METABOLISM OF PHOSPHOENOLPYRUVATE DERIVED FROM GLUCOSE CATABOLISM BY BLOODSTREAM *TRYPANOSOMA CONGOLENSE*

BY

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A thesis submitted in partial fulfilment for the degree of Master of Science (Biochemistry) at the University of Nairobi

1996

DECLARATION

I, Joseph Omondi Odhiambo, hereby declare that this thesis is my original work and has not been presented for a degree in any other University

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ACKNOWLEDGEMENTS

I must thank my first supervisor, Dr. J. K. Kiaira for the support, excellent supervision, patience and encouragement during this study. Without his guidance this work would have not been possible.

I am also very grateful to my other supervisor, Prof. R. M. Njogu for his constructive criticism and the Chairman of the Department of Biochemistry, Prof. D. M. Makawitti for his support, encouragement and patience during this study.

I am very much indebted to the following: all the technical staff of Biochemistry Department who contributed in many ways to my work, Miss Maria Mulindi, Lucy Thairu and Angela Mungai for helping me with the typing and preparation of this thesis, Dr. Andrew Peregrine and Dr. Jonathan Wilkes for allowing me to use ILRI facilities for preparing this thesis and my colleques at ILRI for their encouragement and moral support during the difficult times.

My profound gratitude also goes to my family for their encouragement and moral support and especially my wife Lillian Awino Ojwang.

This study was made possible through a scholarship provided by the University of Nairobi for which I am most grateful.

DEDICATION

To my late father,

Silvester Odhiambo

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ABBREVIATIONS

ADP	Adenosine 5' diphosphate
AK	Acetate kinase
ATP	Adenosine 5' triphosphate
BSA	Bovine serum albumin
CoASH	Coenzyme A (reduced)
CoQ	Coenzyme Q
CN^-	Cyanide
Cs	Citrate synthase
Cys	Cystein
Cyt.	Cytochrome
DEAE-	Diethylaminoethyl-
DHAP	Dihydroxyacetone phosphate
EDTA	Ethylenediamine tetra-acetate
FAD	Flavin adenine dinucleotide
FADH	Reduced flavin adenine dinucleotide
FDP	Fructose diphosphate
F_p	Flavoprotein
FR	Fumarate reductase
GAP	Glyceraldehyde phosphate
x g	Gravitational force
GK	Glycerol kinase
GPDH	Glycerophosphate dehydrogenase
GP	Glycerol phosphate
GPO	Glycerophosphate oxidase
G3P	Glycerol-3-phosphate
G-1,3-P_2	Glycerate 1,3 diphosphate
HEPES	(N[2-Hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid])
α-KG	α-Ketoglutarate

αKGDH	α-Ketoglutarate dehydrogenase
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase
ME	Malic Enzyme
NAD^+	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Oxidised Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetate
Oxid	Oxidized
Pi	Inorganic phosphate
PCA	Perchloric acid
PyrDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
3-PGA	3-Phosphoglycerate
PK	Pyruvate kinase
PS	Phosphate buffered saline
PSG	Phosphate buffered saline glucose
PTA	Phosphotransacetylase
red	Reduced
SHAM	Salicylhydroxamic acid
TAO	Trypanosome alternative oxidase
TCA	Trichloroacetic acid
TPP	Thiamine pyrophosphate
Tris	Tris(hydroxymethyl) aminoethane
$K_3 Fe(CN)^{-3}_6$	Potassium ferricyanide
$K_3 Fe(CN)^{-2}_4$	Potassium ferrocyanide

UNIT ABBREVIATIONS

°C	degree centigrade
cm	centimetre
°F	degree Fahrenheit
g	gramme
i.u	International units
l	litre
M	Molar
m	metre
mol	mole
mg	milligramme
min	minute
mls	millilitres
mM	millimolar
nanomol	nanomoles
µ	micro
µg	microgramme
µl	microlitre
µM	micromolar
µmol	micromoles
V	volts
v/v	volume per volume
w/v	weight per volume

SUMMARY

The main purpose of this study was to establish the pathway(s) by which PEP from glucose catabolism is catabolized, the end products formed in the presence and absence of SHAM, the subcellular localisation of some key enzymes involved in PEP catabolism and to partially characterise PEPCK in bloodstream *T. congolense*.

When the trypanosomes were incubated with glucose as the substrate in the absence of SHAM, the main end products observed were, acetate, glycerol and pyruvate. The amounts observed were 292.9 ± 56.3; 308 ±54 and 154 ± 19 nmoles/30 min/mg protein respectively. Addition of SHAM reduced the production of glycerol, acetate and pyruvate to 165 ± 36; 44.6 ± 30 and 26 ± 2 nmoles/30 min/mg protein respectively. Succinate which was not detectable in the absence of SHAM was found to be 1.32 ± 0.29 nmoles/30 min/mg protein. Lactate was not produced as an end product. It was therefore concluded that, under aerobic conditions, bloodstream *T. congolense* produce glycerol, acetate and pyruvate as the main end products of glucose catabolism. Under anaerobic conditions simulated by addition of SHAM, glycerol, acetate and pyruvate were still produced with succinate as a minor end product.

The rate of respiration was also measured in the presence and absence of SHAM and cyanide. It was observed that SHAM totally inhibited the rate of respiration whereas cyanide had no effect. It was therefore proposed that molecular oxygen is the terminal electron acceptor which reoxidises the reducing equivalents in (NADH) via the trypanosome alternate oxidase (TAO). The bloodstream *T. congolense* appears to have no cytochrome systems which could be inhibited by cyanide but has an alternative oxidase which was inhibited by SHAM.

The amount of pyruvate production was determined in the presence of SHAM, cyanide or in their absence for 30 minutes. It was observed that in the

absence of both SHAM and cyanide, 155±12 nmoles/30 min/mg protein was produced while in the presence of both SHAM and cyanide, only 28 ± 4 nmoles/30 min/mg protein was produced. When SHAM alone was present, 26 ± 8 nmoles/30 min/mg protein was produced while in the presence of cyanide alone 156 ± 12 nmoles/30 min/mg protein was produced. When the production of pyruvate was measured with time under aerobic conditions , there was an increase from 0 to 280 nmoles/mg protein after 3 hrs of incubation. The maximum quantity achieved in the presence of SHAM was 24 nmoles/mg protein. This was achieved after 30 minutes and remained constant for 3 hrs. This was attributed to death and lysis of the trypanosomes after 30 minutes in the presence of SHAM. It was concluded that bloodstream *T. congolense* only possesses trypanosome alternate oxidase (TAO) as a means of oxidising the reducing equivalents.

The activities of the enzymes likely to be involved in the catabolism of PEP derived from glucose oxidation to pyruvate were assayed. The enzymes which had specific activities greater than 36 nmoles/min/mg protein were PEP carboxykinase, NADP-linked malic enzyme and malate dehydrogenase. Those that had specific activities less than 6 nmoles/min/mg protein were pyruvate kinase and NAD-linked malic enzyme. From these observations it was suggested that bloodstream *T. congolense* catabolise PEP to pyruvate via another pathway not involving pyruvate kinase. It was also concluded that it was unlikely that pyruvate could be converted to lactate due to the low specific activity of lactate dehydrogenase of <0.43 nmoles/min/mg protein.

The activities of the enzymes likely to catabolise pyruvate further to either acetate or TCA cycle intermediates were assayed. The enzymes which had specific activities greater than 18 nmoles/min/mg protein were fumarase, pyruvate dehydrogenase, phosphotransacetylase, acetate kinase and malate dehydrogenase. Those that had specific activities less than 2 nmoles/min/mg protein were aconitase, citrate synthase, α-ketoglutarate dehydrogenase and NADP-linked isocitrate dehydrogenase. From these observations, it was

suggested that bloodstream *T. congolense* was unlikely to have a fully functional TCA cycle and that pyruvate could be converted to acetate.

The subcellular localization of some enzymes that could be involved in the catabolism of PEP to pyruvate were determined using various methods. It was observed that PEPCK, MDH and α-GPDH activities were more latent than enolase and NADP-linked malic enzyme. From the pattern of release of PEPCK and MDH which was similar to that of α-GPDH, it was suggested that PEPCK and MDH could be glycosomal while NADP-linked malic enzyme could be cytosolic.

When the activity of PEPCK from the bloodstream *T. congolense* lysate was assayed in the presence of either magnesium or manganese ions, it was observed that the specific activity in the presence of both metal ions was 48 ± 6 nmoles/min/mg protein. Manganese ions only gave activity of 48 ± 6 nmoles/min/mg protein while magnesium ions alone gave activity of 9.0 ± 1.5 nmoles/mg protein. When both metal ions were excluded from the assay mixture, the specific activity was <0.43 nmoles/min/mg protein. It was suggested that bloodstream *T. congolense* PEPCK requires manganese ions for activity. The specific activity of the enzyme was determined in imidazole buffer with pH ranging from 5.6 - 7.6. The activity increased from 2 to a maximum of 48 nmol/min/mg protein at pH 6.6 before gradually falling. From these observations it was concluded that the activity of PEPCK is dependent on the pH.

CHAPTER ONE

LITERATURE REVIEW

1.1 *TRYPANOSOMA CONGOLENSE*

T. congolense belongs to the subgenus *Nannomonas* and genus, *Trypanosoma*. The subgenus *Nannomonas* is characterised by a small size kinetoplast which is typically marginal. The kinetoplast is medium sized and is between 0.7 to 0.8 μm (Hoare, 1972). Development occurs in the midgut and proboscis of tsetse fly of the genus, *Glossina* (Hoare, 1959a; Fairbairn, 1962).

T. congolense causes animal trypanosomosis; also called nagana. Nagana is one of the major animals diseases in Africa. Apart from *T. congolense*, there are other species of Trypanosomes that cause nagana. These include *T. brucei brucei* and *T. vivax* (Hoare, 1970; 1972).

Trypanosoma congolense are small salivarian trypanosomes with a rod-like shape. The average length is 12.2-17.6 μm (Hoare, 1959; Fairbairn, 1962).

1.1.1 Life cycle of *T. congolense*

T. congolense is a digenic parasite whose life cycle involves two hosts. The final host is a vertebrate animal which includes bovines, sheep, goats and equines (Bruce *et al.*, 1959a; Fairbairn, 1962; Hoare, 1957a; 1966). The other host is an invertebrate insect vector (tsetse flies) usually of the genus *Glossina.* Those that are often associated with the transmission of the parasite include:- *G. morsitans*, *G. tachnoides, G. palpalis* and *G. brevipalpis* (Buxton, 1955; Chardome and Peel, 1967).

The parasite is transmitted to the mammalian host through the tsetse fly bite. When an infected tsetse fly bites an animal in the process of feeding, it first inoculates contents of the proboscis which contains anticoagulant before it sucks the blood. The inoculum contains the metacyclic forms of the trypanosomes. The inoculated parasites migrate via the lymphatic to the lymph nodes (Hoare, 1972). They then enter the bloodstream and transform into the bloodstream forms. After transformation, they multiply in large numbers and thereafter invade the intercellular spaces of other tissues (Hoare, 1972).

When a noninfected tsetse fly feeds on an infected animal, it sucks blood containing the bloodstream forms of the trypanosomes. The bloodstream forms in the midgut transform into midgut forms which have alot of similarity to the cultured procyclic forms. They then multiply and migrate to the salivary glands where they transform into epimastigotes (Hoare, 1972). The epimastigotes transform further into metacyclic forms which could be inoculated to animal(s) when the tsetse fly feeds again (Hoare, 1972; Fairlamb and Opperdoes, 1986).

1.2 CARBOHYDRATE METABOLISM

1.2.1 Carbohydrate metabolism in procyclic forms of African trypanosomes

Procyclic forms of African trypanosomes metabolise glucose (Ryley, 1962). However, they prefer proline to glucose as an energy source (Evans & Brown, 1972b). Apart from glucose and proline, the trypanosomes can also catabolise glycerol and 2-oxoglutarate (Srivastava and Bowman, 1971; 1972; Evans and Brown, 1972a; Ford and Bowman, 1973). The end products of glucose catabolism depend on the species of the trypanosome but, they

generally include pyruvate, glycerol, succinate, acetate and CO_2 (Ryley, 1956; 1952).

1.2.1.1 Carbohydrate metabolism in procyclic *T. brucei*

In the subgenus *trypanozoon*, carbohydrate catabolism has been extensively studied in two subspecies. These include procyclic *T. brucei* and *T. rhodesiense* (Ryley, 1956; 1962; Fairlamb and Opperdoes, 1986; Evans and Brown, 1972a). They have been shown to respire on glucose, mannose, fructose, glycerol, proline and 2-oxoglutarate (Ryley, 1962; Ford and Bowman, 1971; Fairlamb and Opperdoes, 1986).

The end products of glucose catabolism in procyclic *T. brucei* under aerobic condition are mainly CO_2, succinate, glycerol and acetate (Ryley, 1956). There is also some malate, alanine and fumarate formed (Evans and Brown, 1972b; Cross *et al.*, 1978).

No carbohydrate store has been reported and in the absence of exogenous substrates, they lose motility with eventual disintegration (Flynn and Bowman, 1973). In one experiment where glucose was included in the incubation medium, motility was maintained and the parasites remained intact for more than four hours (Flynn and Bowman, 1973).

The glycolytic pathway has been shown to be present in procyclic *T. brucei* with most of the glycolytic enzymes occurring in a peculiar organelle called "the glycosome" (Opperdoes *et al.*, 1981; Hart and Opperdoes, 1984). However, several glycolytic enzyme activities in the procyclic trypomastigotes are lower than in the bloodstream forms as indicated below by ratios in brackets. These include hexokinase (1:15), phosphoglucose isomerase (1:7), phosphofructokinase (1:4), fructose bisphosphate aldolase (1:28), glyceraldehyde-3-phosphate dehydrogenase (1:8) and phosphoglycerate kinase (1:8) (Hart *et al.*, 1984; Fairlamb and Opperdoes, 1986); a difference which

could be associated with the changes in the mitochondrion and glycolytic enzymes activities (Fairlamb and Opperdoes, 1986).

T. brucei procyclics have very low activities of pyruvate kinase which is necessary in converting phosphoenolpyruvate formed during glycolysis to pyruvate (Opperdoes and Cottem, 1982). The formation of pyruvate has been proposed to occur via a pathway which involves CO_2 fixation (Opperdoes and Cottem, 1982). CO_2 fixation is catalysed by phosphoenolpyruvate carboxykinase (PEPCK), whose activity has been shown to be significantly high (Opperdoes and Cottem, 1982; Opperdoes *et al.,* 1982; Klein *et al.,* 1975; Bowman *et al.*, 1983). Opperdoes and Cottem (1982) proposed that PEPCK could catalyse the carboxylation of PEP to oxaloacetate in a reversible reaction that requires ADP and CO_2. The oxaloacetate could be reduced to malate through malate dehydrogenase. Malate could be the final product of glycosomal glucose catabolism (Opperdoes and Cottem, 1982). This is summarized in Scheme I. They also proposed that the malate formed could be converted to either alanine, aspartate or succinate, due to the presence of the enzymes alanine aminotransferase present in the cytosol (Kilgour and Godfrey, 1973); fumarase and fumarate reductase (Klein *et al.,* 1975); malate dehydrogenase and aspartate aminotransferase in the cytosol (Opperdoes *et al.,* 1981; Opperdoes and Cottem, 1982).

The pathway(s) by which malate is converted to the final end products namely acetate and succinate has not been confirmed except that the presence of the intermediates malate, fumarate and succinate has been demonstrated by carbon radiolabelling studies (Klein *et al.,* 1975). Opperdoes and Cottem (1982) postulated a pathway which could explain how the final end products of glucose catabolism are formed (Scheme I). The scheme was based on the assumption that no net change in ATP occurs in the glycosomal compartment during glucose metabolism. This implied that all the 3-phosphoglycerate leaving the glycosome must re-enter this compartment as PEP. To fulfil this

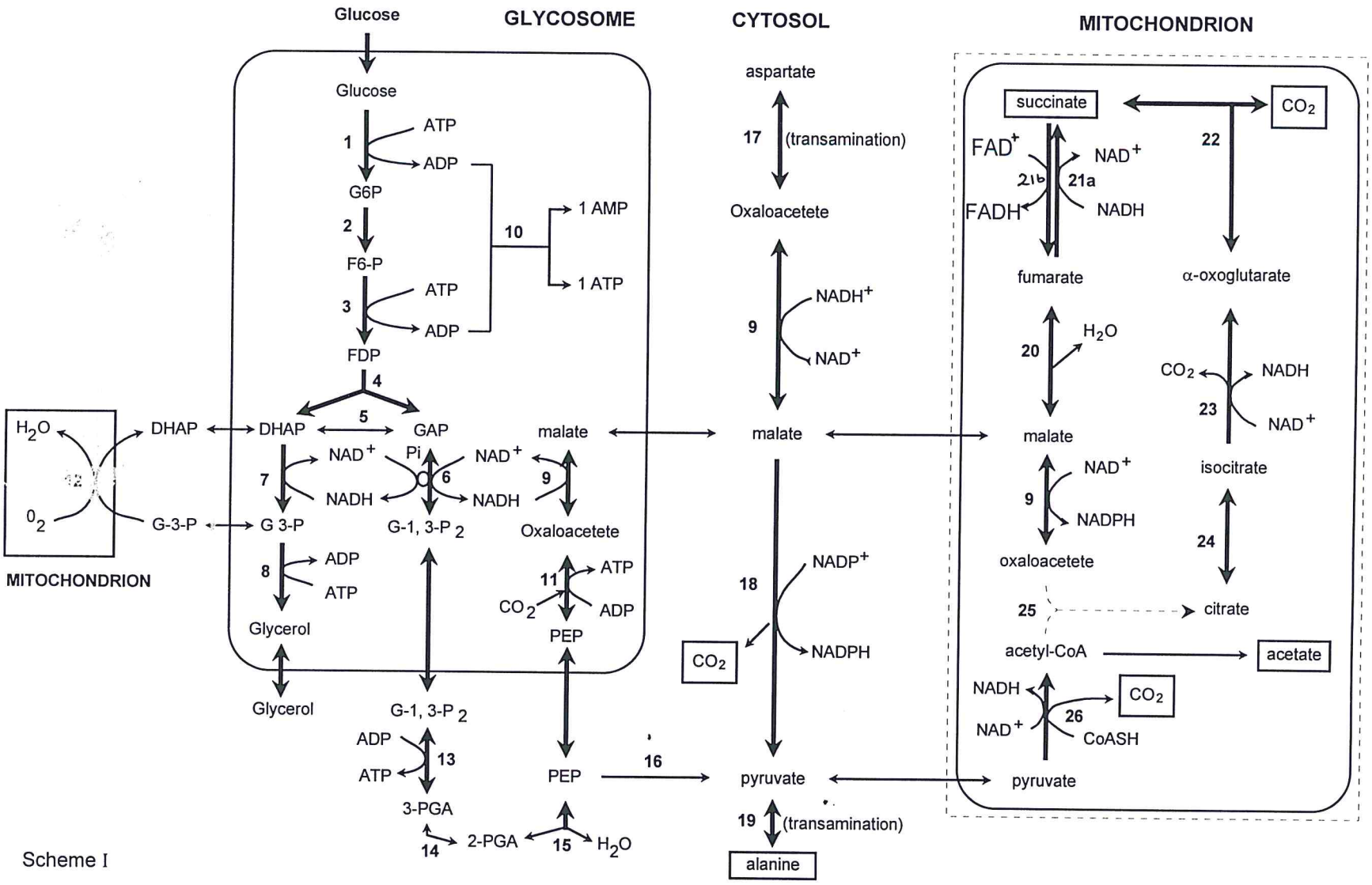
requirement pyruvate kinase should be absent or relatively inactive (as already found by Opperdoes and Cottem, 1982; Callens *et al.*, 1991; Callens and Opperdoes, 1991). The result is that, a significant part of the glycolytically produced PEP is now carboxylated and after two reduction steps it is excreted as succinate (Fairlamb and Opperdoes, 1986; Opperdoes and Cottem, 1982). Similarly, if no net change of NAD^+ occurs then, all NADH produced at the glyceraldehyde 3-phosphate dehydrogenase reaction should be available for the reduction of oxaloacetate to malate, otherwise phosphoenolpyruvate would accumulate.

The formation of acetate from malate is postulated to be via $NADP^+$ linked malic enzyme which converts malate to pyruvate (Scheme I). Pyruvate could then be converted to acetyl CoA catalysed by pyruvate dehydrogenase. Acetyl CoA is converted to acetylphosphate using phosphotransacetylase which is finally converted to acetate by acetate kinase.

The presence of a functional TCA cycle in procyclic *T. brucei* has not been shown except that the activities of a few enzymes of the TCA cycle have been shown. Opperdoes and Cottem (1982) have shown high activities of malate dehydrogenase and fumarate reductase while aconitase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and fumarate hydratase have been shown by Ryley (1962). However, the pathway remains unconfirmed because of failure to demonstrate key metabolites in radioisotope studies like citrate and the insignificant activities of citrate synthase and NAD^+-linked isocitrate dehydrogenase (Evans and Brown, 1972a, b).

1.2.1.2 Carbohydrate metabolism in bloodstream *T. brucei.*

The bloodstream forms of *T. brucei* can utilise glucose, fructose, mannose, 2-oxoglutarate and glycerol as energy sources (Ryley, 1956;


Glucose
GLYCOSOME
CYTOSOL
MITOCHONDRION
Glucose
ATP
ADP
G6P
1 AMP
1 ATP
F6-P
FDP
DHAP
GAP
NAD+
NADH
Pi
malate
Oxaloacetete
G 3-P
G-1, 3-P 2
ADP
ATP
Glycerol
CO2
PEP
3-PGA
2-PGA
H2O
aspartate
(transamination)
Oxaloacetete
NADH+
NAD+
malate
NADP+
NADPH
CO2
pyruvate
(transamination)
alanine
succinate
CO2
FAD+
FADH
NADH
fumarate
α-oxoglutarate
malate
isocitrate
oxaloacetete
citrate
acetyl-CoA
acetate
CoASH
pyruvate
MITOCHONDRION
O2
G-3-P

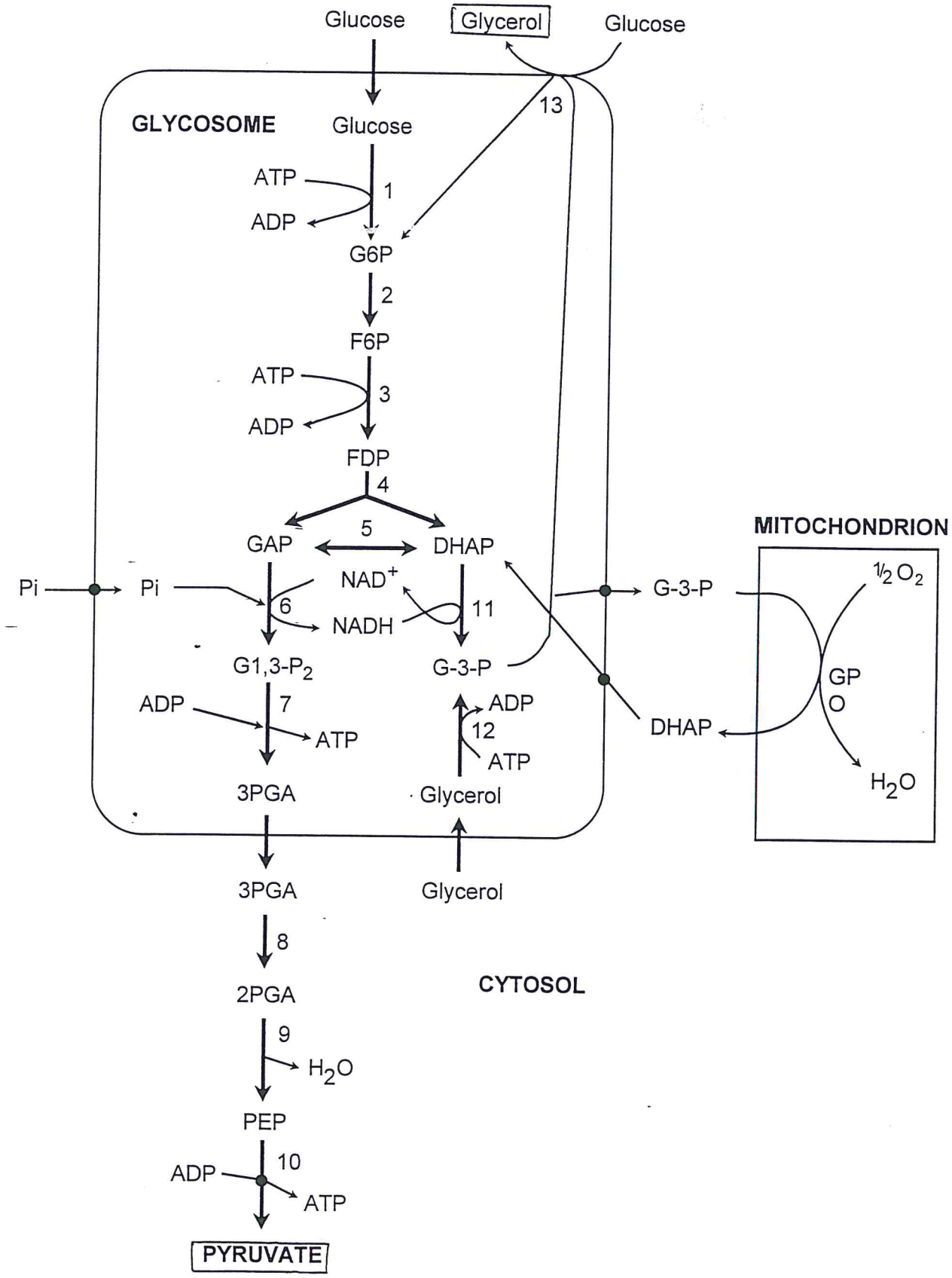
Scheme I

1962; Bienen *et al.,* 1993). They cannot utilise either amino acids or fatty acids for energy production (Bowman and Flynn, 1976).

Under anaerobic condition, glucose is broken down to equimolar amounts of pyruvate and glycerol (Ryley, 1956; Opperdoes *et al.,* 1976).
Aerobic glucose catabolism leads to formation of mainly pyruvate with traces of succinate and CO_2 (Ryley, 1956; Flynn and Bowman, 1973). The bloodstream *T. brucei* has been shown to have a very high rate of respiration on glucose compared to the procyclic forms (Ryley, 1962). Almost all the glycolytic enzymes have been assayed and isolated; some almost in pure forms and characterised. They include hexokinase, Phosphoglucose isomerase, aldolase, 6-phosphofructokinase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase, pyruvate kinase and enolase (Opperdoes and Borst, 1977; Oduro *et al.*, 1980a, b; Hart and Opperdoes, 1984; Risby *et al.*, 1969; Flynn, 1970; Flynn and Bowman, 1974; 1980; Callens *et al.*, 1991; Barnard and Pedersen, 1988; Misset and Opperdoes, 1984; Callens and Opperdoes, 1991; Marchand *et al.*, 1989).
The first seven enzymes of the glycolytic sequence occur in the glycosome (Fairlamb and Opperdoes, 1986; Opperdoes and Borst, 1977).

The bloodstream forms have high activity of pyruvate kinase but they lack lactate dehydrogenase (Dixon, 1966; Risby *et al.*, 1969; Flynn, 1971; Flynn and Bowman, 1974).

The pathway for glucose catabolism is summarized in Scheme II. The Scheme shows the enzymes of glycolysis are in the glycosome. It also shows how the end products of glucose catabolism are formed. The Glycerol phosphate oxidase occurs in the mitochondrion. According to the Scheme, glycerol is formed from glycerol-3-phosphate by the action of Glycerol-3-phosphate : Glucose transphosphorylase which could also phosphorylate glucose to form glucose-6-phosphate


Glucose
Glycerol
Glucose
GLYCOSOME
Glucose
13
ATP
1
ADP
G6P
2
F6P
ATP
3
ADP
FDP
4
5
GAP
DHAP
MITOCHONDRION
Pi
Pi
NAD+
6
NADH
11
G-3-P
½ O2
G1,3-P2
G-3-P
GP
O
ADP
7
ATP
ADP
12
ATP
DHAP
H2O
3PGA
Glycerol
3PGA
Glycerol
8
2PGA
CYTOSOL
9
H2O
PEP
ADP
10
ATP
PYRUVATE

Scheme II

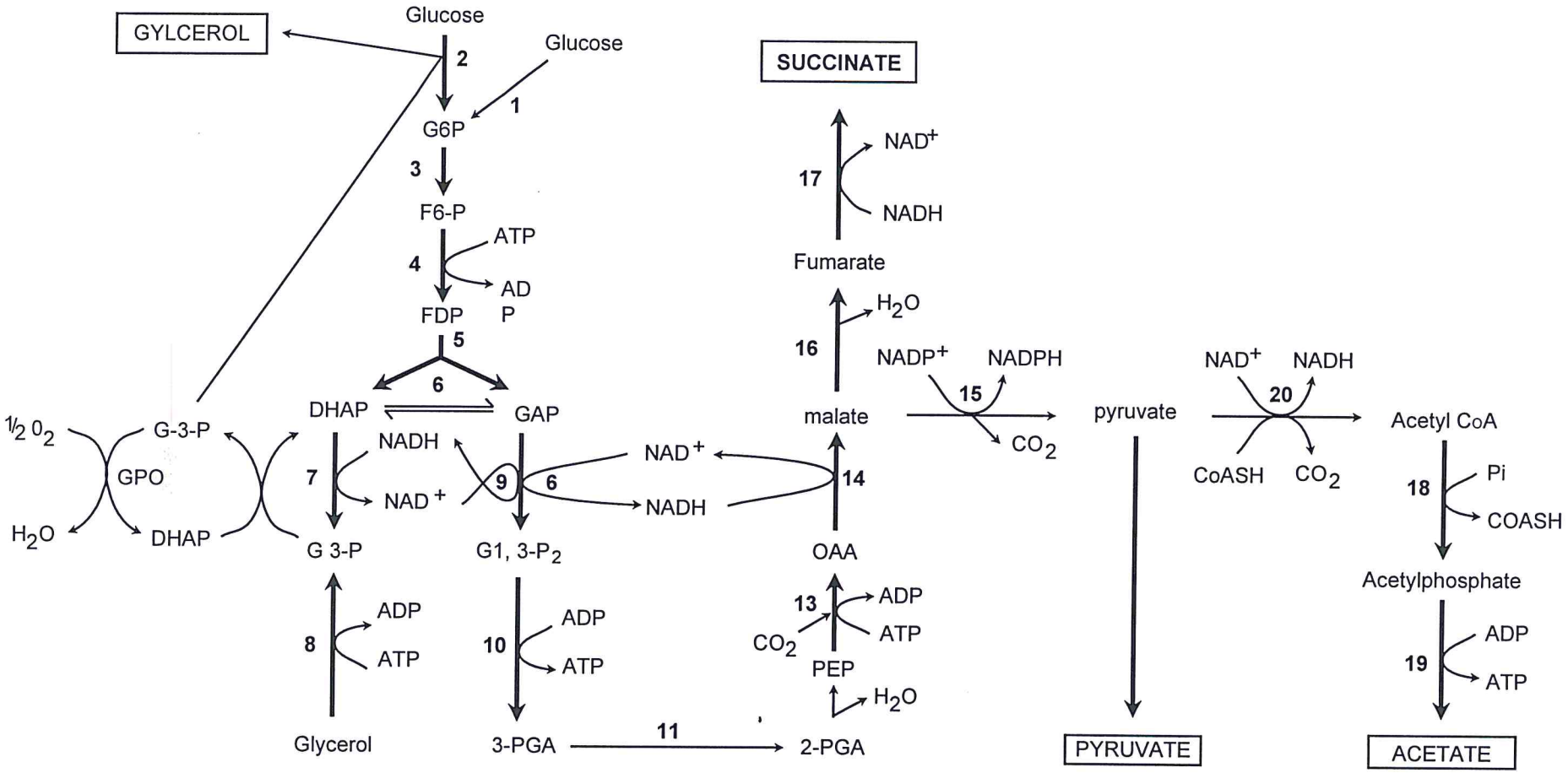
NADH generated in the glycosome during respiration is reoxidised by trypanosome alternate oxidase (TAO) via NAD dependant glycerol-3-phosphate dehydrogenase (Grant and Sargent, 1960; Opperdoes *et al.*, 1977a, b; Fairlamb and Bowman, 1977; Flynn and Bowman, 1973). This is also shown in Scheme II.

1.2.1.3 Carbohydrate metabolism in procyclic *T. congolense*

Unlike procyclic *T. brucei*, very little has been reported on the carbohydrate metabolism in procyclic *T. congolense*. The procyclic *T. congolense* has been shown to respire on glucose (von Brand and Tobie, 1959). However, systematic investigation of the glycolytic pathway and the enzymes involved have not been done despite the early work by von Brand and Tobie (1959) which showed that they catabolise glucose aerobically to pyruvate and acetate. The same authors also reported that small amounts of lactate, succinate and glycerol were excreted under aerobic conditions. Pyruvate accounted for 39% while acetate and CO_2 evolution accounted for 23% each of the total glucose carbons respectively.

When the parasites respire in the presence of SHAM or under anaerobic condition, acetate formation increases along with that of succinate (von Brand and Tobie, 1959; Obungu, 1992). There is also a marked reduction of CO_2 evolution which is thought to be from the decarboxylation of pyruvate to form acetate going to the formation of succinate (von Brand and Tobie, 1959) .

Recent work by Obungu (1992) indicate that the procyclic *T. congolense* has high activities of phosphoenolpyruvate carboxykinase (PEPCK), pyruvate dehydrogenase, succinate dehydrogenase, $NADP^+$ linked malic enzyme, fumarase, fumarate reductase, malate dehydrogenase, acetate kinase and α-ketoglutarate dehydrogenase.


GYLCEROL
Glucose
Glucose
2
1
G6P
3
F6-P
ATP
4
AD
P
FDP
5
6
DHAP
GAP
½ 0₂
G-3-P
GPO
H₂O
DHAP
NADH
7
NAD +
9
6
G 3-P
G1, 3-P₂
NAD +
NADH
14
OAA
8
ADP
ATP
Glycerol
10
ADP
ATP
3-PGA
11
2-PGA
13
ADP
ATP
CO₂
PEP
H₂O
16
H₂O
malate
NADP+
NADPH
15
CO₂
SUCCINATE
17
NAD+
NADH
Fumarate
pyruvate
PYRUVATE
NAD+
NADH
20
CoASH
CO₂
Acetyl CoA
18
Pi
COASH
Acetylphosphate
19
ADP
ATP
ACETATE

Scheme III

Even though some of these enzymes like malate dehydrogenase, α-ketoglutarate dehydrogenase and fumarase are involved in the TCA cycle, it is difficult to infer the presence of a fully functional TCA cycle without demonstrating reasonable activity of key enzyme like citrate synthase and intermediates such as citrate. The possibility of an inactive TCA cycle is suggested by the fact that the parasites are insensitive to flouroacetate and malonate (von Brand and Tobie, 1959). Scheme III shows that the end products are pyruvate, succinate, glycerol and acetate. The NADH produced at the glycereraldehyde 3-phosphate dehydrogenase and succinate dehydrogenase reactions are either reoxidised by the oxaloacetate to malate reaction or DHAP to G-3-P reactions. There is also net production of ATP coming from the reactions catalysed by phosphoglycerate kinase, PEP carboxykinase and acetate kinase.

1.2.1.4 Carbohydrate metabolism in bloodstream *T. congolense.*

The bloodstream *T. congolense* can respire on glucose. The respiration depends on exogenous supply of carbohydrates because it lacks carbohydrate stores (Ryley, 1956). In the absence of exogenous supply of carbohydrate, the parasite losses motility and eventually disintegrates just like procyclic *T. brucei* (Ryley, 1956; Flynn and Bowman, 1973).

The glucose used during aerobic conditions is broken down to acetate, succinate, glycerol and CO_2 (Agosin and von Brand, 1954; Ryley, 1956). These end products of glucose catabolism are similar to the end products formed by procyclic *T. brucei* respiring on glucose aerobically (Ryley, 1956).

The major end product in bloodstream *T. congolense* is acetate which accounts for 33% (Ryley, 1956) and 54% (Agosin and von Brand, 1954) of the glucose carbon used. Succinate accounts for 33%; glycerol (18%) and CO_2

(8%) (Ryley, 1956). Lactate, citrate, propionate or other volatile acids have not been reported (Agosin and von Brand, 1954; Ryley, 1956).

Despite the early work by Agosin and von Brand (1954), Ryley (1956), no systematic investigation into the pathway(s) that could lead to the end products shown above have been undertaken. Neither has there been any attempt to show the presence of the enzymes of glycolysis as has been done in *T. brucei* procyclics which appear to have similar end products.

The end products formed during anaerobic glucose catabolism have been shown to be acetate, glycerol, succinate and CO_2 (Agosin and von Brand, 1954). The acetate formed accounts for 15% of glucose carbons while glycerol and succinate accounts for 31% and 47% respectively (Agosin and von Brand, 1954). The methods used to estimate the end products have since then been improved making it necessary to further investigate with the more specific techniques. The possibility of existence of an active or partial TCA cycle has not been investigated.

1.2.2 Terminal respiratory systems

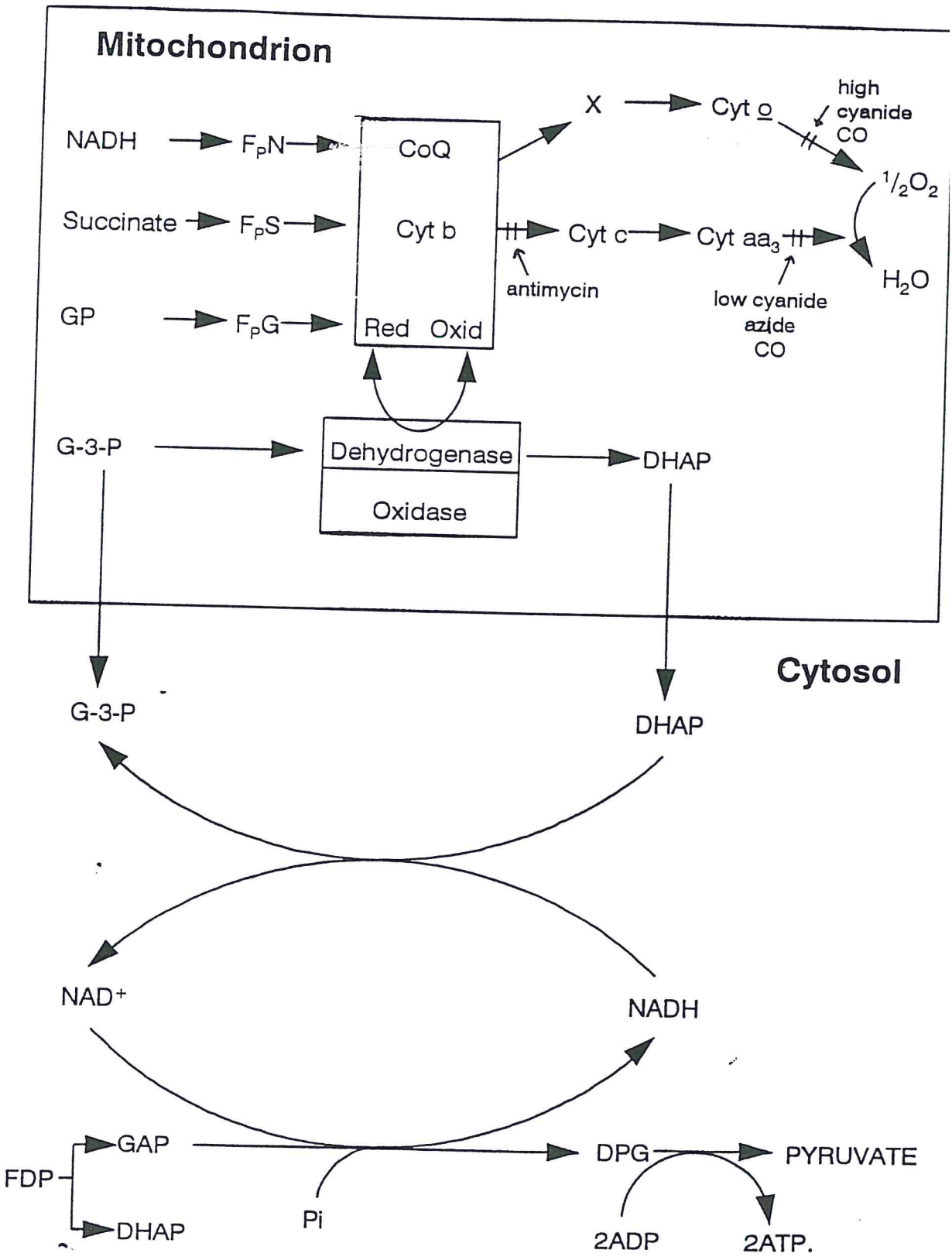
1.2.2.1 Terminal respiratory system in procyclic *T. brucei*

T. brucei procyclics has been shown to respire on both glucose and proline (Tobie *et al.*, 1950). They have oxidases which cannot be completely inhibited by cyanide (Evans and Brown, 1972a). Incomplete inhibition of respiration by cyanide during the utilization of glucose or proline suggests the presence of cytochrome aa_3 and another cytochrome which is insensitive to cyanide. Cytochromes b, c, and c_1 have been shown to be present (Hill, 1976; Bowman and Flynn, 1976). Apart from the presence of the cytochromes, it has been shown that rotenone, which inhibits the electron transfer within NADH-Q reductase complex of the electron transport chain, has 75-90% inhibition

activity (Beattie *et al.,* 1994). This contradicts the earlier observation by Turrens (1989) who proposed that rotenone had no inhibitory effect and concluded that NADH could only be reoxidised via NADH-fumarate reductase to generated succinate. The succinate was in turn oxidised by the mitochondrion respiratory chain. However, there is experimental evidence deduced by Beattie *et al* (1994) on the possibility of existence of an alternative pathway involving NADH-fumarate reductase in the oxidation of NADH as earlier proposed by Turrens (1989).

It has also been observed that antimycin which inhibits electron flow between cytochrome b and c_1 had inhibitory effect on procyclic *T. brucei* thus further suggesting that the electron transport chain in this parasites is not via NADH-fumarate reductase (Beattie *et al.*, 1993). The inhibition studies have implied the presence of at least two terminal oxidases (Evans and Brown, 1973). This is shown in Scheme IV. Njogu *et al.*, (1980) reported that 60% of the respiration on glucose can be inhibited by cyanide and 30% by SHAM. A bout 10% of the respiration is insensitive to a combination of both inhibitors. They, thus proposed a branched pathway to explain their results. This is shown in scheme IV.

These findings are evidence of a possible branched electron transport system as suggested also for procyclic *T. rhodesiense* (Bowman *et al.*, 1972; Newton *et al.*, 1973). Scheme IV shows the possible branched electron transport system for procyclic *T. brucei* as suggested by Njogu *et al* (1980). They suggested that either the NADH generated during glycolysis could reduce DHAP to glycerol-3-phosphate in a reaction catalysed by glycerol-3-phosphate dehydrogenase or reduce flavoproteins in the mitochondria. The glycerol-3-phosphate formed could reduce the GPO which in turn could either be oxidised by molecular oxygen directly to form water or the GPO could be oxidised by cytochrome b-CoQ complex.


Mitochondrion
NADH
F_PN
CoQ
X
Cyt o
high cyanide CO
$^1/_2O_2$
Succinate
F_PS
Cyt b
Cyt c
Cyt aa_3
H_2O
antimycin
low cyanide azide CO
GP
F_PG
Red Oxid
G-3-P
Dehydrogenase
Oxidase
DHAP
Cytosol
G-3-P
DHAP
NAD^+
NADH
FDP
GAP
DHAP
Pi
DPG
PYRUVATE
2ADP
2ATP.

Scheme IV

The reduced cytochrome b-CoQ complex could then be oxidised by molecular oxygen via several steps in the electron transport chain (Njogu *et al.,* 1980). Njogu *et al* (1980) also suggested that cytochrome b-CoQ could be reduced by NADH directly but this has not been verified.

Further evidence of alternative oxidase in procyclic *T. brucei* had been deduced by Evans and Brown (1971; 1972a) when they compared the respiration in bloodstream forms, cultured procyclics and found out that in both forms, respiration was inhibited by diphenylamine which inhibits electron transfer between flavoprotein and cytochrome components (Baker, 1963). Further experiments by Evans and Brown (1971; 1972a) showed that L-glycerol-3-phosphate oxidation in both forms was inhibited by diphenylamine but NADH oxidation in the culture form was inhibited to about half the extent of the corresponding activity in bloodstream forms. They suggested that GPO of bloodstream forms persists during transformation of short stumpy trypanosomes into cultured forms and that alternative pathway of electron transfer from NADH and succinate is developed in culture form of *T. brucei*, which although is partially diphenylamine sensitive, is cyanide insensitive (Evans and Brown, 1971; 1972a).

1.2.2.2 Terminal respiratory system in bloodstream *T. brucei*

Bloodstream *T. brucei* has been shown to be insensitive to cytochrome inhibitors like azide, cyanide or antimycin A (Flynn and Bowman, 1973). They lack cytochromes and rotenone-sensitive NADH dehydrogenase which contradicts the earlier observation by Bienen *et al* (1991), that 100 μM rotenone inhibited the formation of a mitochondrial potential in the transitional bloodstream forms thus indicating the presence of rotenone-sensitive NADH dehydrogenase and also the observation that edited transcripts of

mitochondrially encoded subunits of NADH dehydrogenase complex were present in the short stumpy forms (Flynn and Bowman, 1973; Fairlamb and Bowman, 1977; Fulton and Spooner, 1959; Michelotti and Hajduk, 1987; Bienen *et al.*, 1993; Beattie *et al.,* 1994).

The NADH generated in glycolysis is reoxidised by molecular oxygen through the reaction of glycerol-3-phosphate oxidase system (GPO) via an NAD dependant glycerol-3-phosphate dehydrogenase (Grant and Sargent, 1960). The GPO is composed of at least two components, an *sn*-glycerol-3-phosphate dehydrogenase and an oxidase which are linked by CoQ (Grant and Sargent, 1960, 1961; Clarkson *et al.,* 1989; Bowman and Fairlamb, 1976). They named it α-Glycerol-phosphate oxidase system (GPO) because they thought that it was unique to trypanosomes and non-mitochondria. It was later shown that respiration was mitochondrial in these parasites and depends on plant-like alternative oxidase thus named trypanosome alternative oxidase (TAO)(Opperdoes *et al.*, 1977; Clarkson *et al.,* 1989).

The high rate of respiration on glucose (von Brand, 1951) suggests that glycolysis involves an oxygen consuming process unlike the glycolysis in mammalian cells which does not require O_2. Respiration is not inhibited by cyanide or CO (von Brand, 1951; Ryley, 1956; Fulton and Spooner, 1959). In addition to the lack of cytochromes, it also lacks a fully functional TCA cycle even though insignificant activities of two enzymes, citrate synthase and NAD^+ -linked isocitrate dehydrogenase which are essential for functional TCA cycle activity have been shown (Ryley, 1956; Fulton and Spooner, 1959); a situation that was reconciled by the discovery of L-GPO (Grant and Sargent, 1960). The O_2 is apparently reduced to H_2O since no H_2O_2 has been detected as a free intermediate (Fairlamb and Bowman, 1977b). The GPO is insensitive to inhibition by cyanide and is not mediated by cytochromes (Grant and Sargent, 1960; Flynn and Bowman, 1973, Fairlamb and Bowman, 1977).

The TAO can be inhibited by hydroxamic acids such as salicylhydroxamic acid (SHAM) (Evans and Brown, 1973; Opperdoes *et al.*, 1976; Clarkson *et al.*, 1981). Since it consists of an α-glycerol-phosphate dehydrogenase and a terminal oxidase (Bowman and Fairlamb, 1976), SHAM inhibits the transfer of electrons between the two enzymes. The oxidase is responsible for reducing molecular oxygen to water in a process that is not coupled to ADP phosphorylation (Gutteridge and Coombs, 1977). However, since the parasite is capable of respiring on 2-oxoglutarate in the absence of glucose (Bienen *et al.*, 1993), and has been shown to have a much higher cytoplasmic ATP content/ bloodstream trypanosome cell (Londsdale-Eccles and Grab, 1987; Nolan and Voorheis, 1991); it has been concluded that there could be an alternative oxidase (Bienen *et al.*, 1993; Londsdale-Eccles and Grab, 1987). This is further strengthened by the fact that SHAM was shown to cause 50% decrease in intracellular ATP concentration when glucose was the substrate (Opperdoes *et al.*, 1976a; Nolan and Voorheis, 1991). When 2-oxoglutarate was the substrate SHAM caused 80-90% decrease in intracellular ATP concentration demonstrating the role of the mitochondrion in 2-oxoglutarate metabolism (Bienen *et al.*, 1993). Scheme IV shows the relationship between glycolysis and GPO.

1.2.2.3 Terminal respiration system in procyclic *T. congolense*

The procyclic *T. congolense* respiring on glucose has been shown to be partially sensitive to cyanide (Obungu, 1992). The inhibition by cyanide was about 50% which increased to 85% in the presence of SHAM. But SHAM alone cause 35% inhibition. From these observations he concluded that the procyclic *T. congolense* uses both the electron transport system and the TAO for oxidising the reducing equivalents. The partial sensitivity to a mixture of cyanide, SHAM and antimycin A suggest the presence of cytochrome aa_3,

cytochrome o and TAO in *T. congolense*. This means that there is a possibility of a branched electron transport system coupled to TAO (Obungu, 1992).

1.2.2.4 Terminal respiratory system in bloodstream *T. congolense*

The terminal respiratory system in bloodstream *T. congolense* which also respire on glucose (Ryley, 1956) has not been investigated. However, it has been assumed to lack cytochromes just like the bloodstream forms of *T. brucei*, *T. rhodesiense* and *T. vivax* (von Brand, 1951; Ryley, 1956; Fulton and Spooner, 1959). This was deduced from spectroscopic studies. Apart from the spectroscopic studies on the above species, there has not been any systematic investigation on the oxidation of the NADH produced from glucose catabolism in bloodstream *T. congolense*.

1.3 OBJECTIVES

This study was designed to:-

1. Establish the pathway(s) for the metabolism of PEP derived from the glycolytic reactions to pyruvate, acetate and succinate in bloodstream *T. congolense* by determining the activities of the appropriate enzymes.

2. Establish the amounts of the end products of glucose catabolism under anaerobic conditions simulated by the addition of SHAM.

3. Determine the possible localization of some of the enzymes involved in PEP catabolism in bloodstream *T. congolense* using freeze-thawing and exposure to various amounts of Triton X-100.

4. Partially characterise PEPCK from bloodstream *T. congolense* lysate.

5. Determine the presence or absence of TCA cycle in bloodstream *T. congolense.*

CHAPTER TWO

MATERIALS AND METHODS

2.1 ORGANISMS

Bloodstream *Trypanosoma congolense* IL 3000 was used throughout the study. It was obtained from International Laboratory for Research on Animal Diseases (ILRAD). The parasite was derived from ILC 49 which was originally isolated from a naturally infected cow in Transmara region of Southern Kenya in 1966.

2.2 ANIMALS

Sprague-Dawley rats of about 4-6 months old, weighing 200 - 300 gm were used throughout the study.

2.3 CHEMICALS AND ENZYMES

All the reagents in this study were of analytical grade. They were obtained form British Drug Houses (BDH), Poole England; Whatman Company, U.K.; Boehringer Manheim GmbH, West Germany; Merck A.G. Darnstadt, West Germany and Sigma Chemical Company, St Louis, MO, U.S.A. All the enzymes used in this study were from Sigma Chemical Company.

2.4 IN VIVO GROWTH OF PARASITES AND HARVESTING

The Sprague-Dawley rats were inoculated with a stabilate of IL 3000 bloodstream forms. The parasitaemia was estimated daily on a wet blood smear made on glass slide by viewing under a phase contrast microscope (Paris *et al.*, 1982). Peak parasitaemia in excess of 5×10^7 parasites per ml of blood was observed after 72-96 hours.

The parasites were harvested from the blood drawn from the rats at peak parasitaemia by the method of Lanham and Godfrey (1970). This was done by first anaesthetising the rats using diethylether. The thorax was opened to expose the heart using a pair of scissors. The heart was sprayed with 5% (w/v) trisodium citrate as anticoagulant in phosphate saline glucose (PSG) buffer, pH 8.0 consisting of 56 mM disodium hydrogen phosphate, 3 mM sodium dihydrogen phosphate, 51 mM sodium chloride and 10 mM glucose. It was punctured with a needle fitted to a 20 ml syringe, containing 2 ml of the same anticoagulant. Blood was collected in the syringe while the heart was still beating.

2.5 ISOLATION OF TRYPANOSOMES FROM BLOOD

Trypanosomes were isolated from the blood on a column packed with DEAE-cellulose (Lanham and Godfrey, 1970).

2.5.1 Preparation of DEAE-Cellulose slurry

About 200 gm of pre-swollen DEAE-cellulose from Whatman Company, U.K. was resuspended in about one litre PSG buffer (pH 8.0)

referred in section 2.4. The pH of the slurry was adjusted with HCl while constantly stirring the slurry with a magnetic stirrer. After 1 hour of constant stirring to ensure that all the particles of DEAE - cellulose equilibrated to that pH, the slurry was allowed to settle. The supernatant on top of the thick slurry was decanted carefully and fresh PSG buffer added. The mixture was stirred for about 10 minutes constantly before leaving the slurry to settle at the bottom of the beaker and pouring out the supernatant. This washing was done four times before resuspending the slurry in fresh PSG buffer and storing at 4^0C ready for use.

2.5.2 Column packing, loading and elution

The DEAE-cellulose resuspended in PSG buffer at a ratio of 1:1 was poured in clean sintered glass column of 6 cm internal diameter. The height of the gel in the column depended on the amount of blood to be applied to the column; usually packed at the ratio of 1 cm for every 5 ml of the infected rat blood.

After pouring the gel, all the buffer was allowed to drain and Whatman filter No. 41 paper placed on top of the packed column. Blood containing trypanosomes was diluted with PSG buffer at the ratio of 1:1 and poured slowly on top of the Whatman filter paper so as not to disturb the gel surface. The blood was allowed to enter through the column after which cold PSG buffer was poured slowly onto the piece of paper until the column was full. The eluent was constantly monitored by taking a drop and checking under the microscope until the trypanosomes appeared. The eluent containing the trypanosomes was collected in a 500 ml beaker placed on ice (Lanham and Godfrey, 1970). It was centrifuged at about 1000 xg for 10 minutes at 4^0C and the resulting trypanosome pellet resuspended in fresh PSG buffer.

2.5.3 Counting of the trypanosomes

The trypanosomes resuspended in PSG buffer were counted using an improved Neubauer chamber haemocytometer (Paris *et al.*, 1982). This was done by diluting a small volume of the original suspension in PSG buffer so that the number of parasites in each large square of the haemocytometer could be between 20-100 trypanosomes. The diluted trypanosome suspension was carefully introduced into the haemocytometer counting chamber by touching the edge of a firmly placed coverslip with the tip of a pasture pipette containing the trypanosome suspension. The haemocytometer was placed under a phase contrast microscope (X40 objective lens) and the trypanosomes counted in four large squares (Paris *et al.*, 1982). The number of trypanosomes were estimated using the equation below:-

$N = n/4 \times 10^4 \times D \times V$

Where:-

N = the number of trypanosomes in the undiluted buffer

n = number of trypanosomes counted in 4 large squares of the haemocytometer.

D = Dilution factor.

V = Volume of the undiluted trypanosomes suspension.

2.6 LATENCY DETERMINATION OF VARIOUS ENZYMES

2.6.1 Freeze-thawing of trypanosomes

Trypanosomes in cryo-vial freezing tubes containing PSG (pH 8.0) were frozen in liquid nitrogen for 5 min. The tubes containing (about 2×10^8 trypanosomes/ml) were removed from the liquid nitrogen and thawed in a water bath at 37°C with slight agitation. An aliquot of the sample was removed, put

in 2.5 ml eppendorf tube and centrifuged at 1000 xg for 10 minutes at 4°C. The resulting supernatant was used to determine the various enzymes released. The freeze-thawing was repeated several times and each time the enzyme activities were determined. After the 7th cycle, complete lysis was achieved by adding 0.1% (w/v) Triton X-100 to the remaining sample. This was left on ice for 10 minutes and then centrifuged at 1000 xg for 10 minutes before enzyme activities were determined in the supernatant. The activity determined in the presence of Triton X-100 was taken to be 100% enzyme activity.

2.6.2 Treatment of trypanosomes with Triton X-100

Trypanosomes, resuspended in PSG buffer (pH 8.0) at a concentration of about 2 $x10^8$/ml were aliquoted into various eppendorf tubes and kept on ice. Various quantities of Triton X-100 from the stock solution of 1%(w/v) were added to each tube to give a range of final concentration of 0 to 0.2%. Each tube, clearly marked for the amount of Triton X-100 added was slowly vortexed to mix the content and left on ice for 15 minutes to lyse. The tubes with lysed trypanosomes were centrifuged for 10 minutes at 1000 xg at 4°C. The supernatant was used for enzyme assays.

2.7 ENZYME ASSAYS

The activities of various enzymes were determined by coupling various reactions to the disappearance or appearance of NADH or NADPH. They were measured spectrophotometrically at 340 nm by monitoring change in optical density/ min over a period of 4-10 min on Parkin Elmer 550S UV/Vis spectrophotometer. The total volume of the assay mixture was 1 ml in the cuvettes of 1 cm light path at 25°C. The number of trypanosomes in the stock

suspension was about $2x10^8$/ml. The molar extinction coefficient (ϵ) of reduced nucleotides (NADH and NADPH) at 340 nm was taken as 6.22 x 10^6 cm^{-1} per mole of NADH or NADPH generated or consumed at 25°C (Bergmeyer, 1974). The specific activity of the enzymes was expressed as micromoles/min/mg protein. This was calculated as follows:-

$$S.A = \frac{\Delta OD/min}{\epsilon} \times \frac{V}{v} \times \frac{1}{P}$$

Where;

S.A. = Specific Activity in µmol/min/mg protein.

ΔOD/min = Rate of change of optical density per minute

V = Total volume in the assay cuvette.

v = Volume of the sample used in the assay.

P = Protein concentration in mg/ml.

ϵ = Extinction coefficient of NADH or NADPH at 340 nm

2.7.1 Acetate kinase

The activity was assayed essentially as described by Bergmeyer (1974) in 67 mM triethanolamine buffer (pH 7.4) containing 2 mM PEP, 5 mM ATP, 2 mM NADH, 1.3 mM $MgCl_2$, 2.7 i.u pyruvate kinase/ml, 8.0 i.u LDH/ml, 0.1% (w/v) Triton X-100 and 50 µl of trypanosomes suspension (sample). The temperature of the mixture was allowed to equilibrate to 25°C for at least 3 minutes after which the change in optical density was recorded. The initial rate before the reaction was started by addition of sodium acetate was assumed to be due to ATPase activity. The reaction was started by addition of up to 333 mM

sodium acetate. The rate of decrease in optical density (OD/min) was determined over a period of 6 min. The ΔOD/min for acetate kinase was determined by subtracting the initial ΔOD/min, before the addition of acetate, which was assumed to be due to ATPase from the final ΔOD/min after addition of acetate. The reaction in the cuvettes was as follows:-

$$\text{Acetate} + \text{ATP} \xrightarrow{\text{acetate kinase}} \text{Acetylphosphate} + \text{ADP}$$

$$\text{ATP} \xrightarrow{\text{ATPase}} \text{ADP} + \text{Pi}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{Lactate} + \text{NAD}^+$$

2.7.2 Enolase

The enzyme activity was assayed as described by Bergmeyer (1974). The assay mixture contained 83 mM triethanolamine buffer (pH 7.6), 3.3 mM $MgSO_4$, 2.0 mM NADH, 1.1 mM ADP, 8.5 i.u LDH /ml, 2.7 i.u pyruvate kinase /ml, 0.1% (w/v) Triton X-100, and 20 µl of trypanosome suspension. The mixture was left to equilibrate for two minutes after which glycerate-2-phosphate was added to 2 mM to start the reaction and rate of decrease in optical density (OD/min) monitored for 4 minutes. The reaction in the cuvette was as follows:-

$$\text{Glycerate - 2 - Phosphate} \xrightarrow{\text{enolase}} \text{PEP} + \text{ADP}$$

$$\text{ADP} + \text{PEP} \xrightarrow{\text{pyruvate kinase}} \text{pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{lactate dehydrogenase}} \text{Lactate} + \text{NAD}^+$$

2.7.3 α-Glycerophosphate dehydrogenase (αGPDH)

The enzyme activity was assayed according to Opperdoes *et al* (1977) in a reaction mixture consisting of 50 mM Phosphate buffer (pH 7.2), 1 mM EDTA, 2 mM NADH, 20µl of sample and 0.1% (w/v) Triton X-100. The reaction was started by adding dihydroxyacetone phosphate to 5 mM after 2 minutes of equilibration and the rate of the disappearance of NADH measured by recording the rate of decrease in optical density/ min. The reaction in the cuvette occurred as follows:-

$$DHAP + NADH + H^+ \xrightarrow{\alpha\text{-GPDH}} \text{Glyceraldehyde phosphate} + NAD^+$$

2.7.4 Lactate dehydrogenase (LDH)

The enzyme activity was assayed as described by Bergmeyer (1974) in 48 mM potassium phosphate buffer (pH 7.5) containing 1.8 mM NADH, 0.1% (w/v) Triton X-100 and 50 µl of sample. After stirring, the reaction mixture was left to equilibrate for 2 minutes before starting the reaction by adding pyruvate to 0.5 mM. The rate of decrease in optical density (OD/min) was determined over a period of 6 min. The reaction in the cuvette was as follows:-

$$\text{Pyruvate} + NADH + H^+ \xrightarrow{LDH} \text{Lactate} + NAD^+$$

2.7.5 Phosphoenolpyruvate carboxykinase (PEPCK)

This enzyme activity was assayed according to Opperdoes and Cottem (1982) in 0.1M imidazole buffer (pH 6.6) containing 1.25 mM ADP, 1 mM $MnSO_4$, 50 mM $KHCO_3$, 2 mM NADH, 1 mM glutathione, 50 µl sample. To

start the reaction, up to 1.25 mM PEP was added after 2 minutes of quilibration in the cuvette. However, MDH was not included as described by Opperdoes and Cottem (1982) because the sample itself contained enough MDH to carry on the reaction shown below. The rate of decrease in optical density (OD/min) was determined over a period of 6 min. The reactions that occurred were as follows:-

$$PEP + ADP + CO_2 \xrightarrow{PEPCK} OAA + ATP$$

$$OAA + NADH + H^+ \xrightarrow{MDH} Malate + NAD^+$$

2.7.6 α-Ketoglutarate dehydrogenase

The enzyme activity was assayed according to Overath *et al* (1986) in 150 mM Tris-HCl buffer (pH 7.4) containing 3 mM cystein, 0.2 mM CoASH, 4 mM NAD^+, 0.2 mM thiamine pyrophosphate (cocarboxylase) and 50 µl of sample. The reaction was started by adding oxoglutarate (disodium salt) to 5 mM when the change in OD/min was zero. The rate of increase in optical density (OD/min) was determined over a period of 6 min. The reaction in the cuvette proceeded as follows:-

$$\alpha\text{-Ketoglutarate} + CoASH + NAD^+ \xrightarrow{Cyst.;\ TPP,\ \alpha KGDH} \text{Succinyl CoA} + CO_2 + NADH + H^+$$

2.7.7 Citrate synthase

This was assayed as described by Weeda *et al* (1980b) in 200 mM Tris-HCl buffer (pH 7.8) containing 5 mM EDTA, 5 mM NAD, 0.3 mM Acetyl CoA, 0.1% Triton X-100, 20 µg MDH/ml and 50 µl of sample. The reaction was started by adding DL-malate to 50 mM after 2 minute of equilibration at

25°C. The increase in optical density (OD/min) was determined over a period of 6 min. The reaction in the cuvette proceeded as follows:-

$$\text{L-Malate} + NAD^+ \xrightarrow{\text{malate dehydrogenase}} \text{OAA} + NADH + H^+$$

$$\text{OAA} + \text{Acetyl CoA} + H_2O \xrightarrow{\text{citrate synthase}} \text{Citrate} + \text{CoASH}$$

2.7.8 Aconitase

This enzyme activity was assayed according to Bergmeyer (1974) in Tris-HCl buffer (pH 7.4) containing 10 mM $MgCl_2$, 2 mg isocitrate dehydrogenase/ml, 1 mM $NADP^+$, 0.1%(w/v) Triton X-100 and 50 µl of sample. The reaction was started by adding citrate to 10 mM after 2 minutes of equilibration. The increase in optical density (OD/min) was determined over a period of 6 min. The reactions in the cuvette were as follows:-

$$\text{Citrate} \xrightarrow{\text{Aconitase}} \text{Isocitrate}$$

$$\text{Isocitrate} + NADP^+ \xrightarrow{\text{ICDH}} \alpha\text{-Ketoglutarate} + NADPH + H^+ + CO_2$$

2.7.9 Fumarase

The enzyme activity was assayed as described by Konji (1983) in 100 mM phosphate buffer (pH 7.4), 10 µg citrate synthase/ml, 2 mM NAD^+, 20 µg MDH/ml, 0.1%(w/v) Triton X-100, 50 µl of sample and 1 mM Acetyl CoA. The reaction was started by addition of fumarate to a final concentration of 1.7 mM. The increase in optical density (OD/min) was determined over a period of 6 min. The reaction in the cuvette was as follows:-

$$\text{Fumarate} + H_2O \xrightarrow{\text{fumarase}} \text{Malate}$$

$$\text{Malate} + NAD^+ \xrightarrow{\text{malate dehydrogenase}} \text{OAA} + NADH + H^+$$

$$\text{OAA} + \text{Acetyl CoA} + H_2O \xrightarrow{\text{citrate synthase}} \text{Citrate} + \text{CoASH}$$

2.7.10 Malate dehydrogenase

MDH activity was assayed a
Bergmeyer (1974) but in 94.6 mM pl
mM NADH, 0.1%w/v) Triton X-100 a
equilibration, the reaction was started
rate of decrease in optical density (OD
min. The reaction proceeded as follow

Oxaloacetate + NADH + H^+ ___

2.7.11 Pyruvate dehydrogenas

The assay system was a slight
dehydrogenase described by Overath
contained 150 mM Tris-HCl buffer (pH
pyrophosphate, 0.2 mM coenzyme A, 4
50 µl of sample and sodium pyruvate to
the reaction. The increase in optical de
period of 6 min. The reaction in the cuve

Pyruvate + CoASH + NAD^+ $\xrightarrow[\text{Cys and TPP}]{\text{PD}}$

2.7.12 Pyruvate kinase

Its activity was assayed accordi
triethanolamine buffer (pH 7.5) containi
mM $MgSO_4$, 0.2 mM NADH, 13 mM A
100, 2.5 i.u/ml LDH. To start the

concentration of 1.25 mM after 2 minutes of equilibration. The decrease in optical density (OD/min) was determined over a period of 6 min.

The reaction was as follows:-

$$PEP + ADP \xrightarrow{PK} Pyruvate + ATP$$

$$Pyruvate + NADH + H^+ \xrightarrow{LDH} L\text{-}Lactate + NAD^+$$

2.7.13 Phosphotransacetylase (PTA)

The enzyme activity was assayed according to Bergmeyer (1974) in a medium containing 85.6 mM Tris-acetate buffer (pH 7.4), 1.7 mM glutathione, 0.4 mM CoASH, 19 mM ammonium sulphate, 0.1%(w/v) Triton X-100, 50 µl of sample prepared in 25 mM Tris buffer (pH 8.0), and a final concentration of 7.4 mM acetyl-phosphate to start the reaction. The rate of decrease in optical density (OD/min) was read at 233 nm for over a period of 6 min. The molar extinction coefficient (ϵ) for CoASH at 233 nm was taken to be 4.44×10^6 cm^2 mol^{-1} in the calculation of enzyme activity. The reaction in the cuvette was:-

$$Acetylphosphate + CoASH \xrightarrow{PTA} Acetyl\ CoA + Pi$$

2.7.14 NADP - linked malic enzyme

The activity was assayed according to the method described by Ochoa *et al* (1955) in 70 mM Tris-HCl buffer (pH 7.4), 3.5 mM $MgCl_2$, 5µl of 5 mg/ml $NADP^+$, 0.1%(w/v) Triton X-100, 50 µl of sample and 3.5 mM DL-malate to start the reaction after 2 minutes of equilibration. The increase in optical density (OD/min) was determined over a period of 6 min. The reaction in the cuvette was as follows:-

$$Malate + NADP^+ \xrightarrow{ME} Pyruvate + CO_2 + NADPH + H^+$$

2.7.15 NAD-linked malic enzyme

The assay system was similar to that for NADP-linked malic enzyme except that NADP was replaced with NAD^+. The assay mixture consisted of 70 mM Tris-HCl buffer pH 7.4, 3.5 mM $MgCl_2$, 1 mM NAD^+, 0.1%(w/v) Triton X-100, 50 µl of sample and 3.5 mM DL-malate to start the reaction after 2 minutes of equilibration. The increase in optical density (OD/min) was determined over a period of 6 min. The reaction in the cuvette was as follows:-

$$\text{Malate} + NAD^+ \xrightarrow{\text{ME}} \text{Pyruvate} + CO_2 + \text{NADH} + H^+$$

2.7.16 $NADP^+$-linked isocitrate dehydrogenase

The enzyme was assayed according to the method described by Konji (1983). The assay mixture consisted of 70 mM triethanolamine buffer (pH 7.4) containing 4 mM $MgCl_2$, 0.8 mM NADP, 50 µl of sample and DL-isocitrate to a final concentration of 5 mM to start the reaction after 2 minutes of incubation. The increase in optical density (OD/min) was determined over a period of 6 min. The reaction in the cuvette was as follows:-

$$\text{L-Isocitrate} + NADP^+ \xrightarrow{\text{ICDH}} \alpha\text{-Ketoglutarate} + \text{NADPH} + H^+$$

2.7.17 NAD-linked isocitrate dehydrogenase

This enzyme activity was assayed according to Alp *et al* (1976) in 70 mM triethanolamine buffer (pH 7.4) containing 2 mM ADP, 2 mM NAD^+, 1 mM $MnCl_2$, 10 mM citrate, 50 µl of sample and DL-isocitrate to 5 mM to start the reaction. The increase in optical density (OD/min) was determined for the

first 5 minutes because it is usually very unstable. The reaction in the cuvette was:-

$$\text{L-Isocitrate} + NAD^+ \xrightarrow{\text{ICDH}} \alpha\text{-Ketoglutarate} + CO_2 + NADH + H^+$$

2.8 Respiration of the trypanosomes

The uptake of oxygen was determined using a Clark-type oxygen electrode (Rank Brothers High Street Bottisham - Cambridge, England) polarized at 0.6 V in a magnetically stirred cuvette. The electrode was equilibrated with phosphate buffered saline containing 5 mM glucose (PSG) pH 7.4 for at least 5 minutes at 25°C before starting the experiment. The experiment was started by adding 200µl of $2x10^8$ trypanosomes/ml suspended in PSG (pH 7.4) into the oxygen electrode. The rate of oxygen uptake was monitored on a Sargent-Welch recorder coupled to the electrode. This was done before and after addition 2 mM SHAM and 0.5 mM cyanide. The total volume in the chamber was 2.2 ml and the concentration of oxygen in the media was taken to be 240 nmol/ml (Chance and Williams, 1956).

2.9 METABOLITES ASSAYS

Freshly isolated trypanosomes were resuspended in PSG buffer (pH 8.0) at a concentration of 1×10^9 trypanosomes/ml in a 1.5 ml eppendorf tubes. The trypanosomes were incubated in a waterbath at 37°C for 30 minutes with or without 2 mM SHAM or 0.5 mM CN^-. After 30 minutes of incubation, PCA was added to a final concentration of 7%(v/v) to stop the reaction. The deprotenized samples were neutralised with 6N KOH and centrifuged at 1000 xg for 10 minutes. The control tubes were also subjected to the same treatment

except that the PCA was added immediately the trypanosomes suspension was put in the incubation water bath at 37°C.

Aliquots of the deprotenized samples were used for metabolite assays. The metabolite assays were done at 25°C in cuvettes of 1 cm light path with a total volume of 1 ml. The optical density of either NADH or NADPH was read at either 340 nm or 365 nm in the absence or presence of SHAM respectively on a Perkin Elmer 550S UV/Vis spectrophotometer. This was done due to the interference of SHAM at 340 nm.

The extinction coefficient of 6.22×10^6 or 3.4×10^6 cm^{-1} per mole of NADH used or generated was used in all determinations at 340 and 365 nm respectively (Bergmeyer, 1974), unless otherwise indicated in the text. The metabolite concentrations were estimated using the equation:-

$$C = \frac{\Delta OD}{6.22 \text{ or } 3.4} \times \frac{V}{v} \times \frac{1}{P} \times d$$

Where:-

C = Concentration of respective metabolite in μmoles/30 min/mg protein.

ΔOD = Change in optical density during the assay.

V = Total assay volume.

v = Volume of extract.

P = Protein concentration in mg/ml.

d = Dilution factor

In determination of the initial optical density (OD_1) the reading was always taken after the OD/min reading stabilised at zero before starting the reaction. The time taken depended on each metabolite, while the final optical density (OD_2) was taken when the rate of change of optical density (OD/min) was zero after starting the reaction. The difference in the final and initial

optical densities (OD_2 and OD_1) constituted the ΔOD. This was done for all the metabolites unless otherwise stated in the text.

2.9.1 Pyruvate

This was assayed according to Bergmeyer (1974) in mixture containing 47 mM triethanolamine buffer (pH 7.6), 200 mM NaCl, 2 mM NADH, 50 µl of sample extract and up to a final concentration of 2 µg/ml LDH to start the reaction. The initial optical density (OD_1) was taken when steady optical density was achieved before addition of LDH at 340 nm in the absence of SHAM. The final optical density (OD_2) was taken when there was no further change in optical density after starting the reaction. The change in optical density (ΔOD)was given by OD_1-OD_2. The reaction in the cuvette was as follows:-

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{L-Lactate} + NAD^+$$

2.9.2 Lactate

Lactate was determined using 80 mM Tris/HCl buffer (pH 7.2), containing 200 mM NaCl, 0.2 mM NAD, 100 µl sample extract and 2 µg/ml LDH to start the reaction. The initial optical density (OD_1) at 340 nm in the absence of SHAM was taken before addition of LDH . The final optical density (OD_2) was read when there was no further change in optical density after starting the reaction. The change in optical density (ΔOD) was calculated as (OD_2-OD_1). The reaction in the cuvette was as follows:

$$\text{Lactate} + NAD^+ \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} + H^+$$

2.9.3 Succinate

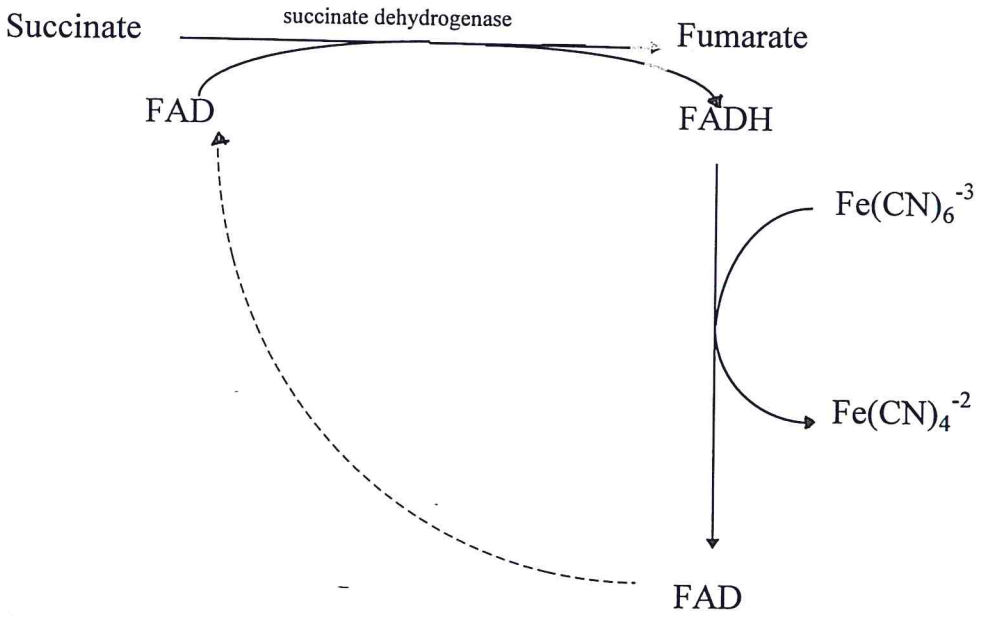
Succinate was determined by slight modification of the method of Singer *et al.,* (1956) from a standard curve constructed using commercial sodium succinate. The curve was constructed with sodium succinate solutions with concentrations ranging from 0 to 1.0 µmoles in a total volume of 3 ml, which was found to give a linear relationship between the optical density and the amount of succinate.

First, buffer 'A' consisted of 100 mM Phosphate buffer (pH 7.6), 1 mM EDTA and 1 gm BSA/l was prepared. Buffer 'B' was then prepared by mixing 2.7 ml of 75 mM $K_3Fe(CN)_6$ (dissolved in buffer 'A'), 1.3 ml of 30 mM KCN (dissolved in buffer 'A') and 36 ml of buffer 'A'.

A total volume of 3 ml of reaction mixture was prepared using 0 to 0.1 ml of the 30 mM sodium succinate (dissolved in buffer 'A'), varying quatities of buffer 'B' and 50 µl of mitochondria suspension (1 mg protein) to start the reaction. The reaction mixture was incubated at 25°C in a waterbath for 60 minutes after which 0.15 ml of 0.25 M HCl was added to stop the reaction. The optical density was read at 455 nm. The optical density was plotted as a function of succinate concentration to give a standard straight line graph that was used to determine the amount of succinate in the experimental samples as described below.

The amount of succinate in the experimental sample was determined by mixing 100 µl of sample extract, buffer 'B' and 50 µl of mitochondria preparation (1 mg protein) to start the reaction. The mitochondria was prepared as described in section (2.9.3.1) and used as a source of succinate dehydrogenase. The reaction mixture was incubated at 25°C in a waterbath for 60 minutes after which 0.15 ml of 0.25 M HCl was added to stop the reaction. The optical density was read at 455 nm. The amount of succinate was determined from the standard curve mentioned above.

The reaction was as follows:-



2.9.3.1 Mitochondria preparation from rat liver

The rat was killed by cervical dislocation. The abdomen was quickly opened to expose the liver which was removed by a pair of scissors. The excess blood was drained using a paper towel after which the liver was weighed and put in a chilled 0.25 M sucrose to make 10% (w/v) homogenate. Homogenisation was done using a dounce homogenizer before spinning at 1000 xg for 10 minutes at 4°C using a sorvall RC-5B refrigerated superspeed centrifuge fitted with SS-34 rotor. The pellet was discarded and the supernatant centrifuged at 25000 x g for 25 minutes at the same conditions as above. The resulting pellet was resuspended in 0.25 M sucrose to wash the mitochondria. The washing was done twice by resuspending the pellet and centrifuging for 15 minutes at the same condition as described above. The final pellet was resuspended in 0.25 M sucrose and protein concentration determined. The

mitochondrial preparation was used as a source of succinate dehydrogenase in succinate determination.

2.9.4 Acetate

This was assayed essentially as described by Bergmeyer (1974) in a reaction mixture consisting of 0.15 M triethanolamine buffer (pH 7.6), 10 mM L-malate, 0.4 mM CoASH, 5.4 mM ATP, 1.33 mM $MgCl_2$, 1 mM NAD^+, 4.0 i.u LDH/ml, 5.0 i.u Phosphotransacetylase/ml, 5.0 i.u Citrate synthase/ml, 5.0 i.u MDH/ml, 5.0 i.u acetate kinase/ml and 100 µl of sample extract. The additions were made sequentially as follows:- First, the buffer containing malate, Mg^{2+}, NAD^+, CoASH, ATP, MDH and the sample extract were pipetted in the 1 ml cuvette. The optical density at 340 nm or 365 nm was left to stabilise and recorded (OD_1). After the steady optical density was achieved, PTA, CS and LDH were added to the cuvette. The contents were mixed by stirring and left to stabilise after which the reaction was started by the addition of 5.0 i.u/ml acetate kinase. The final optical density (OD_2) was recorded when OD/min was zero, which took approximately one hour. The reactions were as follows:-

a) $\text{Acetate} + \text{ATP} \xrightarrow{\text{AK}} \text{Acetylphosphate} + \text{ADP}$

b) $\text{Acetyl Phosphate} + \text{CoASH} \xrightarrow{\text{PTA}} \text{Acetyl CoA} + \text{Pi}$

c) $\text{Acetyl CoA} + \text{OAA} + H_2O \xrightarrow{\text{CS}} \text{Citrate} + \text{CoASH}$

d) $\text{Malate} + NAD^+ \xrightarrow{\text{MDH}} \text{OAA} + \text{NADH} + H^+$

The overall reaction in the cuvette was:-

$$\text{Acetate} + \text{ATP} + NAD^+ + \text{Malate} \xrightarrow[\text{CS, AK.}]{\text{MDH, PTA}} \text{Citrate} + \text{ADP} + H^+ + \text{Pi}$$

$\Delta OD_{(acetate)}$ which was the change in optical density due to acetate in the sample extract was obtained from the formula:-

$$\Delta OD_{(acetate)} = OD_2 - \frac{(OD_1)^2}{OD_2}$$

Where;

OD_1 = Initial value of optical density

OD_2 = Final value of optical density

The amount of acetate was estimated by using the formula below which has been described earlier (sub section 2.8.0).

$$C = \frac{\Delta OD_{(acetate)}}{6.22} \times \frac{V}{v} \times \frac{1}{P}$$

2.9.5 Glycerol

This was assayed using 90 mM triethanolamine buffer (pH 7.4) containing 2 mM $MgS0_4$, 10 mM KCl, 2 mM PEP, 5 mM ATP, 2 mM NADH 25 µg pyruvate kinase/ml, 25 µg LDH/ml, 100 µl of sample and 5 i.u glycerokinase/ml.

The initial optical density (OD_1)was taken before addition of 5 mM ATP at 340 or 365 nm while the final optical density (OD_2) was taken when the change in optical density (OD/min) was zero (after about 28 minutes). The change in optical density due to glycerol (ΔOD) was taken as (OD_1-OD_2). This was used to calculate the amount of glycerol in the sample.

The reaction in he cuvette was as follows:-

Glycerol + ATP $\xrightarrow{GK.}$ Glycerol-3-Phosphate + ADP

ADP + PEP $\xrightarrow{PK.}$ ATP + Pyruvate

Pyruvate + NADH + H^+ $\xrightarrow{LDH}$ Lactate + NAD^+

2.10 PROTEIN DETERMINATION

The amount of protein was determined by the method of Lowry *et al.* (1951). A standard curve was constructed using γ-globulin protein obtained from Sigma chemical company. This was done with γ-globulin protein sample with a concentration ranging from 0 to 60 μg/ml which was found to be within the linear range. First, a solution "A" was prepared by mixing 50 ml of 2% (w/v) anhydrous sodium carbonate, 0.2% (w/v) potassium-sodium tartrate in 0.1 M sodium hydroxide and 0.1 ml of 0.05% (w/v) copper sulphate solution. This was prepared fresh whenever protein concentration was to be determined. A solution "B" was prepared by diluting Folin-phenol (or Folin-Ciocalteu) reagent with distilled water at a ratio of 1:2. To each protein sample an appropriate volume of water was added, followed by 0.77 mls of solution A. This was shaken thoroughly and after 10 minutes 0.08 mls of solution B was added, shaken thoroughly and incubated for 30 minutes. The total assay volume was 1 ml. After 30 minutes the optical densities were read on a Perkin-Elmer 550S UV/Vis spectrophotometer at 578 nm.

CHAPTER THREE

RESULTS

3.1 EFFECT OF METABOLIC INHIBITORS ON RESPIRATION AND END PRODUCTS FROM GLUCOSE CATABOLISM IN BLOODSTREAM *T. CONGOLENSE*

3.1.1 The effect of cyanide and SHAM on respiration

The effect of cyanide and SHAM on the oxygen consumption by bloodstream *T. congolense* was investigated. Figure 1 shows that the addition of cyanide to an incubation mixture of trypanosomes respiring on glucose had no effect on the rate of oxygen consumption. Addition of SHAM to the respiring trypanosomes totally inhibited the oxygen consumption. These results suggest that bloodstream *T. congolense* utilise trypanosome alternate oxidase (TAO) system during the catabolism of glucose just like bloodstream *T. brucei* (Clarkson *et al.*, 1989; Opperdoes *et al.,* 1977; 1987). They further suggest that bloodstream *T. congolense* does not use cytochrome aa_3 which is usually inhibited by cyanide or cytochrome o which is insensitive to cyanide or SHAM (Flynn and Bowman, 1973; Fairlamb and Bowman, 1977; Fulton and Spooner, 1959).

3.1.2 The effect of SHAM on pyruvate production

Pyruvate production by bloodstream *T. congolense* catabolizing glucose was investigated in the presence and absence of 2 mM SHAM. Figure 2 shows that a mean of 150 nmol pyruvate/mg protein was produced within the first 30 minutes in the absence of SHAM while the mean of 25 nmol pyruvate/mg protein was produced in the presence of SHAM during the same period.

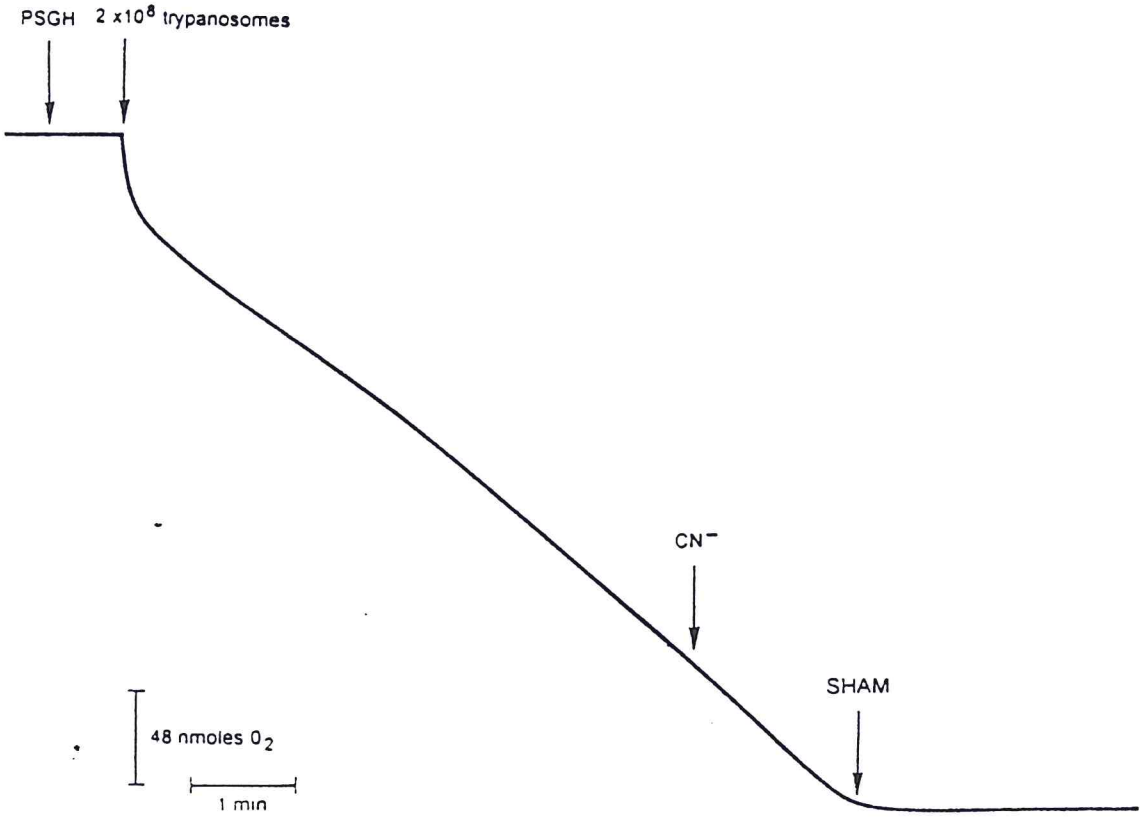


Figure 1

After 60 min of incubation about 180 nmol pyruvate/mg protein was produced in the absence of SHAM while in the presence of SHAM there was no additional increase in pyruvate production. Examination of the sample under a microscope in the presence of SHAM after 30 min indicated that the trypanosomes were lysed while in the absence of SHAM the trypanosomes stayed alive for more than 3 hours. The maximum quantity of pyruvate produced in the absence of SHAM after 180 minutes of incubation was 230 nmol pyruvate/mg protein.

From these results it was concluded that SHAM causes death of the trypanosomes within the first 30 minutes. Incubation of trypanosomes for more than 30 minutes with SHAM did not result into significant increase in pyruvate production because most of the trypanosomes were dead and lysed.

3.1.3 The effect of a combination of SHAM and CN^- on pyruvate production

The effect of a combination of both SHAM and CN^- on pyruvate production was investigated. Table I shows that the mean amounts of pyruvate produced in 30 min was 155 and 26 nmoles/mg protein in the absence and presence of SHAM respectively. This indicated 83% inhibition of pyruvate production by SHAM. The mean production of pyruvate after 30 min in presence and absence of CN^- was 160 and 155 nmoles/mg protein while in the presence of both SHAM and CN^- it was 28 nmoles/mg protein. This indicates that CN^- had a slight stimulatory effect on pyruvate production. SHAM on the other hand inhibits pyruvate production and was lethal to them.

Figure 2

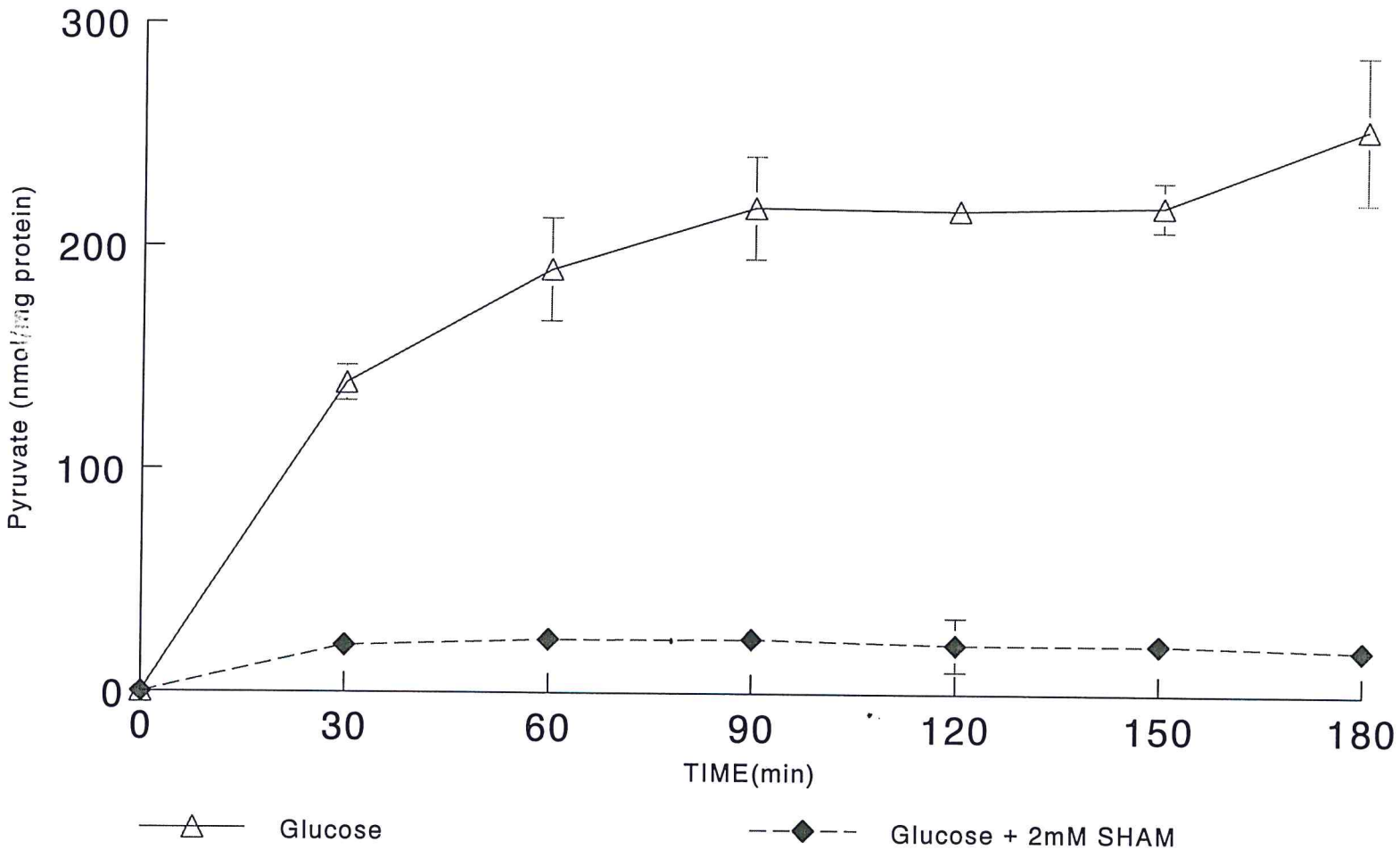

300
200
100
0
Pyruvate (nmol/mg protein)
0
30
60
90
120
150
180
TIME(min)
Glucose
Glucose + 2mM SHAM

Table I

Inhibitor(s) added	**Pyruvate produced (nmoles/30 min/mg protein)**
None	155 ± 12 (4)
SHAM	26 ± 8 (4)
CN^-	156 ± 12 (4)
SHAM and CN^-	28 ± 4 (2)

3.1.4 End products of glucose catabolism

The end products of glucose catabolism were investigated in bloodstream *T. congolense* in the presence and absence of SHAM. Table II shows that the mean glycerol, acetate and pyruvate production after 30 min of incubation in the absence of SHAM were 308, 292.9 and 154 nmoles/mg protein respectively. Lactate and succinate production were significantly low (<1.1 and <0.12 nmoles/mg protein respectively). In the presence of SHAM, there was 165, 44.6, 26.4 and 1.32 nmoles /mg protein of glycerol, acetate pyruvate and succinate produced after 30 min of incubation respectively. Lactate production was still significantly low (<1.1 nmoles/mg protein).

The production of glycerol, acetate and pyruvate was in the molar ratio of 2:2:1 respectively in the absence of SHAM which account for 40%, 38% and 28% of the total end products formed respectively during oxidation of glucose. In the presence of SHAM, the production of succinate, pyruvate, acetate and glycerol were in the molar ratio of 1:20:33:125 respectively. Addition of SHAM resulted in inhibition of production of pyruvate and acetate by 85%. The production of glycerol was inhibited by 50% while succinate production was stimulated.

3.2 ENZYMES FOR THE CATABOLISM OF PHOSPHOENOLPYRUVATE IN BLOODSTREAM *T. CONGOLENSE*

3.2.1 The enzymes involved in catabolism of PEP to pyruvate

In this study, the activities of some enzymes likely to catabolise PEP to pyruvate in bloodstream *T. congolense* were investigated.

Table II.

End Product	Amounts in nmoles/30 min/mg protein MINUS SHAM	PLUS SHAM	Percent Inhibition (%)
pyruvate	154 ± 19 (6)	26.4 ± 2 (4)	85
glycerol	308 ± 54 (4)	165 ± 36 (4)	50
succinate	<0.12 (4)	1.32 ± 0.29 (4)	Stimulated
lactate	<1.1 (4)	<1.1 (4)	0
acetate	292.9 ± 56.3 (4)	44.6 ± 30 (4)	85

They included: pyruvate kinase, PEP carboxykinase, malate dehydrogenase and NAD/NADP linked malic enzymes. Table III(a) shows that the mean activity of pyruvate kinase was 1.1 nmoles/min /mg protein while the mean activities for the other enzymes mentioned above excluding NAD-linked malic enzyme were more than 36.3 nmoles/min/mg protein. It was suggested that the bloodstream *T. congolense* catabolise PEP to oxaloacetate in a reaction catalysed by PEPCK involving CO_2 fixation. The oxaloacetate formed could be reduced to malate in a reaction catalysed by MDH. Finally, the malate could undergo decarboxylation to produce pyruvate in a reaction that is catalysed by NADP-linked malic enzyme.

The possibility of reducing pyruvate to form lactate was also investigated. Table III(a) shows that the activity of lactate dehydrogenase could not be detected (less than 0.43 nmoles/min/mg protein). This suggests that the parasite does not convert significant amount of pyruvate to lactate.

3.2.2 The enzymes involved in the catabolism of pyruvate to acetate

Assuming that all the acetate assayed and shown in Table II was derived from pyruvate, the catabolism of pyruvate to acetate in bloodstream *T. congolense* was investigated. The results are shown in Table III(b). From the Table, the mean activity of pyruvate dehydrogenase was 89.0 nmoles/min/mg protein while phosphotransacetylase and acetate kinase activities were 32.0 and 80.1 nmoles/min/mg protein respectively. It was suggested that the pyruvate produced from PEP could be oxidatively decarboxylated to form acetyl CoA in a reaction catalysed by pyruvate dehydrogenase. The acetyl CoA could then be phosphorylated by phosphotransacetylase to form acetyl phosphate which could finally be dephosphorylated to form acetate by acetate kinase. It was also noted that a pathway involving these enzymes could be a source of reducing

equivalents because NAD is required as coenzyme at the pyruvate dehydrogenase reaction.

3.2.3 The enzymes of the TCA cycle

The possibility of bloodstream *T. congolense* having a functional TCA cycle was investigated. Table III(c) shows that the activities of citrate synthase, aconitase, NAD-linked isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and fumarase were all less than 1.5 nmoles/min/mg protein. It was proposed that the parasite does not oxidise the Acetyl CoA formed from the pyruvate dehydrogenase reaction via the TCA cycle.

3.3 SUBCELLULAR LOCALISATION OF VARIOUS ENZYMES INVOLVED IN PEP CATABOLISM IN BLOODSTREAM *T. CONGOLENSE*

3.3.1 The effect of freeze-thawing on the release of enzymes for the metabolism of PEP to pyruvate

In order to establish the localization of enzymes for the catabolism of PEP to pyruvate, isolated trypanosomes were subjected to several cycles of freeze-thawing. The release of PEPCK, MDH and $NADP^+$ linked malic enzyme which have been shown in section 3.2.1 was investigated. The released activities of these enzyme at each cycle was compared to the activities of enolase and α-GPDH which have been shown to be cytosolic and glycosomal respectively in the bloodstream *T. brucei* (Oduro *et al.*, 1980a). Figure 3(a) shows that the first cycle of freeze-thawing released more than 60% activities of enolase and NADP-ME while less than 25% of MDH, PEPCK, and α-GPDH were released under identical conditions.

Table III(a).

Enzyme	Specific activity in nmoles/min/mg protein
Pyruvate kinase	1.1 ± 0.05 (4)
Lactate dehydrogenase	< 0.43 (4)
PEP carboxykinase	48.0 ± 6.0 (7)
NADP - Malic enzyme	36.3 ± 0.3 (4)
NAD - Malic enzyme	5.0 ± 0.5 (5)
Malate dehydrogenase	502 ± 58 (4)

Table III(c)

Enzyme	Specific activity in nmoles/min/mg protein
Aconitase	0.6 ± 0.02 (4)
Fumarase	18.5 ± 0.7 (5)
Citrate synthase	<0.43 (5)
α-Ketoglutarate dehydrogenase	< 0.43 (5)
NADP-Isocitrate dehydrogenase	1.5 ± 0.4 (4)
NAD-Isocitrate dehydrogenase	<0.43(4)
Pyruvate dehydrogenase	89.0 ± 3 (4)

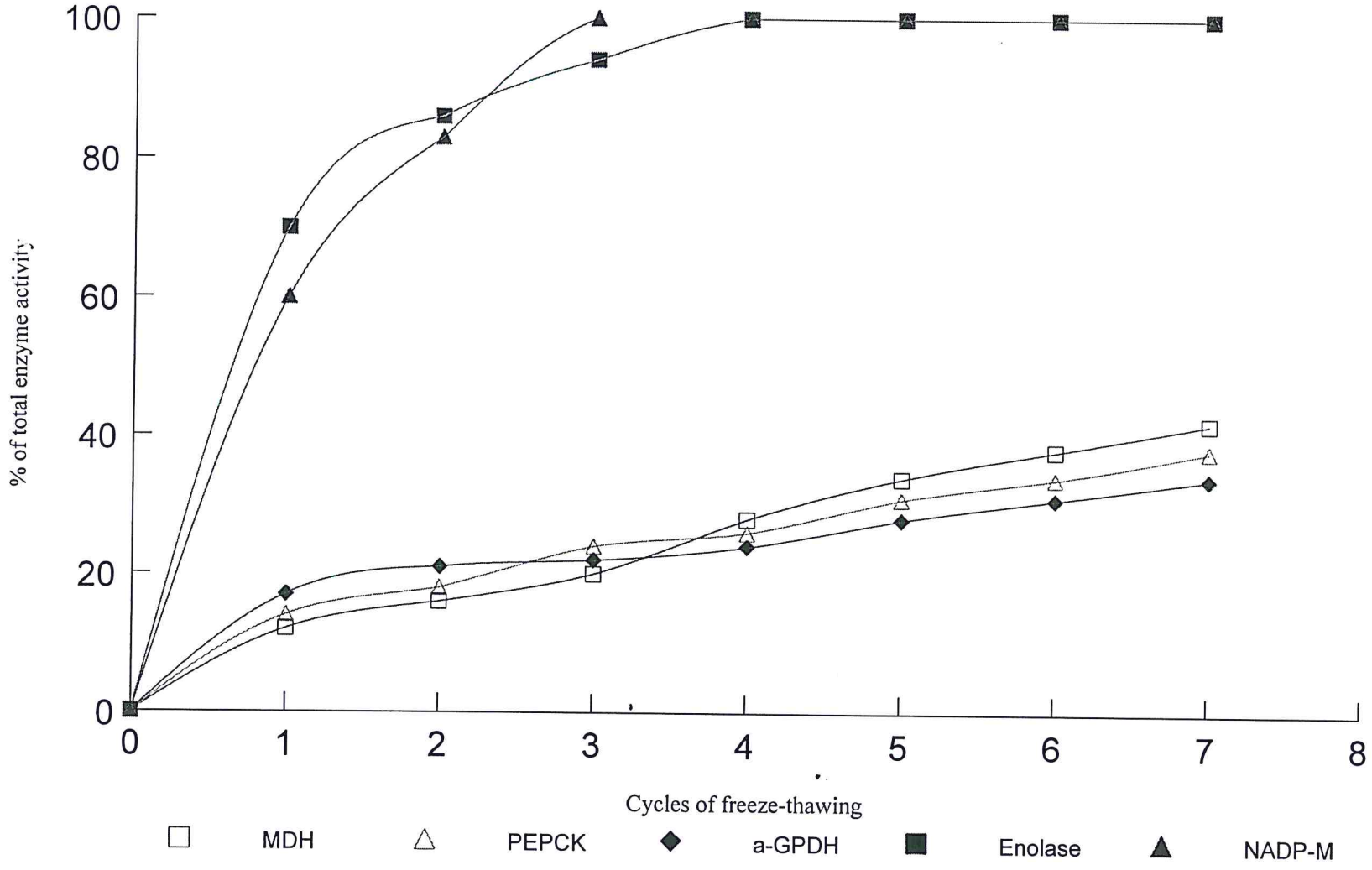
The second cycle of freeze-thawing released more than 85% of enolase and NADP-ME as compared to 30% activities of the other enzymes released. No additional activities of enolase and NADP-ME were released after 3 cycles of freeze-thawing or addition of Triton X-100. This suggests that 100% activities of enolase and NADP-ME were released after the third cycle. The activities of the other enzymes did not increase significantly even after the 7th cycle except on addition of Triton X-100. It is proposed that enolase and NADP-ME are localised in a different compartment from PEPCK, MDH and α-GPDH, because MDH, PEPCK and α-GPDH are more latent than NADP-ME and enolase. It was suggested that NADP-ME and enolase are cytosolic. PEPCK and MDH could be glycosomal.

3.3.2 The effect of increasing concentrations of Triton X-100 on the release of PEPCK and MDH from bloodstream *T. congolense*

To confirm the earlier observation based on several cycles of freeze-thawing, another study was carried out using Triton X-100 which is a non-ionic detergent. The effect of increasing concentrations of Triton X-100 on the release of PEPCK, MDH, enolase and α-GPDH from bloodstream *T. congolense* was investigated. The concentrations of Triton X-100 used were upto 0.1%(w/v).

Figure 3(b) shows that 0.01%(w/v) Triton X-100 released 55% enolase, 7% MDH, 5% PEPCK and 7% α-GPDH activities. 0.02% Triton X-100 released more than 90% of the activities of enolase as compared to 22% PEPCK 24% MDH and 23% α-GPDH released. All the activity of enolase was released after adding 0.03% Triton X-100 but, less than 70% of the activities of PEPCK, MDH and α- GPDH were released. The 100% activities of PEPCK, MDH and α-GPDH were released after adding 0.05% Triton X-100.

Figure 3(a)


100
80
60
40
20
0
% of total enzyme activity
0
1
2
3
4
5
6
7
8
Cycles of freeze-thawing
MDH
PEPCK
a-GPDH
Enolase
NADP-M

These results suggest that PEPCK, MDH and α-GPDH are localised in a different compartment from enolase in bloodstream *T. congolense*. It is apparent that PEPCK, MDH and α-GPDH are more latent than enolase. The results are consistent with the findings of freeze-thawing experiments. It was proposed that these enzymes are glycosomal.

3.3.3 The effect of Triton X-100 on the release of NADP-linked malic enzyme from bloodstream *T. congolense*

The effect of increasing concentrations of Triton X-100 on the release of NADP-linked malic enzyme from bloodstream *T. congolense* was investigated. Enolase and α-GPDH which in section 3.3.1 were proposed to be cytosolic and glycosomal respectively were used as controls. Figure 3(c) shows that 0.01% (w/v) Triton X-100 released 55% enolase, 50% NADP-linked malic enzyme and 5% α-GPDH. Addition of Triton X-100 up to 0.02% further increased the activities of enolase and NADP-malic enzyme to more than 90% while the activities of α-GPDH was less than 20%. No additional increase in the activities of enolase and NADP-linked malic enzyme was observed after the addition of 0.03% Triton X-100, indicating release of 100% of the activities at this concentration of Triton X-100. The activities of α-GPDH continued to increase with the increase of Triton X-100 until after adding 0.05%. These results suggest that enolase and NADP-linked malic enzyme could be localised in the same compartment. α-GPDH could be in another compartment. It was therefore proposed that NADP-ME in bloodstream *T. congolense* is localized in the cytosol. These results are also consistent with the result obtained with freeze-thawing.

Figure 3(b)

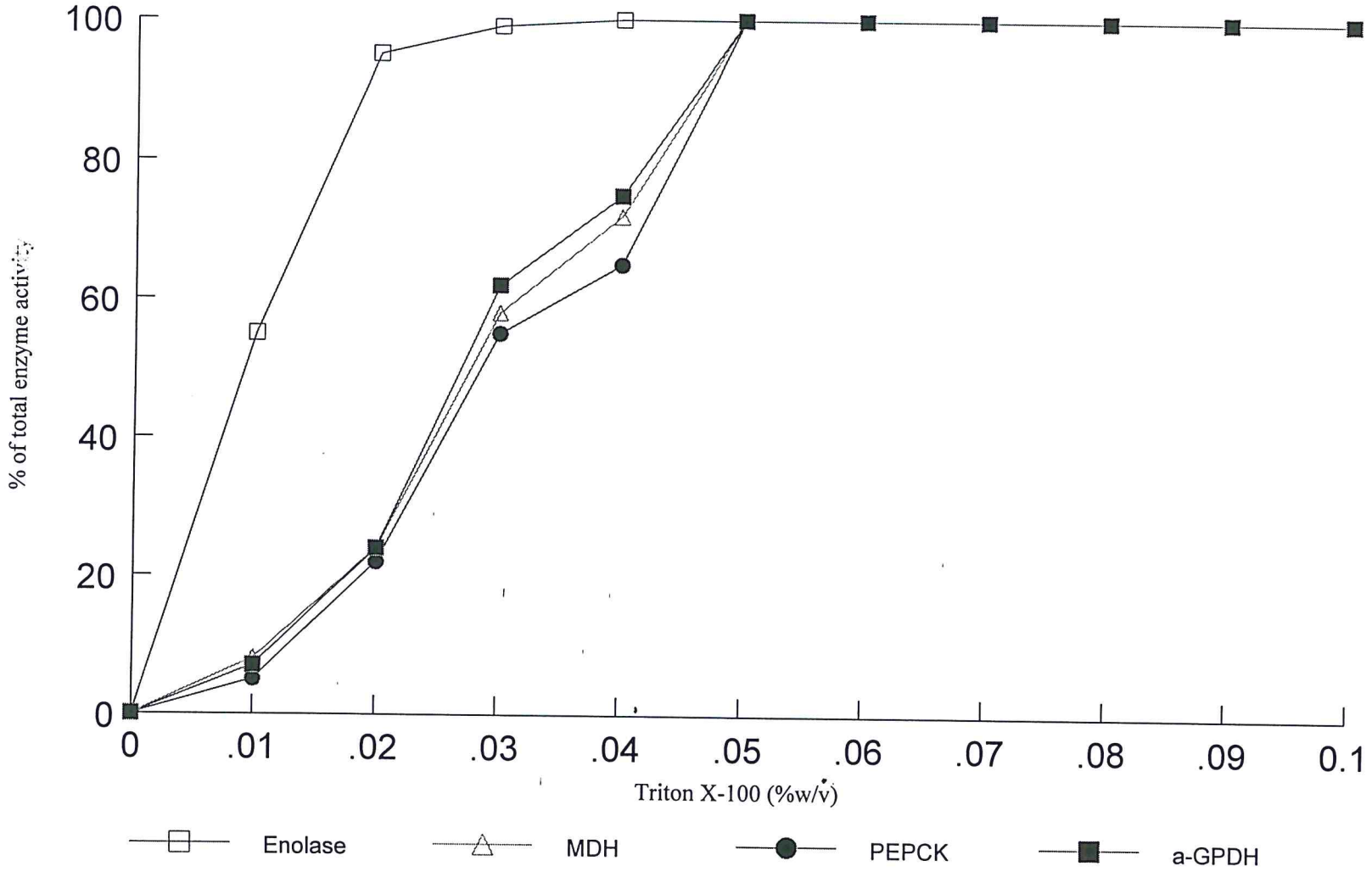
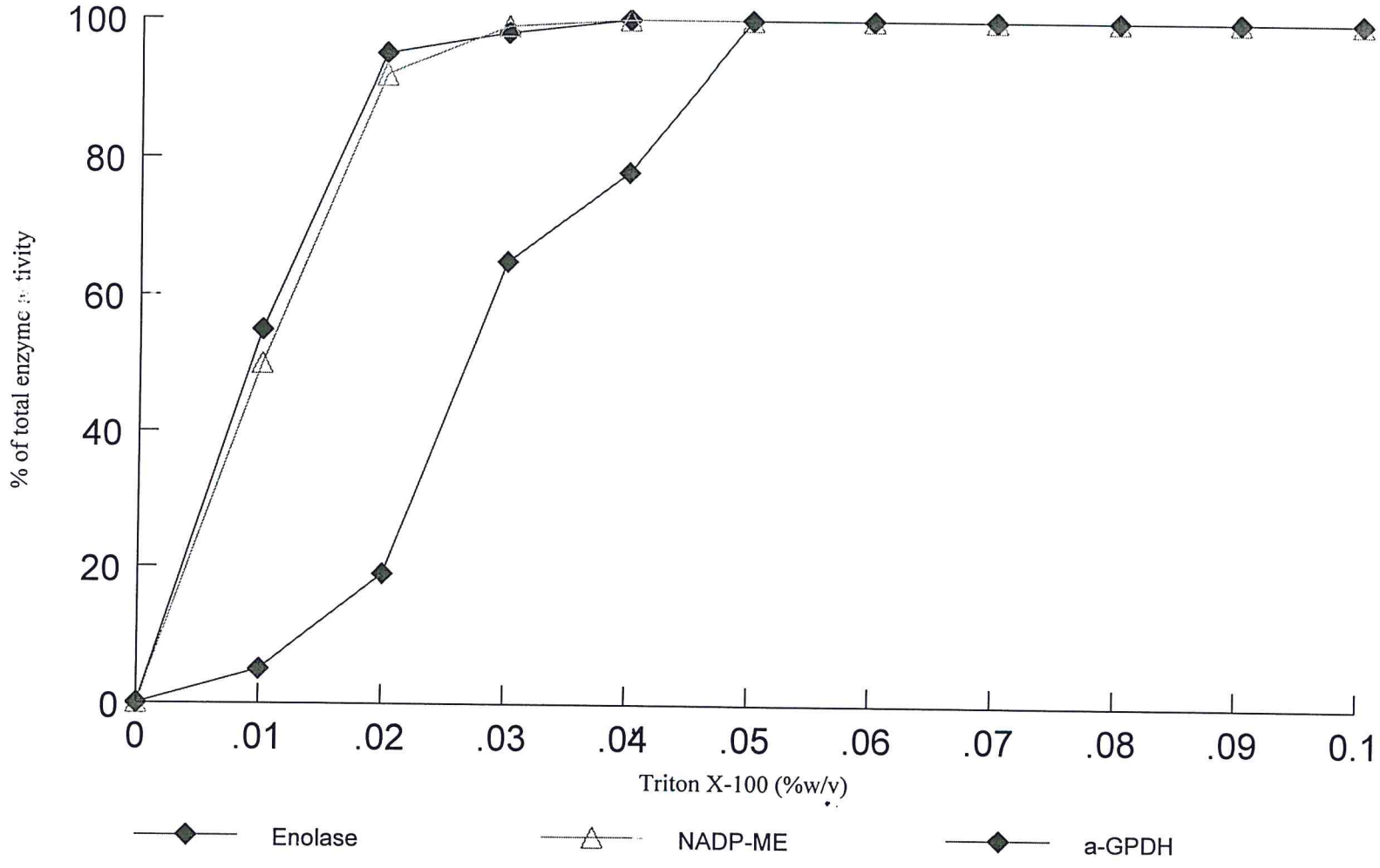

100
80
60
40
20
0
% of total enzyme activity
0
.01
.02
.03
.04
.05
.06
.07
.08
.09
0.1
Triton X-100 (%w/v)
Enolase
MDH
PEPCK
a-GPDH

Figure 3(c)


100
80
60
40
20
0
% of total enzyme activity
0
.01
.02
.03
.04
.05
.06
.07
.08
.09
0.1
Triton X-100 (%w/v)
Enolase
NADP-ME
a-GPDH

3.4.0 PROPERTIES OF PEPCK FROM BLOOD STREAM *T. CONGOLENSE*

3.4.1 The effect of Mn^{2+} and Mg^{2+} ions on the activity of PEP carboxykinase

Due to the absence of significant PK activities, it was proposed that PEPCK should play a major role in the metabolism of glucose in bloodstream *T. congolense*. The possibility of regulating PEPCK activity by manganese and magnesium as reported for procyclic *T. cruzi* lysate (Cannata *et al.*, 1982) was investigated in bloodstream *T. congolense.* Table IV shows that 48 nmoles/min/mg protein was the maximum activity of the enzyme in the assay medium containing 1 mM Mn^{2+} ion. The assay medium containing 1.3 mM Mg^{2+} ions had a specific activity of 9.0 nmol/min/mg protein, which was 5 times lower than the specific activity of the enzyme in presence of Mn^{2+} ion. Addition of both 1 mM Mn^{2+} and 1.3 mM Mg^{2+} ions in the assay medium did not increase the activity of the enzyme above the activity that was observed in the medium containing Mn^{2+} ions only. The absence of both Mn^{2+} and Mg^{2+} ions in the assay medium resulted in decreased enzyme activity of less than 0.43 nmol/min/mg protein. These results suggest that PEP carboxykinase from bloodstream *T. congolense* also requires Mn^{2+} for its full activity just as that from procyclic *T. cruzi.*

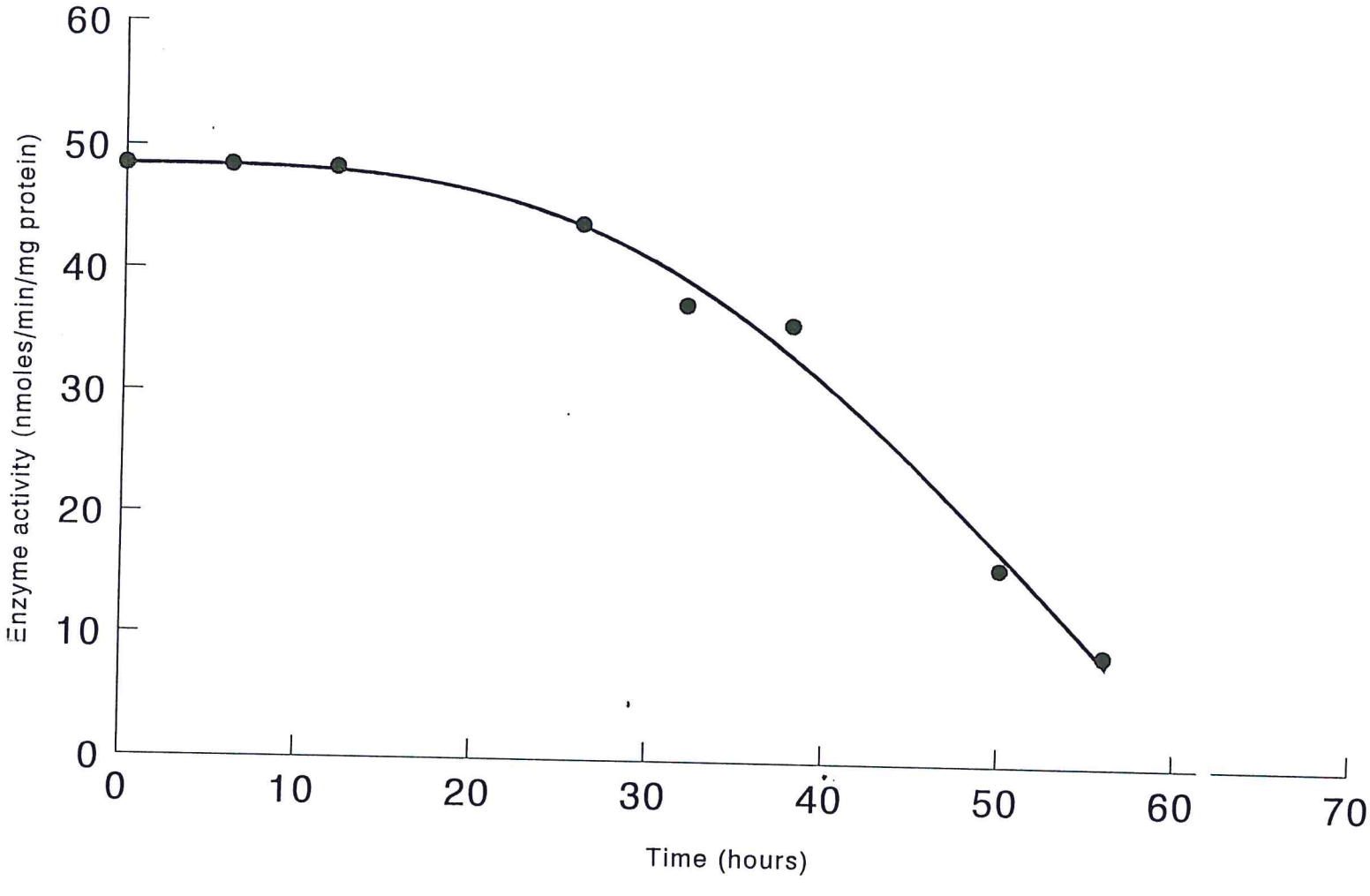
3.4.2 The stability of PEP carboxykinase at 4°C

The stability of PEP carboxykinase from bloodstream *T. congolense* was investigated at 4°C. Figure 4 shows that there was no change in activity of the enzyme within the first 18 hours. After 18 hours the activity started decreasing gradually with time. The activity decreased from 48.0 nmol/min/mg protein to 9.0 nmol/min/mg protein after 54 hrs of storage.

Table IV

Metal ion added	**Specific activity in nmoles/min/mg protein**
Mn^{2+}	48± 6 (4)
Mg^{2+}	9.0 ± 1.5 (4)
$Mn^{2+} + Mg^{2+}$	48 ± 6 (4)
None	<0.43 ± 0.05 (4)

Figure 4

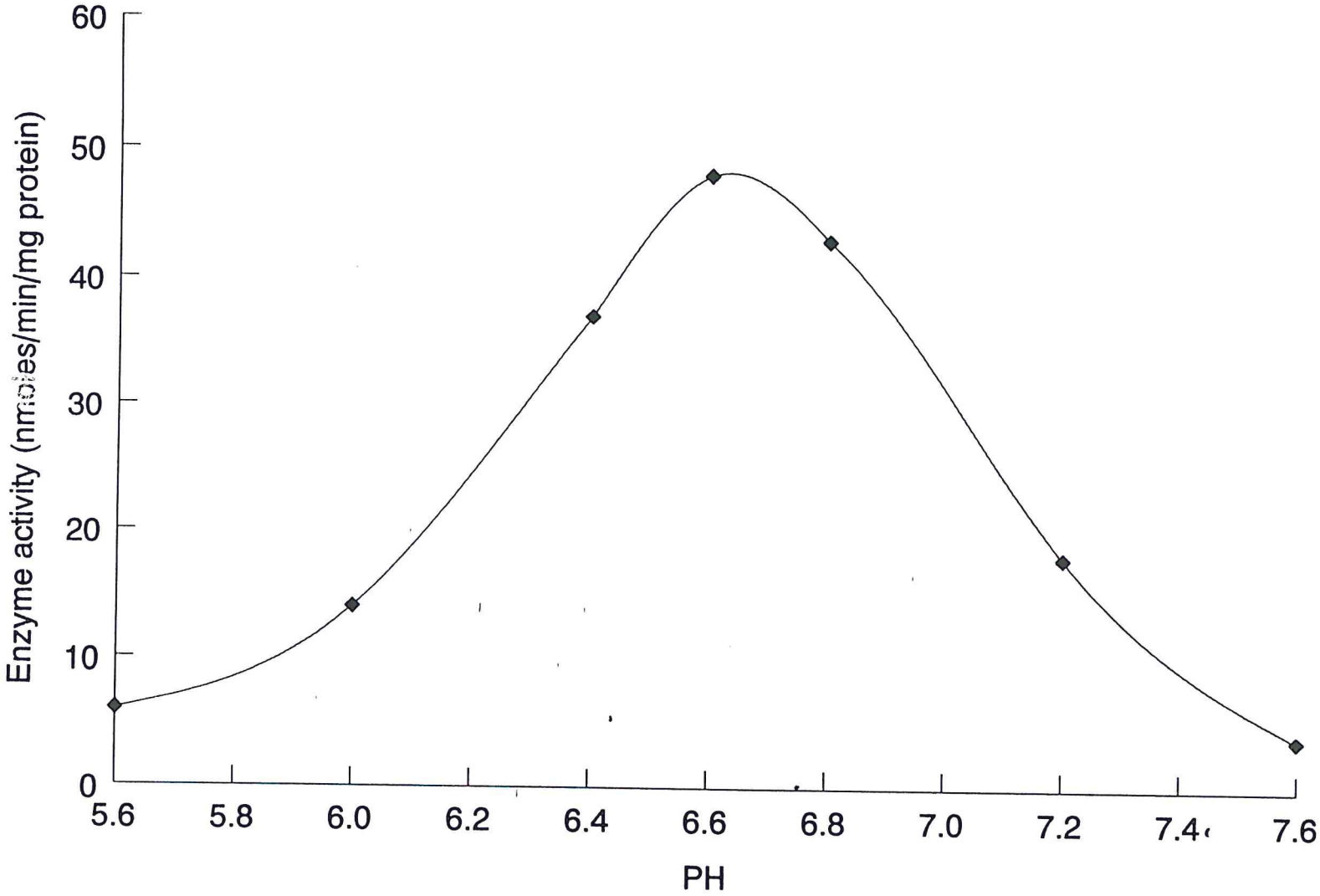

60
50
40
30
20
10
0
Enzyme activity (nmoles/min/mg protein)
0
10
20
30
40
50
60
70
Time (hours)

It was proposed that PEP-carboxykinase from bloodstream *T. congolense* lysate could be stable for a period of less than 18 hours in imidazole buffer (pH 6.6). After 18 hours the activity started reducing drastically. This could be due to the effect of proteases in the crude sample or the bacterial growth.

3.4.3 The optimum pH for PEP carboxykinase enzyme activity from bloodstream *T. congolense.*

To determine whether ionization of PEPCK affects its activity, the enzyme from solubilized trypanosomes was assayed from a pH range of 5.6 to 7.6. Figure 5 shows that there was gradual increase in activity from 6.0 nmol/min/mg protein at pH 5.6 to a maximum of 47.5 nmol/min/mg protein at pH 6.6. The activity of the enzyme then decreased gradually with increase of pH to 4.0 nmoles/min/mg protein at pH 7.6. These results suggest that PEP carboxykinase is pH sensitive when using imidazole buffer, the optimum activity of the enzyme could be realised at a narrow range of pH 6.4 to 7.0. The plots of pH versus enzyme activity was bell-shaped. This observation could suggest that the enzyme has two ionizable groups essential for its activity.

Figure 5


60
50
40
30
20
10
0
Enzyme activity (nmoles/min/mg protein)
5.6
5.8
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
PH

CHAPTER FOUR

DISCUSSION

4.1 Pathway(s) of PEP catabolism and the end products formed in bloodstream *T. congolense* respiring on glucose

The high activity of bloodstream *T. congolense* PEPCK (Table IIIa) could imply that PEP is converted to OAA in a reaction similar to that proposed for procyclic *T. brucei* by Opperdoes and Cottem (1982). This reaction occurs according to equation 1 below:-

1) $PEP + CO_2 + ADP \xrightarrow{PEPCK} OAA + ATP$

2) $OAA + NADH + H^+ \xrightarrow{MDH} Malate + NAD^+$

3) $Malate + NADP^+ \xrightarrow{NADP\text{-}ME} Pyruvate + NADPH + H^+ + CO_2$

4) $Pyruvate + CoASH + NAD^+ \xrightarrow[TPP,\ Cyst]{Pyr\ DH} Acetyl\ CoA + NADH + H^+ + CO_2$

5) $Acetyl\ CoA + Pi \xrightarrow{PTA} Acetylphosphate + CoASH$

6) $Acetylphosphate + ADP \xrightarrow{AK} Acetate + ATP$

The overall reaction is:-

$$PEP + 2\ ADP + Pi + NADP^+ \xrightarrow[Pyr\ DH,\ AK]{MDH,\ NADP\text{-}ME,\ PTA} Acetate + NADPH + H^+ + 2\ ATP + CO_2$$

For this reaction to take place pyruvate kinase activity must be very low otherwise it would compete with PEPCK for PEP. Insignificant activity of pyruvate kinase has also been reported in procyclic *T. brucei* (Opperdoes and Cottem, 1982; Van Schaftingen *et al.,* 1985; Opperdoes, 1987; Callens *et al.,* 1991) and the results in Table IIIa reveal the same range of activity in bloodstream *T. congolense*.

In this study, high activities of NADP-ME, pyruvate dehydrogenase, MDH, phosphotransacetylase and acetate kinase were also observed in bloodstream *T. congolense* (Tables IIIa, IIIb, IIIc). It is proposed that MDH and NADP-ME could catalyse the conversion of oxaloacetate to pyruvate as shown in equations 2 and 3 on page 80. This could account for the pyruvate that was assayed and shown in Table II. The production of pyruvate by bloodstream *T. congolense* was observed for the first time during this investigation. Pyruvate production comprised 20% of the total end products formed under aerobic conditions. Under anaerobic conditions simulated by addition of SHAM, it comprised 11% of the total end products. Pyruvate production has been reported in procyclic *T. congolense* (Obungu, 1992) and bloodstream *T. brucei* (Ryley, 1956; Flynn and Bowman, 1973).

Due to lack of significant LDH activity, pyruvate could be either excreted as an end product or undergo decarboxylation to form acetyl coenzyme A. The Acetyl CoA in bloodstream *T. congolense* is an intermediate in the pathway of acetate formation. Reactions 5-6 above account for this. The ratio of pyruvate to acetate produced was 1:2 (Table II). This implies that 33% of pyruvate from glucose catabolism is not converted to acetate. Perhaps this is due to compartmentation or insufficient activities of pyruvate dehydrogenase. All the enzymes necessary for acetate production from pyruvate have been shown (Table IIIb). The same enzymes have been shown in procyclic *T. congolense* (Obungu, 1992) and procyclic *T. brucei* which also excretes acetate as an end product (Klein *et al.,* 1975; Brown *et al.,* 1983; Opperdoes and

Cottem, 1982). A pathway to account for the end products has been proposed by Fairlamb and Opperdoes (1986) as shown in Scheme I (Chapter 1).

In this study it has been shown that bloodstream *T. congolense* produces glycerol as an end product under both aerobic and anaerobic conditions (Table II). Glycerol formation could result from dephosphorylation of glycerol-3-phosphate by glycerol-3-phosphate : glucose transphosphorylase (Kiaira and Njogu, 1989). Glycerol-3-phosphate results from reduction of DHAP by glycerol-3-phosphate dehydrogenase in an NADH dependant reaction. Glycerol production under aerobic conditions comprised 24% of the total end products formed. Under anaerobic conditions, it comprised 70% of the total end products. Glycerol production under aerobic and anaerobic conditions had been earlier reported in bloodstream and procyclic *T. congolense* and procyclic *T. brucei* (Agosin and von Brand, 1954; Fairlamb and Opperdoes, 1986; Obungu, 1992)

The bloodstream *T. congolense* does not form lactate under aerobic and anaerobic conditions (Table II). The activity of lactate dehydrogenase was insignificant (Table IIIa) which could explain the lack of detectable lactate. The formation of lactate as an end product or the presence of lactate dehydrogenase has not been reported in the African trypanosomes (Agosin and von Brand, 1954; Ryley, 1956; Evans and Brown, 1972b; Cross *et al.,* 1978; von Brand and Tobie, 1959; Opperdoes *et al.*, 1976).

Succinate production under aerobic conditions by bloodstream *T. congolense* was very low (Table II). During anaerobic conditions, there was increased succinate production probably formed from fumarate by the reductive activity of fumarate reductase as proposed in Scheme I. This reaction could result in the oxidation of some NADH from pyruvate dehydrogenase activity while the remaining could be oxidised via the TAO. Fumarate could be formed from malate in a reaction catalysed by fumarase. The formation of succinate as

an end product has been reported by Agosin and von Brand (1954) and Ryley (1956).

From these results, the end products of bloodstream *T. congolense* appears similar to those of procyclic *T. congolense* and procyclic *T. brucei* (Obungu, 1992; Fairlamb and Opperdoes, 1986). It however appears that the mitochondrial pathways are less developed in bloodstream *T. congolense* than in the procyclics.

The overall equation shown on page 80 indicates net formation of CO_2 from the oxidation of PEP to acetate. Carbon dioxide production by procyclic *T. congolense* and procyclic *T. brucei* as an end-product has also been reported (Obungu, 1992; Ryley, 1956) but was not assayed in this investigation.

The production of extra 2 moles of ATP above the classical 2 moles via glycolytic pathway from 1 mole of glucose in bloodstream *T. congolense* indicates that the PEPCK, MDH, NADP- ME, Pyr DH, PTA and AK play a very important role in the provision of metabolic energy.

Bloodstream *T. congolense* did not have significant activities of the TCA cycle enzymes such as citrate synthase, aconitase, α-ketoglutarate dehydrogenase, NAD(P)-isocitrate dehydrogenase and fumarase (Table IIIc) indicating that the first reaction of TCA cycle which results in formation of citrate from acetyl CoA and oxaloacetate does not occur. Since all the TCA cycle enzyme activities have not been shown, it was proposed that the parasite does not rely on TCA cycle for generation of ATP.

The overall equation on page 80 also shows net production of NADPH. Some of the NADPH could be utilised in various biosynthetic pathways while some could undergo trans-hydrogenation reaction to form NADH. The NADH formed could be used as a reducing equivalent by the trypanosome.

It is proposed that glucose is catabolised by bloodstream *T. congolense* in a pathway detailed in Scheme V. The pathway leading to the production of pyruvate in bloodstream *T. congolense* appears similar to that proposed for

procyclic *T. congolense* by Obungu (1992) (refer to Scheme III). It differs from the pathway proposed for bloodstream *T. brucei* because of the high activity of pyruvate kinase. The high activity of PK in the bloodstream *T. brucei* converts PEP to pyruvate. This is unlike in procyclic *T. congolense* which has insignificant amounts of pyruvate kinase to convert PEP to pyruvate.

4.2 Subcellular localization of some enzymes involved in PEP catabolism and the properties of PEPCK from bloodstream *T. congolense*

It is proposed from the results shown in Figures 3a and 3b that PEPCK and MDH are localized in the glycosome of the bloodstream *T. congolense*. The localization of PEPCK and MDH in the glycosome has also been reported for other parasites such as *T. cruzi* epimastigotes and *Crithidia fasciculata* (Cannata *et al.*, 1982). NADP-ME appears to be localized in the cytosol (Figure 3a and c). The localization of these enzymes in one compartment has the advantage of increasing the catalytic efficiency of energy metabolism. It also helps the parasite by ensuring that the nicotinamide nucleotides required for the reduction of OAA to malate and the oxidation of glyceraldehyde 3 phosphate to glycerate 1,3 diphosphate reactions (Scheme V) are available for glycolysis. The glycosomal membrane would be expected to be impermeable to nicotinamide nucleotides besides other adenine nucleotides which is in agreement with Opperdoes and Borst (1977) proposal.

Scheme V shows the proposed localization of the enzymes catalysing glucose metabolism. It is consistent with the results shown in Figures 3a, b and c. The NADP-ME which catalyses the formation of pyruvate from malate generates NADPH. This enzyme appears to be found in the cytosol of bloodstream *T. congolense*.

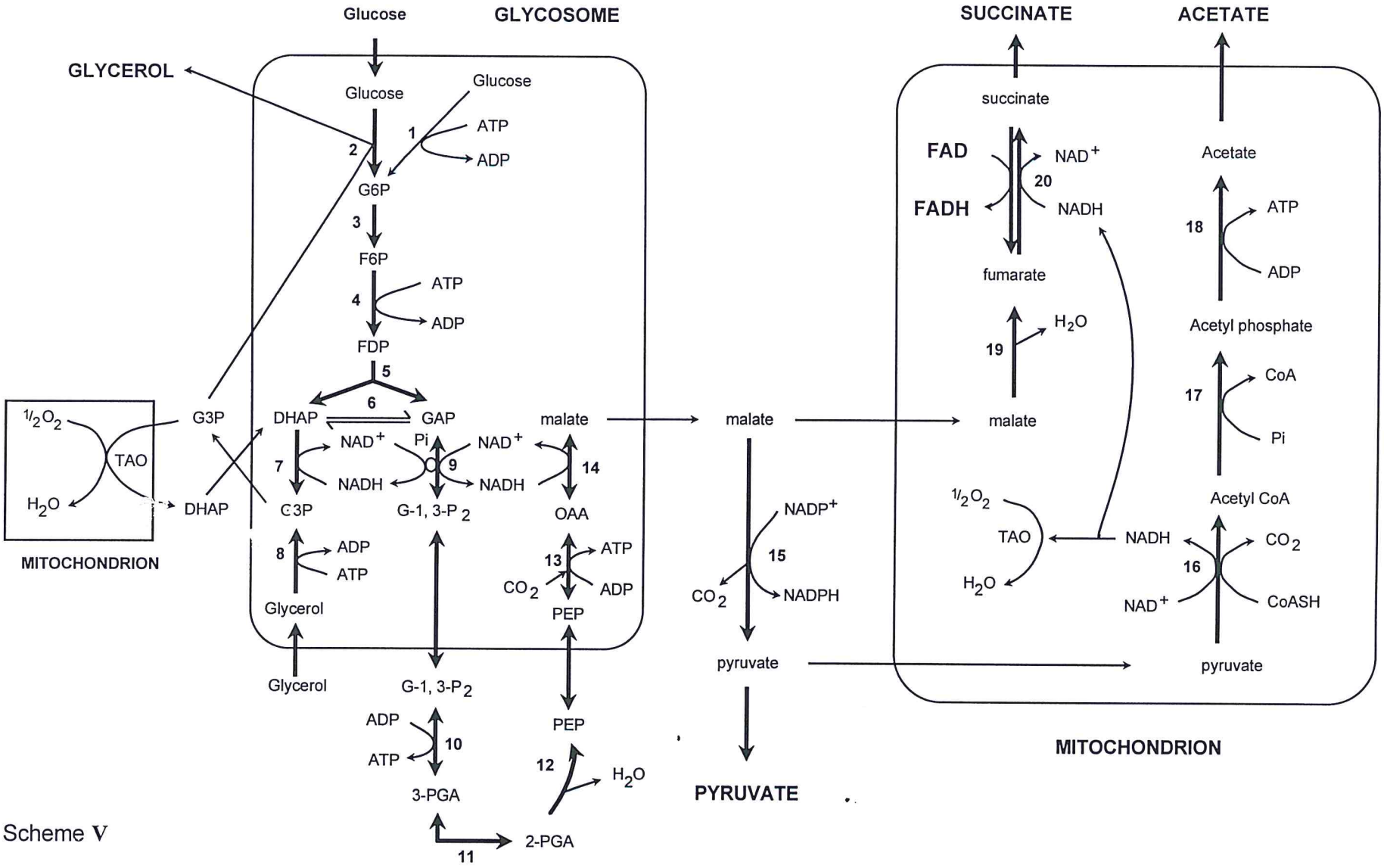
From the results shown on Figure 5 and Table IV about PEPCK of bloodstream *T. congolense* it was proposed that the enzyme has two ionizable dibasic groups essential for the activity. It was further proposed that PEPCK requires Mn^{++} ions which are essential for full activity. The bloodstream *T. congolense* PEPCK appears to be different from that of mammalian cells which requires Mg^{++} ions for full activity (Lowenstain, 1978).

4.3 Terminal Oxidases in Bloodstream *T. congolense*

From the results shown in Figure 4 it is proposed that bloodstream *T. congolense* lacks cyt. aa_3 which is inhibited by cyanide (Grant and Sargent, 1960). This has also been reported for bloodstream *T. brucei* (Fulton and Spooner, 1959). Since the respiration of bloodstream *T. congolense* was totally inhibited by SHAM just as in bloodstream *T. brucei* (Grant and Sargent, 1960), it is proposed that molecular oxygen is the final electron acceptor. SHAM inhibits the transfer of electrons from glycerol-3-phosphate dehydrogenase to glycerol-3-phosphate oxidase of the TAO (Clarkson *et al.*, 1989). From these observations it is proposed that bloodstream *T. congolense* also reoxidises NADH generated in glycolysis via the TAO. This has also been shown in bloodstream *T. brucei* to occur in the mitochondria (Clarkson *et al.*, 1989).

The production of pyruvate was not affected by cyanide (Figure 2 and Table I) confirming that cyanide has no effect on reoxidation of NADH produced during

glucose catabolism. SHAM which inhibits the TAO totally inhibited pyruvate production within 30 minutes as shown in Table I and Figure 1. This is probably because the reoxidation of NADH could not occur as NAD^+ required for the oxidation of GAP to G-1,3-P_2 was not being regenerated. This could account for the death of the trypanosome observed under a microscope within a period of 30 minutes.


Glucose
GLYCOSOME
GLYCEROL
Glucose
Glucose
ATP
ADP
1
2
G6P
3
F6P
4
ATP
ADP
FDP
5
6
DHAP
GAP
G3P
$^1/_2O_2$
TAO
H_2O
DHAP
MITOCHONDRION
NAD+
Pi
NAD+
7
9
NADH
NADH
G3P
G-1, 3-P 2
8
ADP
ATP
Glycerol
Glycerol
G-1, 3-P2
ADP
ATP
10
3-PGA
11
2-PGA
malate
14
OAA
13
ATP
CO_2
ADP
PEP
PEP
12
H_2O
malate
NADP+
15
CO_2
NADPH
pyruvate
PYRUVATE
SUCCINATE
ACETATE
succinate
FAD
FADH
NAD+
20
NADH
fumarate
19
H_2O
malate
Acetate
18
ATP
ADP
Acetyl phosphate
17
CoA
Pi
Acetyl CoA
$^1/_2O_2$
TAO
H_2O
NADH
16
CO_2
NAD+
CoASH
pyruvate
MITOCHONDRION

Scheme V

4.4 CONCLUSIONS

From this study it may be concluded that:-

1. In addition to acetate, succinate and glycerol the bloodstream *T. congolense* excretes pyruvate as end product during glucose catabolism.

2. Bloodstream *T. congolense* do not have a functional TCA cycle, the cytochrome system or the electron transport chain. Instead they have the trypanosome alternate oxidase (TAO) as a terminal oxidase which is insensitive to cyanide but sensitive to SHAM.

3. Bloodstream *T. congolense* has high activity of the PEPCK and very low activity of pyruvate kinase.

4. The PEPCK and MDH in bloodstream *T. congolense* could be localized in the glycosomes to allow efficient in reduction of OAA to form malate by NADH generated from the oxidation of GAP to G-1,3-P_2.

5. The NADP-linked malic enzyme is probably localized in the cytosol.

6. The activity of PEPCK bloodstream *T. congolense* is dependent on manganese ions and its optimum pH is 6.6.

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