from the results of this study and the information from Hajduk (1980) on the electron transport chain. The features of these schemes are discussed below.

It should be noted that many of the data presented in this study are specific activities of enzymes. Such data only give an indication of any one enzyme's importance in metabolism and further work is required in order to measure the flux through pathways, under natural conditions.

4.1.1 Enzymes of glycolysis and the pentose-phosphate shunt

At the start of my studies little was known on the detailed biochemistry of substrate catabolism in herpetomonads. Previous studies (Hajduk 1980, Lopes *et al.* 1983) had shown that oxygen consumption was stimulated by glucose and proline to a small degree in the case of *H. muscarum*, whereas *H. ingenoplastis* oxygen consumption was increased substantially by the presence of glucose, and by proline and succinate to lesser degrees (Hajduk 1980). Since glucose resulted in the greatest increase in the case of *H. ingenoplastis*, which has several features of an anaerobe (Coombs 1990), it was decided to investigate the enzymes likely to be involved in glycolysis and the pentose-phosphate shunt.

My studies have shown that both species of *Herpetomonas* contain high activities of HK, PGI and PFK (Table 3) suggesting that glycolysis is an important pathway in their energy metabolism. 3-Phosphoglycerate kinase activities were also found, as were the first two enzymes of the pentose-phosphate shunt, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, although at lower activities than HK, PGI and PFK. The activities of HK, PGI and PFK found in the herpetomonads are

substantially lower than those in the long slender bloodstream form of *T. brucei*, but are similar to those in the procyclic forms (Fairlamb and Opperdoes 1986). The activities of these enzymes in a variety of *Leishmania* sp. (Martin *et al.* 1974, Coombs *et al.* 1982, Meade et al. 1984, Mottram and Coombs 1984) and *T. cruzi* (Taylor and Gutteridge 1987) are much lower than those found in this study for *H. muscarum* and *H. ingenoplastis*, although in the case of *L. m. mexicana* (Coombs *et al.* 1982) and *L. donovani* (Meade *et al.* 1984) there are differences in the activities of the enzymes between stages of the life cycle. In the case of *T. cruzi*, the activities of the glycolytic enzymes are similar throughout the life cycle (Taylor and Gutteridge 1987).

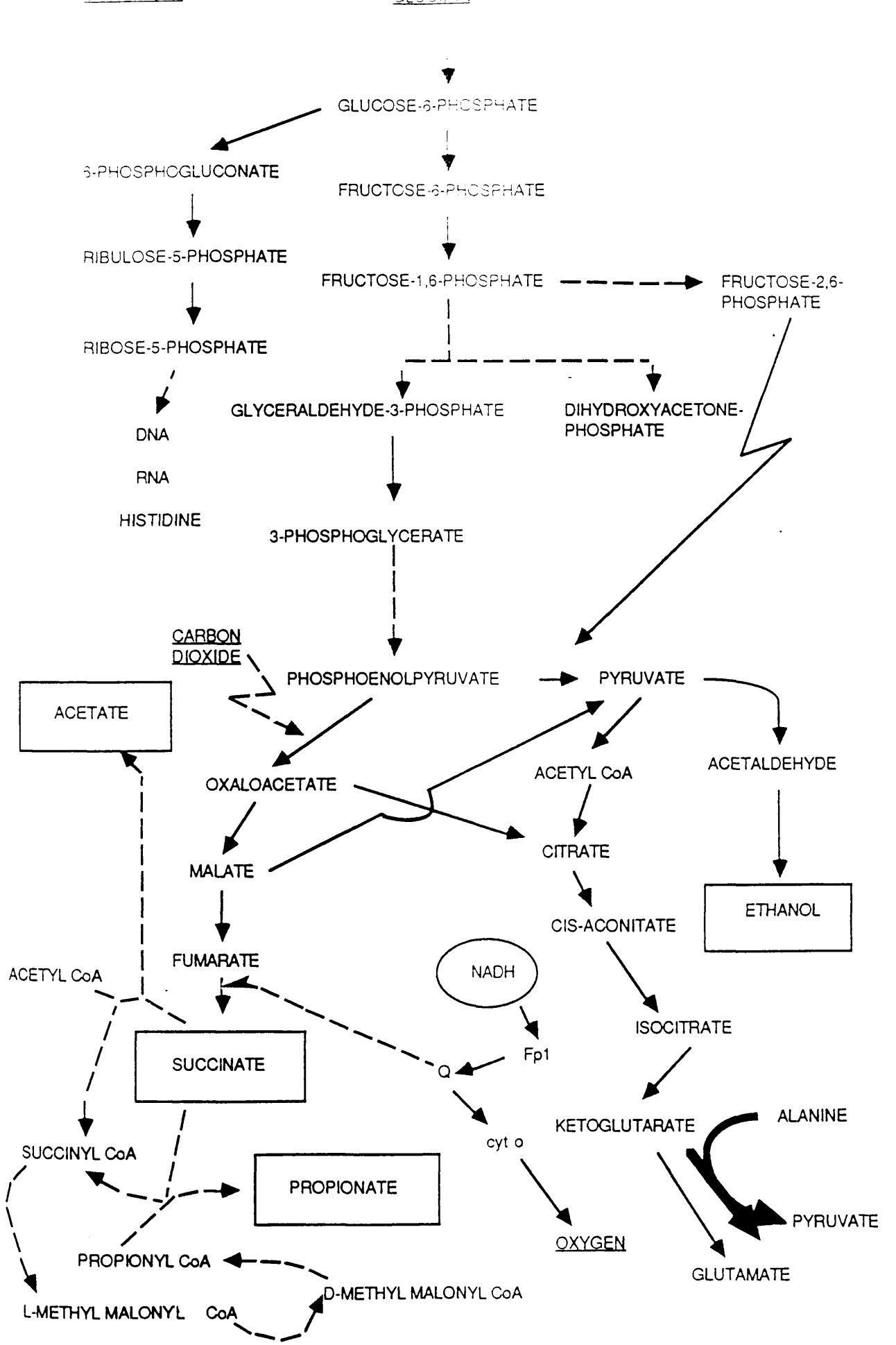
The activity of PGK in the two herpetomonads was found to be much lower than that in either the procyclic trypomastigotes or the long slender bloodstream forms of *T. brucei*, but was, again, higher than that found for four species of *Leishmania* promastigotes (Martin *et al.* 1974), and slightly higher than that found for *T. cruzi* epimastigotes (Taylor and Gutteridge 1987). The data suggest that that the glycolytic flux of the herpetomonads is much lower than that of *T. brucei*, but may be similar to that of *T. cruzi*.

Pyruvate kinase is undetectable in the procyclic forms of *T. brucei*, but is activated by F-2,6-P_2 (Flynn and Bowman 1980, Van Schaftingen *et al.*1985) to give an activity of 1200 nmoles min^{-1}(mg protein)$^{-1}$ (Van Schaftingen *et al.* 1985). Pyruvate kinase is also present in *T. cruzi* at an activity of 20 nmoles min^{-1}(mg protein)$^{-1}$ (Cazzulo *et al.* 1989) and *Leishmania* sp. promastigotes at between 14 and 343 nmoles min^{-1}(mg protein)$^{-1}$ (Martin *et al.* 1974, Mottram and Coombs 1985), and between 4 and 46 nmoles min^{-1}(mg protein)$^{-1}$ in the case of *Leishmania* amastigotes

SCHEME A

Possible pathways of glucose catabolism in *Herpetomonas ingenoplastis*. Unbroken lines represent those enzyme activities which have been shown to be present in this study, as well as that of Hajduk (1980). Broken lines represent pathways which are believed to be present, but for which no data exists. Bold lines indicate pathways which are believed to be preferred, of two possible pathways. Metabolites which are consumed are underlined, while those that are excreted are boxed. Zig-zag lines indicate possible sites of metabolic regulation and the possible regulators.

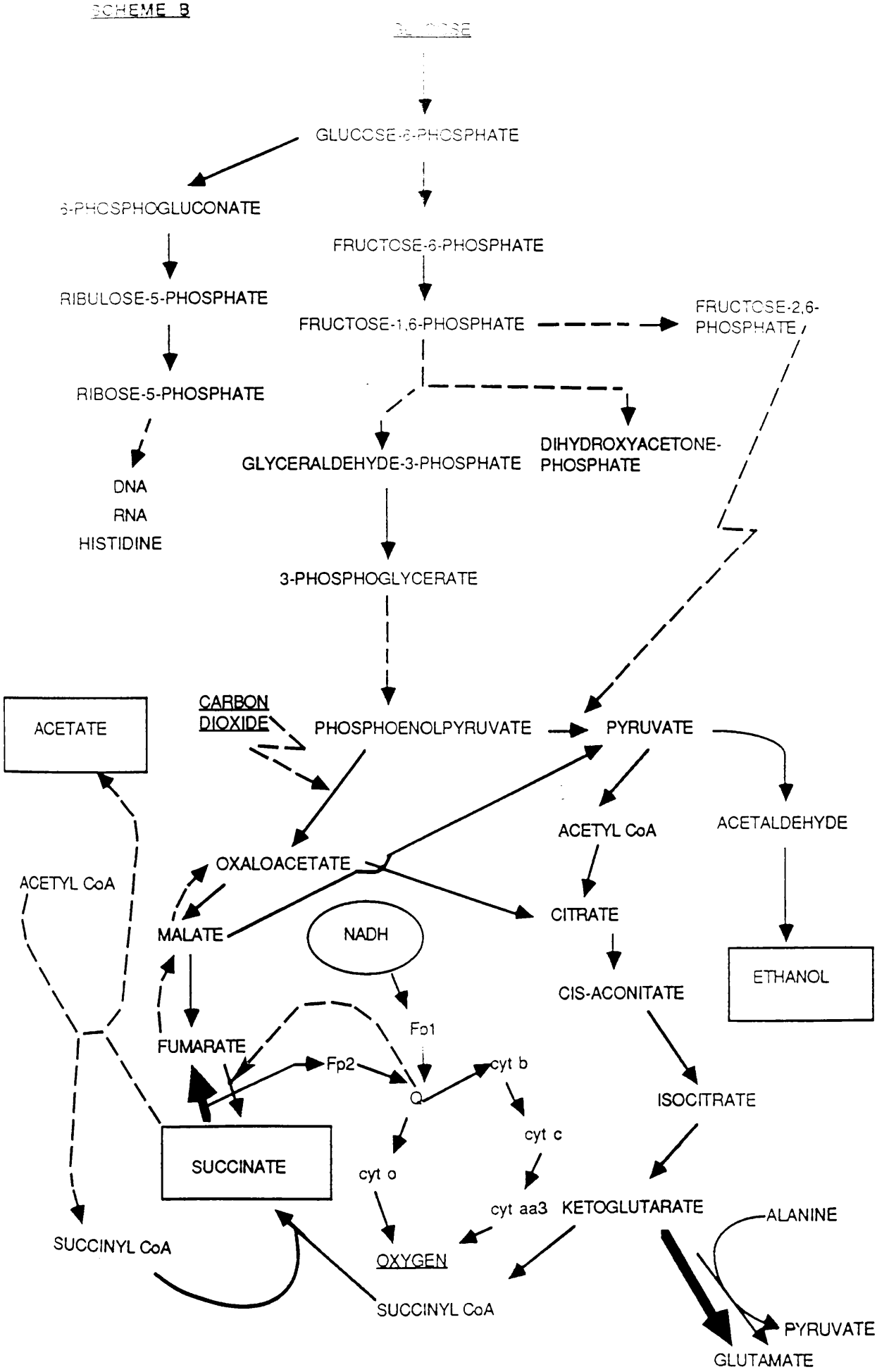
SCHEME A


GLUCOSE
GLUCOSE-6-PHOSPHATE
6-PHOSPHOGLUCONATE
FRUCTOSE-6-PHOSPHATE
RIBULOSE-5-PHOSPHATE
FRUCTOSE-1,6-PHOSPHATE
FRUCTOSE-2,6-PHOSPHATE
RIBOSE-5-PHOSPHATE
GLYCERALDEHYDE-3-PHOSPHATE
DIHYDROXYACETONE-PHOSPHATE
DNA
RNA
HISTIDINE
3-PHOSPHOGLYCERATE
CARBON DIOXIDE
PHOSPHOENOLPYRUVATE
PYRUVATE
ACETATE
ACETYL CoA
ACETALDEHYDE
OXALOACETATE
CITRATE
MALATE
ETHANOL
CIS-ACONITATE
FUMARATE
NADH
ACETYL CoA
ISOCITRATE
SUCCINATE
Fp1
Q
KETOGLUTARATE
ALANINE
cyt o
SUCCINYL CoA
PROPIONATE
PYRUVATE
OXYGEN
PROPIONYL CoA
GLUTAMATE
D-METHYL MALONYL CoA
L-METHYL MALONYL CoA

SCHEME B

Possible pathways of glucose catabolism in *Herpetomonas muscarum*. Unbroken lines represent those enzyme activities which have been shown to be present in this study, as well as that of Hajduk (1980). Broken lines represent pathways which are believed to be present, but for which no data exist. Bold lines indicate pathways which are believed to be preferred, of two possible pathways. Metabolites which are consumed are underlined, while those that are excreted are boxed. Zig-zag lines indicate possible sites of metabolic regulation and the possible regulators.

SCHEME B


GLUCOSE-6-PHOSPHATE
6-PHOSPHOGLUCONATE
FRUCTOSE-6-PHOSPHATE
RIBULOSE-5-PHOSPHATE
FRUCTOSE-1,6-PHOSPHATE
FRUCTOSE-2,6-PHOSPHATE
RIBOSE-5-PHOSPHATE
DNA
RNA
HISTIDINE
GLYCERALDEHYDE-3-PHOSPHATE
DIHYDROXYACETONE-PHOSPHATE
3-PHOSPHOGLYCERATE
ACETATE
CARBON DIOXIDE
PHOSPHOENOLPYRUVATE
PYRUVATE
ACETYL CoA
ACETALDEHYDE
OXALOACETATE
ACETYL CoA
CITRATE
MALATE
NADH
ETHANOL
CIS-ACONITATE
Fp1
FUMARATE
Fp2
cyt b
Q
ISOCITRATE
cyt c
SUCCINATE
cyt o
cyt aa3
KETOGLUTARATE
ALANINE
SUCCINYL CoA
OXYGEN
SUCCINYL CoA
PYRUVATE
GLUTAMATE

(Martin *et al.* 1974, Meade *et al.* 1984, Mottram and Coombs 1985). The activities found in this study (Table 3) were 43 and 150 nmoles min^{-1}(mg protein)$^{-1}$ for *H. muscarum* and *H. ingenoplastis*, respectively. These values are more in line with those for *Leishmania* and *T. cruzi* as opposed to those of bloodstream forms of *T. brucei,* where the enzyme is involved in converting all the glucose to pyruvate, under aerobic conditions (Ryley 1956, Fairlamb and Opperdoes 1986). This suggests, therefore, that the herpetomonads do not obtain all their energetic needs from the glycolytic pathway, as do bloodsream forms of *T. brucei*, but there may be other pathways where glucose is catabolised, forming energy.

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in *H. muscarum* and *H. ingenoplastis* are lower than those of the glycolytic enzymes, suggesting that the main route for glucose catabolism is the Embden-Meyerhof pathway. In *L. donovani* promastigotes there is an active pentose-phosphate shunt, but this plays a small role in glucose catabolism compared with the Embden-Meyerhof pathway (Ghosh and Datta 1971). Enzyme data suggest that the role of the pentose-phosphate shunt is less important in *Leishmania* sp. amastigotes, compared with promastigotes (Coombs *et al.* 1982, Meade *et al.* 1984). *Trypanosoma brucei* bloodstream forms do not have a significant pentose-phosphate shunt (Fairlamb and Opperdoes 1986) as shown by experiments using radiolabelled glucose, as well as studies showing the low activity of glucose-6-phosphate dehydrogenase and the lack of detectable 6-phosphogluconate dehydrogenase activity (Ryley 1962). The pentose-phosphate shunt is much more active in *T. cruzi*, where the Peruvian and Tulahuen strains consume 41% and 28% of glucose, respectively, via the pentose-phosphate shunt (Von Brand 1973, 1979). Glucose consumption by *H. muscarum* and *H. ingenoplastis* appears to be intermediate between the

situations in long slender bloodstream forms of *T. brucei* and *T. cruzi* with respect to the involvement of the pentose phosphate shunt.

The high activities of HK, PFK and PGI show that glucose is important. It is possible, however, that the herpetomonads supplement the energy gained from glucose catabolism *via* the Embden-Meyerhof pathway by utilising amino acids and fatty acids, as do procyclic *T. brucei* and *Leishmania* promastigotes and amastigotes (Martin *et al.* 1974, Coombs *et al.* 1982, Meade *et al.* 1984).

What the pentose-phosphate shunt and the Embden-Meyerhof pathway contribute to glucose catabolism cannot be concluded from the level of enzyme activities alone.This can only be ascertained using radiolabelled glucose and studying the products labelled, and from which part of the glucose molecule they originate.The results of this study suggest, however, that glucose is catabolised more by the Embden-Meyerhof pathway, than by the pentose phosphate shunt.

4.1.2 Analysis of metabolites consumed and produced

The high activities of HK, PGI and PFK, as well as the presence of other enzymes involved in glucose catabolism, suggests that glucose could be a major energy substrate for both species of *Herpetomonas*. The studies on the consumption and production of metabolites confirm this.

My results show that both *H. muscarum* (Table 4) and *H. ingenoplastis* (Table 5) consume glucose during growth in complex medium, under both aerobic and anerobic conditions. Ethanol is a major end product of metabolism during growth under both aerobic and anaerobic conditions, with *H. ingenoplastis* producing greater amounts.

Both herpetomonads consumed glucose and produced ethanol during the log phase of cell growth, with glucose consumption and ethanol production halting during stationary phase (Figures 12a-d). It is interesting to

note that both organisms do not consume all their glucose before, and during, stationary phase, unlike *T. cruzi* epimastigotes (Engel *et al.* 1987). This suggests that there may be one or more other substrates in the complex medium which make important contributions to the growth of both *H. muscarum* and *H. ingenoplastis*, and that they may be exhausted during log phase, causing cell division to halt. Alternately, it may be that the contributing factors to the cessation of cell division are not substrates, but products of metabolism.

However, the difference stated by Coombs (1990) between *H. muscarum* and *H. ingenoplastis*, with respect to cell growth under different gaseous condition, is clearly shown (Figures 12a-d). *Herpetomonas muscarum* grows faster, and therefore consumes glucose and produces ethanol faster, under aerobic, compared with anaerobic conditions, while *H. ingenoplastis* grows equally well under both growth conditions. The fact that *H. ingenoplastis* can grow well under anaerobic conditions makes it an unusual trypanosomatid (Coombs 1990).

The results of metabolite analysis using enzyme-based assays suggested that other than ethanol, very little was produced during growth of the herpetomonads. Neither herpetomonad produced pyruvate, D- or L-lactate. D-lactate is a major end product of glucose catabolism of *T. lewisi* and *Leishmania* sp. (Darling *et al.* 1988), while L-lactate is produced by *T. congolense* and *T. lewisi* (Ryley 1956, Darling *et al.* 1988). As stated earlier pyruvate is a major end product of *T. brucei* bloodstream form glucose catabolism, as well as *C. fasciculata* and *C. oncopelti* catabolism (Ryley 1955, Cazzulo *et al.* 1985).

Glycerol is a minor end product of *H. ingenoplastis* metabolism, while the evidence suggests that *H. muscarum* consumes glycerol during growth. Glycerol stimulates oxygen consumption by intact *H. muscarum* to a similar degree to that of glucose (Lopes *et al.* 1983) and therefore may contribute to

the energy erquirements of this organism. The results presented in this study suggest that this is not the case for *H. ingenoplastis*. In bloodstream forms of *T. brucei,* glycerol is produced under anaerobic conditions as a major end product of glucose catabolism (Ryley 1956, Fairlamb and Opperdoes 1986), but is not produced under aerobic conditions, where pyruvate is the major end product. The glycerol formed by *T. brucei* is believed to be produced by a reversal of glycerol kinase, located in the glycosomes, allowing synthesis of ATP under anaerobic conditions (Opperdoes and Borst 1977, Fairlamb and Opperdoes 1986). Interestingly infective forms of *T. brucei* isolated from the salivary glands of tsetse flies were found to produce pyruvate and glycerol as major end products during glucose catabolism, as well as other unidentified products under aerobic conditions (Njogu and Njindo 1981, Fairlamb and Opperdoes 1986). Bloodstream forms only produced pyruvate. The culture conditions in the above experiment (Njogu and Njindo 1981) required a monolayer of bovine embryonic spleen cells, therefore the cells may have been bloodstream forms, and not infective metacyclics (Fairlamb and Opperdoes 1986). If so, then they did not behave like the control bloodstream forms under the experimental conditions, something which requires further study.

The method, modified from that used by Guerrant and coworkers (1982), using HPLC had the potential for identifying at least 20 SCFAs (Table 6) in one assay. The extraction efficiencies were quite consistent between experiments (Table 7), allowing the quantification of the SCFAs produced. These extraction efficiencies compared well with those found by Guerrant and coworkers (1982), where propionate and L-lactate were found to have extraction percentages of 85% and 25%, respectively, compared to the results found in this study of 92% and 21%, respectively. This method has advantages over enzyme-based assays, which are 'hit-and-miss' with respect to detection, and gas-liquid chromatography, where non-volatile

dicarboxylic acids require a derivatization step before analysis. No derivatization is required to detect SCFAs using the method described in this study. However a disadvantage of this system of detection was its limitation to SCFAs, such that it could not be used to analyse alcohols, amino acids or CO_2. These latter compounds, as well as SCFAs, can be detected using nuclear magnetic resonance spectroscopy. This method analyses the products of metabolism using radiolabelled substrates, allowing the detection of which pathways were involved in the production of the metabolites.

Subsequent experiments revealed another limitation of my HPLC method in quantifying organic acids in complex medium. The method involved quantification of eluted SCFAs using peak height (Figure 14a-c), however analysis of complex media revealed that many peaks ran into each other, resulting in concentrations calculated from these results being inaccurate (Figures 16a-d). This is exemplified by the results of the SCFAs produced during growth of both *H. muscarum* and *H. ingenoplastis* in LIT medium (Figures 15a-d), where the results suggest that succinate is produced only during late log/stationary phase. The succinate peak was hidden by the pyruvate peak, which preceded it, and therefore was not detectable. Thus, while this method can be used to identify and quantify SCFAs, it has limited applications in experiments using complex media. However, the method can be used to identify SCFAs produced and another, e.g. enzyme-based assays, can then be used to quantify the products.

The studies with complex medium using HPLC, while not accurately quantifying the products, did confirm the results found earlier in that there was no evidence for the production of pyruvate, D-lactate or L-lactate by either herpetomonad. Secondly, and more importantly, both *H. muscarum* and *H. ingenoplastis* were shown to produce acetate, succinate and propionate during growth in LIT medium, and the results suggested that the

concentration of propionate produced by *H. ingenoplastis* was greater than *H. muscarum* under anaerobic conditions. This is the first record of any trypanosomatid producing propionate as an end product of metabolism.

Analysis of SCFAs produced using HPLC analysis was shown to be more easily quantitated in the case of the short-term incubations, where there were few and only small 'background' peaks (Figures 17a and b), such that the peaks of any SCFAs produced were clearly separate. Propionate, succinate and acetate were all shown to have distinct elution times, without any overlap of the peaks, allowing accurate quantification to be carried out on the basis of peak height.

The results of analyses of short-term incubation samples showed clearly that *H. ingenoplastis* produces propionate as a major end product during glucose catabolism. The results suggested that *H. muscarum* does not produce propionate as a major end product of glucose catabolism, with none being detected except under anaerobic conditions, where it was detected as a minor end product (Table 9). The propionate detected during long-term experiments, which was found to be produced under both aerobic and anaerobic conditions, may have been due to the catabolism of a compound other than glucose or perhaps due to intrinsic differences between the long-term and short-term experiments, e.g. lower cell densities. What is clear is that there are metabolic differences between growth in complex medium, as shown by the results in the long-term experiments, and short-term incubations, with glucose as the only exogenous carbon source. Any conclusion reached from the results for short-term incubations can only be used to build up a picture of what is happening during growth in the complex medium. This study was concerned with glucose catabolism. Many more studies on fatty acid and amino acid catabolism are required in order to understand the results found for the growth of the herpetomonads in LIT medium.

The results for the short-term incubations suggest that alterations, with respect to glucose catabolism, occur under different gaseous conditions (Table 9). In the case of *H. muscarum,* glucose consumption altered slightly under the four gaseous conditions used. It was lowest under simple anaerobic conditions and greatest under aerobic and anaerobic conditions in the presence of CO_2. While ethanol and propionate were shown to be minor end products of glucose catabolism, acetate and succinate were produced as major end products by pathways which may be controlled by the gaseous conditions. Succinate production was greatest in the presence of CO_2, suggesting that there was a CO_2-fixing pathway in operation, involved in the formation of succinate. This CO_2-fixing pathway may also be linked to acetate production, which is also greatest under the two conditions containing CO_2. The results also suggest that a CO_2-fixing pathway exists in *H. ingenoplastis,* leading to the production of succinate and propionate.

It is interesting to note the presence of a 'reverse Pasteur' effect, where an increase in oxygen tensions lead to an increase in glucose consumption. Intriguingly, increased oxygen tensions also caused an increase in ethanol production. In yeast cells there is a huge increase in ethanol production, and glucose consumption, under anaerobic conditions, compared with aerobic conditions. This 'Pasteur' effect operates in order to maintain NAD^+ and ATP levels using glycolysis as opposed to oxidative phosphorylation, which can only work under aerobic conditions. A reverse Pasteur effect has been reported to exist in *Leishmania* and trypanosomes (Darling *et al.* 1987, 1988, 1989). This effect does not occur with the addition of CO_2 in the case of *Leishmania* (Darling *et al.* 1987, 1988, 1989). The reverse Pasteur effect was not abolished by the presence of CO_2 in the case of *H. muscarum*, although in the case of *H. ingenoplastis* the reverse Pasteur effect was reduced in the presence of CO_2. Unlike yeast, where increased

increased ethanol production is due to a need to maintain redox balance and energy levels under anaerobic conditions, the pathway by which *H. muscarum* and *H. ingenoplastis* produce energy under anaerobic conditions seems to involve the fixation of CO_2 to produce succinate, and therefore possibly acetate or propionate, respectively.

One possible reason for the increased production of ethanol under aerobic conditions by *H. ingenoplastis* could be the increased flux *via* PK into the forward TCA cycle. The results of this study suggests that the presence of CO_2 is important in the formation of succinate in both herpetomonads, especially under anaerobic conditions. It is conceivable, therefore, that under aerobic conditions the major pathway of carbon flux from glucose catabolism is the formation of phosphoenolpyruvate (see Scheme A) and then to pyruvate, *via* PK. Pyruvate can then be catabolised to form acetyl CoA, which enters the TCA cycle, by pyruvate dehydrogenase. A consequence of this flux in to the forward TCA cycle could be that some pyruvate is converted to acetaldehyde and ethanol. Under anaerobic conditions PEPCK would fix CO_2 (see Section 4.1.5), causing the flux to be via the reverse TCA cycle, avoiding the formation of pyruvate, and therefore ethanol.

Hajduk (1980) showed that consumption of oxygen was faster in *H. muscarum*, compared with *H. ingenoplastis.* It may be that the respiratory chain of *H. ingenoplastis* is less effective. Under aerobic conditions alcohol dehydrogenase may have a role in re-oxidising NADH for both glyceraldehyde-3-phosphate dehydrogenase, and pyruvate dehydrogenase, therefore helping maintain glycolytic flux. Under anaerobic conditions, where the electron transport chain would be inactive, PEPCK would serve in both NADH reoxidation to maintain glycolytic flux, and formation of ATP.

4.1.3 Pathways of glucose catabolism

The results of enzyme analysis, in conjuction with the results for short-term incubations, suggest that at least three possible pathways exist by which glucose could be catabolised by *H. muscarum* and *H. ingenoplastis* (see schemes A and B).

As with *C. fasciculata* and *C. oncopelti* (Von Brand 1973, 1979) the herpetomonads produce ethanol during glucose catabolism. This is probably carried out by the two enzymes, pyruvate decarboxylase, which catalyses the formation of acetaldehyde from pyruvate, from the Embden-Meyerhof pathway, and NAD-alcohol dehydrogenase, which reduces the acetaldehyde to ethanol. This pathway is similar to that found in many aerobic organisms, including *C. oncopelti* (Von Brand 1973). An NADP-linked alcohol dehydrogenase is present in *C. oncopelti* and *T. cruzi* (Arauzo and Cazzulo 1989) and is able to use both aromatic and aliphatic aldehydes and alcohols, and may be involved in the detoxification of exogenous and endogenous aldehydes, as opposed the the reoxidation of glycolytic reduced-coenzymes.

Both *H. muscarum* and *H. ingenoplastis* also have an active pyruvate dehydrogenase (Table 11) which converts pyruvate to acetyl CoA, which enters the TCA cycle (Table 13). The activities of all the TCA cycle enzymes, except NAD-ICDH, were present in *H. muscarum*, whereas NAD-ICDH, α-ketoglutarate dehydrogenase, succinyl CoA synthase, succinate thiokinase and SDH were not detected in *H. ingenoplastis*. In *Leishmania* sp., most of the TCA cycle enzymes have been found, the exception being NAD-ICDH (Martin *et al.* 1974, Meade *et al.* 1984), with higher activities of NADP-ICDH,α-ketoglutarate dehydrogenase, SDH and FR in promastigotes compared with amastigotes (Coombs *et al.* 1982, Meade *et al* 1984). Like both *H. muscarum* and *H. ingenoplastis*, the activity of citrate synthase was

very low in promastigotes and, especially, amastigotes and Marr (1980) suggested that this implied a slow TCA cycle, compared with CO_2 fixation pathway. In *T. cruzi*, while there is no evidence for NAD-ICDH, there is an active TCA cycle, which, under aerobic conditions, is used to form succinate and CO_2 as major end products of glucose catabolism (Adroher *et al.* 1988). This TCA cycle is believed to be more active in infective metacyclic forms, than in the epimastigote forms, probably due to the increase in the use of proline as a metabolic substrate by these forms (Adroher *et al.* 1988). In contrast, cultured amastigote-like forms have a repressed TCA cycle, possibly due to the preferential use of glucose, as opposed to proline or other amino acids, as carbon source (Engel *et al.* 1987). Procyclic forms of *T. brucei* have an active TCA cycle, as shown by studies using radiolabelled substrates (Fairlamb and Opperdoes 1986), although the enzyme activity of citrate synthase and NAD-ICDH have not been detected. The latter example shows how the lack of detection of certain enzymes does not mean that these enzymes are not present, merely that they are undetectable with the methods used. However, while a TCA cycle may exist in *H. ingenoplastis*, results from this study suggest that it is much less active than that found in *H. muscarum*.

There was no evidence found in this study for any isocitrate lyase, and therefore no glyoxylate bypass activity, in either *H. muscarum* or *H. ingenoplastis*, although this pathway has been reported in promastigotes of *L. mexicana*, *L. donocani*, *L. tropica* and *L braziliensis* (Mukkada 1977, Simon *et al.* 1978).

The differences in the activities of the TCA cycles in *H. muscarum* and *H. ingenoplastis* are intriguing, and suggest a substantial difference between the two organisms at a metabolic level. While both catabolise glucose, via an active Embden-Meyerhof pathway, to PEP and then to pyruvate, the main pathway to reoxidise NADH in *H. ingenoplastis* may be

NAD-linked alcohol dehydrogenase, whereas *H. muscarum* relies on the active electron transport chain.

Herpetomonas muscarum has a very high activity of NAD-linked glutamate dehydrogenase (over 6-fold greater than *H. ingenoplastis*) (Table 14), which converts α-ketoglutarate, an intermediate of the TCA cycle, into glutamate. The fact that glutamate dehydrogenase was found in *H. ingenoplastis* suggests that the forward TCA cycle is active in this organism. There is evidence that *H. ingenoplastis* may catabolise amino acids. The fact that proline causes an increase in respiration, although small compared with glucose (Hajduk 1980), suggests that this amino acid may be catabolised, possibly employing the glutamate dehydrogenase activity in reverse. Also the very high activity of alanine aminotransferase (9-fold greater than *H. muscarum*) suggests that alanine can be used as a carbon source, feeding carbons into the Embden-Meyerhof pathway at pyruvate. Intracellular pools of alanine in *Leishmania* are believed to be utilised to meet energy needs during starvation (Darling *et al.* 1987), although such intracellular pools are thought unlikely to exist in *H. ingenoplastis* due to the low respiratory rate in the absence of exogenous substrates (Hajduk 1980).

The third pathway by which glycolytic intermediates are further catabolised are by a CO_2-fixation pathway (Table 12). Two CO_2-fixing enzymes were found at high activities in *H. ingenoplastis*, PEPCK and ME. While PEPCK was found at a similar activity to that in *H. muscarum*, this study found the activity of ME to be 10-fold lower in the latter organism. Both these enzymes have been found in a variety of trypanosomatids (Von Brand 1979), but the major CO_2-fixation pathway is believed to be via PEPCK, producing malate, which occurs in the glycosome in all trypanosomatids studied (Coombs *et al.* 1980, Cazzulo *et al.* 1980, Opperdoes and Cottem 1982, Mottram and Coombs 1985). Also present in the glycosomes of *T.*

brucei procyclic forms is MDH, which produces malate from oxaloacetate, formed by PEPCK (Fairlamb and Opperdoes 1986). In these organisms, the malate formed can enter the TCA cycle, be converted to aspartate, or be converted to pyruvate by ME. The pyruvate that is formed is then transaminated to alanine, an end product (Fairlamb and Opperdoes 1986). The results in this study suggest that malate formed via CO_2-fixation would probably enter the TCA cycle, in the cases of both *H. muscarum* and *H. ingenoplastis.* However, the high activity of ME in *H. ingenoplastis* suggests that the malate formed could be converted into pyruvate, as in the procyclic forms of *T. brucei.*

The fact that succinate excretion seems to be associated with CO_2-fixation in both herpetomonads, suggests that products of CO_2-fixation are being fed into the TCA cycle. Of especial interest, in both *H. muscarum* and *H. ingenoplastis*, is the presence of FR activity, 3-fold higher in the latter organism than in the former. In *T. cruzi* epimastigote, CO_2-fixation occurs, producing succinate, *via* the forward TCA cycle, under aerobic conditions, and by the reverse TCA cycle, *via* FR, under anaerobic conditions (Bowman *et al.* 1963). This enzyme has been detected in amastigotes, but not promastigotes, of *L. m. mexicana* (Mottram 1984), along with high activites of PEPCK and MDH, as well as in the procyclic forms of *T. brucei* and in *C. fasciculata* (Baernstein 1953, Ryley 1962, Bacchi *et al.* 1970). That *H. muscarum* has a more active forward TCA cycle, and electron transport chain (Hajduk 1980), while having a less active reverse TCA cycle, compared with *H. ingenoplastis*, as suggested by the SDH/FR ratios, suggests that the operation of the reverse TCA cycle may be important in the abilty of *H. ingenoplastis* to survive well under anaerobic growth conditions (see Section 4.1.5).

4.1.4 Proteinases

While there are no data, from this study, on the characteristics of any internal proteinases of *H. muscarum* and *H. ingenoplastis*, there does seem to be a good correlation between total cellular proteinase and secreted proteinases with respect to pH profile.

The cellular proteinase activity of *H. muscarum* at pH greater than 7.0 (Figure 18) is much less than that of *H. ingenoplastis*. Both *H. muscarum*, and *H. ingenoplastis* proteinase activity peaked between pH 8.0 and 8.9, while extra bands appeared, at 50 and 52.5 kDa, when extracellular extracts are incubated at pH's 8.0 and 8.9, respectively. Also, in the case of *H. muscarum*, the activity of the 105 and 87 kDa bands appeared to be much greater at pH 8.9 (Figure 20). There is no evidence for excretion of proteinases by any other trypanosomatid, although there is an enzyme known as gp63 on the surface of *L. major* promastigotes (Etges *et al.* 1986). This is a zinc metalloproteinase (Bouvier *et al.* 1989). Surface activities have also been found on *T. cruzi*, *Leishmania* sp., *Crithidia* sp. and *Herpetomonas* sp. (Piras *et al* 1985, Coombs *et al.* 1987, Lockwood *et al.* 1987, Bouvier *et al* 1989, Greig and Ashall 1990). However, it is believed that, in the case of *T. cruzi* and *L. major*, these surface activities are involved in the attachment and invasion of the host cells (Bouvier *et al.* 1989, Greig and Ashall 1990).

It is possible that the extracellular activities of *H. muscarum* and *H. ingenoplastis* are involved in nutrition. Certainly fungi excrete proteinases in order to meet nutritional needs (North 1982) and *T. vaginalis* excretes cysteine proteinases of 23 and 60 kDa, which are also believed to be involved in meeting the cells' energetic needs (Lockwood *et al* 1987, North *et al.* 1990).

The identitiy of the proteinases, with respect to class, cannot be concluded, although the 50 and 52.5 kDa bands, from *H. muscarum* and *H.*

ingenoplastis, respectively, are possibly serine proteinases, due to their activity in alkaline pH. Several organisms excrete serine proteinases, including *Onchocerca* (Lackey *et al.* 1989, McKerrowe *et al.* 1989) and *Toxocara canis* (Robertson *et al.* 1989).

The results presented in this study give an indication that proteinases may play a role in the nutrition of the herpetomonads, although much more evidence is required. The activities shown in the gels are low, but there may be a system, as in fungi (North 1982), where proteinase secretion is controlled by the nutritional requirements of the organisms. More study is required to elucidate the role of these enzymes in the metabolism of herpetomonads.

4.1.5 Subcellular organisation of metabolism

Two areas of chemotherapeutic attack against mammalian trypanosomes are glycosomes (Fairlamb and Opperdoes 1986) and the FR activity (Turrens *et al.* 1989).

In *T. cruzi*, 96% of the FR activity is associated with the mitochondrial membrane, along with SDH and the cytochromes (Boveris *et al.* 1986). Fumarate reductase has also been shown to have an important role in the metabolism of *T. brucei* procyclic trypomastigotes (Turrens 1989) where it has been suggested that the NADH-ubiquinione segment of the respiratory chain, found on the inner mitochondrial membrane, is replaced by NADH-FR. This oxidises NADH and is, in turn, oxidised by the respiratory chain, a product being succinate, which is then excreted.

In this study, 54% of the FR was found to be associated with the membrane in the case of *H. ingenoplastis*; 74% in the case of *H. muscarum*, along with 88% of the SDH activity (Table 15). Similar results were found when cells were lysed with alumina, and fractions prepared by differential centrifugation (Tables 16 and 17). The particulate FR activities were not

inhibited by rotenone, malonate or TTFA, which suggests that they are not associated with SDH and the electron transport chain. Certainly FR has an important physiological role in both herpetomonads. The SDH/FR ratio for *H. muscarum* was found to be 5.02, and <0.08 for *H. ingenoplastis*. These values compare with 60 for beef heart (Kimura *et al.* 1967), a tissue which operates under aerobic conditions, 2.3 for the metazoan *Fasciola hepatica* (Lara 1959, Barret 1976), which is a facultative anaerobe, and 0.03 for the obligate anerobe *Micrococcus lactideus* (Warringa *et al.* 1958). The ratio for *T. cruzi* is 0.28, which is suggestive of an important role for FR in the metabolism of this parasite (Boveris *et al.* 1986). Boveris and coworkers (1986) suggested that this enzyme would be important in tissues containing high CO_2 and low oxygen levels, where CO_2 would be fixed and the resulting oxaloacetate formed could enter the reverse TCA cycle *via* malate dehydrogenase. These findings certainly seem to confirm the anaerobic nature of *H. ingenoplastis*, although *H. muscarum* also seems to have certain anaerobic characteristics, including a SDH/FR ratio that is similar to the facultative anaerobe *F. hepatica*. More studies are required to elucidate the significance of FR to both *H. muscarum* and *H. ingenoplastis*. Studying differences in the respiratory chain between the two organisms, which have such different characteristics with respect to growth and oxygen consumption, may lead to a greater understanding of their adaptations to anaerobic conditions. *Trypanosoma cruzi* also has some features of a facultative anaerobe, such as the low SDH/FR ratio, and the presence of superoxide dismutase (Boveris *et al.* 1986), as well as the aerobic features of the presence of a complete cytochrome chain, consisting of cytochromes b, c and aa_3 (Docampo *et al.* 1978). The presence of a cytochrome o has also been suggested, although it's role is believed to unimportant, as respiration is 95% inhibited by antimycin A (Docampo *et al.* 1978). *Herpetomonas muscarum* is similar, in that it posesses a complete

respiratory chain, including cytochrome o, (Hajduk 1980) as well as a relatively similar SDH/FR ratio. The apparent lack of a complete respiratory chain in *H. ingenoplastis*, where cytochrome aa_3 is absent, but cytochromes o, c, and a b-like cytochrome are present (Hajduk 1980) questions its functional significance to this organism.

Differential centrifugation of homogenates of *H. muscarum* (Table 16) and *H. ingenoplastis* (Table 17) revealed that there is substantial particulate HK activities in both cases. Conclusions from these results can only be limited. However, in the case of *H. muscarum*, the patterns of distribution of the enzymes HK and PGI are similar to those found with *L. m. mexicana* promastigotes (Coombs *et al.* 1982, Mottram and Coombs 1985). There were large percentages of particulate HK and PGI activites in P2, with 32% and 19% of the activities in this fraction. This would indicate the possible presence of glycosomes in this organism, as in other trypanosomatids. The difference in the amounts of HK and PGI activities found in P2 also was found with other trypanosomatids. The pututive explanation is that PGI is not believed to be part of the multi-enzyme complex which appears to exist inside the glycosome, and to which many other enzymes belong (Fairlamb and Opperdoes 1986). This multi-enzyme complex is not believed to channel substrates, and its function is not known (Aman and Wang 1986). The majority of the glucose-6-phosphate dehydrogenase was recovered in the soluble fraction, suggesting that it is, in the main, cytosolic. There is some particulate glucose-6-phosphate dehydrogenase activity, which was also reported to occur in *L. m. mexicana* (Mottram and Coombs 1985). This activity may be linked to the glycosome, perhaps allowing glycolytic flux into the pentose phosphate shunt. As well as glycosomes, the P2 fraction of *H. muscarum* may also have contained mitochondrial vesicles, as shown by the presence of FR, SDH and NADH oxidase, possibly representing the electron transport chain. The fact that there was substantial particulate activities of all

three enzymes suggests that that all may be involved in the electron transport chain, perhaps in a similar way to *T. cruzi* (Boveris *et al.* 1986) and *T. brucei* (Turrens 1989). Scheme B shows how fumarate may act as an alternative electron acceptor, receiving electrons from NADH reduction *via* NADH dehydrogenase (oxidase), ubiquinione and fumarate reductase. However considering that FR was not inhibited by rotenone, this suggests that the electron transport chain from NADH to fumarate is not as it is in *Ascaris suum* (Kohler 1985) and that important differences must exist. Certainly this pathway in *H. muscarum* warrants further investigation.

The pattern of subcellular distribution for *H. ingenoplastis* (Table 17) was found to be substantially different from that of *H. muscarum*. Other than FR, little enzyme activity was found in the P2 fraction. The results presented do not neccessarily mean a lack of glycosomes in this organism. Therefore, there may be important differences in the structure of glycosomes, explaining why a different pattern was obtained using the methods described in this study. Certainly HK and MDH do have some particulate activity, which is pelleted by the high speed spin (P3), while glucose-6-phosphate dehydrogenase seems to be cytosolic. Whether the P3 contained glycosomes requires further study. However, it should be noted that when *H. ingenoplastis* were lysed with 0.1% Triton X-100 and centrifuged at 10000g (Figure 22), more than 70% of HK, MDH, PEPCK and 8% of glucose-6-phosphate dehydrogenase were found to be particulate. These results suggest that *H. ingenoplastis* may contain glycosomes, but that they are less stable with respect to the multi-enzyme complex than those of other species, and may require less abrasive techniques to purify them.

4.1.6 The pyruvate kinase of *H. ingenoplastis*

The primary aim of this part of my study was to purify both PK and PEPCK, and to compare them with respect to activity and substrate affinity in

order to provide insight into how metabolic fluxes were regulated at this apparent branch point. However, it proved possible to study only PK.

This enzyme was found to be similar in many ways to PK present in other trypanosomatids. The subunit molecular weight of 63 kDa compares with 59 kDa of the enzyme of *L. major* (Etges and Mukkada 1988), while that of *T. brucei* is a tetramer of 270 kDa (Flynn and Bowman 1981). The subunit molecular weight of the tetrameric PK's of yeast range from 42 and 62 kDa (Flynn and Bowman 1981).

The pH optimum of 7.5, in the absence of F-1,6-P_2, is similar to the 7.0 and 7.6, in the absence and presence of F-1,6-P_2, respectively, in the case of the enzyme in *L. major* promastigotes (Etges and Mukkada 1988).

It seemed that, as in *T. brucei* and *T. cruzi*, F-2,6-P_2 had an important role in the regulation of PK of *H. ingenoplastis*, and therefore in the regulation of the glycolytic flux. Like other trypanosomatids, the PK of *H. ingenoplastis* also showed positive cooperativity in the binding of PEP, and was activated by F-2,6-P_2. The binding of ADP, on the other hand, showed no cooperativity. The sigmoid shape of the curve of activity versus PEP concentration (Figure 30) was abolished by the addition of 1.5 μM F-2,6-P_2. The presence of 5 mM ATP increased the sigmoid nature of the curve, and therefore inhibited the enzyme at subsaturating concentrations of PEP. As in the study of Cazzulo and coworkers (1989) on the PK of *T. cruzi*, the inhibition of the enzyme by ATP was not due to chelation of Mg^{2+}, as the concentration of $MgCl_2$ used in the kinetic studies was about 3-fold greater than the concentration required to saturate the enzyme. Fructose-2,6-bisphosphate is also an important glycolytic regulator in other cells. This compound stimulates all mammalian PFK's, along with AMP, the latter compound having no effect on the PK of *L. major* (Etges and Mukkada 1988) or *H. ingenoplastis* (Table 22). Fructose-2,6-bisphosphate also relieves the

inhibition of PFK exerted by ATP (Van Schaftingen 1987). In fact, the effect F-2,6-P_2 has on the PFK's of other cells is very similar to it's effect on the PK of trypanosomatids. Fructose-2,6-bisphosphate stimulates yeast PFK, lowering the K_m for fructose-6-phosphate, and raising the V_{max}. However, AMP also activates this enzyme, and is synergistic with F-2,6-P_2 (Van Schaftingen 1987). While having no direct effect on the PKs of yeast, *Euglena* and mammalian cells, there is an indirect activation effect by F-2,6-P_2 of PK in these cells. In the case of rat liver cells, insulin activates PFK2, a PFK isoenzyme, via phosphorylation, causing an increase in the concentration of F-2,6-P_2 (Probst and Unthan-Fechner 1985). This, in turn, activates PFK1, causing an increase in F-1,6-P_2, which causes a dephosphorylation of PK, by inhibiting cAMP dependant protein kinase, resulting in increased activity (Probst and Unchan-Fechner 1985).

The enzyme of *H. ingenoplastis* does differ from those in other trypanosomatids in two ways. Firstly, whereas the PK of *L. major* has an absolute requirement for KCl (Etges and Mukkada 1988), the enzyme of *H. ingenoplastis* has an absolute requirement for $MgCl_2$. Secondly, and probably more importantly, the K_m of the PK of *H. ingenoplastis* for ADP (1.41 mM) is much greater than that for other trypanosomatids. In the case of *L. major* it is 0.03 mM, for *T. cruzi* it is 0.34 mM, it is 0.041 mM for *C. fasciculata* and the value for the *T. brucei* enzyme is 0.082 mM (Etges and Mukkada 1988). Therefore the enzyme of *H. ingenoplastis* has an affinity of between 4- and 40-fold less than the enzymes of other trypanosomatids. This relatively low affinity, combined with high environmental CO_2 concentations may result in the glycolytic flux being directed through PEPCK, as opposed to PK, merely due to the mass action effect of CO_2. This could explain the higher succinate, and propionate, concentrations produced when the cells were incubated in the presence of CO_2 under anaerobic conditions. Oxygen must also play a role, probably indirectly, as

under aerobic conditions, in the presence of CO_2, succinate and propionate production was reduced. Carbon dioxide fixation may be used, under anaerobic conditions, in order to produce ATP molecules at PEPCK, and possibly during propionate production.

4.2 The anaerobic nature of *H. ingenoplastis*

The fact that *H. ingenoplastis* grows equally well under both aerobic and anaerobic conditions makes it unusual among trypanosomatids (Coombs 1990). Another indication of its anaerobic nature is the inhibition of growth by the drug metronidazole, a 5-nitroimidazole (Coombs 1990). Metronidazole is commonly used in the treatment of diseases caused by anaerobic protozoa and bacteria, and is believed to be specific against anaerobes (Coombs 1990). In culture this drug kills *Trichomonas vaginalis*, an anaerobic protozoan, at a concentration of 1 $\mu g\ ml^{-1}$, while *H. ingenoplastis* are killed at concentrations of over 20 $\mu g\ ml^{-1}$(Coombs 1990). In contrast, *H. muscarum* survived the effects of metronidazole at concentrations of 100$\mu g\ ml^{-1}$.

This study found that *H. ingenoplastis* contained low activities of both pyruvate: methyl viologen oxidoreductase and NADH: methyl viologen oxidoreductase, and higher activities of pyruvate dehydrogenase. It would be interesting to find if the first two activities represent the enzymes pyruvate: ferredoxin oxidoreductase and NADH: ferredoxin oxidoreductase, respectively, for two reasons. The first reason is that no organism has been found to contain pyruvate dehydrogenase and pyruvate: ferredoxin oxidoreductase, both of which catalyse the conversion of pyruvate to acetyl CoA (Muller 1988). Secondly, both the ferredoxin oxidoreductases are present in trichomonads, such as *T. vaginalis* and *Tritrichomonas foetus*, and

are involved in the reduction of metronidazole in organelles known as hydrogenosomes (Yarlett *et al.* 1985, Thong and Coombs 1987, Muller 1988). These organelles are spherical, and approximately 0.5-1.0 μm in diameter, and in trichomonds, are surrounded by 2 closely opposed membranes (Muller 1988). The major role of these organelles is the conversion of pyruvate to acetate, with the formation of ATP (Muller 1988). This pathway involves the reduction of ferredoxin, which can then be reoxidised by NAD: ferredoxin oxidoreductase (Thong and Coombs 1987), or protons, forming hydrogen, or by metronidazole, to form a one-electron nitro-free radical, which is believed to be the toxic form of the drug (Yarlett *et al.* 1985). Whether such a system exists in *H. ingenoplastis* remains to be seen. Further study requires the elucidation of the pathway to acetate formation in both *H. muscarum* and *H. ingenoplastis*, to see whether pyruvate: ferredoxin oxidoreductase is involved in the activation of metronidazole, in the latter organism, and whether other enzymes, found in trichomonad hydrogenosomes, may also be involved.

Another avenue of study would be into the effects of different gaseous conditions. The conditions I have defined in this study as anaerobic may not be truly anaerobic. Small concentrations of oxygen could have remained, having a dramatic effect on both growth and metabolism of *H. ingenoplastis.* In the case of *T. vaginalis*, which has usually been considered to be an obligate anaerobe (Muller 1988), recent evidence has shown that this organism grows best in the presence of traces of oxygen, and CO_2 levels at 5 mM, and that growth is slowest under anaerobic conditions in the absence of CO_2 (Paget and Lloyd 1990). It is not known why O_2 should have a stimulatory effect on growth, although it has been suggested that O_2 may act as a terminal electron acceptor in the hydrogenosomal electron transport chain (Chapman *et al.* 1986).

It is interesting to note that *T. vaginalis* grown in conditions of trace O_2

and high CO_2 have lower activities of pyruvate: ferredoxin oxidoreductase, than those grown in anaerobic conditions, and may be more resistant to metronidazole (Paget and Lloyd 1990). That lower pyruvate: ferredoxin oxidoreductase activity corresponds with decreased sensitivity to metronidazole in the above study suggests that the very low pyruvate: methyl viologen oxidoreductase activity found in *H. ingenoplastis* is conferring some sensitivity of the organism to metronidazole, but not as much as that found for *T. vaginalis*.

Of especial interest is the formation of propionate by *H. ingenoplastis* as a major end product of glucose catabolism. It is the first trypanosomatid reported to do so. A variety of facultative anaerobes have been found to excrete propionate, e.g. the cestodes *Monieza expansa* (Bryant and Behm 1976), *Spirometra mansonoides* (Pietrzak and Saz 1981), the trematode *Fasciola hepatica* (Barret *et al* 1978, Pietrzak and Saz 1981, Kohler 1985) and the nematodes, *Ascaris* (Kohler 1985, Fields 1987). *Herpetomonas ingenoplastis* produced more propionate under the two anaerobic conditions, during glucose catabolism (Table 9), suggesting that propionate production may be important energetically to this organism.

Propionate production in helminths has been found to be energetically favourable and involves a reversal of the TCA cycle, via MDH and FR, followed by a reversal of the last three enzymes of β-oxidation, methylmalonyl CoA isomerase, methylmalonyl CoA racemase and propionyl CoA carboxylase, the latter reaction yielding ATP.

When *H. ingenoplastis* is incubated in glucose, it is reasonable to assume that PK would be active, due to F-2,6-P_2 activation, as in *T. brucei* bloodstream forms (Van Schaftingen *et al.* 1985, Fairlamb and Opperdoes 1986). Therefore it would seem that there must be other points of control by which flux could be diverted via PK, into the forward TCA cycle, or to ethanol

formation, or diverted via PEPCK to the reverse TCA cycle, forming succinate, and perhaps propionate using a similar pathway to that found in helminths. It may be that CO_2 fixation is important in glycolytic flux regulation. Carbon dioxide can be fixed by PEPCK, forming oxaloacetate and ATP. Under anaerobic conditions the oxaloacetate may then be converted via MDH, fumarase and FR, to form succinate, and perhaps propionate, with extra ATP molecules being generated, perhaps at FR, as in helminths (Fields 1987) and perhaps at propionyl CoA carboxylase. Under aerobic conditions malate may then be decarboxylated, as is believed to occur in *H. diminuta* (McKelvey and Fioravanti 1985), forming pyruvate and NADPH, which may than form ethanol, or enter the forward TCA cycle, or be converted into alanine by a reversal of the alanine aminotransferase activity as occurs in *T. brucei* procyclic forms (Fairlamb and Opperdoes 1986). The relative importance of PK and ME in the formation of pyruvate is something that needs to be investigated, although the fact that F-2,6-P_2 acts as an activator in *H. ingenoplastis* would suggest that PK is the major enzyme involved in pyruvate formation under aerobic conditions. 'Malic' enzyme may have a secondary function in the production of reducing power in the form of NADPH.

The above is speculation. Certainly much more study needs to be done on the metabolism of *H. ingenoplastis*, in order to understand exactly what pathways are important to its survival under anaerobic conditions. For instance, what is the role of the soluble fumarate reductase activity? A similar activity has been found in trichomonads (Muller 1976). However, in this case NADH is not believed to be involved, but rather a flavin mononucleotide. The electron transport chains of both *H. muscarum* and *H. ingenoplastis*, especially the latter, are still not completely understood. More study into any role electron transport may play in metabolism is essential in order to understand any role FR may have. Finally, I have shown that *H.*

ingenoplastis secretes propionate as a major end product of glucose metabolism, the first such report for a trypanosomatid, and that the production of propionate may play an essential role in the survival of *H. ingenoplastis* under anaerobic conditions. Therefore further study on this organism would require the elucidation of the pathway of propionate production and its role in energy metabolism. The above knowledge may lead to the understanding of how enzymes are involved in contributing to the energetic requirements of trypanosomatids as they encounter different gaseous conditions.

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