"GLUCOSE AND PROLINE METABOLISM IN THE *IN VITRO* PROPAGATED PROCYCLIC *TRYPANOSOMA CONGOLENSE*"

BY

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DECLARATION

I, Hosea Victor Odwuor Obungu, 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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DEDICATION

To my parents,

Washington and Clementina Obungu

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ABBREVIATIONS

ADH	Alanine dehydrogenase
ADP	Adenosine 5′ diphosphate
AK	Acetate kinase
Ant. A	Antimycin A
ATP	Adenosine 5′ triphosphate
BSA	Bovine serum albumin
CoASH	Coenzyme A
CoQ	Coenzyme Q (ubiquinone)
CN-	Cyanide
CS	Citrate synthase
Cyst	Cystein
Cyt	Cytochrome
DHAP	Dihydroxyacetone phosphate
DPG	Diphosphoglycerate
EC	Enzyme commission
EDTA	Ethylene diaminetetra acetic acid
FAD	Flavin adenine dinucleotide
FADH	Reduced flavin adenine dinucleotide
FDP	Fructose 1,6-diphosphate
F6P	Fructose-6-phosphate
FR	Fumarate reductase
GAP	Glyceraldehyde phosphate
GDH	Glutamate dehydrogenase
xg	gravitational force
GK	Glycerokinase
GOT	Glutamate oxaloacetate transaminase
GPDH	Glycerophosphate dehydrogenase

GPO	Glycerophosphate oxidase
G6P	Glucose-6-phosphate
GPT	Glutamate pyruvate transaminase
G-3-P	Glycerol-3-phosphate
G 1 3 P_2	Glycerol 1,3 diphosphate
HEPES	(N[2-Hydroxyethyl]piperazine-N´-[2-ethanesulphonic acid])
INT	P-iodonitrotetrazolium violet (2-[4-iodophenyl)-3-(4-nitrophenyl) tetrazolium chloride)
αKG	α-ketoglutarate
αKGDH	α-ketoglutarate dehydrogenase
LDH	Lactate dehydrogenase
M.E.	Malic enzyme
MEM	Minimum essential medium
MOPS	3(N-Morpholino) propane sulphonic acid
M 199	Medium 199
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced Nicotinamide adenine dinucleotide
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetate
Oxid.	Oxidized
Pi	Inorganic phosphate
PCA	Perchloric acid
PDH	Pyruvate dehydrogenase
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyryvate carboxykinase

PGA	Phosphogylcerate
PMS	Phenazine methosulphate
PS	Phosphate buffered saline
PsProline	Phosphate buffered saline proline
PSG	Phosphate buffered saline glucose
PGA	Phosphogycerate
PTA	Phosphotransacetylase
red	Reduced
RPMI 1640	Rosewell Park Memorial Institute 1640
SDM 77	Semi-defined medium 77
SDM 79	Semi-defined medium 79
SHAM	Salicylhydroxamic acid
TCA	Trichloroacetic acid
TPP	Thiamine pyrophosphate
Tris	Tris(hydroxymethyl)aminoethane

UNIT ABBREVIATION

°C	degree centigrade
cm	centimetre
°F	degree Fahrenheit
g	gramme
I.U.	International Units
l	litre
M	Molar
m	metre
mol	moles
mg	milligramme
min	minute
mls	millilitres
mM	millimolar
mm	millimetres
N	Normal
nM	Nanomolar
nanomol	nanomoles
µ	micro
µg	microgram
µl	microlitre
µM	µmolar
µmol	µmoles
V	Volts
v/v	volume per volume
w/v	weight per volume

SUMMARY

The main purpose of this study was to determine the pathways involved in the catabolism of glucose and proline by *in vitro* propagated *Trypanosoma congolense*.

The procyclic *Trypanosoma congolense* were cultured *in vitro* as described by Brun and Schonenberger (1979). Respiration on glucose and proline were investigated. The end products of glucose and proline catabolism were also investigated and the activities of the enzymes, likely to be involved in the catabolism of the same substrates were assayed. The procyclic *T. congolense* respired on both proline and glucose.

When the trypanosomes were incubated with glucose as substrate, it was observed that the rate of oxygen consumption was sensitive to various metabolic inhibitors. Addition of salicylhydroxamic acid (SHAM) alone inhibited the rate of respiration by 36.2 ± 4.2% whereas addition of cyanide alone inhibited the rate of respiration (which was initially 10.3 ± 1.0) by 54.1 ± 5.5%. A combination of SHAM and cyanide inhibited the initial rate of respiration by 85.3 ± 1.6%. It was proposed that SHAM and cyanide inhibit different oxidases. When the parasites which were respiring on glucose were incubated with malonate alone, the rate of respiration was inhibited by 32.3 ± 1.9%. A combination of malonate and SHAM inhibited the rate of initial respiration by 67.1 ± 0.9%. From these observations it was

suggested that some reducing equivalents derived from the oxidation of glucose are channelled to molecular oxygen via succinate dehydrogenase. Incubations in which antimycin A alone was added showed a 39.4 ± 4.2% inhibition of the rate of the respiration. A combination of antimycin A and SHAM resulted in 86.3 ± 4.2% inhibition of the initial rate of respiration. It was suggested that antimycin A inhibits the flow of electrons between cytochrome b and cytochrome aa_3 oxidase. It was further suggested that the rate of respiration insensitive to a combination of antimycin A and SHAM could be due to an alternative oxidase branching prior to cytochrome b.

The activities of the enzymes likely to be involved in the catabolism of phosphoenolpyruvate derived from glucose oxidation were then assayed. The enzymes which had specific activities greater than 60 nmoles/min/mg protein were phosphoenolpyruvate carboxykinase (PEPCK), pyruvate dehydrogenase, succinate dehydrogenase, NADP-malic enzyme, fumarase, fumarate reductase and acetate kinase. Those enzymes which had activities less than 6 nmoles/min/mg protein were pyruvate kinase, lactate dehydrogenase and NAD-linked malic enzymes. From these observations it was suggested that procyclic *T. congolense* catabolise glucose to pyruvate via another pathway not involving pyruvate kinase. It was also concluded that it is unlikely that pyruvate could be converted to lactate.

The end products of glucose catabolism were then determined under both aerobic and anaerobic conditions. Under aerobic conditions the main end products were pyruvate and acetate. Small amounts of succinate could also be detected. Under anaerobic conditions simulated by the addition of SHAM, the major end products were acetate, pyruvate and succinate. Glycerol was also produced in appreciable quantities during anaerobic catabolism of glucose.

When the trypanosomes were allowed to respire on proline as substrate, it was observed that the rate of respiration was also sensitive to various metabolic inhibitors except SHAM. Addition of cyanide alone inhibited the respiration whose initial rate was 12.8 $\pm$ 0.6 by 81.0 $\pm$ 6.7% whereas the addition of malonate alone caused an inhibition of 51.6 $\pm$ 1.6% of the rate respiration. A combination of cyanide and malonate caused an inhibition of 84.9 $\pm$ 6.7% of the initial rate of respiration. It was proposed that some reducing equivalents derived from the catabolism of proline are channelled to the cyanide sensitive cytochrome aa_3 terminal oxidase via the malonate sensitive succinate dehydrogenase. When the parasites were incubated with antimycin A alone the rate of respiration was inhibited by 73.1 $\pm$ 5.9%. A combination of antimycin A and malonate inhibited the respiration by 81.6 $\pm$ 7.6%. From these observations it was suggested that some of the reducing equivalents derived from proline catabolism are oxidized via the malonate sensitive succinate dehydrogenase and cytochromes b and c_1 before

they are eventually reduced by molecular oxygen via the cyanide sensitive cytochrome aa_3 oxidase.

The activities of the enzymes likely to be involved in the catabolism of proline were also assayed. The enzymes that were observed to have specific activities greater than 50 nmoles/min/mg protein were proline dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase, $NADP^+$-linked malic enzyme and glutamate pyruvate transaminase. Glutamate oxaloacetate transaminase and glutamate dehydrogenase were found to have specific activities less than 35 nmoles/min/mg protein. From these results it was suggested that proline could be oxidized to glutamate which is subsequently transaminated with pyruvate to form alanine and α-ketoglutarate. Alanine could be excreted as an end product whereas α-ketoglutarate is channelled to the supposedly partially functional Tricarboxyllic acid (TCA) cycle.

The end products of proline catabolism were then determined under aerobic conditions. The end products detected were alanine and glutamate. Pyruvate and aspartate could not be detected as end products. It was suggested that the pyruvate which could be produced is transaminated immediately with glutamate to form alanine and α-ketoglutarate. Alanine is released whereas a-ketoglutarate is channelled to the partially functional TCA cycle. It is further suggested that the enzyme glutamate pyruvate transaminase could be the enzyme involved in the transamination of pyruvate.

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Trypanosoma congolense is a small salivarian trypanosome with overall lengths ranging from 8 μm to 24 μm with an average of 12.2 - 17.6 μm (Hoare, 1959) or 12.5 - 15.6 μm (Fairbairn, 1962). It has a rod-like shape and possesses a medium sized kinetoplast measuring between 0.7 to 0.8 μm (Hoare, 1972). These parasites have a tendency to adhere to one another, a feature which has been observed in fresh and stained preparations of blood.

T. congolense causes animal trypanosomiasis (nagana) and its infection has become the most predominant in Africa especially in the last twenty years (Hoare, 1972).

1.1.1 Life cycle of *T. congolense*

The life cycle of *T. congolense* involves two hosts : the vertebrate host and the invertebrate host. The invertebrate hosts are the tsetse flies of the genus *Glossina*. Some of the species of *Glossina* capable of transmitting the infection are : *G. morsitans*, *G. tachnoides*, *G. palpalis* and *G. brevipalpis* (Buxton, 1955; Chardome and Peel, 1967).

When an uninfected tsetse feeds on an animal infected by *T. congolense*, it ingests blood containing the short stumpy, long

slender and intermediate forms of the trypanosome. The short stumpy forms transform in the mid-gut of the fly into the mid-gut forms which are identical to the cultured procyclic forms. After multiplication the mid-gut forms migrate into the salivary glands where they undergo transformation into epimastigotes which transform further into metacyclic forms. The metacyclics are infective to susceptible animals (Hoare, 1972; Fairlamb and Opperdoes, 1986).

When the infected tsetse takes a blood meal from an appropriate vertebrate host it inoculates the infective metacyclics. The inoculated parasites migrate via the lymphatics to the lymph nodes and then into the blood stream where they multiply in large numbers and thereafter invade the intercellular spaces of other tissues (Hoare, 1972).

1.1.2 Culturing of procyclic *T. congolense in vitro*

Over the years the procyclic forms of *T. congolense* have been grown in several media. These include Razgha's medium (Reichenew, 1934, 1937) and Brutsaert and Henrard's medium (Henrard and Peel, 1950). Tobie *et al* (1950) also cultured these parasites in a blood-agar medium. Trager (1959) later grew procyclic *T. congolense* in a culture medium containing tsetse fly tissue. The parasite numbers resulting therefrom were not sufficient for metabolic studies.

T. congolense procyclics have also been grown in a semi-defined medium called SDM-77. SDM-77 contains Minimum

Essential Medium (MEM) and Medium 199 (M199) supplemented with 10% foetal calf serum (Brun and Jenni, 1977). When this medium was used to culture *T. congolense* procyclics it was found that in as much as the parasites could easily be adapted into the culture medium, it was not possible to sub-culture them continuously for long. The bloodstream forms (used to initiate the culture) transformed and started to multiply but after 2 to 3 subcultures they became swollen and contained large vacuoles. Eventually they stopped dividing and finally died off (Brun and Jenni, 1977).

A modification of SDM-77 has recently been developed and has proved more suitable than SDM 77 in culturing procyclic *T. brucei* (Brun and Schonenberger, 1979). The main alterations in the new SDM-79 is the use of lower concentrations of glucose and higher concentrations of pyruvate, bicarbonate, adenosine and guanosine; addition of folic acid and a group of amino acids known to be present in the tsetse haemolymph (Cunningham and Slater, 1974).

It is possible that *T. congolense* procyclics may also be able to grow better in this modified medium (SDM-79) than they did in SDM-77.

1.2 ENERGY METABOLISM IN TRYPANOSOMES

Both bloodstream and the procyclic forms of trypanosomes can derive their energy from the catabolism of glucose, mannose, fructose and glycerol (Ryley, 1962; Fairlamb and

Opperdoes, 1986). In addition to glucose the procyclics derive energy from proline catabolism (Srivastava and Bowman, 1971, 1972; Evans and Brown, 1972a).

1.2.1 Carbohydrate metabolism

1.2.1.1 Carbohydrate metabolism in bloodstream forms

All bloodstream forms of trypanosomes catabolize various carbohydrate sugars as a source of energy. The end products of glucose catabolism depend on the species of the trypanosome.

1.2.1.1.1 Carbohydrate metabolism in bloodstream forms of *T. congolense*

Bloodstream forms of *T. congolense* have a fairly high rate of oxygen and sugar consumption. Their respiration is not blocked by cyanide (Von Brand and Johnson, 1947; Von Brand *et al*, 1950). These parasites also have a high respiratory quotient and the main metabolic end products accounting for nearly all the degraded glucose include acetate, pyruvate and CO_2. Succinate is also produced in trace amounts (Agosin and Von Brand, 1954).

Ryley (1956) has also reported that the motility of trypanosomes depends on the supply of extracellular glucose and other utilizable carbohydrates. They degrade glucose to pyruvate CO_2, succinate, and acetate. They also produce negligible quantities of glycerol, ethanol and formate. However, there has been no

evidence of excretion of lactate, citrate, malate, propionate or other volatile acids by bloodstream forms of *T. congolense* (Agosin and Von Brand, 1954; Ryley, 1956). These products have not been confirmed by others.

1.2.1.1.2 Carbohydrate metabolism in bloodstream forms of *T. brucei*

Trypanosoma brucei respire on glucose, fructose, mannose and glycerol (Ryley, 1956; 1962). They cannot respire on amino acids or fatty acids (Bowman and Flynn, 1976).

These parasites have been found to have microbody-like organelles called glycosomes (Opperdoes and Borst, 1977). Glycosomes in bloodstream *T. brucei* contain the first seven enzymes of the glycolytic sequences (Fairlamb and Opperdoes, 1986). These are shown in Scheme I.

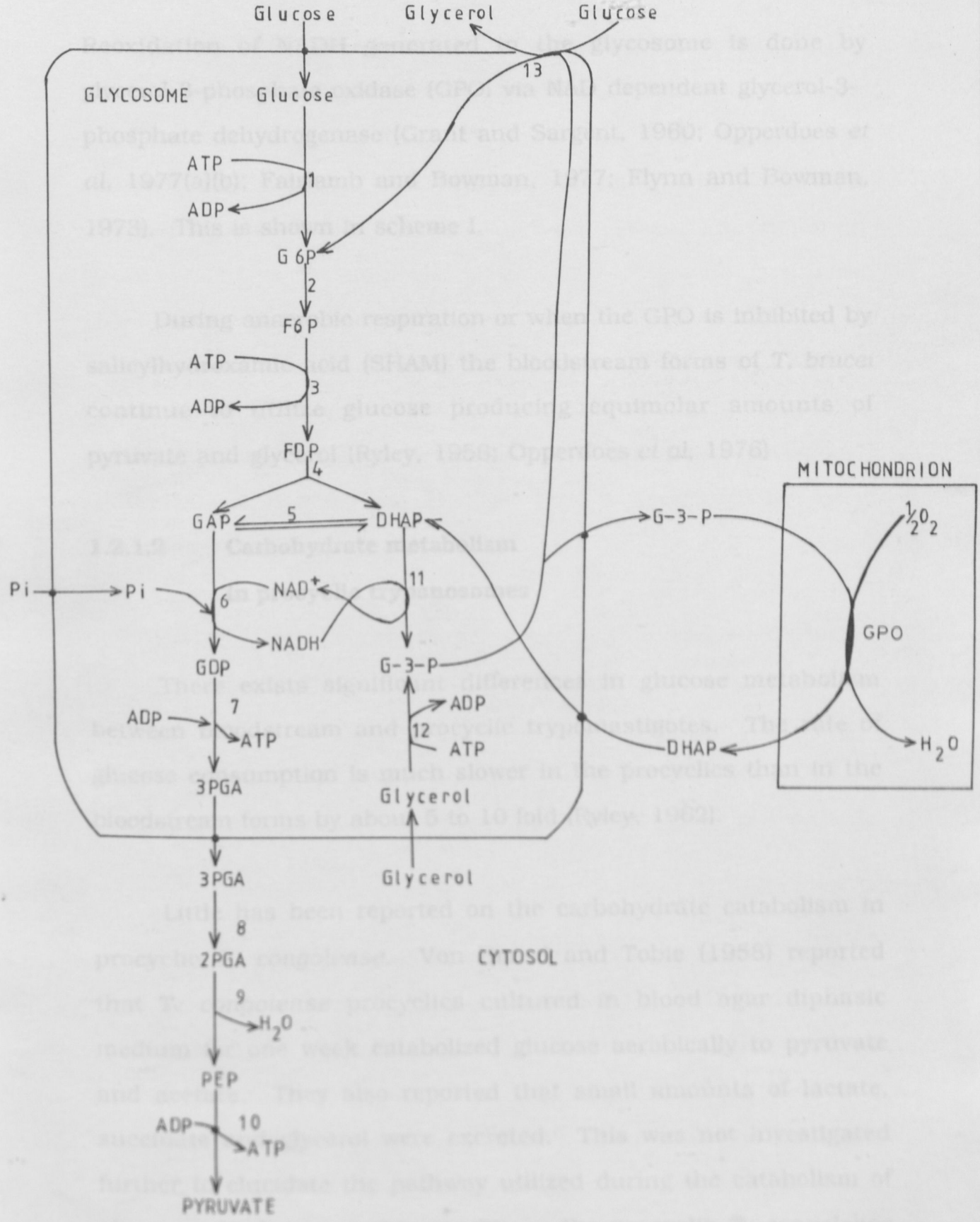
The bloodstream forms of *T. brucei* have a very high rate of respiration on glucose. Glucose is metabolized almost exclusively to pyruvate. All the enzymes of the glycolytic pathway have been identified and most of them isolated in pure form. These are hexokinase, glucosephosphate isomerase, 6 phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase, phosphoglycerol kinase, pyruvate kinase and enolase (Opperdoes and Borst, 1977; Oduro *et al*, 1980 (a) (b); Hart and Opperdoes, 1984). Pyruvate cannot be metabolized to lactate because these parasites lack lactate dehydrogenase (Dixon, 1966).

Scheme I

Pathways of glucose catabolism by bloodstream *Trypanosoma brucei.* (Source: Kiaira & Njogu, 1989)

The enzymes shown are:-

1. hexokinase
2. phosphoglucose isomerase
3. phosphofructokinase
4. aldolase
5. triose phosphate isomerase
6. glyceraldehyde-3-phosphate dehydrogenase
7. phosphoglycerate kinase
8. phosphoglycerate mutase
9. enolase
10. pyruvate kinase
11. glycerol-3-phosphate dehydrogenase
12. glycerol kinase
13. glycerol-3-phosphate : glucose transphosphorylase


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

Scheme I

Reoxidation of NADH generated in the glycosome is done by glycerol-3-phosphate oxidase (GPO) via NAD dependent glycerol-3-phosphate dehydrogenase (Grant and Sargent, 1960; Opperdoes *et al*, 1977(a)(b); Fairlamb and Bowman, 1977; Flynn and Bowman, 1973). This is shown in scheme I.

During anaerobic respiration or when the GPO is inhibited by salicylhydroxamic acid (SHAM) the bloodstream forms of *T. brucei* continue to utilize glucose producing equimolar amounts of pyruvate and glycerol (Ryley, 1956; Opperdoes *et al*, 1976)

1.2.1.2 Carbohydrate metabolism in procyclic trypanosomes

There exists significant differences in glucose metabolism between bloodstream and procyclic trypomastigotes. The rate of glucose consumption is much slower in the procyclics than in the bloodstream forms by about 5 to 10 fold (Ryley, 1962).

Little has been reported on the carbohydrate catabolism in procyclic *T. congolense*. Von Brand and Tobie (1958) reported that *T. congolense* procyclics cultured in blood agar diphasic medium for one week catabolized glucose aerobically to pyruvate and acetate. They also reported that small amounts of lactate, succinate and glycerol were excreted. This was not investigated further to elucidate the pathway utilized during the catabolism of glucose. Under anaerobic conditions the procyclic *T. congolense* catabolized glucose mainly to pyruvate, acetate and succinate. Small amounts of glycerol were also reportedly excreted (Von Brand and

Tobie, 1958). Further investigations were not made regarding the stoichiometry of these products.

On the other hand a lot more has been reported on carbohydrate metabolism in procyclic *Trypanosoma brucei* and procyclic *Trypanosoma rhodesiense*. Both of these can respire on glucose, mannose, fructose and glycerol (Fairlamb and Opperdoes, 1986). The end products of these carbohydrates metabolism in *T. brucei* and *T. rhodesiense* procyclics are CO_2, succinate and acetate (Ryley, 1962). Cross *et al* (1975) reported that 17 and 8% of the glucose carbon used by procyclic *T. brucei* could be recovered as succinate and alanine, respectively. This, however, has not been investigated in detail.

Under anaerobic conditions procyclic *T. rhodesiense* utilized both glucose and glycerol only in the presence of CO_2 (Ryley, 1962). During such anaerobic conditions most of the glucose carbon utilized was recovered mostly as succinate and to a lesser extent as acetate. Lactate has not been detected as an end product (Fairlamb and Opperdoes, 1986).

Several glycolytic enzyme activities are lower in the procyclic trypomastigotes than in the bloodstream forms. This difference could also reflect changes in the mitochondrial and glycolytic enzyme activities (Fairlamb and Opperdoes, 1986). Hart and Opperdoes (1984) showed the activities of most of the glycolytic enzymes in *T.b. brucei* procyclics except for pyruvate kinase, lactate dehydrogenase and pyruvate dehydrogenase.

Very low activities of NAD-linked isocitrate dehydrogenase and citrate synthase have been observed (Evans and Brown, 1972(b). Enzyme activities of the glyoxylate cycle; isocitrate lyase, malate synthase, alanine glyoxylate aminotransferase and for anaplerotic reactions; NAD-linked malic enzyme and pyruvate carboxylase are also insignificant (Opperdoes and Cottem, 1982). TCA cycle enzyme activities greater than 2 nanomol/min/mg protein include malate dehydrogenase and fumarate reductase (Opperdoes and Cottem, 1982). Enzyme activities that appear fairly high are of NADP-linked malic enzyme, glutamate pyruvate transaminase (GPT) glutamate oxaloacetate transaminase (GOT) (Evans and Brown, 1972(a); Opperdoes and Cottem 1982), phosphoenolpyruvate carboxykinase, adenylate kinase and glycerol-3-phosphate dehydrogenase. (Visser *et al.* 1981). No similar report has been provided on procyclic *T. congolense*.

The changes in metabolic end-products during glucose metabolism in the glycolytic reactions from pyruvate to the formation of CO_2, succinate and acetate can be correlated with changes in other enzyme activities (Fairlamb and Opperdoes, 1986). In particular, very low activities of pyruvate kinase can be detected in procyclic *T brucei* ($\leq$ 2 nanomol/min/mg protein; Opperdoes and Cottem, 1982). This contrasts sharply with the high specific activity reported in bloodstream forms of 1200-900 nanomol/min/mg protein (Oduro *et al*, 1980a; Flynn and Bowman, 1980). In addition, there is a large increase in the specific activity of glycosomal phosphoenolpyruvate carboxykinase suggesting that the glycosome plays an important role in CO_2 fixation in procyclic trypomastigotes (Fairlamb and Opperdoes, 1986).

Ryley (1956, 1962) has inferred the presence of a functional TCA cycle in procyclic trypomastigotes. However, this situation still remains unclear because while aconitase, α-oxoglutarate dehydrogenase, succinate dehydrogenase, fumarate hydratase and malate dehydrogenase activities have been shown (Ryley, 1962), citrate synthetase and NAD-dependent isocitrate dehydrogenase activities have not been shown.

Conversion of PEP derived from glucose metabolism to malate, fumarate and succinate via CO_2 fixation has been demonstrated by carbon radiolabelling studies (Klein *et al*, 1975) Fumarate reductase has been detected in particulate fractions from homogenates of *T. brucei* procyclics (Klein *et al*, 1975).

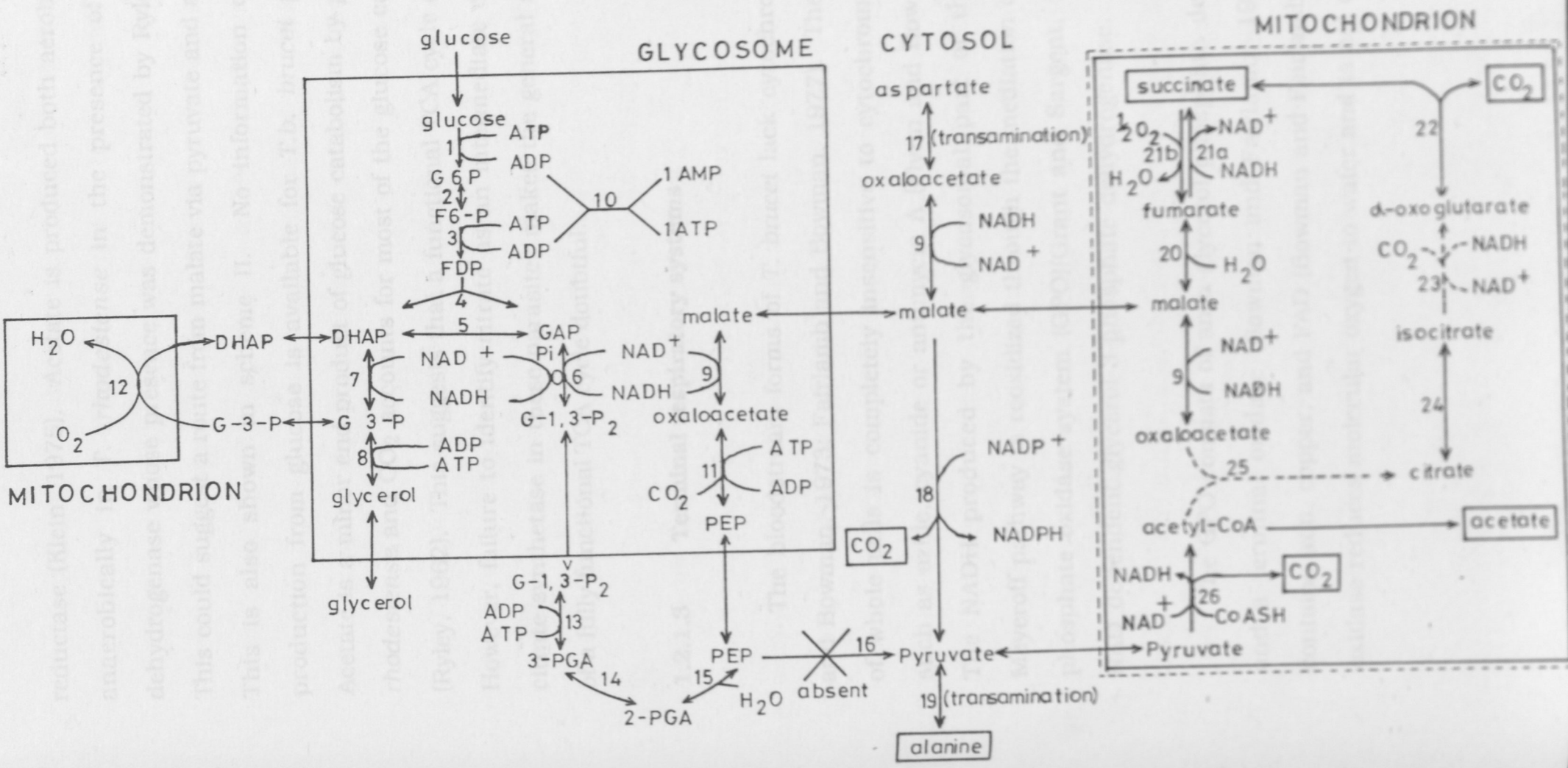
The pathways by which malate, the final product of glycosomal metabolism is converted to final end products of glucose metabolism have not been confirmed. Evans and Brown (1972(a) and Cross *et al* (1975) have reported that *T. brucei* procyclics can convert glucose to alanine. This conversion is presumed to be via the pathway involving decarboxylation of malate catalysed by cytosolic malic enzyme; this is followed by a transamination with a suitable amino acid transferase found in these organisms (Kilgour and Godfrey, 1973). These are represented in scheme II. This scheme also shows that aspartate (though it has not been reported as an end product) could function in a shuttle between the mitochondrion and the cytoplasm (Opperdoes *et al*, 1981; Opperdoes and Cottem, 1982). Malate could be converted to succinate via fumarate through the action of fumarase and fumarate

Scheme II

Pathways of glucose metabolism in procyclic trypomastigotes. End products of aerobic or anaerobic metabolism are enclosed in boxes. Enzymes whose presence are not certain have been indicated by dashed lines. The enzymes shown are:

1. Hexokinase
2. Phosphoglucose isomerase
3. Phosphofructokinase
4. Aldolase
5. Triose phosphate isomerase
6. Glyceraldehyde-3-phosphate dehydrogenase
7. Glycerol-3-phosphate dehydrogenase
8. Glycerol kinase
9. Malate dehydrogenase
10. Adenylate kinase
11. Phosphoenolpyruvate carboxykinase
12. Glycerol-3 phosphate oxidase
13. Phosphoglycerate kinase
14. Phosphoglycerate mutase
15. Enolase
16. Pyruvate kinase
17. Aspartate aminotransferase
18. Malic enzyme
19. Alanine amino transferase
20. Fumarate hydratase
21 (a) Fumarate reductase
21 (b) Succinate dehydrogenase
22. α-ketoglutarate decarboxylase
23. Isocitrate dehydrogenase
24. Aconitase
25. Citrate synthetase
26. Pyruvate dehydrogenase

(Source : Fairlamb and Opperdoes, 1986)


glucose
GLYCOSOME
CYTOSOL
MITOCHONDRION
glucose
ATP
ADP
1
G 6 P
2
F6-P
ATP
ADP
3
FDP
4
10
1 AMP
1ATP
5
DHAP
GAP
Pi
NAD +
NADH
7
6
G-1, 3-P2
8
ADP
ATP
glycerol
G 3-P
12
H2O
O2
G-3-P
DHAP
MITOCHONDRION
malate
NAD+
NADH
9
oxaloacetate
11
ATP
ADP
CO2
PEP
aspartate
17
(transamination)
oxaloacetate
NADH
NAD +
9
malate
NADP +
18
NADPH
CO2
G-1, 3-P2
ADP
ATP
13
3-PGA
14
2-PGA
15
H2O
PEP
16
absent
Pyruvate
19
(transamination)
alanine
succinate
CO2
22
1/2 O2
NAD+
21b
21a
H2O
NADH
fumarate
α-oxoglutarate
CO2
NADH
23
NAD+
20
H2O
malate
isocitrate
NAD+
9
NADH
24
oxaloacetate
25
citrate
acetyl-CoA
acetate
NADH
CO2
NAD+
26
CoASH
Pyruvate

Scheme II

reductase (Klein, 1975). Acetate is produced both aerobically and anaerobically in *T. rhodesiense* in the presence of pyruvate dehydrogenase whose presence was demonstrated by Ryley, (1962). This could suggest a route from malate via pyruvate and acetyl CoA. This is also shown in scheme II. No information on acetate production from glucose is available for *T.b. brucei* procyclics. Acetate is a minor end product of glucose catabolism by procyclic *T. rhodesiense* and CO_2 accounts for most of the glucose carbon used (Ryley, 1962). This suggests that a functional TCA cycle could exist. However, failure to identify citrate as an intermediate or to detect citrate synthetase in these parasites makes the general assumption of a fully functional TCA cycle doubtful.

1.2.1.3 Terminal respiratory systems

The bloodstream forms of *T. brucei* lack cytochromes (Flynn and Bowman, 1973; Fairlamb and Bowman, 1977). The respiration of whole cells is completely insensitive to cytochrome inhibitors such as azide, cyanide or antimycin A (Flynn and Bowman, 1973). The NADH produced by the glycosomal part of the Embden-Meyeroff pathway is reoxidized through the mediation of glycerol-3-phosphate oxidase system (GPO)(Grant and Sargent, 1960) via an NAD dependent glycerol-3-phosphate dehydrogenase.

The GPO consists of an α-glycerol-phosphate dehydrogenase and a terminal oxidase (Bowman and Fairlamb, 1976). It also contains iron, copper, and FAD (Bowman and Fairlamb, 1976). The oxidase reduces molecular oxygen to water and is not coupled to

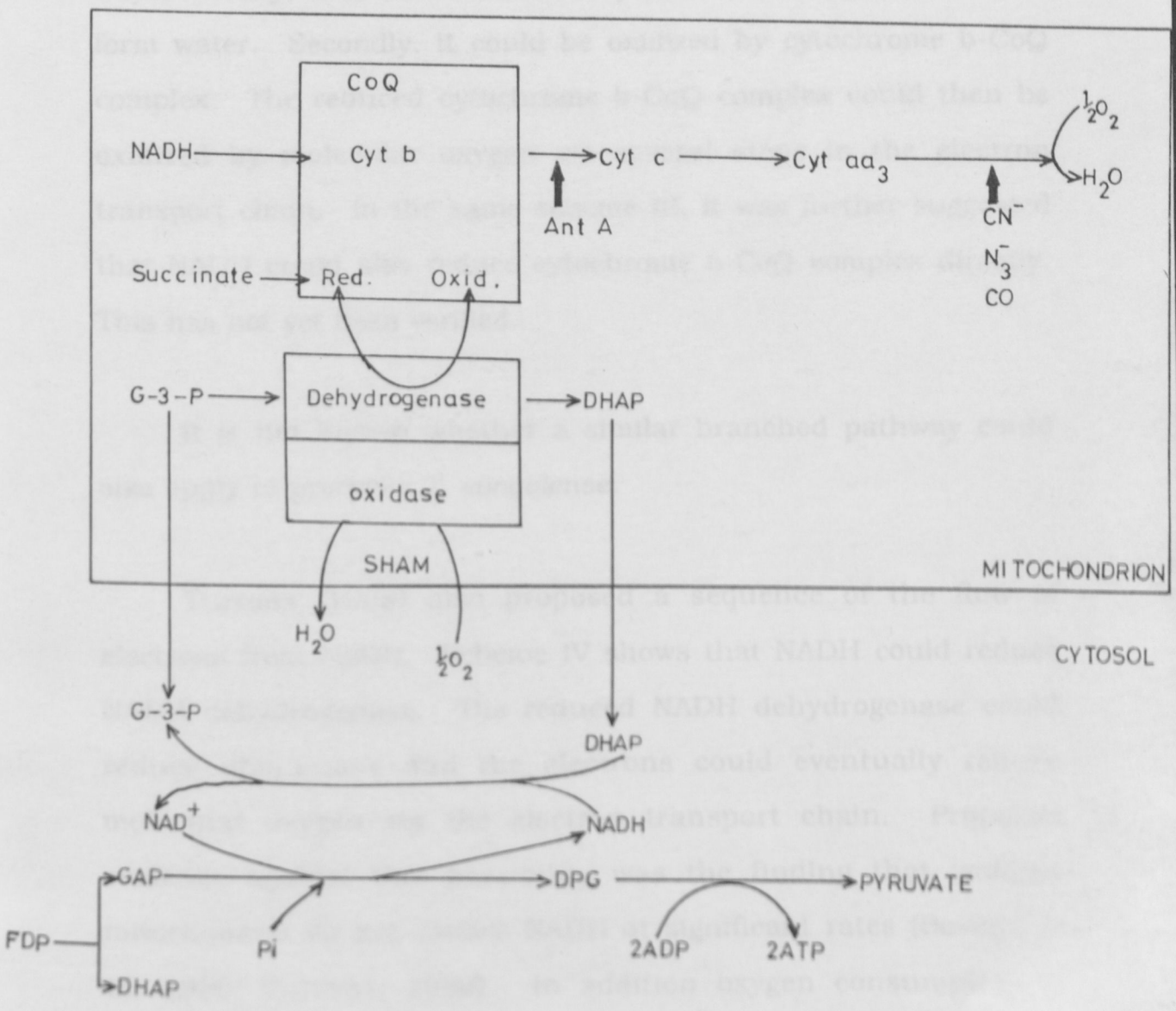
ADP phosphorylation (Gutteridge and Coombs, 1977). This terminal respiratory system is unique in that respiration is not sensitive to inhibition by cyanide and is not mediated by cytochromes (Grant and Sargent, 1960; Flynn and Bowman, 1973; Fairlamb and Bowman, 1977). The GPO can be inhibited by hydroxamic acids such as salicylhydroxamic acid (SHAM) (Evans and Brown, 1973; Opperdoes *et al*, 1976; Clarkson *et al*, 1981).

The procyclic forms posses cytochromes b, c, c_1, a and a_3 (Hill, 1976; Bowman and Flynn, 1976). *T. brucei* procyclics have 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, 1972(a). Compounds such as amytal or rotenone that usually inhibit site one of electron transport chain in most aerobic cells have no effect on the respiration on glucose or proline (Gutteridge and Coombs, 1977). An electron transport chain mediated by cytochromes has been proposed for *T.b. rhodesiense* procyclics (Ryley, 1962). Evidence has also been produced for the presence of the cytochrome system in *T. mega* (Hill, 1976) and in *Crithidia fasciculata* (Hill and White, 1968; Hill, 1972). Inhibitor studies have indicated that *T.b. brucei* procyclics possess at least two terminal oxidases (Evans and Brown, 1973). Njogu *et al* (1980) have reported that 60% of the respiration on glucose can be inhibited by cyanide and 30% of it by SHAM. About 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 III. They proposed that the reducing equivalents (NADH) produced in glycolysis could reduce

Scheme III

Reoxidation of reducing equivalents generated from the catabolism of glucose by *T.b. brucei* procyclics trypomastigotes.

(Source : Njogu *et al*, 1980).


CoQ
NADH
Cyt b
Cyt c
Cyt aa3
½O2
H2O
Ant A
CN⁻
N3⁻
CO
Succinate
Red.
Oxid.
G-3-P
Dehydrogenase
DHAP
oxidase
SHAM
MITOCHONDRION
H2O
½O2
CYTOSOL
G-3-P
DHAP
NAD+
NADH
GAP
DPG
PYRUVATE
FDP
Pi
2ADP
2ATP
DHAP

Scheme III

DHAP to glycerol-3-phosphate in a reaction catalysed by glycerol-3-phosphate dehydrogenase. The glycerol-3-phosphate could then reduce the GPO and the reduced GPO could be reoxidized in two ways. Firstly, it could be oxidized by molecular oxygen directly to form water. Secondly, it could be oxidized by cytochrome b-CoQ complex. The reduced cytochrome b-CoQ complex could then be oxidized by molecular oxygen via several steps in the electron transport chain. In the same scheme III, it was further suggested that NADH could also reduce cytochrome b-CoQ complex directly. This has not yet been verified.

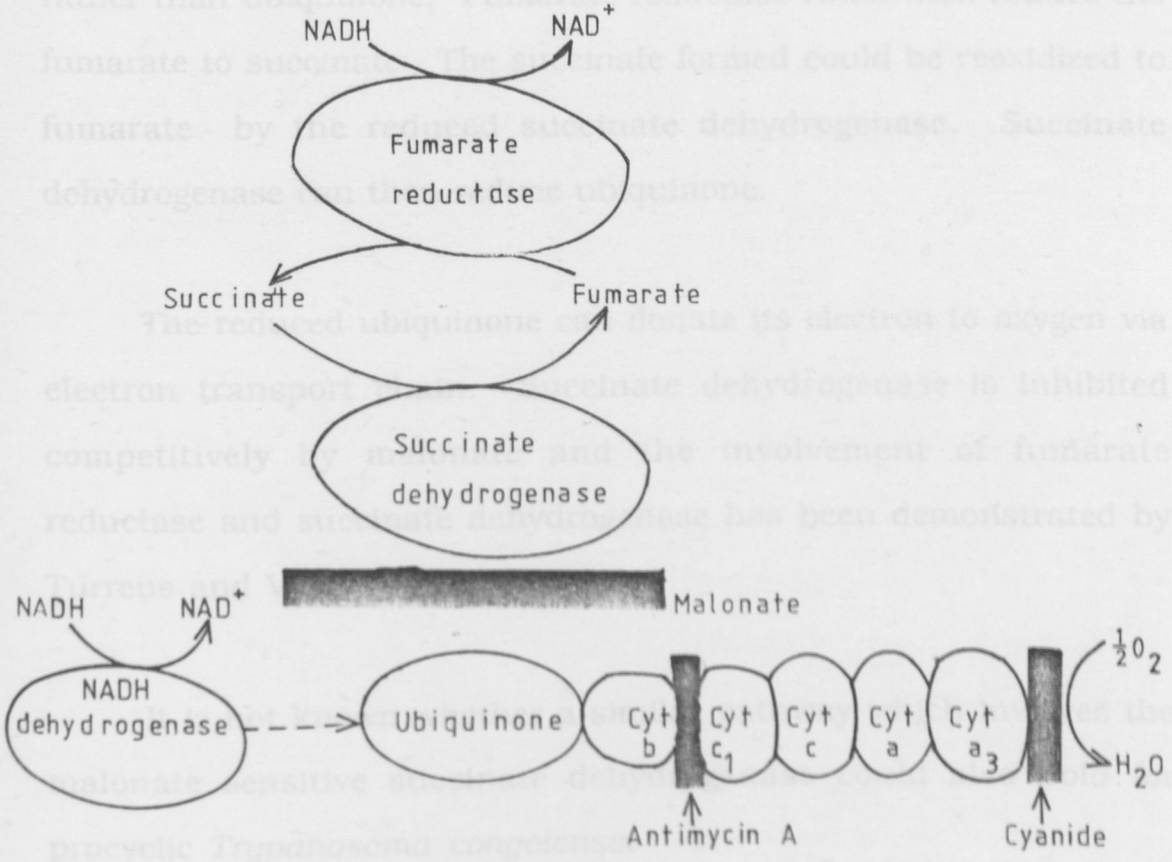
It is not known whether a similar branched pathway could also apply to procyclic *T. congolense*.

Turrens (1989) also proposed a sequence of the flow of electrons from NADH. Scheme IV shows that NADH could reduce NADH dehydrogenase. The reduced NADH dehydrogenase could reduce ubiquinone and the electrons could eventually reduce molecular oxygen via the electron transport chain. Proposed evidence against this possibility was the finding that isolated mitochondria do not oxidize NADH at significant rates (Boveris *et al*, 1986; Turrens, 1989). In addition oxygen consumption is insensitive to amytal or rotenone which usually inhibit the NADH dehydrogenase (Gutteridge and Coombs, 1977). Turrens and Villalta (1989) suggested that the lack of inhibition of respiration by either amytal or rotenone could be due to the fact that NADH dehydrogenase is different from that in mammalian cells. Scheme IV also shows that alternatively oxygen consumption could be dependent on succinate oxidation rather than NADH oxidation.

Scheme IV

An alternative branched pathway for the re-oxidation of reducing equivalents from the catabolism of glucose in the mitochondria of *Trypanosoma cruzi* epimastigotes and *T. brucei* procyclic trypomastigotes.

(Source : Turrens and Villalta, 1989)


NADH
NAD+
Fumarate reductase
Succinate
Fumarate
Succinate dehydrogenase
Malonate
NADH
NAD+
NADH dehydrogenase
Ubiquinone
Cyt b
Cyt c1
Cyt c
Cyt a
Cyt a3
½O2
H2O
Antimycin A
Cyanide

Scheme IV

Evidence by Turrens (1989) supports the use of succinate as an intermediate in the reoxidation of NADH. The NADH dehydrogenase could transfer electrons to fumarate reductase rather than ubiquinone. Fumarate reductase could then reduce the fumarate to succinate. The succinate formed could be reoxidized to fumarate by the reduced succinate dehydrogenase. Succinate dehydrogenase can then reduce ubiquinone.

The reduced ubiquinone can donate its electron to oxygen via electron transport chain. Succinate dehydrogenase is inhibited competitively by malonate and the involvement of fumarate reductase and succinate dehydrogenase has been demonstrated by Turrens and Villalta (1989).

It is not known whether a similar pathway which involves the malonate sensitive succinate dehydrogenase could also hold for procyclic *Trypanosoma congolense*.

1.2.2 PROLINE METABOLISM

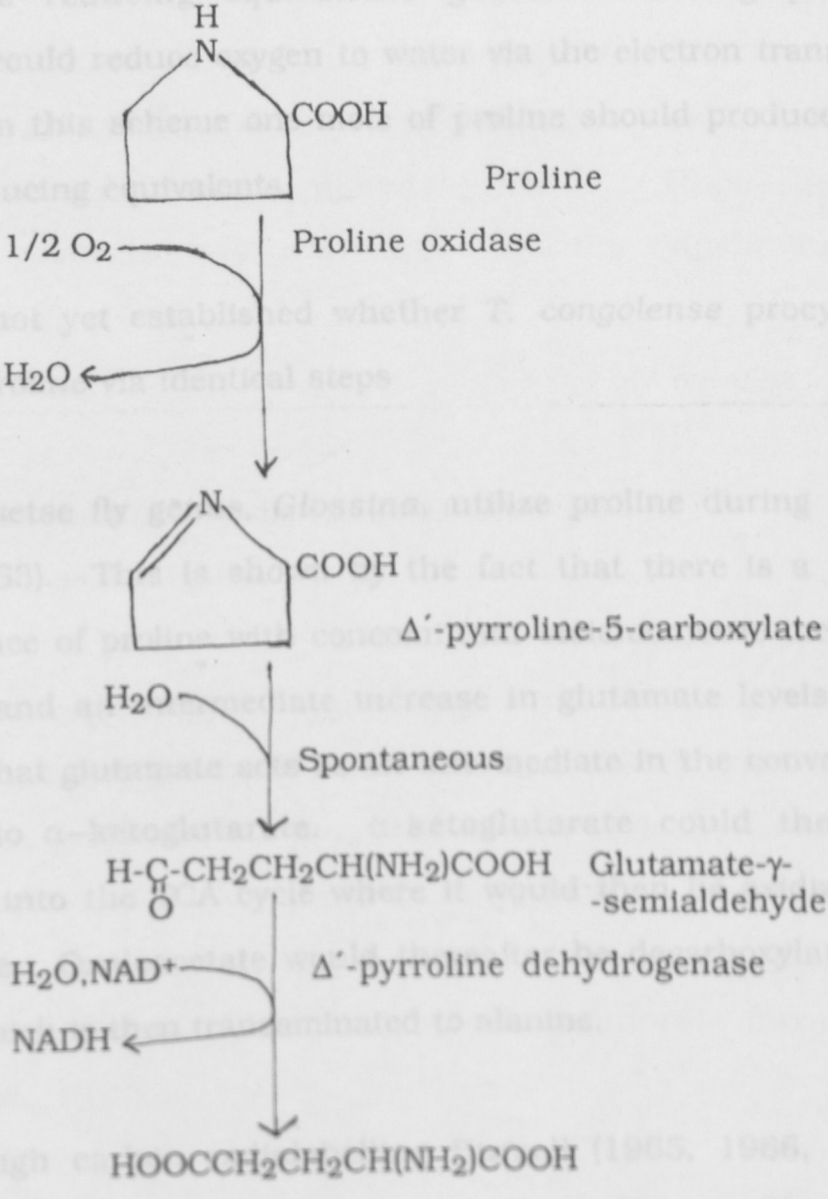
1.2.2.1 Proline metabolism in non-trypanosomatids

Proline is oxidized in mammals in two steps. This is represented in Scheme V. The first step is catalysed by proline oxidase and yields Δ´-pyrroline-5-carboxylate. This is followed by a spontaneous reaction involving the addition of water to yield glutamate semi-aldehyde. Glutamate semi-aldehyde is then oxidized by Δ´-pyrroline dehydrogenase to yield L-glutamate; NAD^+

Scheme V

The pathways of proline catabolism in mammals

(Source : Lehninger, 1975)


H
N
COOH
Proline
1/2 O2
Proline oxidase
H2O
N
COOH
Δ´-pyrroline-5-carboxylate
H2O
Spontaneous
H-C-CH2CH2CH(NH2)COOH
O
Glutamate-γ-
-semialdehyde
H2O,NAD+
Δ´-pyrroline dehydrogenase
NADH
HOOCCH2CH2CH(NH2)COOH
Glutamate

is also reduced in the process to give NADH. The glutamate produced undergoes transamination to give α–ketoglutarate. The resulting α–ketoglutarate can then be channelled into the TCA cycle. The reducing equivalents generated during proline catabolism could reduce oxygen to water via the electron transport chain. From this scheme one mole of proline should produce two moles of reducing equivalents.

It is not yet established whether *T. congolense* procyclics catabolize proline via identical steps

The tsetse fly genus, *Glossina*, utilize proline during flight Bursell (1963). This is shown by the fact that there is a rapid disappearance of proline with concomittant stoichiometric increase in alanine and an intermediate increase in glutamate levels. He suggested that glutamate acts as an intermediate in the conversion of proline to α–ketoglutarate. α-ketoglutarate could then be channelled into the TCA cycle where it would then be oxidized to oxaloacetate. Oxaloacetate would thereafter be decarboxylated to pyruvate which is then transaminated to alanine.

Through carbon radiolabelling Bursell (1965, 1966, 1977) confirmed the presence of the proline alanine pathway. Glutamate is the first intermediate in this pathway followed by succinate and then pyruvate. Alanine and CO_2 are end products (Bursell and Slack, 1976).

The enzymes of this pathway which have been noted to be very active include proline dehydrogenase and alanine aminotransferase (Crabtree and Newsholme, 1970, 1975; Bursell, 1975). NAD-linked malic enzyme has also been found to be active (Hoek *et al*, 1976; Norden and Matanganyidze, 1977, 1979). Another enzyme in this proline catabolism pathway in the tsetse is Δ´-pyrroline-5-carboxylate dehydrogenase. The reducing equivalents generated are oxidized through the cytochrome aa_3 oxidase (Bender, 1975).

1.2.2.2 Proline metabolism in procyclic trypomastigotes

The procyclic trypanosomes respire on proline unlike the bloodstream forms (Fairlamb and Opperdoes, 1986). The rate of respiration on proline by the procyclics is fairly high (Srivastava and Bowman, 1971, 1972; Evans and Brown 1972(a)). Respiration on proline in cell free homogenates is upto two times higher than that on succinate, glycerol-3-phosphate or α–ketoglutarate (Srivastava and Bowman, 1971).

Proline is present in high concentrations in the insect haemolymph (Bursell, 1978, 1981) and would therefore seem a likely physiological substrate for the trypanosomes *in vivo* (Fairlamb and Opperdoes, 1986).

When procyclic *T. brucei* are utilizing proline, they apparently lack a fully functional TCA cycle (Fairlamb and Opperdoes, 1986).

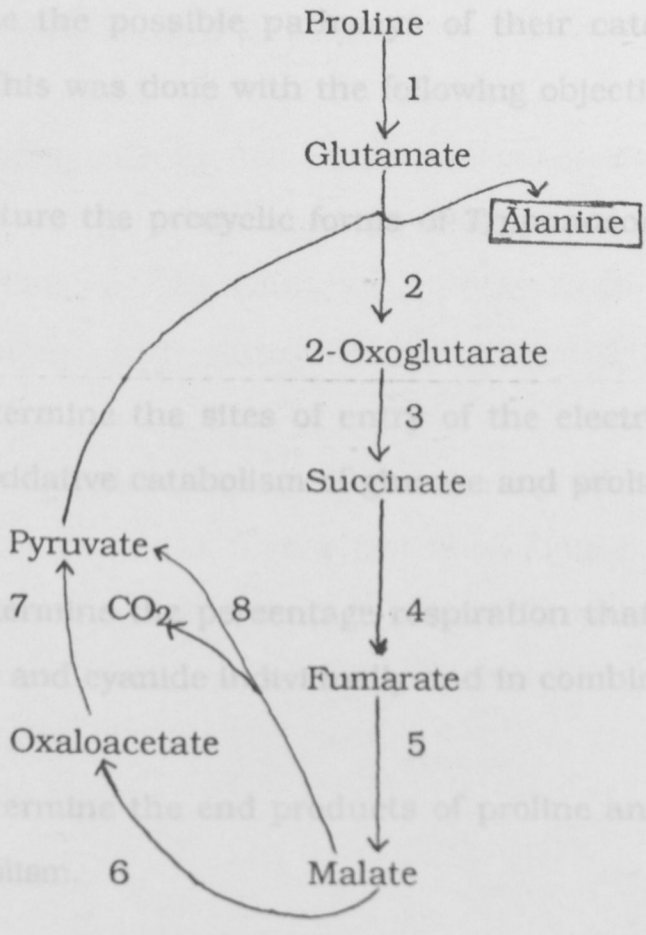
The partial TCA cycle present accounts for the CO_2 evolved and the energy produced. This is because the NADH generated is presumed to be re-oxidized by the respiratory chain with the concomittant synthesis of ATP (Fairlamb and Opperdoes, 1986).

Proline is catabolized mainly to CO_2, alanine and aspartate (Gutteridge and Coombs, 1977). The presence of the enzyme alanine aminotransferase is critical for the growth on proline and is in fact present in very high quantities. Its presence explains why alanine is one of the end products (Gutteridge and Coombs, 1977). Evans and Brown (1972(a) proposed a pathway for the catabolism of proline by procyclic *T. brucei*. This is shown in scheme VI. In this scheme the enzyme alanine aminotransferase catalyses the transamination of glutamate and pyruvate with the concomittant production of alanine.

Scheme VI

The pathway of proline catabolism by the procyclic *T. brucei* trypomastigotes (Evans and Brown, 1972a). The suggested names for the enzymes 1-8 are as follows:-

1. Proline dehydrogenase
2. Glutamate pyruvate transaminase
3. α-ketoglutarate dehydrogenase and succinylCoA synthetase
4. Succinate dehydrogenase
5. Fumarase
6. Malate dehydrogenase
7. Oxaloacetate decarboxylase
8. Malic enzyme or malate decarboxylase



(Source : Evans and Brown, 1972a)

1.3. AIMS AND OBJECTIVES

This study was designed to establish whether procyclic *Trypanosoma congolense* can catabolize glucose and proline and if so determine the possible pathways of their catabolism in these parasites. This was done with the following objectives:-

1. To culture the procyclic forms of *Trypanosoma congolense in vitro.*

2. To determine the sites of entry of the electrons derived from the oxidative catabolism of glucose and proline.

3. To determine the percentage respiration that is sensitive to SHAM and cyanide individually and in combination.

4. To determine the end products of proline and glucose catabolism.

CHAPTER TWO

MATERIALS AND METHODS

2.1 CHEMICALS AND ENZYMES

The reagents in this study were of analytical grade. They were obtained from British Drug Houses, Poole England; Sigma Chemical Company, St. Louis, MO, U.S.A.; E. Merck A.G Darmstadt, West Germany and Boehringer Manheim GmBH, West Germany.

All the cell culture reagents were of cell culture grade and were purchased from Sigma Chemical Company, St. Louis MO, U.S.A.. The serum used for supplementing the cell culture medium was obtained from Flow Laboratories, U.K. (Cat. No. 29-101-54, Lot No. 106126).

2.1.1 Buffers

The following two buffers were used routinely in this study:-

(i) Phosphate buffered saline glucose consisting of 51 mM sodium chloride, 20 mM disodium hydrogen phosphate, 2 mM disodium hydrogen phosphate, 2 mM sodium dihydrogen phosphate and 10 mM glucose.

(ii) Phosphate buffered saline proline consisting of 51 mM sodium cloride, 20 mM disodium hydrogen phosphate,

2 mM sodium dihydrogen phosphate and 10 mM proline.

2.2 ORGANISMS

Trypanosoma congolense IL 3000 procyclics were used throughout this study. The bloodstream forms of the parasites were obtained from International Laboratory for Research on Animal Diseases (ILRAD). This parasite IL 3000 was derived from ILC 49 which was isolated from a cow in the Transmara region in 1966. The bloodstream forms of IL 3000 were maintained in adult Sprague-Dawley rats with weekly serial passages. Approximately $1x10^6$ cells/ml of these were each time inoculated into culture as described below and allowed to transform into procyclic forms.

2.3 CULTURING OF PROCYCLIC *TRYPANOSOMA CONGOLENSE*

2.3.1 Sterilization of the glassware

The glassware routinely used during trypanosome culture consisted of : beakers of sizes ranging from 10 mls to 250 mls; glass pipettes of 5 ml capacity and measuring cylinders of sizes ranging from 5 mls to 100 mls. All the glassware were of pyrex brand.

They were first soaked in chromic acid overnight and then washed thoroughly with lysol (detergent) and rinsed 4 to 5 times with tap water to remove the detergent. They were then rinsed four to five times with distilled water and dried at 100°C. They

were then removed and the mouth of each wrapped tightly with an aluminium foil. An indicator tape was fixed along the neck of each piece of glassware. Small pieces of clean cotton wool were inserted into the glass pipettes through the constricted end. The pipettes were then put in a cylindrical metallic container which was then capped. The container was wrapped with aluminium foil and an indicator tape stuck on it. All the glassware were heat-sterilized in an oven at 160°C for 2 hours. After this time the indicator tape developed dark stripes indicating that sterilization was complete. The oven was switched off and the glassware left to cool slowly at room temperature. The glassware were then ready for use. They were unwrapped only when they were about to be used.

2.3.2 Sterilization of media, storage bottles, water and NaOH

All the bottles to be used were of pyrex brand. They were washed thoroughly with lysol (detergent) and rinsed with tap water 4 to 5 times to remove the detergent and then rinsed with distilled water. They were then dried in the oven at 80°C for about one hour. These bottles were then screw-capped and their openings wrapped with pieces of aluminiun foil. A piece of an indicator tape was then stuck on the necks of the bottles. These bottles were autoclaved at 250°F for 30 minutes after which the tape developed dark stripes indicating that sterilization was complete.

Deionized water was also put in some of these bottles which were then sterilized as described above.

In one of the bottles was put 2 N NaOH which had earlier been prepared in deionized water. It was also sterilized in the same way as described above.

After sterilization for 30 minutes the autoclave was switched off and the bottles allowed to cool slowly. They were then ready for use.

2.3.3 Heat inactivation of the serum

The commercially obtained foetal bovine serum was inactivated by heating in a water bath at 56°C for 30 minutes. It was then allowed to cool at room temperature and later aliquoted into several sterile plastic tubes of 50 mls capacity.

The serum was then frozen at -20°C and removed only when required.

2.3.4 Preparation of the culture media

The culture media (SDM-79) was prepared as described by Brun and Schonenberger (1979). However, MEM powder (containing factor 14) was replaced by RPMI 1640. The following media components were weighed in a sterile beaker:- 7.0 g RPMI 1640, 2.0 g M 199, 1 g glucose; 8.0 g HEPES; 5.0 g MOPS; 2.0 g $NaHCO_3$; 100 mg Na-pyruvate; 200 mg alanine; 100 mg L-arginine 70 mg L-methionine; 80 mg L-phenylanine; 600 mg L-proline; 60 mg L-serine; 160 mg L-taurine; 300 mg L-glutamine; 350 mg L-threonine; 100 mg L-tyrosine; 10 mg adenosine; 10 mg guanosine;

50 mg glucosamine-HCl; 4 mg folic acid; 2 mg p-aminobenzoic acid and 0.2 mg biotin. These were then dissolved in sterile deionized water. To this mixture was added 8.0 ml of MEM amino acids (50x) without glutamine and 6.0 mls of MEM non-essential amino acids (100 x). The pH was adjusted with sterile 2 N NaOH to 7.3 and the medium made up to one litre with sterile deionized water. The medium was then filter sterilized using Nalgene millipore filter of pore size 0.22 μm.

Before use, the medium was supplemented with 20% of heat inactivated foetal bovine serum.

The volume of culture media prepared each time was 500 mls. This lasted for about 3-4 weeks at 4°C.

2.3.5 *In vivo* growth of parasites

Bloodstream *T. congolense* were maintained in adult Sprague-Dawley rats by weekly passages. The infected rats were housed in cages in a well ventilated room at a temperature of about 25°C. The rats were fed on a normal pellet diet and water.

2.3.6 Initiation of *in vitro* procyclic *T. congolense* culture

Blood from an infected rat with rising parasitaemia was removed aseptically as follows. First the rat was doused in 70% ethanol and then anaesthetized under diethyl ether and the thorax opened to expose the heart. Into a sterile 5 ml syringe fitted with a sterile needle (19G) was drawn 625 units of heparin as

anticoagulant. The exposed heart was punctured with the needle and 2-4 mls of blood was drawn into the syringe.

The collected infected blood was then aseptically transferred into a sterile screw capped centrifuge tube. About 6-8 mls of SDM-79 was then added into the tube. The tube was centrifuged at about 1000 x g for 5 minutes at 4°C. About 1 ml of the supernatant was then aseptically transferred to 5 mls of SDM-79 in a sterile T-25 Falcon flask. Approximately $1x10^6$ trypanosomes/ml were each time used to initiate a culture. The flask was then screw-capped tightly and incubated at 28°C.

2.3.7 Maintenance of procyclic *T. congolense* in culture

After 48 hours from the time of initiation into culture, the parasites were subcultured and the number of trypanosomes estimated on an improved Neubauer haemocytometer. Subsequently they were maintained in culture with two weekly subcultures. Subculturing was done whenever the colour of the medium in the flask turned to almost colourless. To subculture, approximately 2.5 mls of the trypanosome suspension was transferred into a sterile T. 25 Falcon flask followed by addition of 2.5 ml of SDM-79 into each of the flasks. The volume of each culture was 5 mls.

The parasites were incubated for at least 7 days before they were used in any biochemical experiment.

2.4 HARVESTING OF TRYPANOSOMES

About 2-5 mls of the trypanosome suspension was aseptically removed from each flask when the trypanosome density was about $1x10^7$ cells/ml. These were centrifuged at about 1000 x g at 4°C for 15 minutes. The resulting pellet was washed at least five times with about 9.5 mls of either PSG or PS Proline depending on the experiment to be performed. It was then suspended in appropriate buffer and the trypanosomes counted on an improved Neubauer haemocytometer.

2.5 RESPIRATION EXPERIMENTS

Oxygen uptake was determined using a Clark-type electrode (Rank Brothers High Street Bottisham-Cambridge, England) polarized at -0.6 V in a magnetically stirred cuvette. The rate of oxygen uptake was monitored on a Sargent-Welch recorder coupled to the electrode. The electrode was left to equilibrate with either PSG or PS proline for at least 5 minutes at 25°C before starting the experiment. One hundred to three hundred microlitres of trypanosome suspensions was then added into the chamber housing the oxygen electrode and respiration was determined before and after addition of various inhibitors.

PSG buffer containing 10 mM glucose or PS proline containing 10 mM proline was used routinely in respiration experiments. The total volume of buffer used was 1.2 mls.

The concentration of oxygen in the incubation media was taken to be 240 nmol/ml (Chance and Williams, 1956).

2.6 Enzyme Assays

For enzyme assays the trypanosomes were suspended in PSG buffer (unless otherwise indicated in the text). Triton X-100 was added to a final concentration of 0.1% (v/v) (Opperdoes *et al*, 1976). The lysate was centrifuged at about 1000 g for 10 minutes at 4°C. The supernatant obtained from this centrifugation was used for all enzyme assays. This is referred to as the sample in the following assay descriptions. Calculations for specific activities of the enzymes were done according to Bergmeyer (1974) according to the following equation unless otherwise indicated in the text.

$$C = \frac{\Delta A/min}{\varepsilon} \times \frac{V}{v} \times \frac{1}{P}$$

where

C = specific activity of the enzyme in µmols/min/mg protein.

ΔA/min = Rate of change of absorbance per minute

v = Volume of the sample used in the assay

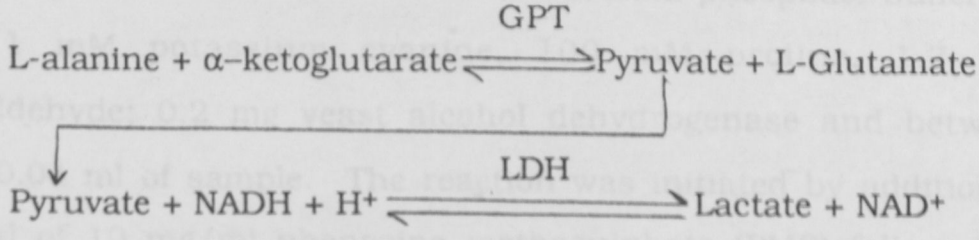
V = Total volume in the assay cuvette

P = Protein concentration in mg/ml

ε = Extinction coefficient of NADH or NADPH at 340 nm (6.22×10^6 cm^2/mol or 6.22 cm^2/µmole). This value was used as the extinction coefficient in all calculations unless otherwise indicated in the text.

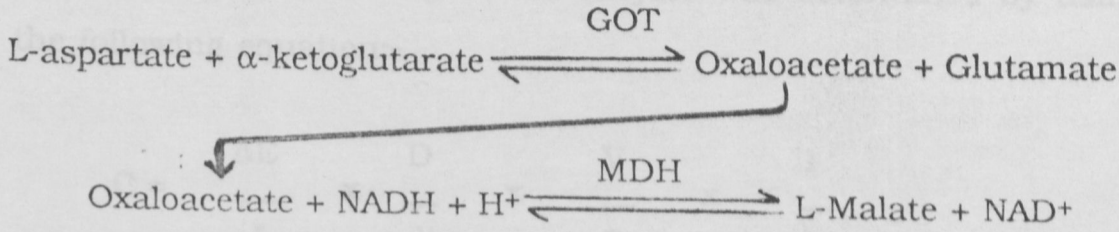
2.6.1 Glutamate Pyruvate Transaminase (E.C. 2.6.1.2)

This enzyme was assayed according to Bergmeyer (1974) in 80 mM phosphate buffer pH 7.4 containing; 0.8 M L-alanine; 0.18 mM NADH; 3.7 I.U/ml lactate dehydrogenase and 0.02 ml of the sample. This mixture was left to stand for 3 minutes. The reaction was started by addition of 18 mM α–ketoglutarate solution. The total volume of the assay mixture was 3 ml. After mixing the rate of decrease of absorbance was read at 340 nm. The assay was based on the following reaction.



2.6.2 Glutamate oxaloacetate transaminase (GOT) (E.C. 2.6.1.1)

This was assayed according to Bergmeyer (1974) in a cuvette containing; 80 mM phosphate buffer pH 7.4; 200 mM L-aspartate; 0.18 mM NADH; 7.5 I.U./ml of MDH; 3.7 I.U./ml LDH and 0.1 ml of the sample. The mixture was allowed to stand for 5 minutes. The reaction was started by the addition of α-ketoglutarate solution to a final concentration of 12 mM. The total assay volume was 3 ml. The rate of decrease in the absorbance was read at 340 nm. The assay was based on the following reaction:-



2.6.3 Proline Dehydrogenase

This enzyme was assayed as described by Olembo (1980) based on the reduction of p-iodonitrotetrazolium violet (2-[4--iodophenyl]-3-[4-nitrophenyl]-5-phenyl tetrazolium chloride; INT). The assay mixture consisted of 50 mM sodium phosphate buffer pH 7.5; 1 mM potassium cyanide, 100 mM proline, 1.7 mM acetaldehyde; 0.2 mg yeast alcohol dehydrogenase and between 0.01-0.04 ml of sample. The reaction was initiated by addition of 100 µl of 10 mg/ml phenazine methosulphate (PMS) followed by 200 µl of 5 mg/ml INT. The final volume of the incubation mixture was 1.5 ml. The mixture was incubated at room temperature for 10 minutes. Control incubations were treated in the same way except that proline was omitted. The incubations were terminated by the addition of 1.5 ml of 10% (w/v) TCA. A volume of 4 mls of ethyl acetate was then added and the mixture shaken vigorously for approximately 30 seconds. The tubes were then left to stand until ethyl acetate layer cleared. The ethyl acetate layer was removed with a Pasteur pipette and its absorbance read at 490 nm against ethyl acetate as blank. Molar extinction coefficient for the reduction of INT in ethyl acetate was taken to be 20.1×10^3 M^{-1} cm^1 or 20.1 $cm^2/\mu mol$ (according to Pennington, 1961).

The specific activity of the enzyme was determined by using the following equation:-

$$C = \frac{\Delta E}{\varepsilon} \times \frac{D}{v} \times \frac{V}{p} \times \frac{1}{t}$$

Where C = specific activity of the enzyme in µmoles/min/mg protein

ΔE = Difference in the values of absorbance between the test sample and control ample

ε = Extinction coefficient of INT in ethyl acetate at 490 nm which was taken to be $20.1 \times 10^3\ M^{-1}\ cm^{-1}$ or $20.1 \times 10^6\ cm^2/mol$ or $20.1\ cm^2/\mu mol$ (according to Pennington, 1961).

V = Total incubation volume which was 1 ml

v = Volume of the sample

D = Dilution factor = volume of ethyl acetate added

P = Protein concentration

t = Duration of incubation in minutes

2.6.4 Succinate dehydrogenase (E.C. 1.3.99.1)

This enzyme was assayed as described by Njagi (1987) in a reaction mixture containing 50 mM potassium phosphate buffer pH 7.4, 50 mM sodium succinate, 25 mM sucrose and between 0.02 to 0.04 ml of the sample. The reaction was started by addition of 100 µl of 10 mg/ml PMS followed by 200 µl of 5 mg/ml INT. The final

volume of the incubation medium was 1.5 ml. This was then incubated for 10 minutes at room temperature (25°C). Control samples were treated in a similar manner except for the omission of sodium succinate. The incubations were terminated with 1.5 mls 10% (v/v) TCA. Ethyl acetate (4.0 ml) was then added into each tube and then shaken vigorously for abut 30 seconds. The tubes were left to stand until the ethyl acetate layer was clear. The top layer consisting of ethyl acetate was then removed carefully with a pasteur pipette and its absorbance read at 490 nm against ethyl acetate as a blank. The molar extinction coefficient for the reduction of INT in ethyl acetate was taken to be 20.1 x 10^3 M^{-1} cm^{-1} (Pennington, 1961).

The specific activity of the enzyme was calculated using the same equation as that used for proline dehydrogenase.

2.6.5 α-Ketoglutarate dehydrogenase (E.C. 1.2.4.2)

This enzyme was assayed as described by Overath *et al* (1986) in a reaction mixture consisting on 150 mM Tris-HCl buffer pH 7.4, 3 mM cysteine, 0.2 mM coenzyme A, 4 mM NAD^+, 100 µl of sample and 0.2 mM thiamine pyrophosphate (cocarboxylase). The reaction was started by the addition of 5 mM sodium oxoglutarate to a final concentration of 5 mM. The final volume of the assay mixture was 3 ml. The rate of increase of absorbance was read at 340 nm. The assay was based on the following reaction.

$$\alpha\text{-ketoglutarate} + NAD^+ + CoASH \xrightarrow[TPP,\ Cyst]{\alpha KGDH} SuccinylCoA + NADH + CO_2$$

2.6.6 NADP-linked malic enzyme (E.C. 1.1.1.40)

This enzyme was assayed as described by Ochoa *et al* (1955) in 70 mM Tris-HCl buffer pH 7.4 containing 3.5 mM $MgCl_2$, 5 µl of 5 mg/ml $NADP^+$ and 0.1 ml of sample. The reaction was started by addition of DL malate to a final concentration of 3.5 mM. The total assay volume was 3 ml. The rate of increase of absorbance was read at 340 nm.

The assay was based on the following reaction:-

$$\text{Malate} + NADP^+ \xrightarrow{\text{M.E}} \text{Pyruvate} + CO_2 + NADPH + H^+$$

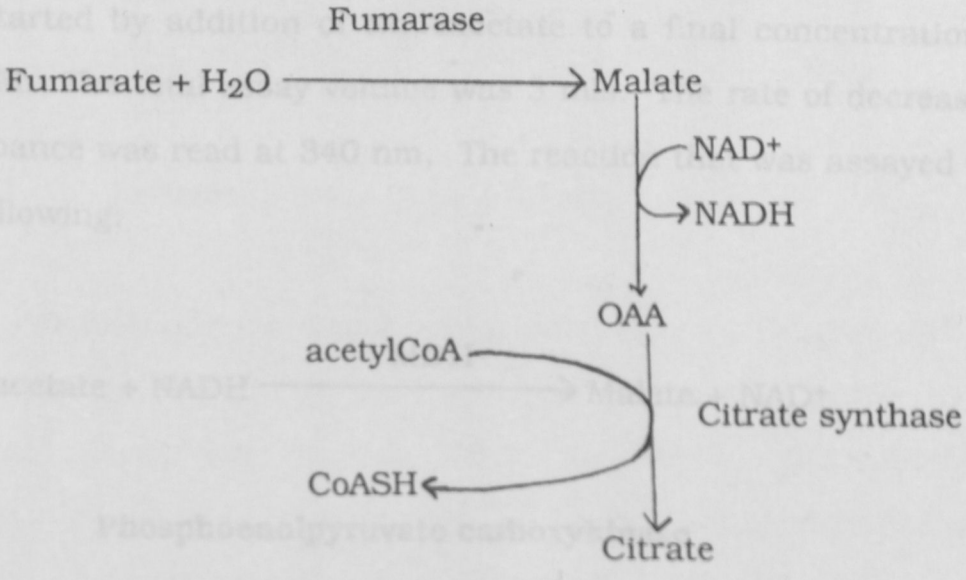
2.6.7 NAD-linked malic enzyme

The assay medium for this enzyme was similar to that for the assay of NADP-linked malic enzyme above except that $NADP^+$ was replaced with NAD^+. The assay mixture contained 70 mM Tris-HCl buffer pH 7.4, 3.5 mM $MgCl_2$, 1 mM NAD^+ and 0.1 ml of the sample. The reaction was started by the addition of DL-malate to a final concentration of 3.5 mM; the total assay volume was 3 ml. The rate of increase in absorbance was read at 340 nm. The assay was based on the following reaction:-

$$\text{Malate} + NAD^+ \xrightarrow{\text{M.E}} \text{Pyruvate} + CO_2 + NADH + H^+$$

2.6.8 Fumarase (E.C. 4.2.1.2)

This was assayed as described by Konji (1983) in 100 mM phosphate buffer pH 7.4, 10 µg/ml citrate synthase, 2mM NAD, 20 µg/ml malate dehydrogenase, 1 mM acetylCoA and 0.1 ml of the sample. The reaction was started by addition of fumarate at a final concentration of 1.7 mM. The final assay volume was 3 ml. The rate of increase in absorbance was read at 340 nm. The assay was based on the reaction below:-



2.6.9 Fumarate Reductase

This enzyme was assayed as described by Klein *et al* (1975) in 0.1M Tris-HCl buffer pH 8.0 containing 0.18 mM NADH and 50 µl of the sample. The reaction was started by the addition of sodium fumarate (pH 8.0) to a final concentration of 0.01 M. The total assay volume volume was 3 mls. The progress of the reaction was

followed by reading the rate of decrease of absorbance at 340 nm. The reaction that was assayed was as follows :-

$$\text{Fumarate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{Fumarate reductase}} \text{Succinate} + \text{NAD}^+$$

2.6.10 Malate dehydrogenase (E.C. 1.1.1.37)

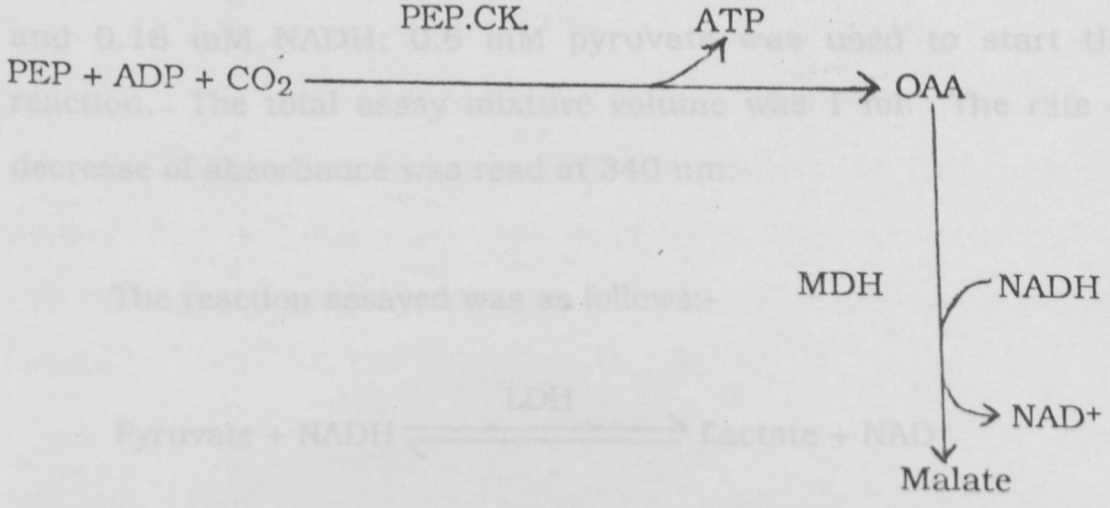
This enzyme was assayed essentially as described by Bergmeyer (1974) but in 94.6 mM phosphate buffer pH 7.5 containing 0.2 mM NADH and 20 µl of the sample. The reaction was started by addition of oxaloacetate to a final concentration of 0.5 mM. The total assay volume was 3 mls. The rate of decrease of absorbance was read at 340 nm. The reaction that was assayed was the following:

$$\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+$$

2.6.11 Phosphoenolpyruvate carboxykinase

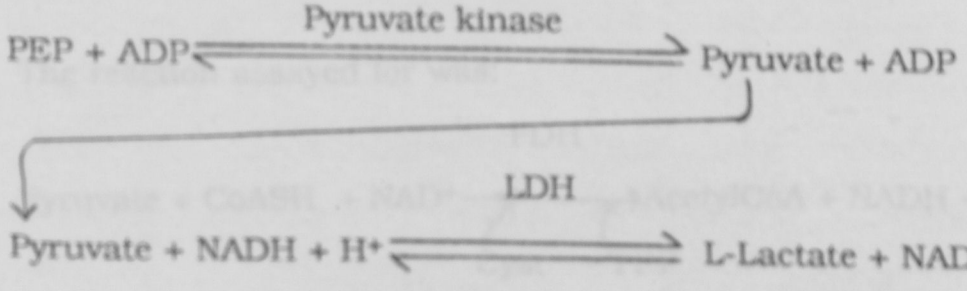
This enzyme was assayed according to Opperdoes and Cottem (1982) in an assay mixture containing 0.1 M imidazole buffer pH 6.6; 50 mM $KHCO_3$, 1.25 mM ADP, 1 mM manganous sulphate, 0.2 mM NADH, 1 mM glutathione and 50 µl sample. The reaction was started with PEP at a final concentration of 1.25 mM. The final volume of the assay mixture was 1 ml. MDH was not included in the mixture as that in the sample itself proved sufficient to carry on the reaction.

The rate of decrease of absorbance was read at 340 nm. The reaction being assayed was:-

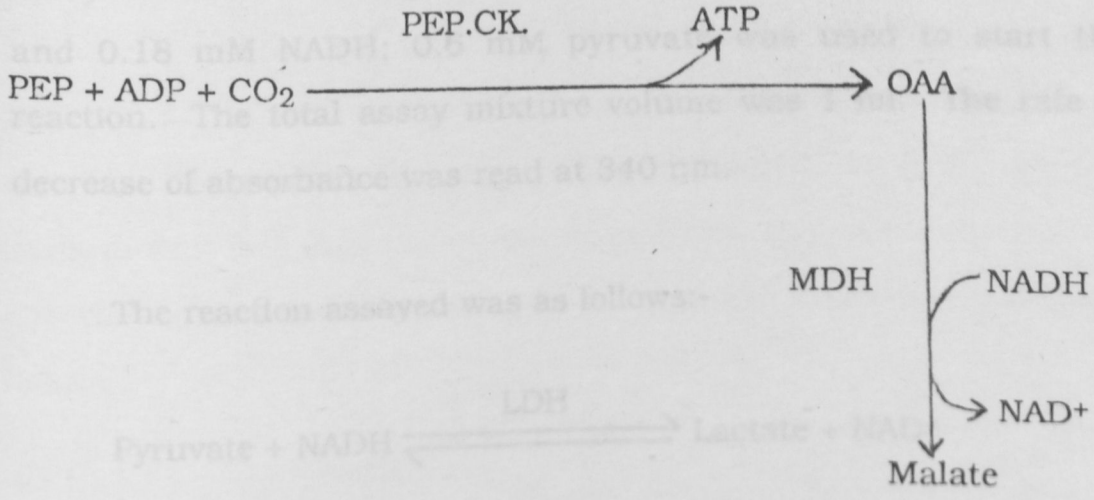


2.6.12 Pyruvate Kinase (E.C. 2.7.1.40)

Pyruvate kinase was assayed according to Bergmeyer (1974) in a medium containing 97.5 mM triethanolamine buffer pH 7.5, 92.5mM potassium chloride, 16.2 mM $MgSO_4$, 0.2 mM NADH, 13mM ADP 20 µl of sample and 2.5 IU/ml lactate dehydrogenase. The reaction was started by addition of 1.25 mM PEP at a final concentration of 1.25 mM to give a total volume of 1 ml. The rate of decrease in absorbance was read at 340 nm. The reaction being assayed was as follows:-

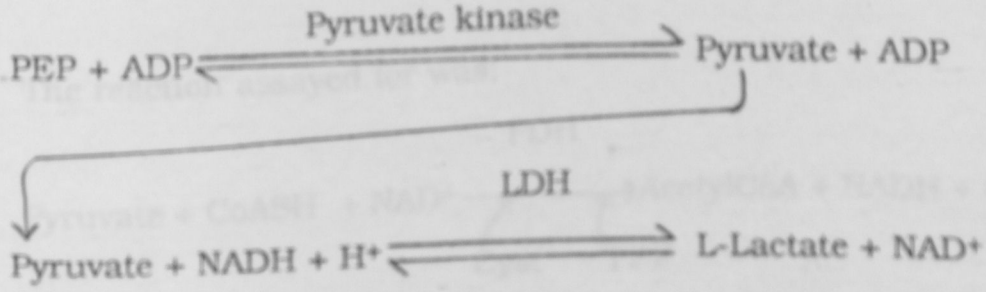


The rate of decrease of absorbance was read at 340 nm. The reaction being assayed was:-



2.6.12 Pyruvate Kinase (E.C. 2.7.1.40)

Pyruvate kinase was assayed according to Bergmeyer (1974) in a medium containing 97.5 mM triethanolamine buffer pH 7.5, 92.5mM potassium chloride, 16.2 mM $MgSO_4$, 0.2 mM NADH, 13mM ADP 20 µl of sample and 2.5 IU/ml lactate dehydrogenase. The reaction was started by addition of 1.25 mM PEP at a final concentration of 1.25 mM to give a total volume of 1 ml. The rate of decrease in absorbance was read at 340 nm. The reaction being assayed was as follows:-



2.6.13 Lactate Dehydrogenase (E.C. 1.1.1.27)

This was assayed as described by Bergmeyer (1974) in an assay mixture containing 48 mM potassium phosphate buffer pH 7.5 and 0.18 mM NADH; 0.6 mM pyruvate was used to start the reaction. The total assay mixture volume was 1 ml. The rate of decrease of absorbance was read at 340 nm.

The reaction assayed was as follows:-

$$\text{Pyruvate} + \text{NADH} \underset{}{\overset{\text{LDH}}{\rightleftharpoons}} \text{Lactate} + \text{NAD}^+$$

2.6.14 Pyruvate Dehydrogenase

This enzyme was assayed in a modified assay system similar to that for α-ketoglutarate dehydrogenase as described by Overath *et al* (1986).

The assay medium contained 150 mM Tris-HCl buffer pH 7.4, 3 mM cystein, 0.2 mM coenzyme A, 4 mM NAD^+ 0.2 mM thiamine pyrophosphate, and 5.4 mM sodium pyruvate. The reaction was started by addition of 0.01 ml of the sample to give a total assay volume of 1 ml. The rate of increase in absorbance was read at 340 nm.

The reaction assayed for was:

$$\text{Pyruvate} + \text{CoASH} + \text{NAD}^+ \xrightarrow[\text{Cyst, TPP}]{\text{PDH}} \text{AcetylCoA} + \text{NADH} + \text{Co}_2$$

2.6.15 Glycerokinase (E.C. 2.7.1.30)

Glycerokinase was assayed according to Bergmeyer (1974) in glycine-hydrazine buffer, pH 9.8 (182 mM glycine and 0.91 mM hydrazine) containing 1.82 mM $MgCl_2$, 0.41 mM NAD^+, 3.0 mM glycerol, 3.6 I.U/ml glycerol-3-phosphate dehydrogenase and 20 µl sample. The reaction was started by the addition of 1.35 mM ATP (sodium salt) to a final assay volume of 1 ml. The rate of increase of absorbance was read at 340 nm. The reaction measured was as follows:-

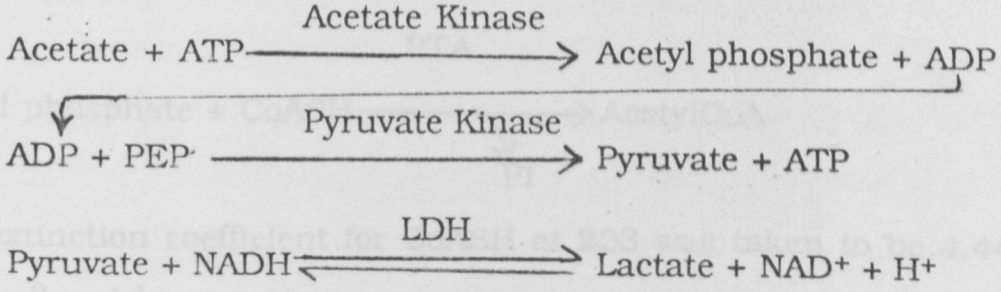
$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + \text{NAD}^+ \xrightarrow{\text{GPDH}} \text{DHAP} + \text{NADH} + \text{H}^+$$

2.6.16 Acetate Kinase (E.C. 2.7.2.1)

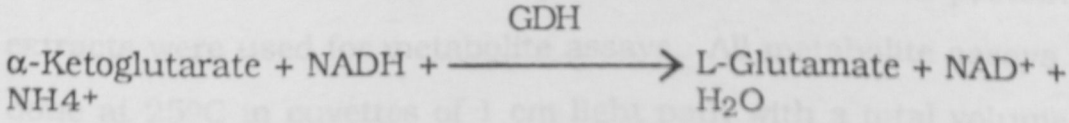
This enzyme was assayed essentially as described by Bergmeyer (1974) in 67 mM triethanolamine buffer pH 7.6 containing 5.4 mM ATP (sodium salt), 1.1 mM phosphoenol pyruvate, 0.32 mM NADH, 1.33 mM $MgCl_2$, 2.7 I.U/ml pyruvate kinase, 4.8 I.U/ml myokinase, 8.3 I.U/ml lactate dehydrogenase and 20 µl of the sample. This mixture was left to equilibrate and the reaction started only when the rate of change of absorbance was zero. This was to enable the ATPase activity come to completion. The reaction was then started with 333 mM sodium acetate. The final volume of the assay mixture was 1 ml. The rate of decrease of

absorbance was read at 340 nm. The reactions in the cuvette that was being assayed for were as follows:-



2.6.17 Glutamate dehydrogenase (E.C. 1.4.4.1.3)

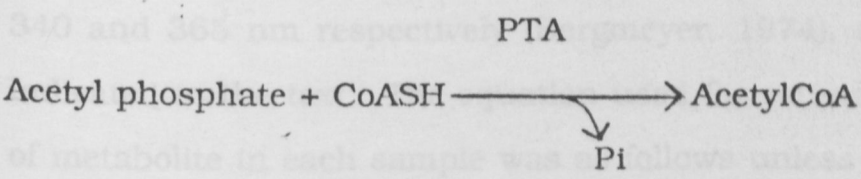
This was assayed as described by Overath *et al* (1986). The reaction mixture contained 50 mM Tris/HCl buffer pH 7.4, 50 mM ammonium chloride, 10 mM sodium oxoglutarate and 0.2 mM NADH. The reaction was started by the addition of 20 µl sample. The total assay volume was 1 ml and the rate of decrease of absorbance measured at 340 nm. The reaction that was assayed for was as follows:-



2.6.18 Phosphotransacetylase (E.C. 2.3.1.8)

This was assayed according to Bergmeyer (1974) in 85.6 mM Tris-acetate buffer pH 7.4 containing 1.7 mM glutathione, 0.4 mM CoASH, 19 mM ammonium sulphate and 20 µl sample (suspended in 25 mM tris buffer pH 8.0). The reaction was started with acetyl phosphate whose final concentration was 7.4 mM. The total assay

volume was 1 ml. The rate of increase of absorbance was read at 233 nm. The reaction assayed for was:-



The extinction coefficient for CoASH at 233 was taken to be 4.44 x 10^6 cm^2 mol^{-1}.

2.7 METABOLITE ASSAYS

When the end products of either glucose or proline catabolism were to be determined, then trypanosome incubations were carried out in a Dubnoff metabolic shaking incubator in a 5 ml bottle. The final volume of the incubation mixture was 0.2 ml. Metabolism was stopped by deproteinization with perchloric acid to a final concentration of 7% (v/v). The deproteinized samples were neutralized with 6 N KOH. Aliquots of the neutralized protein free extracts were used for metabolite assays. All metabolite assays were done at 25°C in cuvettes of 1 cm light path with a total volume of 1 ml. Absorbances were read on a Perkin-Elmer 550 UV/VIS spectrophotometer.

The end products of glucose catabolism were measured in the absence and presence of SHAM. This represented aerobic and anaerobic modes of metabolism respectively. Whenever SHAM was absent then all the readings of the generation or reduction of NADH were taken at 340 nm, but when SHAM was present then the readings were taken at 365 nm.

An 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 equation used for estimating the amount of metabolite in each sample was as follows unless otherwise stated in the text.

$$C = \frac{\Delta E}{6.22 \text{ or } 3.4} \times \frac{V}{v} \times \frac{1}{p}$$

Where C = concentration of respective metabolite in µmoles/30 min/mg protein

ΔE = change in absorbance during the assay

V = total assay volume

v = volume of the extract

In all cases the absorbance was allowed to stabilize and the initial reading taken (E_1). At the end of the reaction i.e. when the rate of change of absorbance was zero, the final reading (E_2) was also taken. The difference between the final reading and the initial reading (E_2-E_1) gave ΔE.

2.7.1 Pyruvate

Pyruvate was assayed according to Bergmeyer (1974) in an assay mixture consisting of 477 mM triethanolamine buffer pH 7.6; 4.8 mM EDTA; 0.12 mM NADH and 0.05 ml of the neutralized extract. Before the reaction was started the absorbance was left to stabilize and the initial reading of the absorbance taken at 340 nm. The reaction was started by addition of 2.75 I.U/ml of LDH. The final assay volume was 1 ml. The final reading of absorbance was taken at 340 nm when the rate of change of absorbance was zero. The reaction in the assay was as below:-

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

2.7.2 Alanine

This was assayed as described by Olembo (1980) in a medium consisting of 92 mM glycine 70 mM hydrazine buffer pH 10, 2 mM NAD^+ and 0.02 ml of the neutralized extract. The reaction was started by the addition of 0.38 I.U/ml of alanine dehydrogenase (free from ammonium ions). The final volume of the assay mixture was 1 ml. Before starting the reaction the absorbance was allowed to stabilize and then the initial reading was taken. The final reading of the absorbance was taken when the rate of change of absorbance was zero (after about one hour). The absorbances for every determination was read at 340 nm. The reaction in the assay cuvette was:-

$$\text{L-alanine} + NAD^+ + H_2O \xrightleftharpoons{ADH} \text{Pyruvate} + NADH + NH_4^+$$

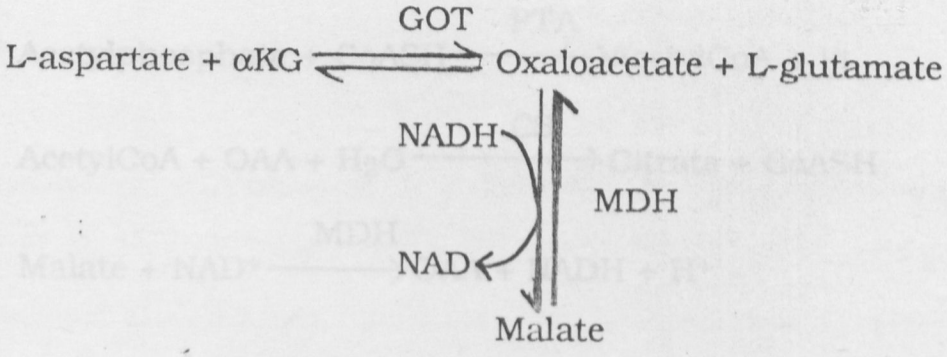
2.7.3 Glutamate

Glutamate was assayed according to Bergmeyer (1974) in an assay medium containing 290 mM glycine - 232 mM hydrazine buffer pH 9.0, 0.97 mM ADP and 1.6 mM NAD^+. The reaction was started by 4.5 I.U/ml glutamate dehydrogenase (ammonium ions free). The total volume of the assay mixture was 1 ml. The initial value of the absorbance was read before the start of the reaction and the final one read after about one hour when the rate of change of absorbance was zero. All measurements were done at 340 nm. The reaction assayed for was:-

$$\text{L-glutamate} + NAD^+ + H_2O \xrightleftharpoons[ADP]{GDH} \alpha\text{-ketoglutarate} + NH_4^+ + NADH$$

2.7.4 Aspartate

This was assayed in 61.5 mM sodium phosphate buffer pH 7.2 containing 0.18 mM NADH 20 µl sample and 3 mM α-ketoglutarate. The reaction was started by addition of 17 I.U/ml each of malate dehydrogenase and glutamate oxoglutarate transaminase. The final assay volume was 1 ml. The final absorbance was read when the rate of change of absorbance was zero (after about one hour). The reaction was



The values of the absorbances were read at 340 nm.

2.7.5 Acetate

Acetate was assayed essentially as descried by Bergmeyer (1974) in a reaction mixture consisting of 0.15 M triethanolamine buffer pH 7.6, 10 mM L-malate, 0.40 mM CoASH, 5.4 mM ATP, 1.33 $MgCl_2$ 1mM NAD^+, 4.0 I.U/ml LDH, 5.0 I.U/ml MDH, 5.0 I.U/ml phosphoacetyl transferace, 5.0 I.U/ml citrate synthase, 5.0 I.U/ml acetate kinase and the extract. The reaction was started by addition of acetate kinase. The sequence of addition was:- First the buffer containing malate, Mg^{2+}, NAD^+, CoASH, ATP, MDH and extract was pipetted in the cuvette. The absorbance was left to stabilize and absorbance, E_1, read at 340 or 365 nm. Next to be added was 5.0 I.U/ml of PTA, CS and LDH. After the absorbance had stabilized then the reaction was started by addition of 5.0 I.U/ml of acetate kinase. The final assay volume was 1 ml. The final absorbance, E_2, was taken at 340 or 365 nm after the reaction had stopped which was approximately one hour. The reaction beng assayed for were:-

(a) Acetate + ATP $\xrightarrow{AK}$ Acetyl phosphate + ADP

(b) $\text{Acetylphosphate} + \text{CoASH} \xrightarrow{\text{PTA}} \text{AcetylCoA} + \text{Pi}$

(c) $\text{AcetylCoA} + \text{OAA} + H_2O \xrightarrow{\text{CS}} \text{Citrate} + \text{CoASH}$

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

The overall reaction in the cuvette was :-

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

The amount of acetate was estimated by using the formula below which has been described earlier (see sub section 2.7).

$\Delta E_{acetate}$ which is the change in absorbance due to acetate in the extract was calculated as follows:-

$$\Delta E_{acetate} = \frac{E_2 - E_1}{E_2}^2$$

$$C = \frac{\Delta E_{acetate}}{6.22} \times \frac{V}{v} \times \frac{1}{P}$$

where E_2 = final value of absorbance

E_1 = initial value of absorbance

2.7.6 Glycerol

Glycerol was assayed in a medium containing 0.19 M glycine-0.69 M hydrazine buffer pH 9.5, 5 mM $MgSO_4$, 2.3 mM NAD^+, 5 I.U/ml of glycerokinase and glycerol-3-phosphate dehydrogenase and 0.02 ml of the extract. The reaction was started by addition of 4 mM ATP. The final assay volume was 1 ml. The initial value of absorbance was read 340 or 365 nm before starting the reaction while the final value of absorbance was taken at the end of the reaction. The reaction in the cuvette was as follows:-

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + \text{NAD}^+ \xrightarrow{\text{GPDH}} \text{DHAP} + \text{NADH} + \text{H}^+$$

2.7.7 Succinate

Succinate was assayed in a medium containing 50 mM potassium phosphate buffer pH 7.4, 25 mM sucrose, 1mM KCN and 0.02 ml of the extract. To this was added 0.025 ml of 10 mg/ml PMS, 0.05 ml of 10 mg/ml INT. The reaction was started by addition of 0.1 mg protein of rat liver mitochondria (to provide the enzyme succinate dehydrogenase). The final volume of the incubation mixture was 1 ml. The mixture was incubated at room temperature for 30 minutes. Control incubations were treated in the same way except that rat liver mitochondrial protein was not added. The incubations were terminated by the addition of 1 ml of

10% trichloroacetic acid. A volume of 4 mls ethyl acetate was added into each tube and the mixture shaken vigorously for approximately 30 seconds. The tubes were then left to stand until the ethyl acetate layer cleared. The ethyl acetate layer was removed with a pasteur pipette and its absorbance read at 490 nm against ethyl acetate as a blank. All incubations with and without SHAM were read at this wavelength.

The extinction coefficient was taken to be 20.1×10^6 cm^{-1} mol^{-1}) (Pennington, 1961).

The amount of succinate produced was estimated as follows:-

$$C = \frac{\Delta E}{20.1} \times \frac{V}{v} \times \frac{D}{p}$$

where C = Amount of succinate produced in µmoles/30 min/mg protein

20.1 = Extinction coefficient of INT in ethyl acetate at 490 nm

V = Total incubation volume

v = Volume of the extract used in the assay

D = This is the dilution factor caused by addition of a known volume of ethyl acetate. This volume is equivalent to D.

2.8 PROTEIN DETERMINATION

The amount of protein was determined by the method of Lowry *et al* (1951). A standard curve was constructed using crystalline bovine serum (fraction V-essentially fatty acid free) obtained from Sigma Chemical Company. The curve was constructed with standard BSA samples ranging from 0 µg to 40 µg which was found to lie within the linear range. A solution, A was perepared by mixing 50 ml of 2% (w/v) anhydrous sodium carbonate, 0.02% (w/v) potassium-sodium tartrate in 0.1 M sodium hydroxide and 1.0 ml of 0.05% (w/v) copper sulphate solution. This was done 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. After ten minutes 0.08 mls of solution B was added, shaken thoroughly and incubated for thirty minutes. The total assay volume was 1 ml. After thirty minutes the absorbances were read using a Perkin Elmer 550 - UV/vis spectrophotometer at 578 nm.

CHAPTER THREE

GLUCOSE CATABOLISM IN PROCYCLIC *TRYPANOSOMA CONGOLENSE*

3.1 EFFECT OF VARIOUS METABOLIC INHIBITORS ON RESPIRATION BY *TRYPANOSOMA CONGOLENSE* PROCYCLICS UTILIZING GLUCOSE AS SUBSTRATE

3.1.1 Respiration on glucose and the effect of SHAM

The effect of SHAM on the oxygen consumption by *T. congolense* procyclics was investigated. Figure 1(a) shows that addition of SHAM to an incubation containing respiring trypanosomes caused a decrease in the rate of oxygen consumption. The figure shows that the rate of respiration decreased from 10.0 to 6.37 nmol/min/mg protein. From six separate determinations the rate of oxygen consumption decreased on the average from 10.3 $\pm$ 1.0 to 6.6 $\pm$ 0.5 nmol/min/mg protein. This represented a percentage inhibition of 36.2 $\pm$ 4.2% (table 1). These results suggest that procyclic *T. congolense* utilize glycerol-3-phosphate oxidase system during the catabolism of glucose. The same oxidase system has also been proposed in *T. b. brucei* procyclics (Njogu *et al*, 1980). Apparently SHAM could not inhibit oxygen consumption completely. This has also been observed in *T.b. brucei* procyclics (Njogu *et al.*, 1980).

Table 1

The effect of various metabolic inhibitors on the rate of oxygen consumption of *T. congolense* procyclics utilizing glucose.

Isolated trypanosomes were washed 5 times and suspended in PSG. They were then introduced into the electrode chamber also pre-equilibrated with 1.2 mls of PSG. The rates of respiration before and after the addition of various metabolic inhibitors were then noted as indicated in figures 1(a) - (i). The results are expressed as mean plus or minus the standard deviation. The numbers in brackets indicate the total number of separate determinations.

Table 1

Initial Rate of oxygen consumption nmol/min/ mg protein	Inhibitor added	Rate of oxygen consumption after addition of inhibitor nmol/min/mg protein	Percentage Inhibition
10.3 ± 1.0 (6)	SHAM	6.6 ± 0.5 (6)	36.2 ± 4.2 (6)
11.1 ± 0.3 (5)	KCN	5.1 ± 0.5 (5)	54.1 ± 5.5 (5)
10.9 ± 0.3 (6)	KCN + SHAM	1.6 ± 0.2 (6)	85.3 ± 1.6 (6)
10.6 ± 0.4 (5)	Malonate	7.1 ± 0.2 (5)	32.3 ± 1.9 (5)
10.8 ± 0.3 (3)	Malonate + SHAM	3.5 ± 0.2 (3)	67.1 ± 0.9 (3)
10.6 ± 0.8 (5)	Antimycin A	6.4 ± 0.8 (5)	39.4 ± 4.2 (5)
10.7 ± 0.9 (4)	Antimycin A+ SHAM	1.5 ± 0.5 (4)	86.3 ± 4.2 (4)
10.4 ± 0.4 (3)	Antimycin_ A+ Malonate	6.2 ± 0.3 (3)	40.7 ± 0.8 (3)
10.5 ± 0.6 (3)	Rotenone	10.0 ± 0.9 (3)	5.0 ± 3.8 (3)

Figure 1 (a) **The effect of SHAM on the rate of oxygen consumption by *Trypanosoma congolense* procyclics utilizing glucose as substrate.**

A suspension containing 1.1 x 10^8 trypanosomes (equivalent to 1.07 mg protein) was introduced into the chamber housing the oxygen electrode containing 1.2 mls PSG. A steady (linear) rate of oxygen consumption was noted. SHAM was then added to a final concentration of 0.5 mM (Njogu *et al*, 1980) and the new rate of oxygen consumption recorded as shown.

Figure 1(b) **Effect of KCN on rate of oxygen consumption by *T. congolense* procyclics utilizing glucose as substrate**

A suspension containing 7.6 x 10^7 trypanosomes (equivalent to 0.82 mg protein) was introduced into the chamber housing the oxygen electrode containing 1.2 mls PSG. A steady rate of oxygen consumption was noted. KCN was added into the chamber to a final concentration of 0.1 mM (Njogu *et al*, 1980) and the new rate of oxygen consumption recorded as shown.

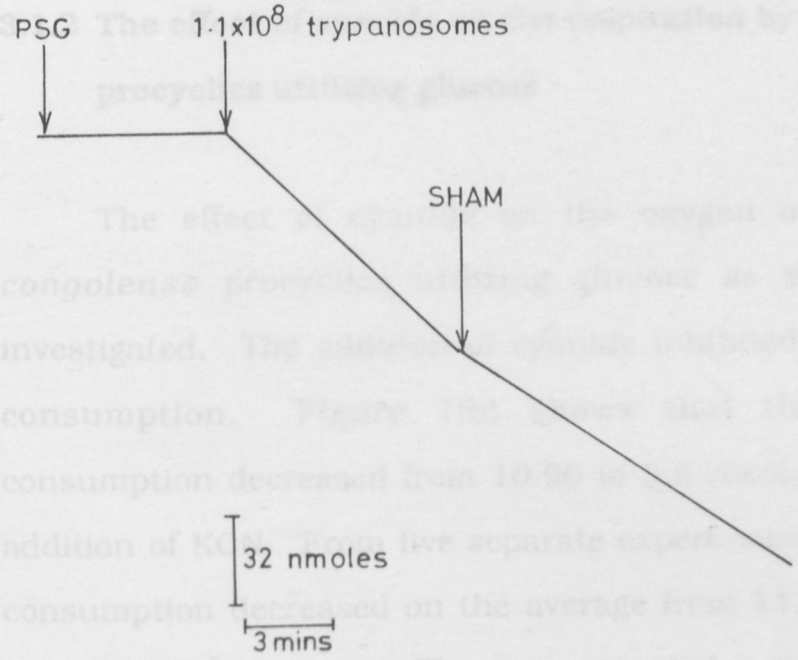


Figure 1(a)

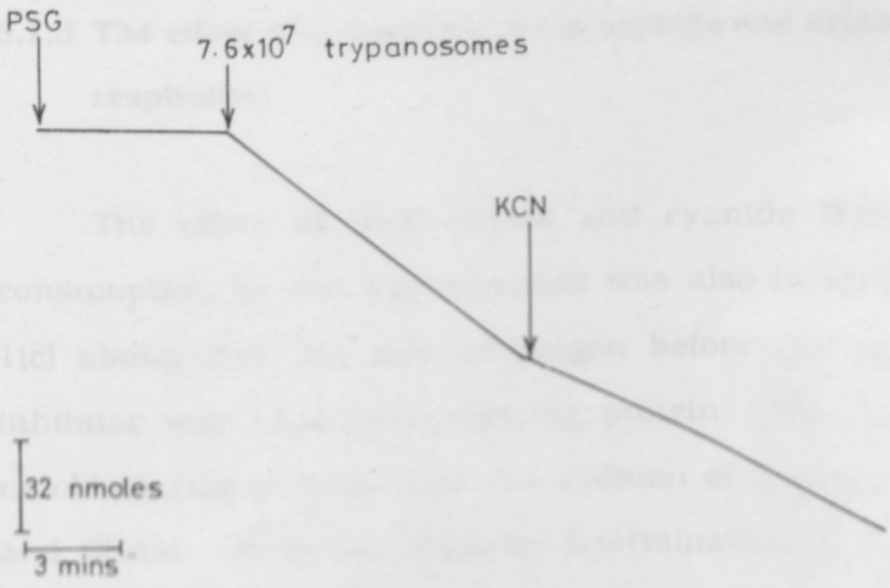


Figure 1(b)

3.1.2 The effect of cyanide on the respiration by *T. congolense* procyclics utilizing glucose

The effect of cyanide on the oxygen consumption by *T. congolense* procyclics utilizing glucose as substrate was also investigated. The addition of cyanide inhibited the rate of oxygen consumption. Figure 1(b) shows that the rate of oxygen consumption decreased from 10.90 to 5.6 nmol/min/mg protein on addition of KCN. From five separate experiments the rate of oxygen consumption decreased on the average from 11.1 $\pm$ 0.2 to 5.1 $\pm$ 0.5 nmol/min/mg protein. These represented a percentage inhibition of 54.1 $\pm$ 5.5% (Table 1). This observation suggests the presence of cytochrome aa_3 oxidase which is usually inhibited by cyanide. Incomplete inhibition of oxygen consumption by cyanide has also been observed in *T. brucei* procyclics (Njogu *et al*, 1980).

3.1.3 The effect of a combination of cyanide and SHAM on the respiration

The effect of both SHAM and cyanide (KCN) on oxygen consumption by the trypanosomes was also investigated. Figure 1(c) shows that the rate of oxygen before the addition of any inhibitor was 11.0 nmol/min/mg protein. This rate fell to 1.3 nmol/min/mg protein upon the addition of a combination of KCN and SHAM. From six separate determinations, a combination of SHAM and KCN had the effect of decreasing the rate of oxygen consumption from 10.9 $\pm$ 0.3 to 1.6 $\pm$ 0.3 nmol/min/mg protein. This represented a percentage inhibition of 85.3 $\pm$ 1.6%. These

Figure 1(c) Effect of a combination of SHAM and KCN on the oxygen consumption by *T. congolense* procyclics utilizing glucose as substrate.

A suspension containing 1.1 x 10^8 trypanosomes was introduced into the oxygen electrode chamber pre-equilibrated with 1.2 mls of PSG. The rate of respiration before and after addition of a combination of SHAM and KCN to a final concentration of 0.5 mM and 0.1 mM respectively was recorded as shown. The trypanosomes added were equivalent to 1.07 mg protein.

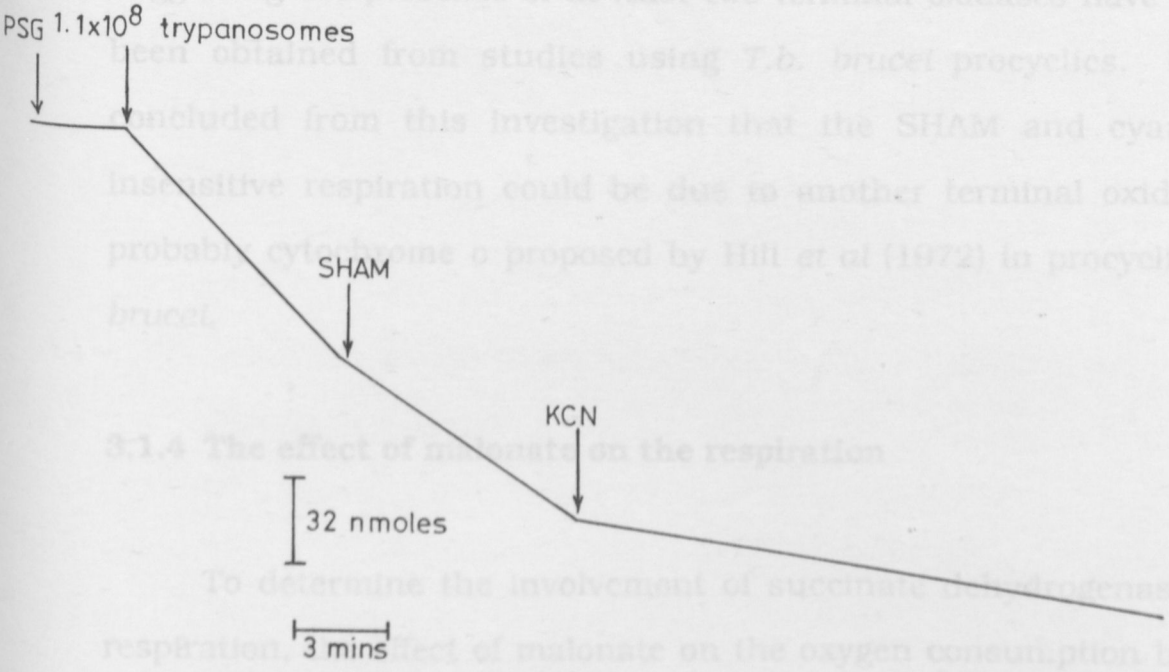


Figure 1(c)

are shown in Table 1. These results suggest that procyclic *T. congolense* possess at least two terminal oxidases : the SHAM sensitive oxidase and the cyanide sensitive oxidase. The SHAM sensitive oxidase is insensitive to cyanide and vice versa. Results suggesting the presence of at least two terminal oxidases have also been obtained from studies using *T.b. brucei* procyclics. It is concluded from this investigation that the SHAM and cyanide insensitive respiration could be due to another terminal oxidase, probably cytochrome o proposed by Hill *et al* (1972) in procyclic *T. brucei*.

3.1.4 The effect of malonate on the respiration

To determine the involvement of succinate dehydrogenase in respiration, the effect of malonate on the oxygen consumption by *T. congolense* procyclics incubated with glucose was investigated. During sequential additions of malonate it was observed that there was a gradual decrease in the rate of oxygen consumption. Addition of upto 25 mM of malonate caused maximum inhibition. Further addition of malonate did not cause additional decrease in the rate of oxygen consumption. Figure 1 (d)(i) shows plots of the rate of oxygen consumption against malonate concentration during the catabolism of glucose. Addition of malonate had an inhibitory effect on the rate of oxygen utilization. Figure 1 (d)(ii) shows that before addition of 25 mM malonate the rate of respiration was 11.0 nmol/min/mg protein. After addition of malonate the rate fell to 7.3 nmol/min/mg protein. From six separate determinations malonate had the effect of reducing the rate of oxygen consumption

Figure 1 (d) (i) : Effect of increasing concentrations of malonate on the rate of oxygen consumption of procyclic *T. congolense* utilizing glucose.

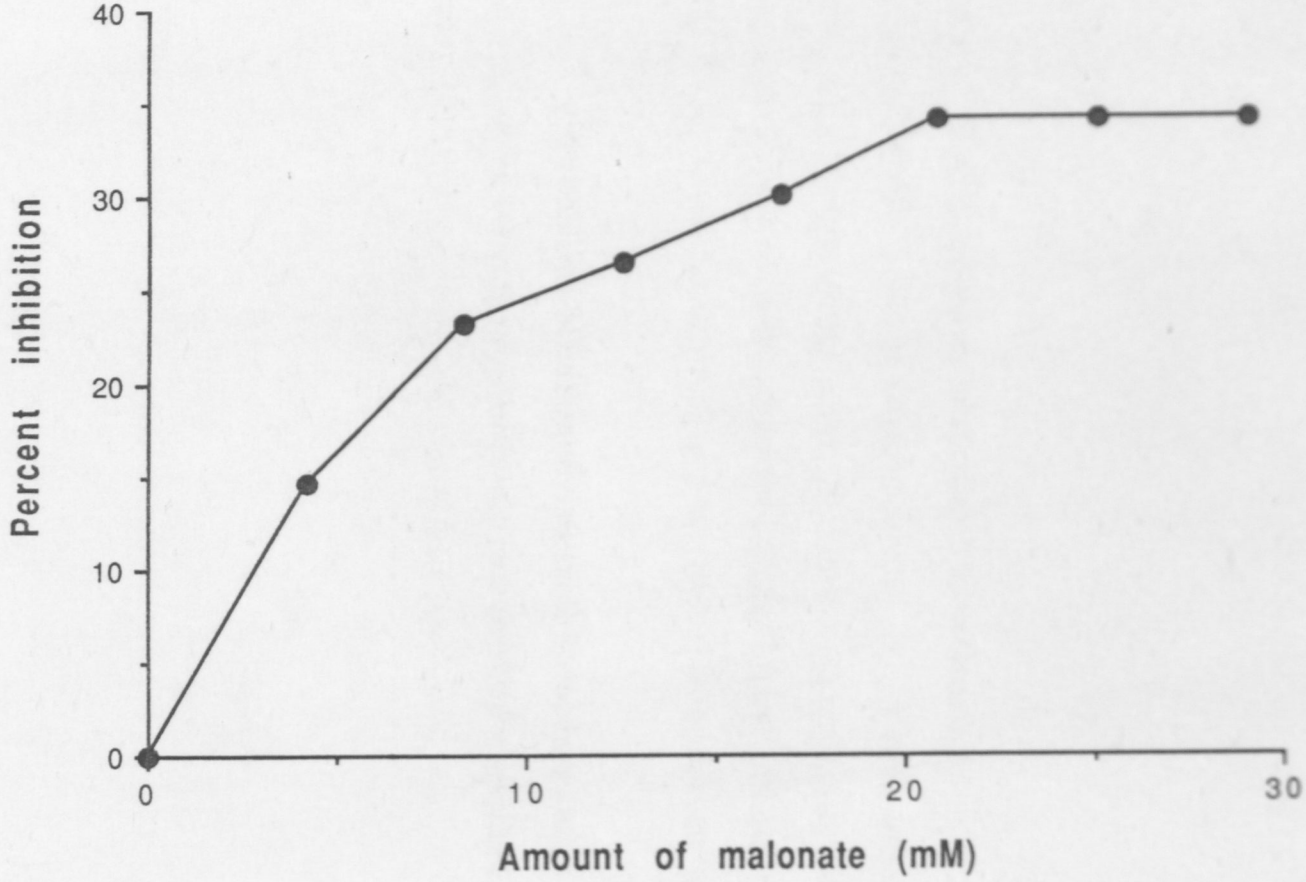

Percent inhibition
40
30
20
10
0
0
10
20
30
Amount of malonate (mM)

Figure 1(d)(i)

Figure 1(d)(ii) Effect of malonate on the oxygen consumption by *T. congolense* procyclics utilizing glucose as substrate

A suspension containing 6.1 x 10^7 trypanosomes (equivalent to 0.6 mg protein) was introduced into the oxygen electrode chamber pre-equilibrated with 1.2 mls of PSG and the rate of respiration noted. A final concentration of 25 mM malonate was then added and the rate of respiration determined.

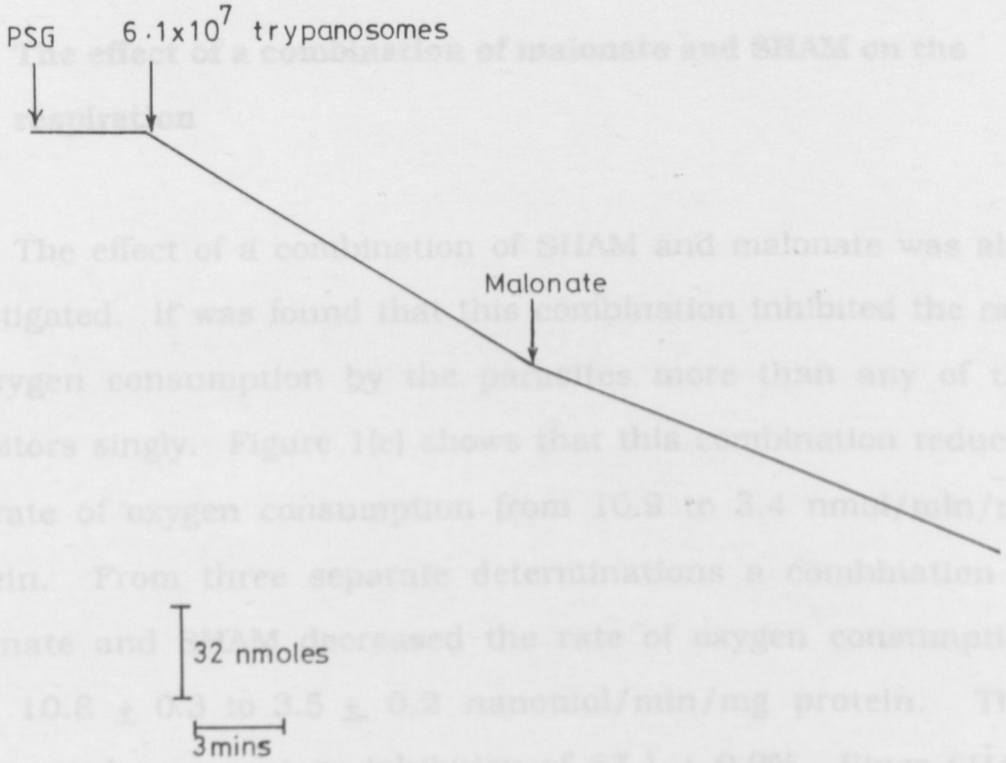


Figure 1(d)(ii)

These results suggest that succinate dehydrogenase (which is competitively inhibited by malonate) is involved in the catabolism of glucose in *T. congolense* procyclics. It could be inferred that reducing equivalents derived from glucose catabolism are oxidized by molecular oxygen via succinate dehydrogenase.

3.1.5 The effect of a combination of malonate and SHAM on the respiration

The effect of a combination of SHAM and malonate was also investigated. It was found that this combination inhibited the rate of oxygen consumption by the parasites more than any of the inhibitors singly. Figure 1(e) shows that this combination reduced the rate of oxygen consumption from 10.9 to 3.4 nmol/min/mg protein. From three separate determinations a combination of malonate and SHAM decreased the rate of oxygen consumption from 10.8 $\pm$ 0.3 to 3.5 $\pm$ 0.2 nanomol/min/mg protein. This represented a percentage inhibition of 67.1 $\pm$ 0.9%. Since SHAM alone inhibits the respiration by 36.2 $\pm$ 4.2% and malonate alone by 32.3 $\pm$ 1.9% (Table 1), it could be suggested that the GPO and the succinate dehydrogenase catalyse the reduction of different substrates (reducing equivalents).

3.1.6 The effect of antimycin A on the respiration

The effect of antimycin A alone on the respiration was also investigated. Figure 1(f) shows that addition of antimycin A reduced the rate of oxygen consumption from 10.7 to 7.2 nmol/min/mg protein. From five separate determinations it was

Figure 1(e) Effect of a combination of malonate and SHAM on the rate of oxygen consumption by *T. congolense* procyclics utilizing glucose as substrate.

A suspension containing 3.9 x 10^7 trypanosomes (equivalent to 0.44 mg protein) was introduced into the chamber of oxygen electrode pre-equilibrated with 1.2 mls PSG and the rate of oxygen uptake noted. Twenty five mM of malonate was then added followed with the addition of 0.5 mM SHAM. The rate of oxygen consumption was again noted.

Figure 1(f) Effect of antimycin A on the rate of oxygen consumption by *T. congolense* procyclics utilizing glucose as substrate

A suspension containing 3.4 x 10^7 trypanosomes (equivalent to 0.37 mg protein) was introduced into the chamber pre-equilibrated with 1.2 mls PSG. The rate before and after addition of 10 µl of 1 mg/ml antimycin A were then recorded as shown.

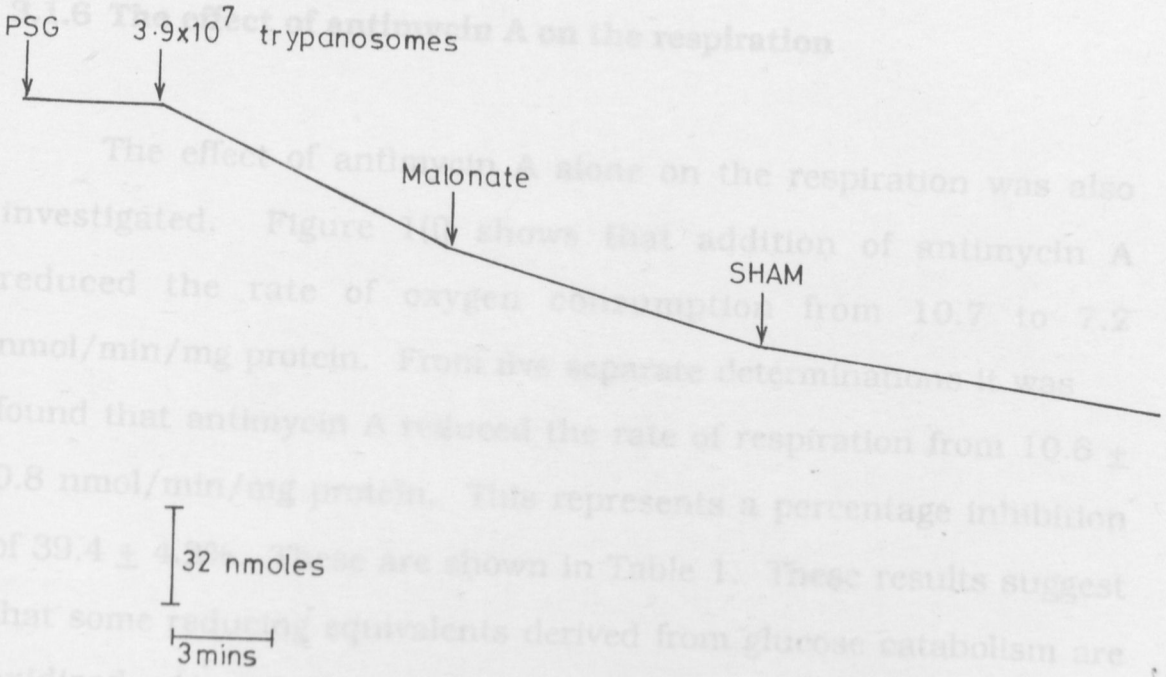


Figure 1(e)

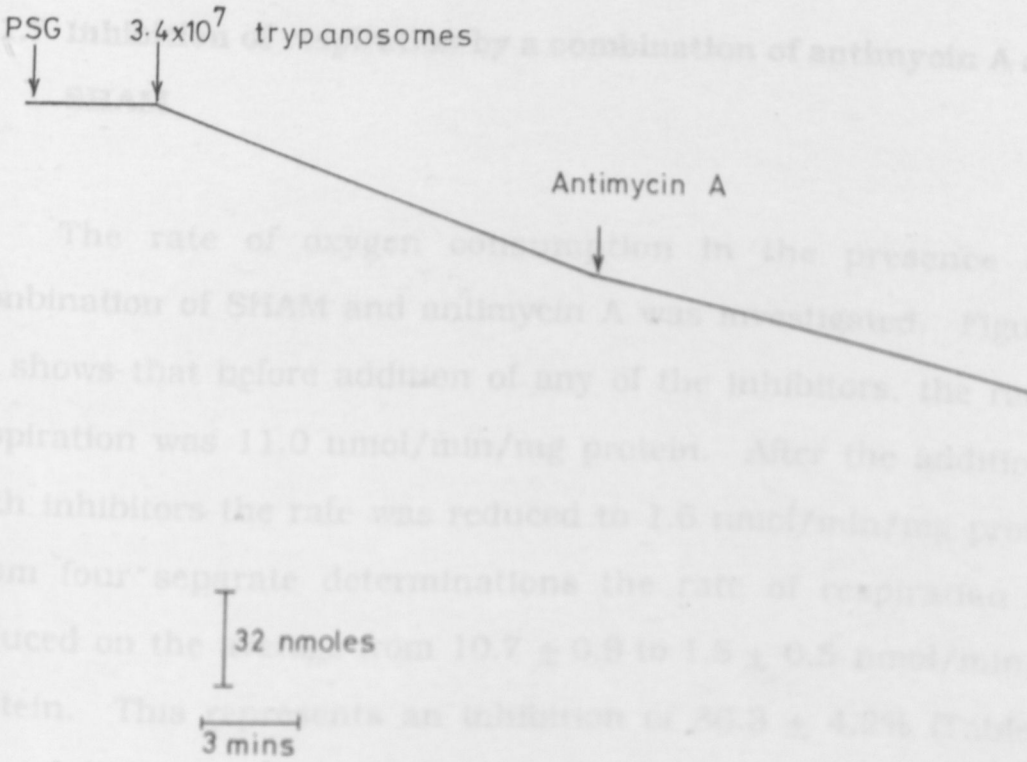


Figure 1(f)

3.1.6 The effect of antimycin A on the respiration

The effect of antimycin A alone on the respiration was also investigated. Figure 1(f) shows that addition of antimycin A reduced the rate of oxygen consumption from 10.7 to 7.2 nmol/min/mg protein. From five separate determinations it was found that antimycin A reduced the rate of respiration from 10.6 ± 0.8 nmol/min/mg protein. This represents a percentage inhibition of 39.4 ± 4.2%. These are shown in Table 1. These results suggest that some reducing equivalents derived from glucose catabolism are oxidized via cytochrome b and cytochrome c_1. Incomplete inhibition of oxygen consumption by antimycin A has also been observed in *T. brucei* procyclics (Njogu *et al*, 1980). It is suggested that an electron transport chain with cytochrome b and cytochrome c_1 is present in procyclic *T. congolense*.

3.1.7 Inhibition of respiration by a combination of antimycin A and SHAM

The rate of oxygen consumption in the presence of a combination of SHAM and antimycin A was investigated. Figure 1 (g) shows that before addition of any of the inhibitors, the rate of respiration was 11.0 nmol/min/mg protein. After the addition of both inhibitors the rate was reduced to 1.6 nmol/min/mg protein. From four separate determinations the rate of respiration was reduced on the average from 10.7 ± 0.9 to 1.5 ± 0.5 nmol/min/mg protein. This represents an inhibition of 86.3 ± 4.2% (Table 1). The inhibition here was apparently similar to that observed in the presence of a combination of KCN and SHAM. These results

Figure 1(g) Effect of a combination of SHAM and antimycin A on the respiration by procyclic *T. congolense* catabolizing glucose.

A suspension containing 7.3 x 10^7 trypanosomes (equivalent to 0.74 mg protein) was introduced into the chamber housing the oxygen electrode containing 1.2 ml PSG and the rate of respiration noted. Ten microlitres of 100 mM SHAM was then added followed with the addition of 10 µl of 10 mg/ml antimycin A. The rate of respiration was then determined.

Figure 1(h) Effect of a combination of malonate and antimycin A on the rate of respiration by *T. congolense* procyclics utilizing glucose as substrate

A suspension containing 3.5 x 10^7 trypanosomes (equivalent to 0.47 mg/ protein) was introduced into the oxygen electrode chamber containing 1.2 ml PSG and the rate of respiration noted. After this 25 µl of 1 M malonate was added into the chamber followed by 10 µl of 10 mg/ml antimycin A. The rate of oxygen uptake was again noted.

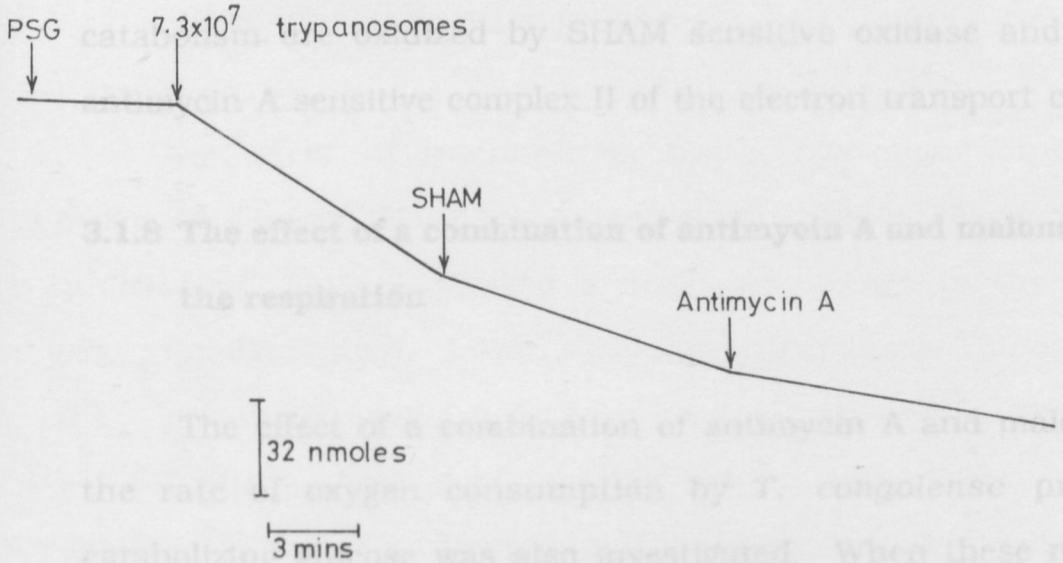


Figure 1(g)

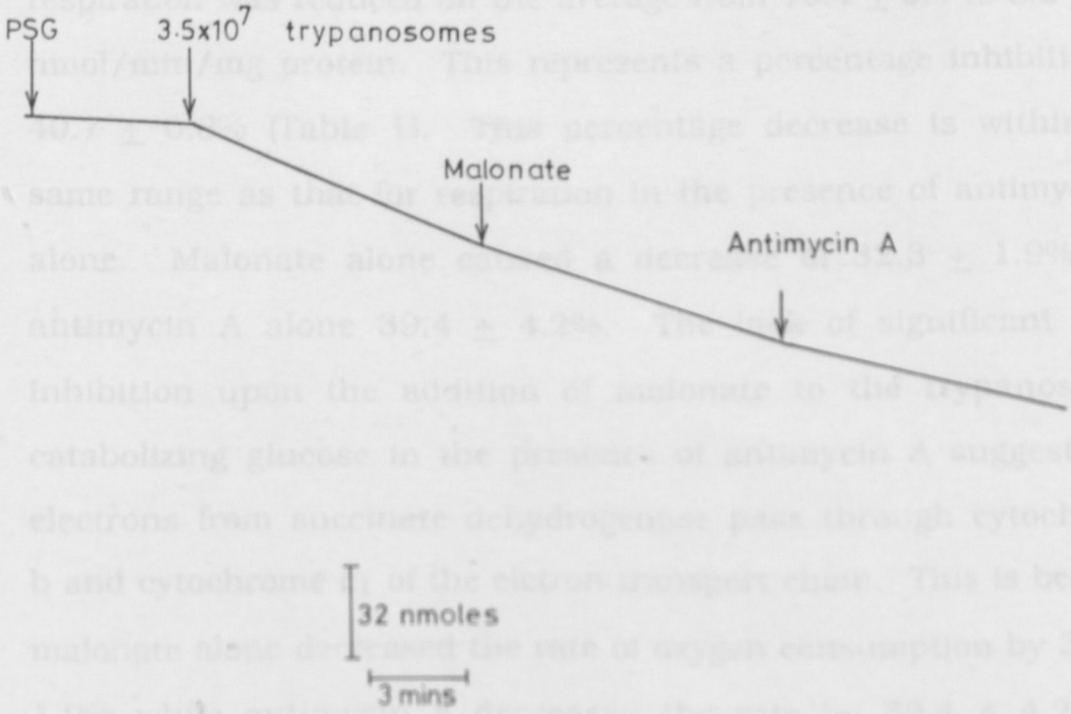


Figure 1(h)

suggest that most of the reducing equivalents derived from glucose catabolism are oxidized by SHAM sensitive oxidase and via the antimycin A sensitive complex II of the electron transport chain.

3.1.8 The effect of a combination of antimycin A and malonate on the respiration

The effect of a combination of antimycin A and malonate on the rate of oxygen consumption by *T. congolense* procyclics catabolizing glucose was also investigated. When these metabolic inhibitors were added, they caused a decrease in the rate of oxygen consumption. Figure 1(h) shows that addition of these inhibitors reduced the rate of respiration from 10.5 to 6.2 nmol/min/mg protein. From three separate determinations, the rate of respiration was reduced on the average from 10.4 ± 0.4 to 6.2 ± 0.3 nmol/min/mg protein. This represents a percentage inhibition of 40.7 ± 0.8% (Table 1). This percentage decrease is within the same range as that for respiration in the presence of antimycin A alone. Malonate alone caused a decrease of 32.3 ± 1.9% and antimycin A alone 39.4 ± 4.2%. The lack of significant extra inhibition upon the addition of malonate to the trypanosomes catabolizing glucose in the presence of antimycin A suggest that electrons from succinate dehydrogenase pass through cytochrome b and cytochrome c_1 of the elctron transport chain. This is because malonate alone decreased the rate of oxygen consumption by 32.3 ± 1.9% while antimycin A decreased the rate by 39.4 ± 4.2%, in combination the two inhibited oxygen consumption by 40.7 ± 0.8% (Table 1).

3.1.9 The effect of rotenone on the respiration by procyclic *T. congolense* catabolising glucose

The effect of rotenone on the oxygen consumption by procyclic *T. congolense* was investigated. Figure 1(i) shows that addition of rotenone caused a negligible change in the rate of oxygen consumption. From 3 separate experiments rotenone had the effect of decreasing the rate of oxygen consumption from 10.5 $\pm$ 0.6 to 10.0 $\pm$ 0.9 nmol/min/mg protein. This represented a decrease in the rate of oxygen consumption by 5.0 $\pm$ 3.8% (Table 1). These results suggest that either procyclic *T. congolense* do not have the NADH dehydrogenase segment in their electron transport chain or that the NADH dehydrogenase in these parasites is modified such that rotenone does not have any effect on it. It could also suggest that electrons derived from glucose catabolism are channelled to molecular oxygen via another site and not through the NADH dehydrogenase.

Figure 1 (i) : **Effect of rotenone on the respiration by procyclic *T. congolense* catabolizing glucose**

A suspension containing 2.56 x 10^7 trypanosomes (equivalent to 0.33 mg protein) was introduced into the chamber housing the oxygen electrode containing 1.2 ml PSG and the rate of respiration noted. Rotenone was then added at a final concentration of 5 μM. The rate of the respiration was again then determined.

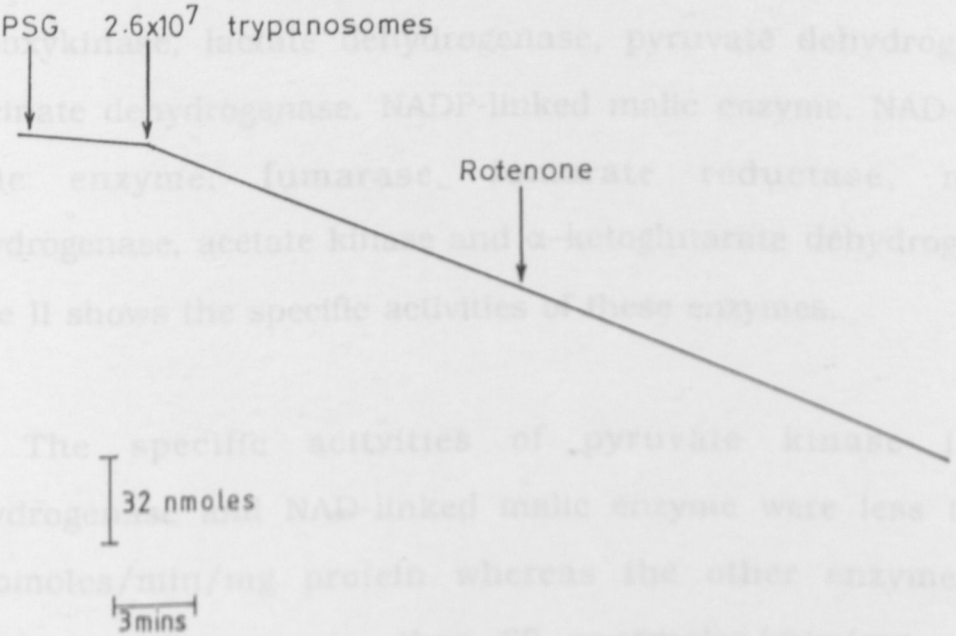


Figure 1 (i)

3.2 ENZYMES FOR THE CATABOLISM OF PHOSPHOENOLPYRUVATE DERIVED FROM GLYCOLYTIC INTERMEDIATES IN PROCYCLIC *T. CONGOLENSE*

All mammalian parasites catabolize glucose via the classical Embden-Meyeroff pathway to phosphoenolpyruvate. Thereafter each organism has adopted different metabolic mechanisms for the reoxidation of NADH generated from the oxidation of glyceraldehyde-3-phosphate (GAP) to 1,3 diphosphoglycerate (Fairlamb, 1989).

In this study the activities of some enzymes likely to be involved in the catabolism of phosphoenolpyruvate in procyclic *T. congolense* was investigated. The enzymes whose activities were investigated were pyruvate kinase, phosphoenolpyruvate carboxykinase, lactate dehydrogenase, pyruvate dehydrogenase, succinate dehydrogenase, NADP-linked malic enzyme, NAD-linked malic enzyme, fumarase, fumarate reductase, malate dehydrogenase, acetate kinase and α–ketoglutarate dehydrogenase. Table II shows the specific activities of these enzymes.

The specific acitvities of pyruvate kinase lactate dehydrogenase and NAD-linked malic enzyme were less than 6 nanomoles/min/mg protein whereas the other enzymes had specific activities greater than 60 nanomoles/min/mg protein. These observations suggest that *T. congolense* procyclics do not catabolize phosphoenolpyruvate via the pyruvate kinase and lactate dehydrogenase at significant rates. Phosphoenolpyruvate could be catabolized via another pathway. Further catabolism of

phosphoenolpyruvate may involve phosphoenolpyruvate carboxykinase, pyruvate dehydrogenase, succinate dehydrogenase, fumarase, NADP-linked malic enzyme, fumarase, fumarate reductase, malate dehydrogenase, acetate kinase and α-ketoglutarate dehydrogenase. This will discussed further in a later section.

Table II

Specific activities of some enzymes likely to be involved in the catabolism of phosphoenolpyruvate by procyclic *Trypanosoma congolense.*

Isolated trypanosomes were washed five times in PSG and then lysed with 0.1% Triton X100. The samples were then centrifuged at 1000 g at 4°C and the resulting supernatant assayed for the enzyme activities shown above. The assay temperatures for all determinations was 25°C. The results are expressed as mean plus or minus the standard deviation. The numbers in brackets represent the total number of separate determinations.

Enzyme	Specific activity in nmoles/min/mg protein
Pyruvate kinase	5.7 ± 0.9 (4)
PEP carboxykinase	61.3 ± 9.8 (4)
Pyruvate dehydrogenase	82.3 ± 8.1 (3)
Lactate dehydrogenase	4.5 ± 1.3 (3)
Succinate dehydrogenase	69.8 ± 10.1 (6)
NADP-linked malic enzyme	79.6 ± 4.3 (5)
NAD-linked malic enzyme	2.0 ± 1.0 (3)
Fumarase	66.7 ± 3.7 (6)
Fumarate reductase	72.0 ± 3.5 (5)
Malate dehydrogenase	1105.0 ± 6.6 (4)
Acetate kinase	95.8 ± 6.6 (4)
α–ketoglutarate dehydrogenase	61.4 ± 4.3 (5)
Glycerokinase	61.2 ± 2.1 (3)

Table II

3.3 END PRODUCTS OF GLUCOSE CATABOLISM

The end products of aerobic glucose catabolism were pyruvate, acetate and succinate. During anaerobic glucose catabolism (simulated by the addition of SHAM), the end products detected were pyruvate, acetate, succinate and glycerol. Table III shows the end products of glucose catabolism in the presence and absence of SHAM.

In the absence of SHAM the mean rates of pyruvate, acetate and succinate production were 0.218, 0.425 and 0.048 μmoles/30 min/mg protein respectively. In the presence of SHAM the mean rates of production of pyruvate, acetate and succinate were 0.095, 0.144 and 0.096 μmoles/30 min/mg protein respectively. The addition of SHAM also led to formation of glycerol at a mean rate of 0.147 μmoles/30 min/mg protein. Thus the addition of SHAM caused a decrease in the rates of production of pyruvate and acetate but it led to an increase in the rate of production of succinate.

From the above data during the aerobic catabolism of glucose the molar ratio of the mean rates of production of pyruvate: acetate:succinate was 9:18:2. From the data above during the catabolism of glucose in the presence of SHAM the molar ratio of the mean rates of production of pyruvate:acetate:succinate:glycerol was 2:3:2:3. It was proposed that the procyclic *T. congolense* might have a similar mechanism for glycerol production as bloodstream *Trypanosoma brucei* (Kiaira and Njogu, 1989).

Table III : End Products of glucose catabolism by procyclic *T. congolense* in the presence and absence of SHAM

Every sample of freshly isolated trypanosomes was divided into two. One of these was deproteinized immediately with PCA (i.e. at zero time). Both of these were then incubated in PSG for 30 minutes. The previously undeproteinized portion was then deproteinized with PCA after the incubation period. They were then processed for metabolite assays as described in materials and methods. All the results were corrected for zero time. The results are expressed as mean plus or minus standard deviation. The figures in brackets represent the total number of separate determinations.

Table III : End Products of glucose catabolism by procyclic *T. congolense* in the presence and absence of SHAM

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End Product	Rate of production in μmoles/30 min/mg protein	
	NO SHAM	PLUS SHAM
Pyruvate	0.218 ± 0.018 (6)	0.095 ± 0.010 (7)
Acetate	0.425 ± 0.023 (4)	0.144 ± 0.016 (7)
Glycerol	Not detected	0.147 ± 0.007 (6)
Succinate	0.048 ± 0.006 (7)	0.096 ± 0.019 (7)

Table III

3.4 DISCUSSION

3.4.1 Terminal Oxidases and the electron transport chain in procyclic *T. congolense* catabolizing glucose

The NADH formed during the catabolism of glucose in the bloodstream and procyclic *Trypanosoma brucei* is reoxidized by molecular oxygen (Fairlamb and Opperdoes, 1986). The reduction of molecular oxygen involves the GPO alone or the GPO in combination with cytochrome aa_3 oxidase and another oxidase probably cytochrome o (Hill, 1972).

In this study procyclic *Trypanosoma congolense* also respired on glucose just as *T. brucei* procyclics (Njogu *et al*, 1980) indicating that molecular oxygen could be a terminal electron acceptor.

Rotenone had no effect on the respiration (Figure 1(i)). This has also been reported in the case of procyclic *T. brucei* (Gutteridge and Coombs, 1977). Turrens and Villalta (1989) proposed that this observation could be due to the possibility that the segment of the electron transport chain between NADH-dehydrogenase and ubiquinone is different from that in mammalian cells or that the oxygen consumption by the trypanosomes is dependent on succinate oxidation rather than NADH oxidation. However, the activity of NADH-dehydrogenase has been shown (Turrens and Villalta, 1989). In addition the transcription product of a gene that might code for a sub-unit of NADH dehydrogenase from maxi-circles of *T. brucei* kinetoplasts has been isolated (Faegin *et al*, 1986). Moreover an NADH-dehydrogenase activity

(determined as NADH-ferricyanide reductase) has been shown to be present in *T. cruzi* and which is also insensitive to rotenone (Boveris *et al*, 1986) but its specific function is not clear. The NADH dehydrogenase may transfer electrons to NADH linked fumarate reductase rather than ubiquinone (Turrens and Villalta, 1989). It would appear that procyclic *Trypanosoma congolense* could also reduce molecular oxygen using a similar mechanism as that proposed in procyclic *T. brucei* and epimastigote *T. cruzi*. This is represented in Scheme IV. During glucose catabolism the reducing equivalents from NADH could be channelled to fumarate to produce succinate which can in turn be reoxidized by the malonate sensitive succinate dehydrogenase. The reduced succinate dehydrogenase then transfers the reducing equivalents to ubiquinone and eventually to molecular oxygen in the electron transport chain. This contrasts with the case in mammalian cells where the reducing equivalents from NADH are channelled via NADH dehydrogenase to ubiquinone and eventually to molecular oxygen. Malonate was observed to inhibit the respiration by 30.4-34.2%. This indicates that at least some reducing equivalents are reoxidized through succinate dehydrogenase.

The observation that 48.6-59.6% of the respiration was sensitive to KCN (Table I) suggests that some reducing equivalents must be oxidized via the cytochrome aa3. This has also been observed in other trypanosomatids (Hill, 1972, 1976). The lack of complete inhibition suggests the presence of at least one other terminal oxidase. Addition of SHAM increased the percentage inhibition from 48.6-59.6% to 83.7-86.9% (Table 1). SHAM alone caused an inhibition of 32.0-40.4%. It is proposed that procyclic *T.*

(determined as NADH-ferricyanide reductase) has been shown to be present in *T. cruzi* and which is also insensitive to rotenone (Boveris *et al*, 1986) but its specific function is not clear. The NADH dehydrogenase may transfer electrons to NADH linked fumarate reductase rather than ubiquinone (Turrens and Villalta, 1989). It would appear that procyclic *Trypanosoma congolense* could also reduce molecular oxygen using a similar mechanism as that proposed in procyclic *T. brucei* and epimastigote *T. cruzi*. This is represented in Scheme IV. During glucose catabolism the reducing equivalents from NADH could be channelled to fumarate to produce succinate which can in turn be reoxidized by the malonate sensitive succinate dehydrogenase. The reduced succinate dehydrogenase then transfers the reducing equivalents to ubiquinone and eventually to molecular oxygen in the electron transport chain. This contrasts with the case in mammalian cells where the reducing equivalents from NADH are channelled via NADH dehydrogenase to ubiquinone and eventually to molecular oxygen. Malonate was observed to inhibit the respiration by 30.4-34.2%. This indicates that at least some reducing equivalents are reoxidized through succinate dehydrogenase.

The observation that 48.6-59.6% of the respiration was sensitive to KCN (Table I) suggests that some reducing equivalents must be oxidized via the cytochrome aa_3. This has also been observed in other trypanosomatids (Hill, 1972, 1976). The lack of complete inhibition suggests the presence of at least one other terminal oxidase. Addition of SHAM increased the percentage inhibition from 48.6-59.6% to 83.7-86.9% (Table 1). SHAM alone caused an inhibition of 32.0-40.4%. It is proposed that procyclic *T.*

congolense have also the GPO system as a terminal oxidase because it is the GPO that is usually inhibited by SHAM (Opperdoes *et al*, 1976). This is consistent with observations in other trypanosomatids (Fairlamb and Opperdoes, 1986). There was, however, 13.1-16.3% of the respiration which was insensitive to a combination of KCN and SHAM. A similar observation was made in procyclic *T. brucei* (Hill, 1972, 1976, 1977; Njogu, *et al*, 1980).

When antimycin A was added to the respiring procyclic *T. congolense* it inhibited this respiration by 35.2-43.6%. It was interesting to note that a combination of SHAM and antimycin A did not result in complete inhibition. This combination inhibited the respiration by 82.1-90.5%. Thus about 9.5-17.9% was still insensitive to a combination of antimycin A and SHAM. This suggests that the cyanide/SHAM insensitive respiration is also insensitive to antimycin A. The cyanide/SHAM insensitive respiration in procyclic *T. brucei* has been attributed to cytochrome o (Ray and Cross, 1972; Hill, 1972). Hill (1972) proposed a branched electron transport chain where cytochrome o branches off from the cytochrome b before the antimycin A sensitive site. The cyanide/SHAM and antimycin A /SHAM insensitive respiration in procyclic *T. congolense* could also be attributed to cytochrome o.

It was interesting to note that malonate did not inhibit the oxygen consumption as much as antimycin A. This could be due to the presence of trypanosome membrane barrier which led to inability to establish sufficient concentration of the added malonate to the succinate dehydrogenase active site. It is also possible that

some electrons are channelled to ubiquinone through a rotenone insensitive NADH dehydrogenase.

From their results, Njogu *et al* (1980) proposed that the electron transport chain in procyclic *T. brucei* is branched and has at least two terminal oxidases : cytochrome aa_3 and the GPO. The electron transport chain could branch at the ubiquinone region and be reduced by the G-3-P dehydrogenase component of the GPO (Scheme III). From this investigation it is not possible to say conclusively whether the electron transport chain and GPO in procyclic *T. congolense* are branched or parallel. It is, however, possible that procyclic *T. congolense* also have a branched electron transport chain similar to that of procyclic *T. brucei* though this was not investigated. Table III shows that when the GPO was inhibited with SHAM the trypanosomes did not form glycerol equivalent to the total molar amounts of pyruvate, acetate and succinate. In fact the total pyruvate, acetate and succinate to glycerol molar ratio was 3:1.

This indicates that glycerol-3-phosphate (G-3-P) or NADH generated from the oxidation of GAP was being oxidized in the presence of SHAM leading to the formation of pyruvate, acetate and succinate. This oxidation must be catalyzed by another enzyme other than the GPO. In the presence of SHAM G-3-P may transfer electrons directly to the electron transport chain via ubiquinone as in eukaryotic mitochondria (Lehninger, 1975). This is discussed in detail in later sections.

3.4.2 Pathways of catabolism of phosphoenolpyruvate derived from glucose oxidation

In their studies, Opperdoes and Cottem (1982) reported that glucose consumption in procyclic *T. brucei* is accompanied by CO_2 fixation. This explains the excretion of significant amounts of succinate under both aerobic and anaerobic conditions (Bowman, 1974; Ryley, 1962). Opperdoes and Cottem (1982) observed high activities of PEP-carboxykinase and the NADP linked malic enzyme. Klein *et al* (1974) also provided evidence for the presence of phosphoenolpyruvate carboxykinase in *Crithidia fasciculata*, *Trypanosoma mega* and the procyclic form of *T. brucei*. They reported that the most significant enzymes of PEP catabolism were PEP carboxykinase and malic enzyme.

In this investigation using procyclic *T. congolense* it was observed that PEP carboxykinase had significant activity. The low activities of pyruvate kinase and lactate dehydrogenase (Table II) suggest that they play a minor role and that PEP carboxykinase plays a predominant role during glucose catabolism (Table II). It is, therefore, proposed that procyclic *T. congolense* catabolize glucose to PEP which then undergoes CO_2 fixation according to the following reaction:

$$PEP + CO_2 + ADP \xrightarrow{PEP.CK} OAA + ATP$$

The OAA formed could be the substrate for malate dehydrogenase which also had very high activity. This would result

in the production of malate. Malate then undergoes oxidative decarboxylation to form pyruvate in a reaction dependent on either NAD or NADP. Since no significant activity of NAD-linked malic enzyme was detected it is possible that it does not contribute towards pyruvate formation. This would imply that NADP-linked malic enzyme is the major enzyme that contributes towards pyruvate formation. Some of the pyruvate is excreted and the rest oxidised in the mitochondria to acetylCoA via the pyruvate dehydrogenase catalysed reaction. The acetylCoA could be converted to acetate as follows:-

$$\text{AcetylCoA + Pi} \xrightarrow{\text{PTA}} \text{Acetyl phosphate + CoASH} \quad (1)$$

$$\text{Acetylphosphate + ADP} \xrightarrow{\text{AK}} \text{Acetate + ATP} \quad (2)$$

A similar pathway of acetate formation has been proposed for *Trichomonas vaginalis* (Steinbuchel and Muller, 1986 (a) and (b)). It was, however, not possible to demonstrate the presence of the activity of the enzyme PTA in procyclic *T. congolense* using the method described in Materials and Methods.

Some of the malate could also be converted to fumarate and then reduced to succinate via the reaction catalysed by fumarase and fumarate reductase as shown below:

$$\text{Malate} + H_2O \xrightarrow{\text{fumarase}} \text{Fumarate} \qquad (1)$$

$$\text{Fumarate} + NAD^+ \xrightarrow{FR} \text{Succinate} + NADH \qquad (2)$$

The results of this investigation are consistent with Scheme VII. It is a modification of Scheme II. This scheme shows that the reducing equivalents (NADH) generated from the oxidation of GAP may be reoxidised by DHAP to form G-3-P. Under aerobic conditions or in the absence of SHAM the G-3-P could be oxidised by molecular oxygen to form water and DHAP. This could explain the sensitivity to SHAM during the catabolism of glucose. This suggests that there is no accumulation of G-3-P and hence glycerol cannot be produced under aerobic conditions. However, because of the interconversion of DHAP and GAP, the NAD would still be reduced to NADH. The NADH generated is then reoxidized to NAD via the GPO.

Under anaerobic conditions or when the GPO was inhibited by SHAM, the GPO system could no longer reoxidize the NADH. This leads to an accumulation of G-3-P. G-3-P could then be transphosphorylated by the glycerol-3-phosphate : glucose transphosphorylase (Kiaira & Njogu, 1989) with the concomittant production of glycerol. The NADH produced from the oxidation of GAP could be reoxidized by the malate dehydrogenase reaction with the concomittant production of malate from OAA (Scheme VII).

It is proposed that glucose is catabolized by procyclic *T. congolense* in a pathway detailed in Scheme VII. The malate

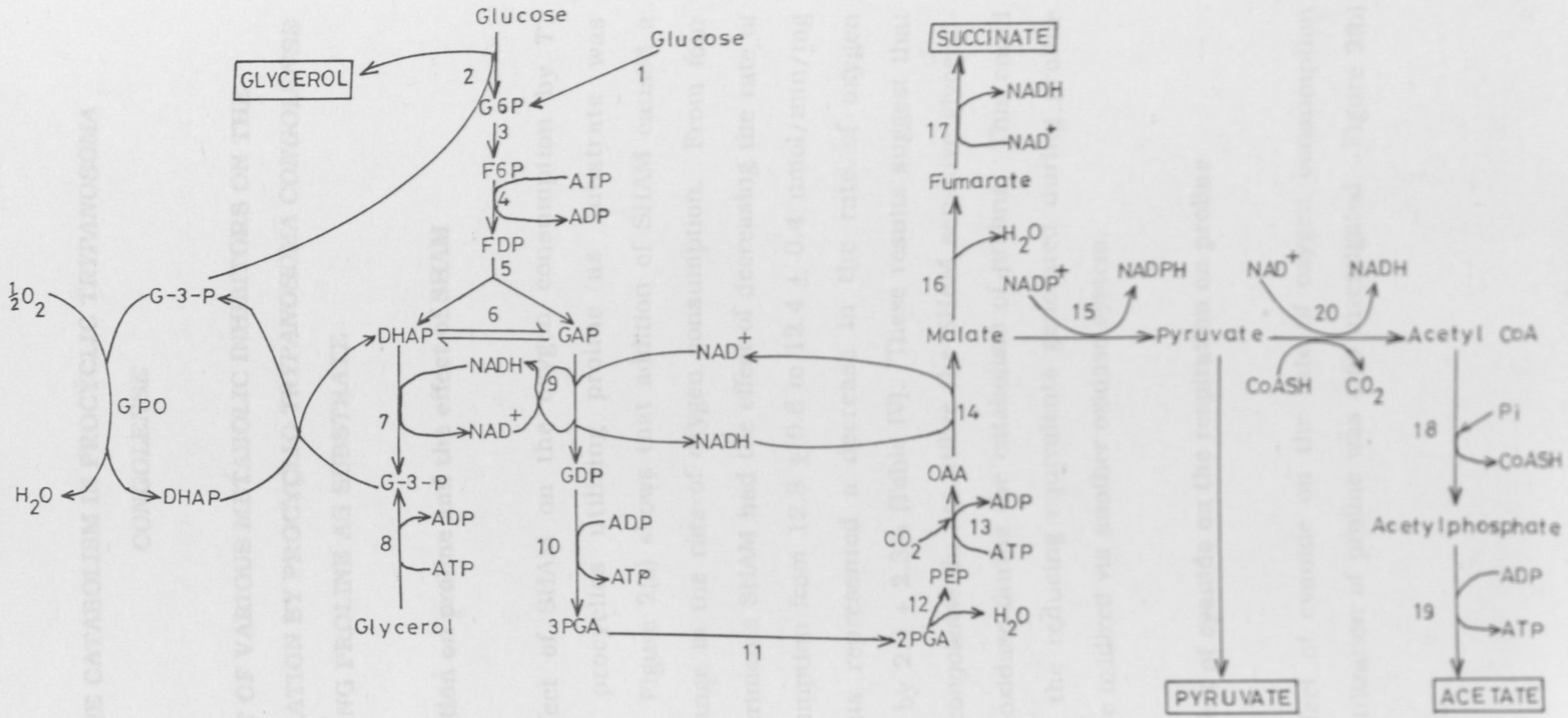
dehydrogenase reaction and the glycerol-3-phosphate dehydrogenase reactions both of which reoxidize NADH would seem to be competing reactions during anaerobic conditions.

Scheme VII

Proposed pathway for the catabolism of glucose by procyclic *Trypanosoma congolense.* [Adapted from Fairlamb & Opperdoes (1986) and Kiaira and Njogu (1989)].

The enzymes shown are:-

1. Hexokinase
2. Glycerol-3-phosphate : Glucose transphosphorylase
3. Phosphoglucose isomerase
4. Phosphofructokinase
5. Aldolase
6. Triose phosphate isomerase
7. Glycerol-3-phosphate dehydrogenase
8. Glycerokinase
9. Glyceraldehyde 3-phosphate dehydrogenase
10. Phosphoglycerate kinase
11. Phosphogycerate mutase
12. Enolase
13. PEP carboxykinase
14. Malate dehydrogenase
15. NADP-linked malic enzyme
16. Fumarase or fumarate hydratase
17. Fumarate reductase
18. Phosphotransacetylase or phosphoacetyl transferase
19. Acetate kinase
20. Pyruvate dehydrogenase


Glucose
Glucose
GLYCEROL
2
1
G6P
3
F6P
ATP
4
ADP
FDP
5
½O2
G-3-P
6
DHAP
GAP
NADH
NAD+
9
7
NAD+
NADH
GPO
H2O
DHAP
G-3-P
GDP
ADP
8
ATP
10
ADP
ATP
Glycerol
3PGA
11
2PGA
SUCCINATE
NADH
17
NAD+
Fumarate
H2O
16
NADP+
NADPH
15
Malate
Pyruvate
NAD+
NADH
20
CoASH
CO2
Acetyl CoA
14
OAA
ADP
CO2
13
ATP
PEP
12
H2O
18
Pi
CoASH
Acetylphosphate
ADP
19
ATP
PYRUVATE
ACETATE

Scheme VII

CHAPTER FOUR

PROLINE CATABOLISM IN PROCYCLIC *TRYPANOSOMA CONGOLENSE*

4.1 EFFECT OF VARIOUS METABOLIC INHIBITORS ON THE RESPIRATION BY PROCYCLIC *TRYPANOSOMA CONGOLENSE* UTILIZING PROLINE AS SUBSTRATE

4.1.1 Respiration on proline and the effect of SHAM

The effect of SHAM on the oxygen consumption by *T. congolense* procyclics utilizing proline as substrate was investigated. Figure 2(a) shows that addition of SHAM caused a negligible change in the rate of oxygen consumption. From four separate experiments SHAM had the effect of decreasing the rate of oxygen consumption from 12.8 ± 0.6 to 12.4 ± 0.4 nmol/min/mg protein. This represented a decrease in the rate of oxygen consumption by 2.9 ± 2.3% (Table IV). These results suggest that procyclic *T. congolense* do not utilize the SHAM sensitive glycerol-3-phosphate oxidase during the catabolism of proline. This could suggest that the reducing equivalents generated during proline catabolism are oxidized via another oxidase system.

4.1.2 The effect of cyanide on the respiration on proline

The effect of cyanide on the rate of oxygen consumption during the utilization of proline was also investigated. Figure 2(b)

Table IV

The effect of various metabolic inhibitors on the rate of oxygen consumption of *T. congolense* procyclics utilizing proline.

Isolated trypanosomes were washed five times and suspended in PS proline. They were then introduced into the oxygen electrode chamber also pre-equilibrated with 1.2 mls of PS proline. The rates of respiration before and after the addition of various metabolic inhibitors were then noted as shown in figures 2(a) - (g). The results are expressed as mean plus or minus the standard deviation. The numbers in brackets indicate the total number of separate determinations.

Table IV

Initial Rate of oxygen consumption in nmol/min /mg protein	Inhibitor added	Rate of oxygen consumption after addition of inhibitor in nmol/ min/mg protein	Percentage inhibition
12.8 ± 0.6 (4)	SHAM	12.4 ± 0.5 (4)	2.9 ± 2.3 (4)
13.5 ± 1.2 (4)	KCN	2.5 ± 1.0 (4)	81.0 ± 6.7 (4)
12.8 ± 0.5 (6)	Malonate	6.2 ± 0.4 (6)	51.6 ± 1.6 (6)
12.9 ± 1.8 (3)	Antimycin A	3.5 ± 1.3 (3)	73.1 ± 5.9 (3)
12.8 ± 0.5 (3)	Antimycin A+ Malonate	2.4 ± 1.0 (3)	81.6 ± 7.6 (3)
12.0 ± 0.7 (4)	Malonate + KCN	1.8 ± 0.9 (4)	84.9 ± 6.7 (4)
11.3 ± 1.0 (3)	Rotenone	11.0 ± 1.2 (3)	2.4 ± 2.1 (3)

Figure 2(a) : Effect of SHAM on oxygen consumption by *T. congolense* procyclics utilizing proline as substrate

A suspension containing 6.6 x 10^5 trypanosomes was introduced into the chamber containing the oxygen electrode pre-equilibrated with 1.2 mls of PS proline. The rate of oxygen consumption before and after the addition of SHAM was recorded as shown. SHAM was added to give its final concentration of 0.5 mM. The trypanosomes added into the chamber were equivalent to 0.32 mg protein.

Figure 2 (b) : Effect of cyanide on the rate of oxygen uptake by *T. congolense* procyclics utilizing proline as substrate

A suspension containig 9.9 x 10^6 trypanosomes was introduced into the chamber containing the oxygen electrode pre-equilibrated with 1.2 mls PS proline and the rate of oxygen consumption before and after the addition of 0.1 mM KCN as shown. The trypanosomes added were equivalent to 0.24 mg protein.

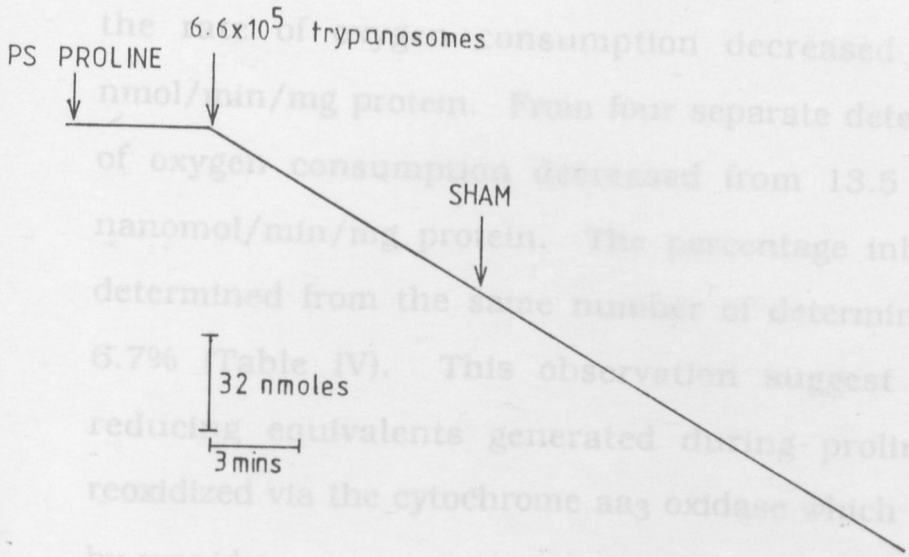


Figure 2 (a)

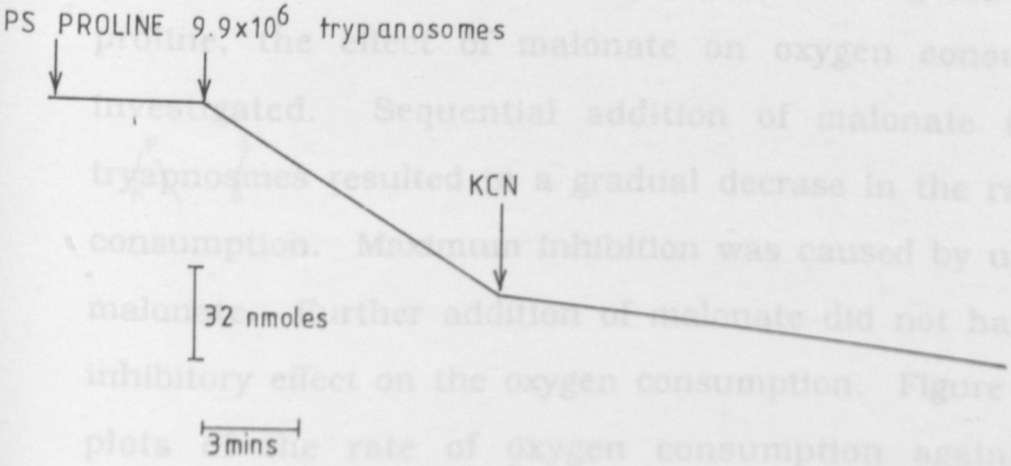


Figure 2 (b)

the rate of oxygen consumption decreased from 14.9 to 3.0 nmol/min/mg protein. From four separate determinations the rate of oxygen consumption decreased from 13.5 ± 1.2 to 2.5 ± 1.0 nanomol/min/mg protein. The percentage inhibition by cyanide determined from the same number of determinations was 81.0 ± 6.7% (Table IV). This observation suggest that most of the reducing equivalents generated during proline catabolism are reoxidized via the cytochrome aa_3 oxidase which is usually inhibited by cyanide.

4.1.3 Effect of malonate on the respiration of proline

To determine the involvement of succinate dehydrogenase on respiration by *T. congolense* procyclics during the utilization of proline, the effect of malonate on oxygen consumption was investigated. Sequential addition of malonate to respriring tryapnosmes resulted in a gradual decrase in the rate of oxygen consumption. Maximum inhibition was caused by upto 21.0 mM malonate. Further addition of malonate did not have additional inhibitory effect on the oxygen consumption. Figure 2(c)(i) shows plots of the rate of oxygen consumption against malonate concentration during the catabolism of proline. Similar observation had earlier been made during the catabolism of glucose. Figure 2(c)(ii) shows that the 21 mM malonate caused a decrease in the rate of oxygen consumption from 13.3 to 6.4 nmol/min/mg protein. From six separate determinations the rate of oxygen consumption was decreased by malonate from 12.8 ± 0.5 to 6.2 ± 0.4 nmol/min/mg protein. This represented a decrease of 51.6 ± 1.6%

Figure 2(c)(i) : Effect of increasing concentration of malonate on the rate of oxygen consumption by procyclic *T. congolense* utilising proline

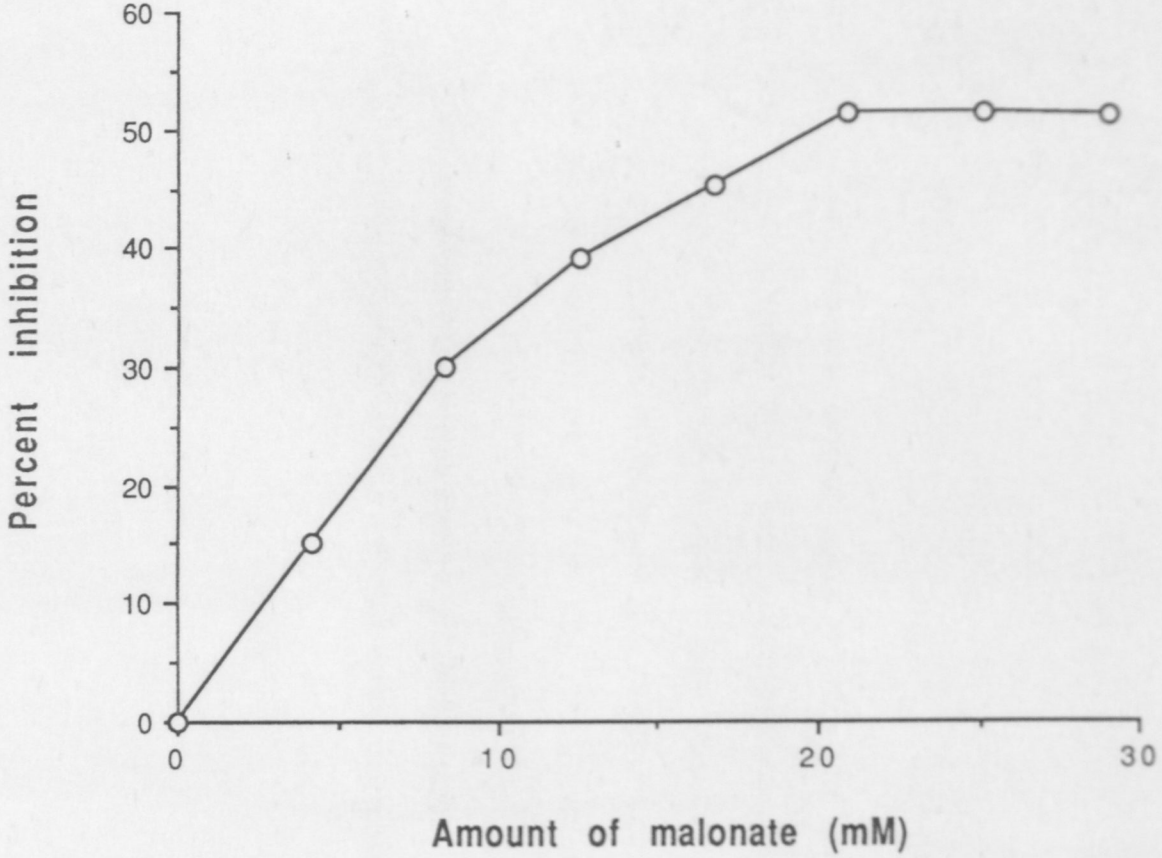

Percent inhibition
60
50
40
30
20
10
0
0
10
20
30
Amount of malonate (mM)

Figure 2(c)(i)

Figure 2(c)(ii) : Effect of malonate on oxygen consumption by *T. congolense* procyclics catabolizing proline

A suspension containing 4.3 x 10^7 trypanosomes was introduced into the chamber containing the oxygen electrode pre-equilibrated with 1.2 mls of PS proline. The rate of respiration before and after the additon of 25 mM malonate was recorded as shown. The trypanosomes added were equivalent to 0.32 mg protein.

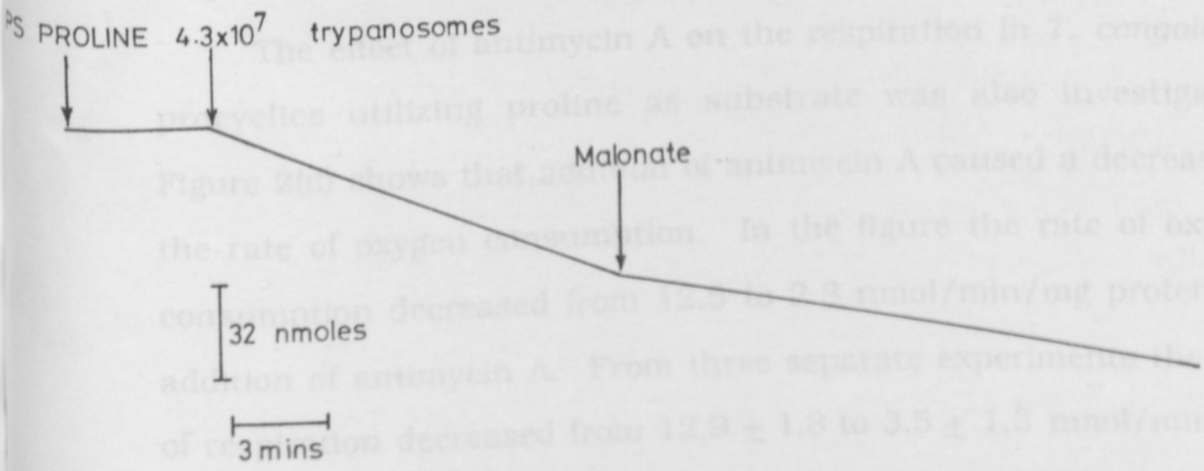


Figure 2 (c)(i)

was decreased by malonate from 12.8 ± 0.5 to 6.2 ± 0.4 nmol/min/mg protein. This represented a decrease of 51.6 ± 1.6% (Table IV). From this result it is suggested that there could be an involvement of succinate dehydrogenase during the catabolism of proline by *T. congolense* procyclics. Since the inhibition by malonate was incomplete, it is possible that not all the reducing equivalents derived from proline catabolism are oxidized via succinate dehydrogenase.

4.1.4 The inhibition of the respiration by antimycin A

The effect of antimycin A on the respiration in *T. congolense* procyclics utilizing proline as substrate was also investigated. Figure 2(d) shows that addition of antimycin A caused a decrease in the rate of oxygen consumption. In the figure the rate of oxygen consumption decreased from 12.5 to 2.8 nmol/min/mg protein on addition of antimycin A. From three separate experiments the rate of respiration decreased from 12.9 ± 1.8 to 3.5 ± 1.3 nmol/min/mg to protein. This represented a percentage decrease of 73.1 ± 5.9% (Table IV). From these observations it is suggested that a significant percentage of reducing equivalents derived from proline catabolism are oxidized by oxygen via cytochrome b and cytochrome c_1 which is normally inhibited by antimycin A.

4.1.5 Inhibition of respiration by a combination of malonate and antimycin A

Respiration on proline by procyclic *T. congolense* in the absence and presence of a combination of malonate and antimycin A

Figure 2(d) : Effect of antimycin A on the rate of oxygen consumption by procyclic *T. congolense* utilizing proline as substrate

A suspension containing 8.9 x 10^7 trypanosomes was introduced into the oxygen electrode chamber pre-equilibrated with 1.2 mls of PS proline. The rate of respiration before and after addition of 10 µl of antimycin A was recorded as shown. The trypanosomes added were equivalent to 0.71 mg protein.

Figure 2(e) : Effect of a combination of malonate and antimycin A on the rate of oxygen consumption by *T. congolense* procyclics utilizing proline as substrate

A suspension containing 1.2 x 10^8 trypanosomes was introduced into the chamber containing the oxygen electrode pre-equilibrated with 1.2 mls PS proline. The rate of respiration was then recorded. After this 25 mM malonate was added into the chamber to a final concentration of 25 mM followed by addition of 10 µl antimycin A (stock = 1 mg/ml). The rate of oxygen consumption before and after the addition was recorded as shown.

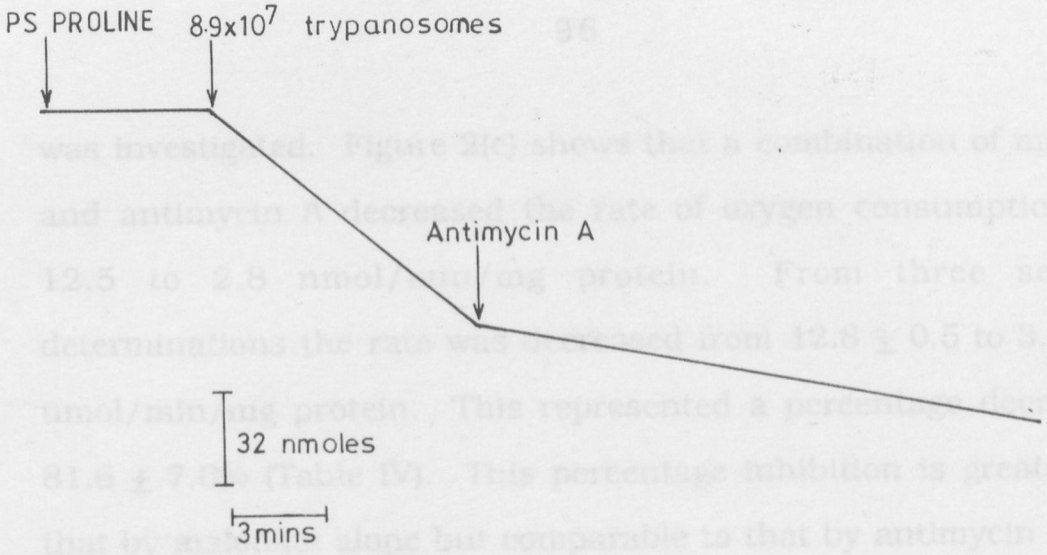


Figure 2 (d)

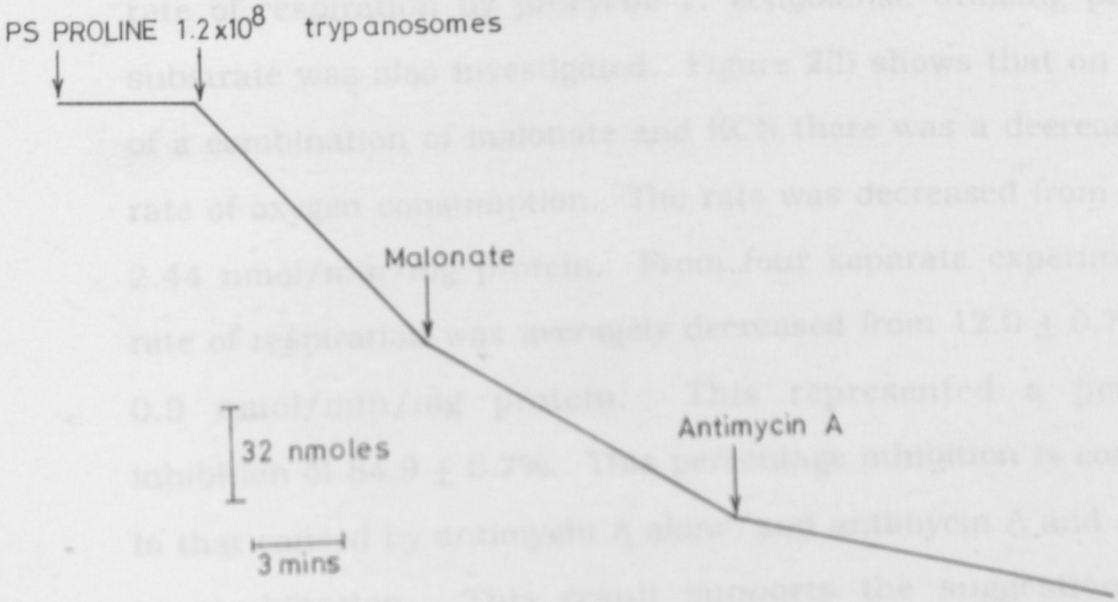


Figure 2 (e)

was investigated. Figure 2(e) shows that a combination of malonate and antimycin A decreased the rate of oxygen consumption from 12.5 to 2.8 nmol/min/mg protein. From three separate determinations the rate was decreased from 12.8 ± 0.5 to 3.4 ± 1.0 nmol/min/mg protein. This represented a percentage decrease of 81.6 ± 7.6% (Table IV). This percentage inhibition is greater than that by malonate alone but comparable to that by antimycin A alone which is 73.1 ± 5.9% (Table IV). It could be suggested from these results that the reducing equivalents derived from proline catabolism are oxidized via the malonate sensitive succinate dehydrogenase and cytochromes b and c_1.

4.1.6 Inhibition of respiration by a combination of malonate and KCN

The effect of a combination of malonate and cyanide on the rate of respiration by procyclic *T. congolense* utilizing proline as substrate was also investigated. Figure 2(f) shows that on addition of a combination of malonate and KCN there was a decrease in the rate of oxygen consumption. The rate was decreased from 11.96 to 2.44 nmol/min/mg protein. From four separate experiments the rate of respiration was averagely decreased from 12.0 ± 0.7 to 1.8 ± 0.9 nmol/min/mg protein. This represented a percentage inhibition of 84.9 ± 6.7%. This percentage inhibition is comparable to that caused by antimycin A alone, and antimycin A and malonate in combination. This result supports the suggestions under sections 4.1.3 and 4.1.5 that some reducing equivalents derived from the catabolism of proline are channelled to the cyanide

sensitive cytochrome aa_3 terminal oxidase via the malonate sensitive succinate dehydrogenase.

4.1.7 Respiration on proline and the effect of rotenone

The effect of rotenone on the oxygen consumption by *T. congolense* procyclics utilizing proline as substrate was investigated. Figure 2(g) shows that addition of rotenone caused a negligible change in the rate of oxygen consumption. From three separate experiments rotenone had the effect of decreasing the rate of oxygen consumption from 11.0 $\pm$ 1.2 to 2.4 $\pm$ 2.1% (Table IV). These results suggest that either procyclic *T. congolense* do not possess a functional NADH dehydrogenase or that the NADH dehydrogenase present is modified in such a way that it can not be inhibited by rotenone. On the other hand these results could suggest that electrons derived from proline catabolism are reoxidized by molecular oxygen via another pathway not involving NADH dehydrogenase.

Figure 2(f) : Effect of a combination of malonate and KCN on the rate of oxygen uptake by procyclic *T. congolense* utilizing proline as substrate

A suspension containing 1.2 x 10^8 trypanosomes was introduced into the oxygen electrode chamber pre-equilibrated with 1.2 mls of PS proline. The rate of oxygen consumption before and after the additions of inhibitors was recorded as shown. The final concentration for malonate and KCN were 25 mM and 0.1 mM respectively.

Figure 2 (g) Effect of rotenone on the rate of oxygen uptake by procyclic *T. congolense* utilizing proline as substrate

A suspension containing 2.6 x 10^8 trypanosomes was introduced into the chamber containing the oxygen electrode pre-equilibrated with 1.2 mls PS proline. The rate of respiration was then recorded. After this rotenone was added at a total concentration of 5 μM and the rate of oxygen uptake again noted. The trypanosomes added were equivalent to 2.6 mg protein.

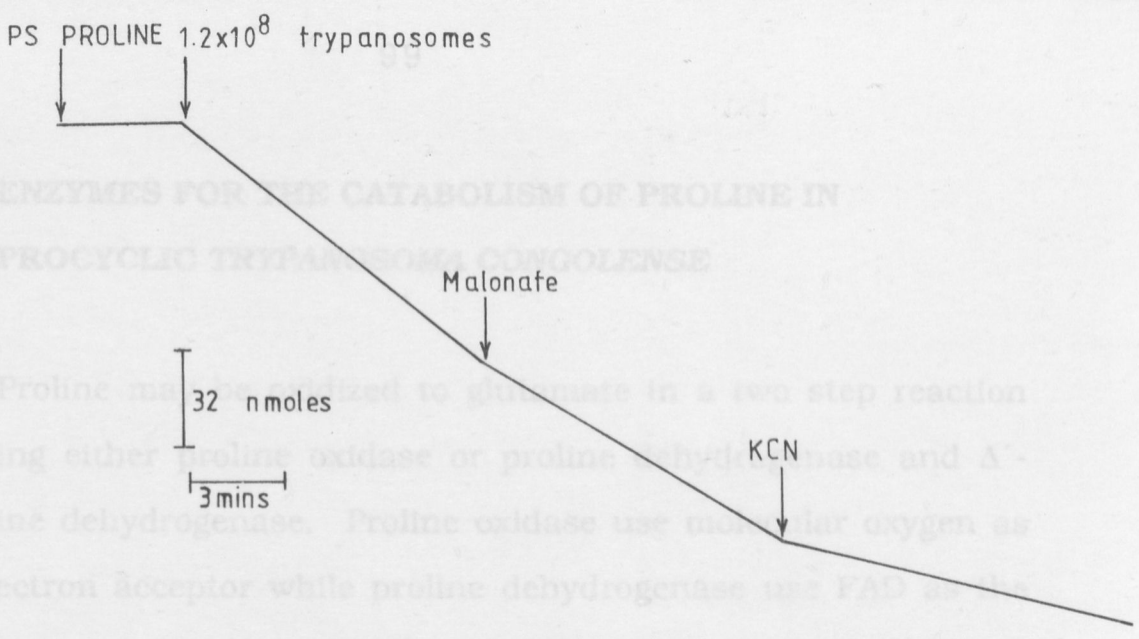


Figure 2(f)

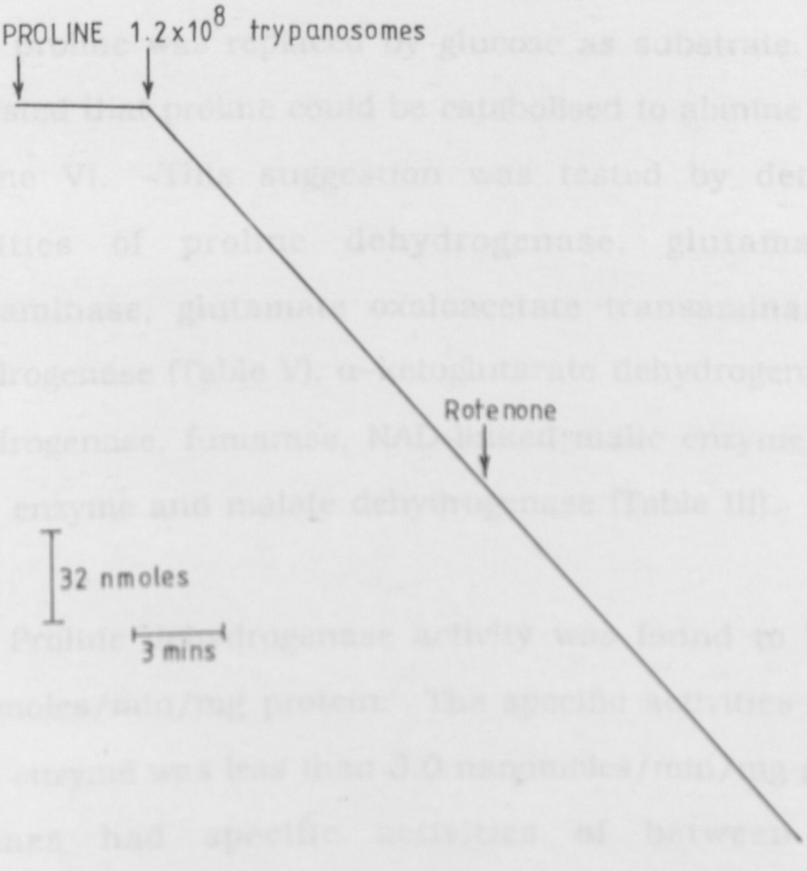


Figure 2(g)

4.2 ENZYMES FOR THE CATABOLISM OF PROLINE IN PROCYCLIC *TRYPANOSOMA CONGOLENSE*

Proline may be oxidized to glutamate in a two step reaction involving either proline oxidase or proline dehydrogenase and Δ´-pyrroline dehydrogenase. Proline oxidase use molecular oxygen as the electron acceptor while proline dehydrogenase use FAD as the electron acceptor (Scheme V and VI). Proline oxidase is not sensitive to inhibitors of the respiratory chain.

In sections 3.1 and 4.1 it was shown that about 15% of respiration is insensitive to SHAM, antimycin A and cyanide during the utilization of glucose or proline. It was proposed that this respiration was not due to proline oxidase because it was observed when proline was replaced by glucose as substrate. It was also suggested that proline could be catabolised to alanine as outlined in scheme VI. This suggestion was tested by determining the activities of proline dehydrogenase, glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, glutamate dehydrogenase (Table V), α–ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase, NAD-linked malic enzyme, NADP-linked malic enzyme and malate dehydrogenase (Table III).

Proline dehydrogenase activity was found to be 55.2 ± 4.8 nanomoles/min/mg protein. The specific activities of NAD-linked malic enzyme was less than 3.0 nanomoles/min/mg protein. Other enzymes had specific activities of between 30 - 1170 nanomoles/min/mg protein.

Table V : Specific activities of some enzymes likely to be involved in the catabolism of proline by procyclic *Trypanosoma congolense.*

Isolated trypanosomes were washed five times in PSG and then lysed with 0.1% Triton X100. The samples were then centrifuged at 1000 g at 4°C and the resulting supernatant assayed for the enzyme activities shown. The assay temperature for all determinations was 25°C. The results are expressed as mean plus or minus the standard deviation. The numbers in brackets represent the total number of separate determinations.

Table V

Enzyme	Specific Activity nmoles/min/mg protein
Proline dehydrogenase	55.2 ± 4.8 (6)
Glutamate dehydrogenase	11.1 ± 3.0 (3)
Glutamate pyruvate transaminase	92.2 ± 8.7 (4)
Glutamate oxoglutarate transaminase	34.7 ± 3.8 (5)

4.3 END PRODUCTS OF PROLINE CATABOLISM

The end products of aerobic proline catabolism were alanine and glutamate. Table VI shows the rates of production of the end products of aerobic proline catabolism.

The mean rates of production of alanine and glutamate were 0.0472 and 0.081 μmoles/30 min/mg protein respectively. This represented a molar ratio of the mean rates of production of alanine : glutamate of 1:1.7. Aspartate was however not detected as an end product when the same samples were assayed enzymatically.

It was suggested that the procyclic *Trypanosoma congolense* might have a similar mechanism for catabolizing proline as proposed in procyclic *Trypanosoma brucei* by Evans and Brown (1972a).

From these results it is observed that more glutamate is produced compared to alanine. This suggests that there could be a rate limiting (regulatory) step after the production of glutamate. This could slow down the rate of further catabolism of glutamate such that this metabolite accumulates.

Table VI : End products of aerobic proline catabolism by procyclic *T. congolense*

Every sample of freshly isolated trypanosomes was divided into two. One of these was deproteinized immediately with PCA (i.e. at zero time). Both of these were then incubated in PS proline for 30 minutes. The previously undeproteinized portion was then deproteinized with PCA after the incubation period. They were then processed for metabolite assays as described in materials and methods. All the results were corrected for zero time. The results are expressed as mean plus or minus standard deviation. The figures in brackets represent the total number of separate determinations.

End product	Rate of production in μmoles/30 min/mg protein
Alanine	0.0472 ± 0.003 (6)
Aspartate	Not detected
Glutamate	0.081 ± 0.006 (7)

Table VI

4.4. DISCUSSION

4.4.1 Terminal oxidases and the electron transport chain in procyclic *T. congolense* catabolizing proline

In this study procyclic *T. congolense* respired on proline (Figure 2a-g) just like procyclic *T. brucei* (Evans and Brown, 1972a). This suggests that molecular oxygen could be a terminal electron acceptor. Rotenone which normally inhibits site one of the mitochondrial electron transport chain (Lehninger, 1975) had no effect on the respiration. The explanation for this observation is similar to that made with glucose as substrate (Section 3.4). It would therefore seem that a similar mechanism for the channelling of electrons from NADH to the next acceptor, ubiquinone, is used during proline catabolism.

When SHAM was added to the respiring trypanosomes, it did not have any significant effect on the rate of oxygen consumption. This suggests that the GPO is not used for the oxidation of reducing equivalents generated during proline catabolism by procyclic *T. congolense*. This was different from the observation made when SHAM was added when the parasites were catabolizing glucose (Section 3.4).

Malonate inhibited the respiration by 50.0-53.2%. This suggests either that not all the reducing equivalents are oxidized via succinate dehydrogenase or that malonate did not achieve maximum inhibition as was explained in section 3.4. However, this percentage inhibition was more than that observed during the

catabolism of glucose by the same parasites. This could be because some of the reducing equivalents which would have otherwise been reoxidized by molecular oxygen through the GPO are now being channelled to molecular oxygen through the electron transport chain.

The observation that 74.3-87.7% of the respiration was sensitive to KCN suggests that most of the reducing equivalents derived from proline oxidation are reoxidized via the cytochrome aa_3 which is the one sensitive to cyanide. A similar observation was also made during the respiration by the procyclic *T. congolense* when glucose was used as the substrate (Section 3.4). The lack of complete inhibition by cyanide suggests that during proline catabolism by procyclic *T. congolense*, not all the electrons reduce molecular oxygen through cytochrome aa_3 but some reduce oxygen via at least one more oxidase. This other oxidase(s) could, however, not be the GPO as addition of SHAM did not result in any significant inhibition.

When antimycin A was added to the procyclic *T. congolense* catabolizing proline, it had the effect of inhibiting the respiration by 67.2-79.2%. This suggests that a significant percentage of reducing equivalents derived from proline catabolism are oxidized by oxygen via cytochrome b and cytochrome c_1. It was interesting to note that neither KCN nor antimycin A could inhibit the respiration completely. The KCN insensitive respiration could be due to the cytochrome o proposed earlier in section 3.4.

It is therefore proposed that the reducing equivalents derived from proline catabolism by procyclic *T. congolense* are reoxidized by molecular oxygen. Between 50.0 to 53.2% (Table IV) of these reducing equivalents are channelled to ubiquinone through succinate dehydrogenase and eventually to cytochrome aa_3. This was the percentage respiration sensitive to malonate. Approximately 12.3 to 25.7% of the reducing equivalents are reoxidized by another oxidase probably cytochrome o. It is also possible that the rest of the reducing equivalents are channelled to cytochrome aa_3 via a somehow modified NADH dehydrogenase (insensitive to rotenone). The GPO appears not to play a role in the reoxidation of these reducing equivalents during the catabolism of proline.

It is suggested that during the catabolism of proline by procyclic *T. congolense* a respiratory chain similar to that proposed by Turrens and Villalta (1989) is used (Scheme IV). It would seem that procyclic *T. congolense* use a similar respiratory chain during the catabolism of both glucose and proline except that during glucose catabolism the GPO plays a significant role.

4.4.2 Pathways of proline catabolism in procyclic *T. congolense*

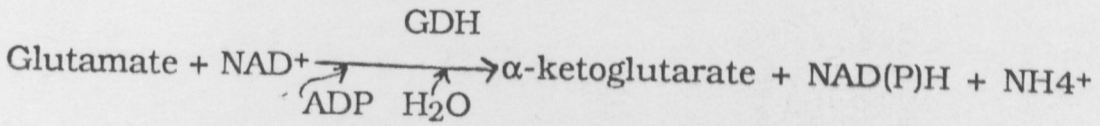
Evans and Brown (1972a) proposed that in procyclic *T. brucei*, proline is catabolized as shown in Scheme VI.

In this study using procyclic *T. congolense* it was observed that proline dehydrogenase activity was 55.2 nmols/min/mg protein (Table IV). It is proposed that procyclic *T. congolense*

catabolize proline to glutamate. The glutamate produced then undergoes transamination with pyruvate to give alanine in a reaction catalysed by glutamate pyruvate transaminase. The GPT activity was found to be significant and relatively higher than the activity of proline dehydrogenase. Alanine is excreted whereas α-ketoglutarate is catabolized through some reactions of the TCA cycle during which it is converted to succinate through the α-ketoglutarate dehydrogenase catalysed reaction. The activity of α-ketoglutarate dehydrogenase was found to be comparable also to that of proline dehydrogenase as both were higher than 55 nmol/min/mg protein (Table V). Succinate is oxidised to malate through reactions catalysed by succinate dehydrogenase and fumarase. Both succinate dehydrogenase and fumarase activities were more than 55 nmol/min/mg protein (Table II). Malate can then undergo oxidative decarboxylation to form pyruvate in a reaction dependent on either NAD or NADP-linked malic enzyme. Since no significant activity of NAD-linked malic enzyme was detected it is possible that it does not contribute towards pyruvate formation. The NADP-linked malic enzyme whose activity was higher than that of proline dehydrogenase could then be the one involved in the production of pyruvate. This is the pyruvate which is transaminated with glutamate to give alanine. It is not clear from this investigation whether pyruvate could also be produced from malate in a two step reaction involving malate dehydrogenase and oxaloacetate decarboxylase as the presence of the activity of this enzyme was not investigated.

This investigation also revealed very low values of glutamate dehydrogenase (Table V). It seems that the oxidation of glutamate

to α-ketoglutarate catalysed by glutamate dehydrogenase according to the following reaction is not a preferred pathway by procyclic *T. congolense*.



Opperdoes and Cottem (1982) also reported low activities of glutamate dehydrogenase in procyclic *T. brucei*.

The finding that glutamate oxaloacetate transaminase (GOT) activity was significant though less than that of GPT suggested that aspartate could have been an end product. However, when it was assayed for it could not be detected (Table VI). It is proposed that GOT could be used by the parasite to generate oxaloacetate and α-ketoglutarate from aspartate oxidation. This aspartate could be obtained from either the host or from the parasites' protein breakdown.

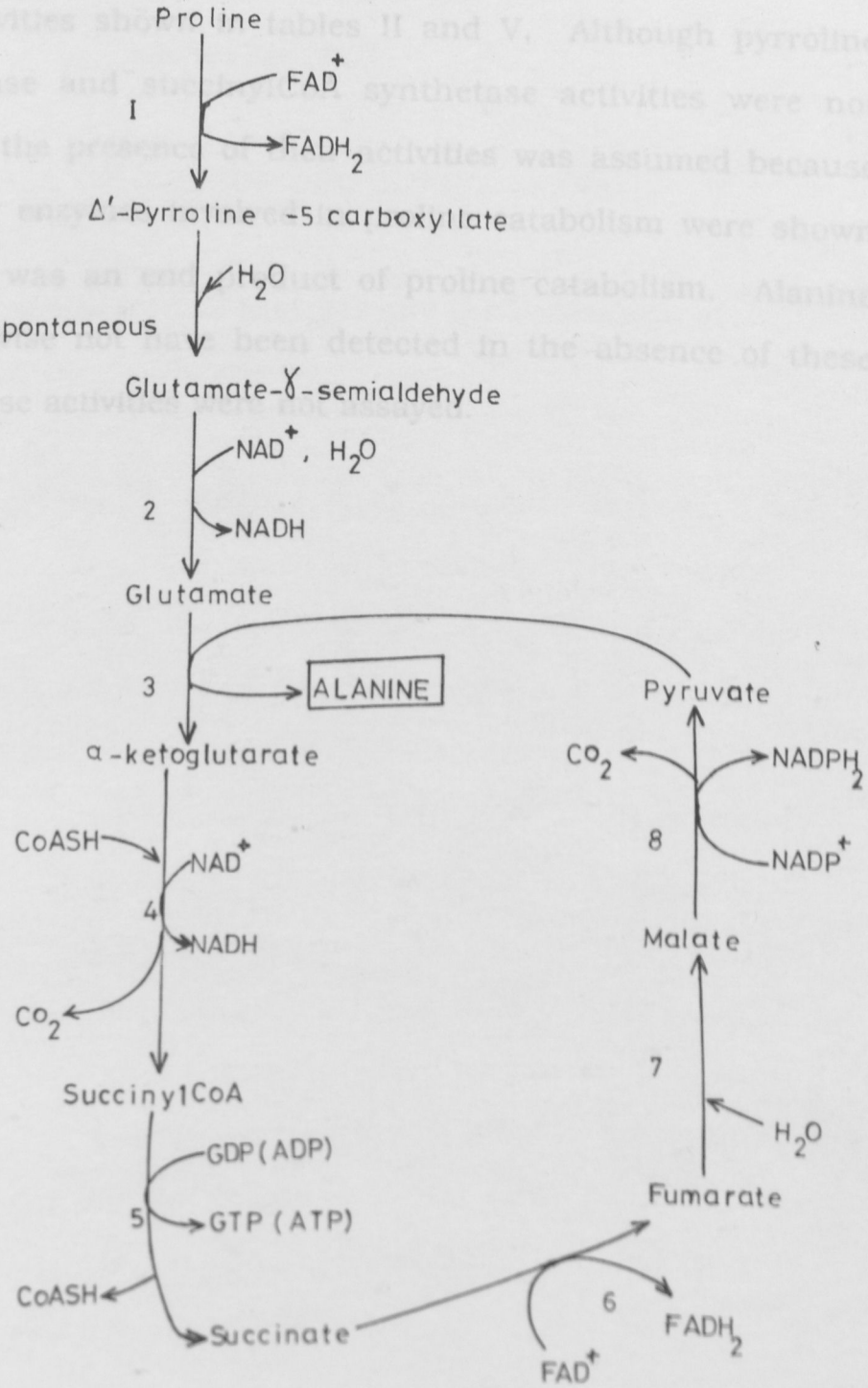
From the results of this investigation it is proposed that procyclic *T. congolense* catabolize proline in the pathways shown in Scheme VIII. The pathways are a modification of Scheme VI proposed by Evans and Brown (1972a). In their scheme Evans and Brown (1972a) did not show the intermediary steps during the catabolism of proline to alanine. Scheme VIII shows that proline is oxidized by FAD in a reaction catalysed by proline dehydrogenase which utilizes FAD as a coenzyme. In this scheme the possible cofactors, coenzymes and the intermediary steps of proline breakdown are shown. The pathways are based on the

Scheme VIII

Proposed pathways for the catabolism of proline in procyclic *T. congolense*. Adapted from Evans and Brown (1972a).

The enzymes shown are:-

1. Proline dehydrogenase
2. Δ^1-pyrroline dehydrogenase
3. Glutamate pyruvate transaminase
4. α-ketoglutarate dehydrogenase
5. SuccinylCoA synthetase
6. Succinate dehydrogenase
7. Fumarase
8. NADP-linked malic enzyme.


Proline
FAD+
1
FADH2
Δ'-Pyrroline -5 carboxyllate
H2O
Spontaneous
Glutamate-γ-semialdehyde
NAD+, H2O
2
NADH
Glutamate
3
ALANINE
Pyruvate
α-ketoglutarate
CO2
NADPH2
8
NADP+
CoASH
NAD+
4
NADH
Malate
CO2
7
H2O
Succiny1CoA
GDP(ADP)
5
GTP (ATP)
Fumarate
CoASH
6
FADH2
Succinate
FAD+

Scheme VIII

enzyme activities shown in tables II and V. Although pyrroline dehydrogenase and succinylCoA synthetase activities were not investigated the presence of their activities was assumed because all the other enzymes involved in proline catabolism were shown and alanine was an end product of proline catabolism. Alanine would otherwise not have been detected in the absence of these enzymes whose activities were not assayed.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSIONS

5.1 Reoxidation of Reduced Coenzymes and the Production of ATP by Procyclic *T.. congolense*

From the proposed pathways of glucose and proline catabolism in schemes VII and VIII respectively, reduced coenzymes are produced. These are NADH, NADPH and $FADH_2$. The NADH produced according to these pathways is reoxidized via the electron transport chain and GPO. It enters the electron transport chain via fumarate reductase (scheme IV). This would ordinarily result in 2 moles of ATP per mole of NADH oxidized (Lehninger, 1975). However, because of the SHAM/cyanide insensitive oxidase (section 3.4 and 4.4) less than 2 moles of ATP could be produced per mole of NADH oxidized by molecular oxygen. Some NADH generated from the catabolism of glucose are oxidized by molecular oxygen via the SHAM sensitive GPO (Scheme III). This oxidation through the GPO is, however, not coupled to ATP production (Grant and Sargent, 1960).

The NADPH produced during the catabolism of both glucose and proline through the malic enzyme (NADP-linked) could be used for biosynthesis or it could also be used for energy production. However, NADPH cannot normally be channelled directly to the electron transport chain for eventual oxidation by molecular oxygen. It is, therefore, proposed that there is an NADP transhydrogenase (pyridine-nucleotide transhydrogenase). This transhydrogenase

catalyses the transfer of electrons from NADPH to NAD^+ (Lehninger, 1975). NADH is then reoxidized through the electron transport chain with the concomittant production of ATP.

During proline catabolism some $FADH_2$ is generated which could be reoxidized via the electron transport chain. $FADH_2$ could possibly enter the electron transport chain at the coenzyme Q (ubiquinone) through the malonate sensitive succinate dehydrogenase. Ordinarily this would lead to production of 2 moles of ATP per mole of $FADH_2$ oxidized. However, because of the SHAM/cyanide insensitive respiration less than 2 moles of ATP per mole of $FADH_2$ oxidized could be produced.

5.2 CONCLUSIONS

From this study the following conclusions can be made:-

1. Procyclic *T. congolense* respire on both glucose and proline as substrates. These substrates are therefore useful sources of energy.

2. The end products of aerobic glucose catabolism are pyruvate, acetate and succinate while the end products of anaerobic catabolism of glucose are pyruvate acetate succinate and glycerol. During proline catabolism the main end product is alanine with glutamate also accumulating.

3. The reducing equivalents generated during the catabolism of proline are oxidized by a cyanide sensitive oxidase. During

glucose catabolism the reducing equivalents are oxidized by a SHAM sensitive GPO and cyanide sensitive oxidases. In both cases there is always some residual respiration not sensitive to either cyanide or SHAM or combination of both.

4. This study suggests that with more research it would be possible to target both glucose and proline catabolism reactions for chemotherapeutic attack. This is because the two substrates are useful sources of energy. Such a method of control of the insect stage of the parasite would be desirable as the drugs would not have to be administered to the mammal with possible subsequent toxic side effects. This would go a long way in controlling animal trypanosomiasis.

5. It is recommended from this study that the enzymes identified to be involved in the metabolism of the two substrates be studied further with a view to localising and characterizing them. It would also be prudent to investigate whether the procyclics utilize any other sugar or amino acid in preference to glucose and proline respectively.

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