" PATHWAYS OF GLYCEROL METABOLISM IN THE BLOODSTREAM AND CULTURED INSECT FORMS OF TRYPANOSOMA BRUCEI BRUCEI"

By

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A thesis submitted in fulfilment for the Degree of Doctor of Philosophy in Biochemistry in the University of Nairobi.

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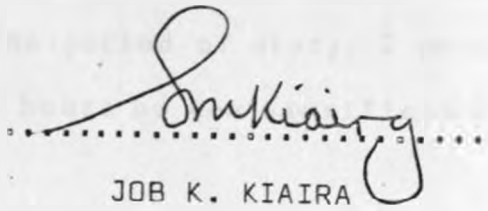
DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.



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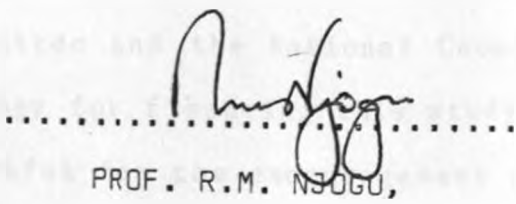
This thesis has been submitted for examination with my approval as University Supervisor.

PROF. R.M. NJOGU



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ABBREVIATIONS

ADP	Adenosine 5'diphosphate
AMP	Adenosine 5'monophosphate
ATP	Adenosine 5'triphosphate
ATPase	Adenosine 5'triphosphatase
Asp	Aspartate
BSO	Buthionine sulphoxime
Cyt	Cytochromes
DEAE-cellulose	Diethylaminoethyl cellulose
DFMO	Difluoromethyl ornithine
DNA	Deoxyribonucleic acid
E.C	Enzyme Commission
EDTA	Ethylenediaminotetraacetic acid
ENOL	Enolase
FAD	Oxidised flavin adenine dinucleotide
$FADH_2$	Reduced flavin adenine dinucleotide
FBS	Foetal bovive serum
Fru	Fructose
$Frul,6P_2$	Fructose 1,6-bisphosphate
Fru6P	Fructose 6-phosphate
Glc	Glucose
Glc6P	Glucose 6-phosphate
Glu	Glutamate

Gly	Glycine
GOT	Glutamate oxaloacetate transaminase
GPO	Glycerol 3-phosphate oxidase
GPT	Glutamate pyruvate transaminase
Gra3P	Glyceraldehyde 3-phosphate
$Gri1,3P_2$	Glycerate 1, 3-bisphosphate
Gri2P	Glycerate 2-phosphate
Grnp	Glycerone phosphate (Dihydroxyacetone Phosphate)
Gro	Glycerol
Gro3P	Glycerol 3-phosphate
Gro3PDH	Glycerol 3-phosphate dehydrogenase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
PI	Isoelectric pH
I.U	International Units
kDa	Kilo Daltons
kJ	Kilojoules
LDH	Lactate dehydrogenase
Mann	Mannose
MEM	Minimum essential medium
MDH	Malate dehydrogenase
MOPS	3-(N-morpholino) propanesulphonic

	acid
NAD	Oxidised nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Oxidised nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetate
$OH^{\cdot -}$	Hydroxyl radical
$O_2^{\cdot -}$	Superoxide anion
PABA	P-aminobenzoic acid
PEPyr	Phosphoenoylpyruvate
Pi	Inorganic phosphate
Pro	Proline
Pyr	Pyruvate
RPMI 1640	Rosewell Park Memorial Institute medium 1640
$S_{0.5}$	Concentration of substrate x giving half-maximal velocity.
SAM	S-adenosyl methionine
SHAM	Salicylhydroxamic acid
SOD	Superoxide dismutase
T.b.brucei	**Trypanosoma brucei brucei**
TCA	Tricarboxylic acid

Tra	Triethanolamine
Tris	Tris(hydroxymethyl)aminomethane
$T(SH)_2$	Reduced trypanothione
TS_2	Oxidised trypanothione
Wt	Weight
Vmax	Maximum velocity of an enzymatic process

Unit Abbreviations

m	meter
ml	millilitre
h	hour
kj	kilojoule
M	molar concentration
mg	milligram
min	minute
mol	mole
pH	- log H^+ concentration
PK	- log K'
S	second
L	litre

Prefixes

Multiple	Prefix	Abbreviation
10^3	kilo	K
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n

Summary of thesis

The in vitro propagated midgut forms, proventricular forms, midgut-like forms, metacyclic forms derived from tsetse salivary glands and the blood stream forms of Trypanosoma brucei brucei had high activities of the glycerol kinase and other enzymes for glycerol metabolism. These enzyme activities may account for the ability of these trypanosomes to use glycerol as substrate under aerobic condi tions. Of these trypanosome stages, only the blood stream and the in vitro propagated metacyclic forms derived from tsetse salivary glands produce glycerol from glucose under anaerobic conditions (Njogu and Nyindo, 1981), although all the stages had significant glycerol kinase activity.

The production of pyruvate, glycerol, and glycerol3-phosphate by digitonin-permeabilized bloodstream forms of T.b.brucei was studied with glucose or glycolytic intermediates as substrates.
Under aerobic conditions hexose phosphates gave maximum glycolysis in the presence of 40-60 micrograms of digitonin/10^8 trypanosomes while the triosephosphates gave it at 20-30 micrograms of digitonin/10^8 trypanosomes. In the presence of salicylhydroxamic acid (SHAM) and glycolytic intermediates, permeabilized trypanosomes produced equimolar amounts of pyruvate

and Gro3P but glucose catabolism under the same conditions produced equimolar amounts of pyruvate and glycerol plus Gro3P It is proposed that glycerol production from glucose in intact trypanosomes is regulated by ATP/ADP ratios and that ATP and ADP have a carrier at the glycosomal membrane. Glycerol kinase in digitonin permeabilized **T.b.brucei** catalysed ATP production at fairly high concentrations of Gro3P and ADP. The $S_{0.5}$ for Gro3P in permeabilized trypanosomes was 33.2 $\pm$ 5.8 mM. Pyruvate production from glucose in the presence of SHAM was significantly inhibited by more than 10 mM Gro3P indicating that high concentrations of Gro3P may also inhibit some glycolytic reaction(s). It was observed that high concentrations of Gro3P inhibited the Gro3P dehydrogenase. The apparent Ki for unpurified Gro3P dehydrogenase in the presence of GrnP was 29.5 mM. It was therefore concluded that the concentrations of Gro3P that can effectively transphosphrylate ADP also inhibit the reoxidation of NADH, thus inhibiting glycolysis. Aerobically, intact bloodstream trypanosomes catabolised glucose, fructose, mannose and glycerone to pyruvate. The rates of pyruvate production in micromoles/h/10^8 trypanosomes were: glucose, 6.0., fructose, 6.8., mannose, 5.0., and glycerone, 1.3. In the presence of SHAM, pyruvate production was reduced by

half, and equimolar amounts of pyruvate and glycerol were produced from glucose and mannose. SHAM caused about 85% and 100% decrease of pyruvate production from fructose and glycerone respectively, although equimolar amounts of pyruvate and glycerol were produced from fructose. In addition, it caused 50-70% decrease of glycolytic intermediates and 40-50% decrease of ATP concentration from glucose catabolism, whereas it caused 70-90% decrease of ATP production from fructose catabolism. It also caused 80% decrease of triosephosphates level and 81% decrease of the already low level of ATP from glycerone catabolism. There was a 2 to 2.5, 4 to 5.5 and 2.5 to 4 fold increase in the level of ADP, AMP and Gro3P respectively from glucose catabolism after the addition of SHAM. The level of Gro3P from fructose and mannose catabolism was in the same range as from glucose. Gro3P production from glycerone in the presence of SHAM was about half of that from glucose. Gro3P concentration decreased rapidly in trypanosomes pre-incubated with fructose for 20-30 min or with glycerone for 5 min after the addition of glucose or mannose but not D-galactose or 2-deoxy D-glucose. There was a slight decrease in Gro3P level in trypanosome pre-incubated with SHAM and glycerone for 5 minutes after the addition of fructose. It is proposed that Gro3P transphosphory-

lates with a hexose under the catalysis of the novel enzyme, Gro3P : glucose phosphotransferase. Pyruvate production under aerobic conditions from glucose, fructose and mannose was inhibited 80-90% by 10 micrograms oligomycin/10^8 trypanosomes but was not affected from glycerol catabolism. This inhibition lead to a rapid initial accumulation of Glc6P, Fru6P, Frul,$6P_2$, GrnP, Gra3P, Gro3P, ADP and AMP but a decrease in Gri2P, PEPyr and ATP from glucose but not from glycerol catabolism.

Exogenous ATP or ADP did not alleviate oligomycin inhibited glycolysis. In addition oligomycin did not inhibit pyruvate production from glycolytic intermediates in digitonin-permeabilized trypanosomes. Increase in some glycolytic intermediates after the addition of oligomycin could be also obtained by addition of a combination of SHAM, glycerol, and ATP to the trypanosomes. These results are discussed.

CHAPTER I

1.1 INTRODUCTION

The bloodstream forms of **Trypanosoma brucei brucei** are entirely dependent on glycolysis for energy production (Bowman, 1976). They lack lactate dehydrogenase, a functional Krebs cycle and the cytochrome systems (Dixon, 1966; Fulton and Spooner, 1959; Ryley, 1956). These trypanosomes produce mainly pyruvate from glucose under aerobic conditions but under anaerobic conditions they produce pyruvate and glycerol in equimolar amounts (Ryley, 1956). Schemes to account for ATP production during anaerobiosis have been proposed (Opperdoes and Borst,1976; Clarkson and Brohn,1976) So far none of these schemes has been shown to be operational in **T.b.brucei**. Another scheme was proposed by Opperdoes and Borst (1977) to account for ATP production and compartmentation of glycolytic enzymes. It involves the use of the reverse glycerol kinase activity to form ATP and glycerol from ADP and Gro3p. Although the reverse glycerol kinase activity has been demonstrated **in vitro** it has not been demonstrated **in vivo** or in isolated intact glycosomes.A peculiar observation related to glycolysis was made by Miller and Klein (1980). They reported inhibition of respiration and pyruvate production from glucose by oligomycin. This observation was

rather unusual because the the long slender bloodstream trypanosomes lack a functional TCA cycle and cytochromes (Ryley, 1956; Fulton and Spooner, 1959). Whether there is a link between the mechanism of glycerol production and oligomycin sensitivity of glycolysis is not known.Other trypanosome stages do also catabolise glucose but it's not yet documented whether they produce glycerol during anaerobiosis. (Gutteridge and Coombs, 1976). These are the midgut forms believed to be similar to the cultured procyclic forms, proventricular forms, epimastigote forms and the metacyclic forms. The main problem has been the inability to obtain sufficient number of trypanosomes for study (Gutteridge and Coombs, 1976).

Other than the bloodstream forms of **T.b.brucei** the only other reported trypanosome stage that produces glycerol from glucose is the cultured metacyclic forms derived from tsetse fly salivary glands (Njogu and Nyindo, 1981). The metacyclic forms have also been propagated (Hirumi, 1979) during **in vitro** cyclic transformation of the bloodstream forms but no studies on glycolysis have been done because the metacyclic forms density was only 1% of the total trypanosome population in the cultures.

1.1.1 Objectives of the study

The aim of the study was to:

(i) determine the conditions that favour the propagation of large numbers of viable metacyclic trypanosomes by each of the two culture methods reported by Nyindo et al (1979) and Hirumi et al (1979).

(ii) investigate further the pathway of glycerol metabolism by the cultured trypanosomes derived from tsetse salivary glands.

(iii) provide more information on the pathways of glycerol production during anaerobiosis in the bloodstream forms and hence the possible mechanism of net ATP production.

(iv) determine the relationship between glycerol production and oligomycin sensitivity of glycolysis in the bloodstream forms during anaerobiosis.

1.2 LITERATURE REVIEW

1.2.1 The causative agents of African trypanosomiases

African human trypanosomiasis is caused by **Trypanosoma gambiense** and **Trypanosoma rhodesiense**. The equivalent disease in animals is called Nagana and is caused by **T.brucei, T.vivax** and **T. congolense. T.brucei** is morphologically identical to **T. gambiense** and **T.rhodesiense** but (by definition) is not pathogenic to man. These very similar organisms are regarded as subspecies to **T.brucei sensu latu** and designated **T.b.brucei, T.b.gambiense and T.b.rhodesiense** (Hoare, 1972; Vickerman, 1965). Massamba and Williams (1984) have shown that these morphologically identical trypanosomes can be differentiated by nucleic acid hybridization. They showed **T.b.brucei** nuclear DNA fragments can be used as probe in hybridization analysis to diffrentiate the trypanosome species and subspecies. Tait **et al** (1984) showed that the enzyme peptidase C can be used to differentiate **T.b.brucei** and **T.b.rhodesiense** from **T.b.gambiense** stocks. Peptidase C activity was not detected or was very low in **T.b.gambiense** stocks.

Due to the apparent similarity between the subspecies of **T. brucei, T.b.brucei** is used in preliminary reserch as a model for the other two subspecies patho-

genic to man.

There are three distinct cell types of T.brucei found in the blood of infected animals. Short stumpy, long slender and the intermediate trypomastigotes. The short stumpy forms appear to be the ones which survive in the insect vector when it takes a blood meal from an infected animal (Wijers and Willet, 1960) and these later transform to the midgut forms. The long slender forms predominate in the blood during infection and are thought to cause the pathology observed in infected hosts.

1.2.2 The importance of African trypanosomiasis

Both T.b.gambiense and T.b.rhodesiense cause primarily a disease of the central nervous system (CNS) commonly known as sleeping sickness. The disease occurs in two clinical epidemiological patterns. The chronic Gambian type caused by T.b.gambiense and the acute Rhodesian type caused by T.b.rhodesiense. In both types the infection is initiated by a period of blood infection followed by invasion of the cerebrospinal fluid by the trypanosomes with involvement of the CNS. In case of the Gambian disease death may occur several years after onset and in less than a year in the case of the Rhodesian disease if untreated.

The immune response of human hosts appear to be

mainly humoral. The phagocytes play a subsidiary role. After a rise of parasitemia, there is an abrupt decline of the trypanosome number due to the action of trypanolytic antibodies which destroy the parasites. The phagocytes dispose of the parasite remains. The trypanosomes which survive proceed to multiply initiating a new wave of parasitemia and the process is repeated. (Ross and Thompson, 1910, Hoare, 1972).

The antibodies produced in the course of infection eliminate the trypanosomes during the crisis but some of the parasites undergo antigenic change which renders them resistant to the original antibodies. This enables them to continue to multiply until most of them are destroyed by the production of specific antibody by the host. This process usually continues until the host's defence is overcome resulting in death.

There is no evidence of the existence of innate immunity to infection with **T.b.gambiense** or **T.b.rhodesiense** although some people exhibit considerable degree of tolerance. Of the **T.brucei** species it is **T.b.brucei** which causes the disease (Nagana) in wild animals and livestock. All laboratory animals are susceptible to infection. The severity of Nagana depends on the hosts' species, nutrition and environmental conditions. There is no evidence of acquired

immunity to infection with **T.b.brucei**. The changes in parasitemia with time are as for **T.b.gambiense** and **T.b.rhodesiense**. (Ross and Thompson, 1910).

1.2.3 In vivo cyclic development of T. brucei

When tsetse of **Glossina morsitans** species takes **T.brucei** infected blood , the trypanosomes are passed after 30 min via the proventriculus to the midgut (Moloo and Kutuzu, 1970). The trypanosomes transform to form with less pronounced undulating membrane which have kinetoplast situated halfway between the posterior end of the body and the nucleus. A free flagellum is present in all the forms. These are referred to as the midgut forms. After 12 days their mitochondria appear enlarged. Some of them appear caught on folds of the peritrophic membrane. They come to lie within the ectoperitrophic space after penetration of the apex of the folds. (Ellis and Evans, 1977). Penetration of gut cells follow, and by 18-21 days the midgut trypomastigotes are found multiplying within the cytoplasm of the same cells. Some eventually come to lie between the basement of the gut cells and the membrane of the hemocole (Evans and Ellis, 1975). Apparently many trypomastigotes penetrate the gut cell and no phagocytosis occurs. Establishment of the trypanosomes in the gut cells is

followed by migration to the lumen of the salivary glands where many metacyclic trypomastigotes which are infective to mammals appear.**T.brucei** does not always complete its development in the insect vector. Trypanosomes may get established in the gut but fail either to invade the salivary glands or to develop in the gut, or existing salivary gland infection may die out (Hoare, 1972). Such trypanosome strains are nontransmissible by tsetse except by mechanical inoculation.

1.2.4 **Metabolic changes during cyclic in vivo development**

None of the these stages have been isolated in quantities enough for biochemical studies. Only preliminary work has been carried out using small numbers of midgut forms (Gutteridge and Coombs, 1976).

1.2.5 **Cyclic development of T. brucei in vitro**

Complete **in vitro** cyclic development of T.b.brucei was first reported by Hirumi et al (1979). They used TC 221 population derived from the monomorphic strain stock 427. The cultures containing the epimastigotes and metacyclic forms were infective to Balb c mice. It was not possible to induce the majority of the trypanosomes to transform to the metacyclic form . There was a maximum of 1% infective trypanosomes in these cultures judging from infectivity to

Balb/c mice after intraperitoneal inoculation of various trypanosome numbers.

1.2.6 Metabolic changes during in vitro cyclic transformation

The report by Hirumi et al (1979) opened way to the study of biochemical characteristics of the various trypanosome stages during cyclic transformation. No biochemical work has so far been reported regarding these organisms.

1.2.7 The procyclic trypomastigotes

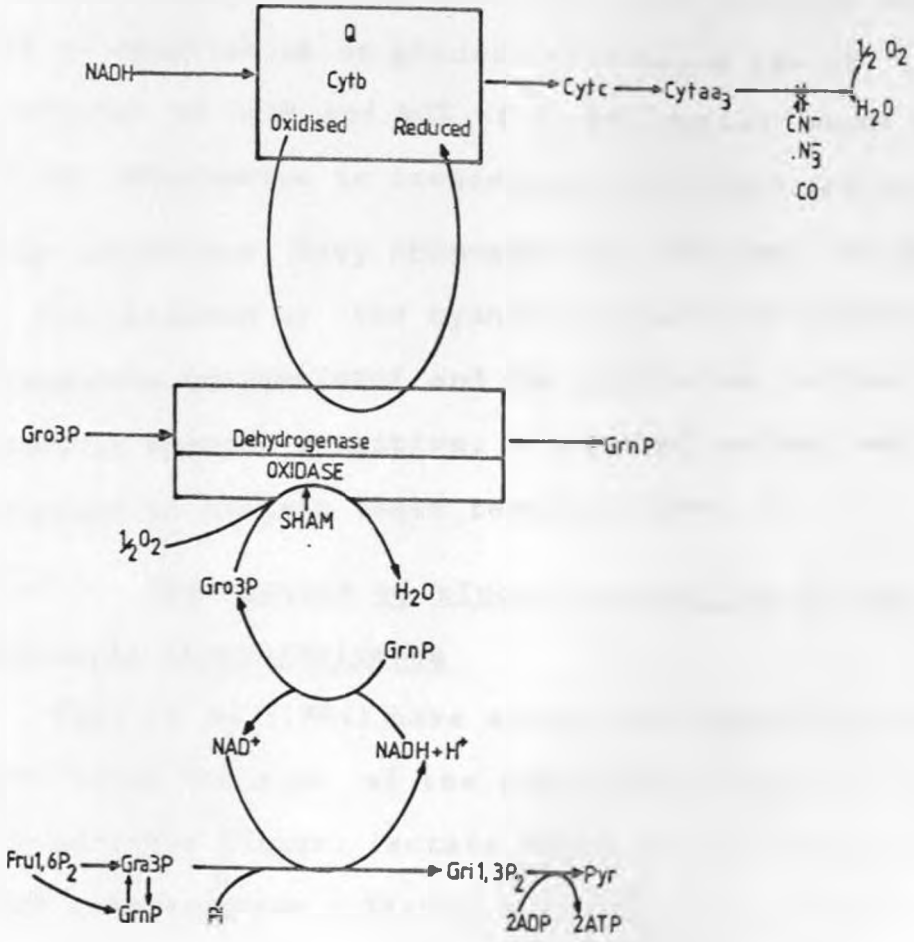
1.2.7.1 In vitro propagation of the procyclic trypomasigotes

When the bloodstream forms of T.brucei species are introduced in a semi-defined medium and incubated at 28°C (Brun and Schonenberger, 1979), they transform to the procyclics. These are not infective to mammals and are somewhat morphologically similar to the midgut forms.

1.2.7.2 Respiration of the procyclic trypomastigotes

The procyclic trypomastigotes can respire on proline or glucose but not on aspartate, methionine, serine or glutamate. Cyanide does not completely inhibit the respiration on proline or glucose (Evans and Brown, 1972). Compounds such as amytal and rotenone that usually inhibit site I of electron tran-

Scheme I: Reoxidation of reducing equivalents generated from the catabolism of glucose by the T.b.brucei procyclic trypomastigotes (Njogu et al, 1980).


NADH
Q
Cytb
Oxidised
Reduced
Cytc
Cytaa3
½ O2
H2O
CN⁻
N3⁻
CO
Gro3P
Dehydrogenase
OXIDASE
GrnP
SHAM
½O2
H2O
Gro3P
GrnP
NAD+
NADH+H+
Fru1,6P2
Gra3P
GrnP
Pi
Gri1,3P2
Pyr
2ADP
2ATP

sport chain in most aerobic cells have no effect on the respiration on glucose or proline (Gutteridge and Coombs, 1976). Njogu et al (1980) have reported that 30% of respiration on glucose or proline can be inhibited by SHAM and 60% of it by cyanide. About 10% of the respiration is insensitive to a combination of both inhibitors. They proposed that this may be due to the presence of the cyanide insensitive glycerol-3phosphate oxidase(GPO) and the cytochrome oxidase which is cyanide sensitive. A branched pathway was proposed to explain their results(Scheme I).

1.2.7.3 The pathway of glucose catabolism in the procyclic trypomastigotes

Hart et al (1984) have shown the presence of the activities for most of the glycolytic enzymes except for pyruvate kinase, lactate dehydrogenase and pyruvate dehydrogenase . Enzyme activities of the TCA cycle that are extremely low are: NAD^+- linked isocitrate dehydrogenase and citrate synthase (Evans and Brown, 1972). Enzyme activities of the glyoxylate cycle; isocitrate lyase, malate synthase, alanine glyoxylate aminotransferase, and for the anaplerotic reactions; $NADP^+$ linked glutamate dehydrogenase and pyruvate carboxylase are also insignificant. (Opperdoes and Cottem, 1982). TCA cycle enzyme activities

Scheme II: The pathway of glucose catabolism and CO_2 fixation by the **T.b.brucei** procyclic trypomastigotes (Opperdoes and Cottem,1982).

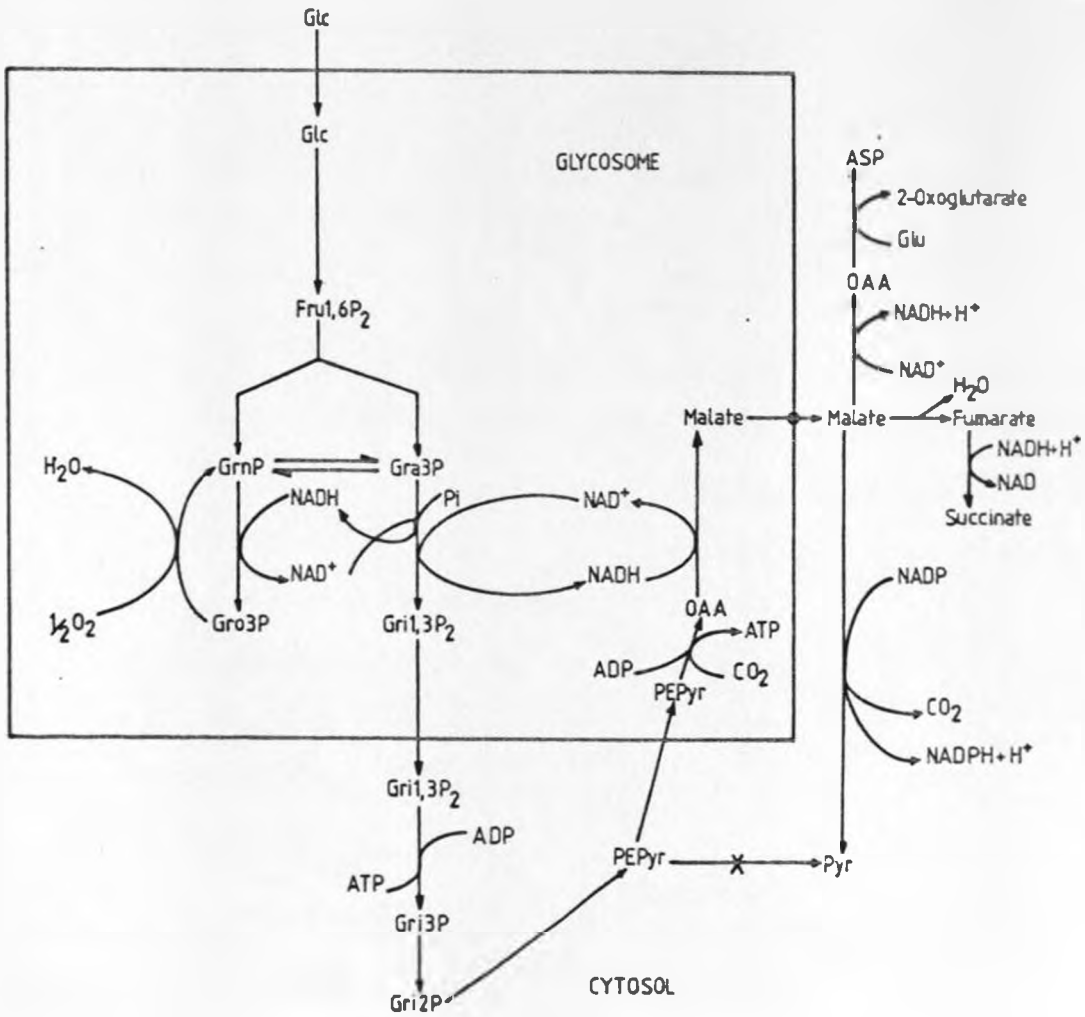

Glc
Glc
GLYCOSOME
Fru1,6P2
H2O
GrnP
Gra3P
NADH
Pi
NAD+
NAD+
NADH
½O2
Gro3P
Gri1,3P2
Malate
OAA
ATP
ADP
CO2
PEPyr
Gri1,3P2
ADP
ATP
Gri3P
Gri2P
CYTOSOL
PEPyr
Pyr
ASP
2-Oxoglutarate
Glu
OAA
NADH+H+
NAD+
H2O
Malate
Fumarate
NADH+H+
NAD
Succinate
NADP
CO2
NADPH+H+

Table I: Some enzyme activities of oxidative pathways of glucose in the bloodstream and procyclic forms of T.b.brucei. Units of activities are nanomoles/min/mg protein

References

(a) Hart et al (1984).

(b) Opperdoes and Cottem (1982).

(c) Opperdoes and Steiger (1981).

(d) Opperdoes and Borst (1977).

(e) Opperdoes et al (1977).

(f) Opperdoes et al (1984).

(g) Oduro et al (1980b).

Enzymes	Bloodstream forms	procyclic-forms
Hexokinase	760(a)	55(a)
Phosphoglucose-isomerase	780(a)	135(a)
Aldolase	33(a)	120(a)
Triosephosphate isomerase	98(a)	1370(a)
Glyceraldehyde-phosphate dehydrogenase	130(a)	11(a)
Glycerol kinase	1240(a)	1590(a)
Glycerol3-phosphate dehydrogenase	300(a)	290(a)
Enolase	80(d)	200(b)
Pyruvate kinase	1600(g)	2(b)
Succinate dehydrogenase	ND(e)	10(e)
Fumarate hydratase	0.3(e)	66(b)
Fumarate reductase	ND(b)	72(b)
Malate dehydrogenase	66(a)	212(a)
Malic enzyme(NADP)	--	35(b)
Alanine amino-transferase	523(c)	52(c)
Adenylate kinase	95(a)	87(a)
Total ATPase	170(f)	190(f)
Oligomycin sensitive ATPase	20(f)	40(f)

more than 2 nanomoles/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, alanine aminotransferase, aspartate amino transferase (Evans and Brown, 1972; Opperdoes and Cottem, 1982), phosphoenoylpyruvate carboxykinase, adenylate kinase, glycerol kinase and glycerol3-phosphate dehydrogenase (Opperdoes and Cottem, 1982).Table I shows some enzyme activities of oxidative pathways of glucose in the procyclic forms of **T.b.brucei**. Opperdoes and Cottem (1982) proposed the pathway of glucose catabolism shown in scheme II. It was assumed that the glycosome is impermeable to small charged compounds such as malate, fumarate, succinate, oxaloacetate and the glycolytic intermediates.

1.2.7.4 The pathway of proline catabolism

Based on some enzyme activities, end-products of proline catabolism and respiration studies a pathway for proline catabolism has been proposed (Evans and Brown, 1972). Scheme III is a summary of the proposed pathway. The pathway for the electrons derived from reaction (1), (3) (4) and (6) to the electron transport chain has not been established.

It is also unclear as to whether there is an OAA decarboxylase or a malic enzyme that decarboxylates

Scheme III: The pathway of proline catabolism by the procyclic trypomastigotes (Evans and Brown, 1972). The suggested names for the enzymes numbered 1 - 8 are as follows:_

(1) Proline dehydrogenase

(2) Glutamate pyruvate transaminase

(3) 2-Oxoglutarate dehydrogenase and succinyl CoA synthetase

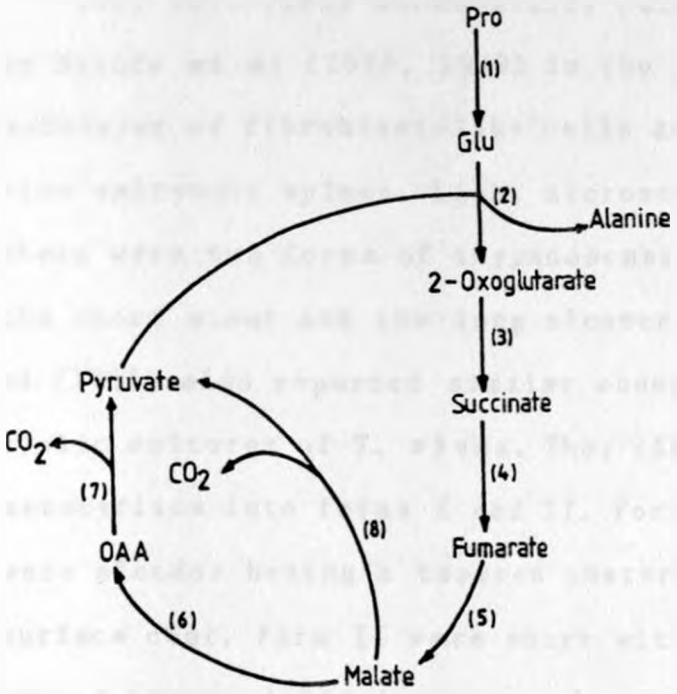
(4) Succinate dehydrogenase

(5) Fumarase

(6) Malate dehydrogenase

(7) OAA decarboxylase

(8) Malic enzyme or malate decarboxylase


Pro
(1)
Glu
(2)
Alanine
2-Oxoglutarate
(3)
Succinate
(4)
Fumarate
(5)
Malate
(6)
OAA
(7)
CO_2
Pyruvate
(8)
CO_2

malate to pyruvate.

1.2.8 The metacyclic trypomastigotes

1.2.8.1 In vitro propagation of the metacyclic forms of T.b.brucei derived from tsetse salivary glands

They were first successfully cultivated at 28°C by Nyindo et al (1978, 1979) in the presence of a monolayer of fibroblast-like cells derived from bovine embryonic spleen. Light microscopy showed that there were two forms of trypanosomes in the cultures; the short stout and the long slender forms. Hirumi et al (1985) also reported similar observations in metacyclic cultures of T. vivax. They classified T.vivax metacyclics into forms I and II. Form I trypanosomes were slender having a tapered posterior and without a surface coat. Form II were short with round posterior end, a terminal kinetoplast and a surface coat. They proposed that form I is the immature stage and form II is the mature metacyclic stage. On the other hand the T.b.brucei short stout forms (Form II) lacked the thick surface coat in contrast to the T.vivax forms which were very distinct from the long slender forms although they were infective to Balb/c mice (Nyindo et al, 1980). The latter authors (Nyindo et al, 1980) proposed that the short stout forms represented the immature metacyclic forms and the long slender forms the mature metacyclics.Both forms of T.b.brucei had

peroxisome-like organelles similar to the glycosomes observed in the bloodstream forms. These organelles were assumed to be the glycosomes. The number of glycosomes in the long slender forms appeared higher than in the stout forms.

1.2.8.2 Respiration and the catabolism of glucose

Metabolic studies revealed some unusual characteristics (Njogu and Nyindo, 1981). The parasites could not respire on proline or any of the TCA cycle intermediates. On incubation with glucose as substrate the end products were pyruvate and glycerol in the molar ratio of 3:1. When they were incubated with SHAM, respiration was completely inhibited and equimolar amounts of pyruvate and glycerol were produced. The rate of respiration on glucose in the absence of SHAM was 86-96 nanoatoms O /min/10^8 trypanosomes. This is less than that of the bloodstream forms of 150 nanoatoms O /min/10^8 trypanosomes. No other investigations were done and much of the glucose metabolism in these parasites remain unknown.

1.2.9 **The bloodstream trypomastigotes**

1.2.9.1 **In vitro cultivation of the animal infective bloodstream forms of T.b. brucei.**

These were first cultured successfully by Hirumi et al (1977) in the presence of fibroblast-like cells derived from bovine blood buffy coat in HEPES buf-

fered RPMI 1640 supplemented with 20% v/v foetal bovine serum. The same experiment can now be repeated in the presence of fibroblasts derived from other bovine organs (embryonic) and Microtus montanus embryonic fibroblasts (Hirumi, 1979, Brun et al, 1981).Baltz et al (1985) showed that fibroblast-like cells are unnecessary for the maintenance of these trypanosomes. They devised a medium consisting of MEM, hypoxanthine, thymidine, mercaptoethanol and bovine serum. This was later modified by Dusenko et al (1985) who showed that L-cystein could replace the feeder layer and mercaptoethanol. The optimal concentration of L-cystein depends on the trypanosome concentration.Despite this success in the trypanosome cultivation, the major source of trypanosomes for metabolic studies is the blood of heavily infected laboratory animals. In vitro trypanosomes culture has been used more in screening of potential trypanocides (Borowy et al, 1985).

1.2.9.2 Carbohydrate catabolism in the bloodstream trypomastigotes of T.b. brucei.

The bloodstream forms of T.brucei species aerobically catabolize large quantities of D-glucose to pyruvate, the predominant end-product (Grant and Fulton, 1957; Brohn and Clarkson, 1978). They cata-

bolize glucose at a rate 50 times that of most mammalian cells (Von Brand, 1951). Grant and Fulton (1957) showed that **T.b. rhodesiense** catabolize D-glucose to approximately equimolar amounts of pyruvate and glycerol during anaerobiosis. They observed that with (1-^{14}C) glucose, pyruvate was labelled only in the methyl carbon. Carboxyl-labelled pyruvate was obtained using (3:4 - $^{14}C_2$) glucose. During anaerobiosis the radioactivity in glycerol was much higher than in pyruvate, whereas during aerobiosis the radioactivity in both compounds was the same. They proposed that glucose is catabolised through the Embden-Meyerhof pathway in these trypanosomes. Ryley (1956) had reported that **T.b.rhodesiense** are insensitive to inhibitors of the electron transport system. Further results showed that the bloodstream forms of **T.brucei** species are dependent on glycolysis for their energy supply since they do not have cytochromes (Ryley, 1956; Fulton and Spooner, 1959). Reoxidation of NADH under aerobic conditions is achieved mainly via the GPO. The GPO is thought to be unique to the salivarian trypanosomes. It catalyses the reduction of O_2 in a reaction not coupled to ADP phosphorylation (Grant and Sargent, 1960).

$1/_2O_2$ + Gro3P $\rightarrow$ Grnp + H_2O

The GPO consists of an oxidase and a dehydrogenase

component (Fairlamb and Bowman, 1977). Opperdoes et al (1977) observed that it co-purified with mitochondrial ATPase. They concluded that it must be localised in the mitochondria contrary to previous reports (Miller, 1975) that it is localised in microbody-like structures: the glycosomes.

When the GPO is inhibited by SHAM or during anaerobiosis, the trypanosomes produce equimolar amounts of pyruvate and glycerol (Opperdoes et al, 1976; Brohn and Clarkson, 1976; Grant and Fulton, 1957). However the inhibition of the GPO is not lethal to the trypanosomes incubated with glucose as substrate. In the absence of glucose they become motionless after 5 min and then disintegrate. Exposure of the trypanosomes utilizing glucose as substrate to a combination of SHAM and glycerol **in vivo** and **in vitro** is trypanocidal (Clarkson and Brohn, 1976; Brohn and Clarkson, 1978; Fairlamb et al, 1977). This finding has led to some research attention in the hope of producing new trypanocidal drugs that would completely inhibit glycolysis.

1.2.9.2.1 Compartmentation of glycolytic intermediates and anaerobic glycolysis in T. b. brucei

Opperdoes and Borst (1977) proposed that the enzymes of glycolysis are inside the glycosome and that these enzymes catalyze the reactions:

1. Glc + 2GrnP + 2Pi $\longrightarrow$ 2Gro3P + 2Gri3P and

2. Gro + 2GrnP + Pi $\longrightarrow$ 2Gro3P + Gri3P

The formation of these products has been confirmed using isolated glycosomes (Oduro et al, 1980). These authors proposed that Gri3P can leave the glycosome to the cytosol where it is converted to pyruvate with concomitant ATP production according to the equation:

3. Gri3P + ADP $\rightarrow$ ATP + pyruvate + H_2O

The oxidation of Gro3P in the promitochondrion is catalyed by GPO according to the equation:

4. Gro3 + $1/_2O_2$ $\rightarrow$ Gro3P + H_2O

Due to the latency of the glycolytic enzymes catalysing the reactions 1 and 2, they proposed that the glycosomal membrane is permeable to glucose and glycerol but not to NAD^+, adenine nucleotides and the glycolytic intermediates. To account for the entry and exit of some metabolites, they proposed the presence of the translocators for Gro3P, GrnP, Gri3P and Pi. They argued that when reaction 4 above is inhibited or during anaerobiosis, glucose is converted to

pyruvate and glycerol according to reaction 5 and 6 shown below believed to be catalysed by the reverse activity of the glycerol kinase only at high concentration of Gro3P and ADP inside the glycosome. These authors did not demonstrate unequivocally that the reverse glycerol kinase activity resides inside

5. Glc + ADP + Pi $\longrightarrow$ Gro +Pyr + ATP + H_2O

6. Gro3p + ADP $\longrightarrow$ Gro + ATP

the glycosome. The results of Hammond et al (1985) show no latency of the reverse glycerol kinase activity of isolated glycosomes in contrast to other glycosomal enzymes. It was further argued that the glycosome is self sustaining and thus there should be no change in the glycosomal levels of ATP, ADP and AMP during anaerobiosis. Glycerol and ATP production (reaction 6) would thus be regulated by the concentration of Gro3P. Decrease in cytosolic ATP would be expected. Theoretically ATP would be expected to fall by half in the cytosol. It has been shown that during anaerobiosis there is total increase in cellular ADP and AMP but a decrease in ATP (Hammond and Bowman, 1980; Visser and Opperdoes, 1980). It is not known whether this decrease of ATP is a reflection of what happens in the cytosol, the glycosome or in both compartments.

The translocators for P_i shown in scheme IVA and the triosephosphate isomerase at the glycosomal membrane have

also not been demonstrated experimentally, possibly due to lack of adequate method for the preparation of functionally intact glycosomes suitable for metabolite transport studies. The glycosomes prepared so far appear unsuitable. They have lower activities of some glycolytic enzymes (Oduro et al, 1980; Hammond et al, 1985b, Opperdoes et al 1984) and can utilize exogenous NADH (Aman and Wang, 1986).

1.2.9.2.2 Thermodynamics of anaerobic glycolysis in T.b.brucei.

Reaction (5) shown above is thermodynamically possible under standard conditions. The standard free energy is - 31 kJ/mol (Hammond and Bowman, 1980b). Reverse activity of the glycerol kinase has been shown in vitro (Hammond et al 1985; Hammond and Bowman, 1980b) but not in vivo. Under standard conditions reaction (6) appears thermodynamically unfavourable because the standard free energy is positive and large (+22 kJ/mol). Theoretically a thermodynamically unfavourable reaction cannot be the last reaction of a metabolic pathway even if all the proceeding reactions were thermodynamically favourable unless it is coupled to a thermodynamically favourable reaction. There is evidence that the glycerol kinase reaction is not coupled to any of the ATP utilizing reactions; the phosphofructokinase and the

Scheme IVA: A schematic representation of the compartmentation of the glycolytic pathway in the bloodstream trypomastigotes of T.b. **brucei** (Visser **et al**,1981).

The names for the enzymes numbered 1-13 are:-

(1) Hexokinase (E.C 2.7.1.1)

(2) Phosphoglucose isomerase (E.C 5.3.1.9)

(3) Phosphofructokinase (E.C 2.7.1.11)

(4) Aldolase (E.C.4.1.2.7)

(5) Triosephosphate isomerase (E.C 5.3.1.1)

(6) Gra3p dehydrogenase (E.C 1.2.1.12)

(7) Gro3p dehydrogenase (E.C 1.1.1.8)

(8) Phosphoglycerate kinase (E.C 2.7.2.3)

(9) Glycerol kinase (E.C 2.7.1.30)

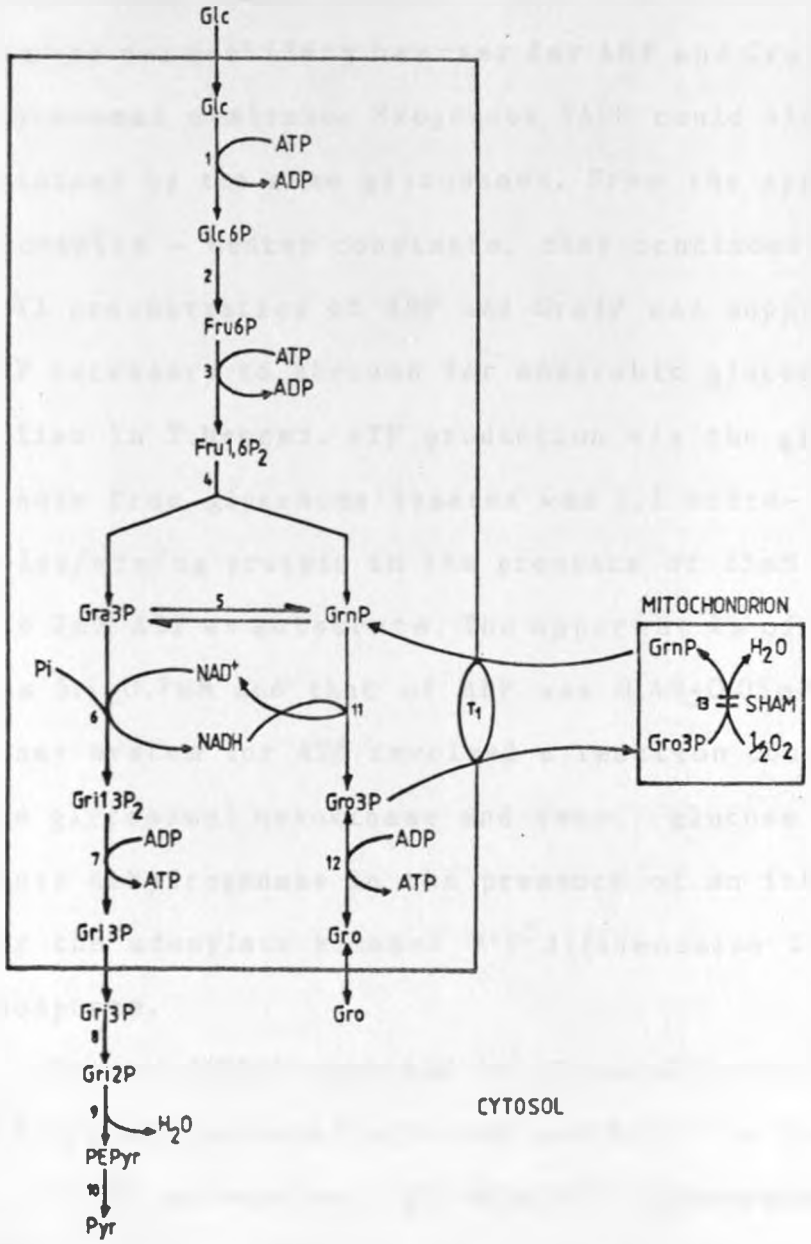
(10) Phosphoglyceromutase (E.C 2.7.5.3)

(11) Enolase (E.C 4.2.1.11)

(12) Pyruvate kinase (E.C 2.7.1.40)

(13) Glycerolphosphate oxidase (not classified)

T1 is the Grnp/Gro3p shuttle system .


Glc
Glc
ATP
1
ADP
Glc6P
2
Fru6P
ATP
3
ADP
Fru1,6P2
4
Gra3P
5
GrnP
MITOCHONDRION
Pi
NAD+
6
11
NADH
T1
GrnP
H2O
13
SHAM
Gro3P
½O2
Gri13P2
Gro3P
ADP
7
ATP
ADP
12
ATP
Gri3P
Gro
Gri3P
8
Gro
Gri2P
9
H2O
PEPyr
10
Pyr
CYTOSOL

hexokinase reaction (Hammond et al, 1985). In addition, using isolated glycosomes, these authors observed no permeability barrier for ADP and Gro3P at the glycosomal membrane. Exogenous NADH could also be oxidised by the same glycosomes. From the apparent Michaelis - Menten constants, they concluded that the cell concentration of ADP and Gro3P can supply the ATP necessary to account for anaerobic glucose metabolism in **T.brucei**. ATP production via the glycerol kinase from glycosome lysates was 1.2 micromoles/min/mg protein in the presence of 23mM Gro3P and 2mM ADP as substrate. The apparent Km of Gro3P was 5.1±0.7mM and that of ADP was 0.49±0.05mM. Their assay system for ATP involved a reaction coupled to the glycosomal hexokinase and yeast glucose 6-phosphate dehydrogenase in the presence of an inhibitor for the adenylate kinase: P^1P^5di(adenosine 5') pentaphosphate.

Kiaira (1983) reported no glycerol production in **T.b.brucei** incubated with ADP and Gro3P in the presence of 60 micrograms digitonin/10^8 trypanosomes, and iodoacetamide to inhibit glyceraldehyde 3-phosphate dehydrogenase. Gro3P accumulated from the catabolism of the hexose phosphates incubated with SHAM but without iodoacetamide. It was proposed that glycerol production depends on the integrity of the glycosomal

membrane.

1.2.9.2.3 Glycerol production from glucose catabolism

1.2.9.2.3.1 The mechanism of glycerol production under anaerobic conditions in T.b.brucei.

To explain the net synthesis of ATP under anaerobic conditions four possible hypotheses were proposed. One has already been discussed in the previous subsections and shown in scheme IVA.

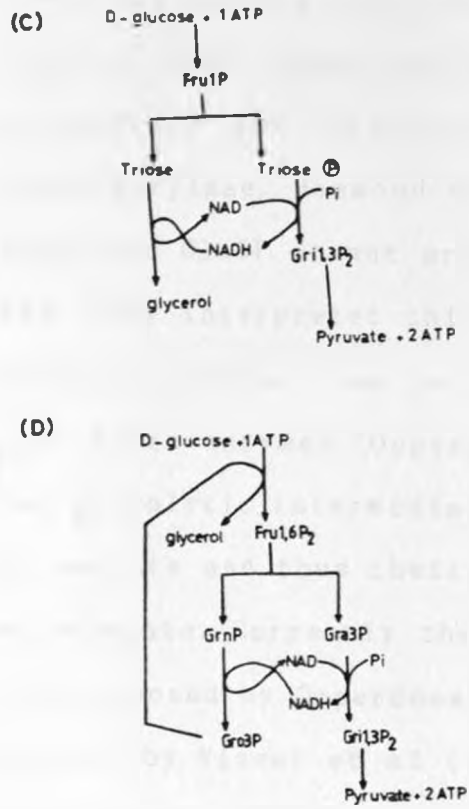
Clarkson and Brohn (1976) proposed scheme IVB They proposed that a monophosphate aldolase could cleave FrulP to GrnP and glyceraldehyde. The Grnp could then be reduced by NADH generated from the glyceraldehyde 3-phosphate dehydrogenase reaction during the oxidation of Gra3P. The formation of Gra3P would be via a novel Gra:Gro3P transphosphorylase. The presence of some FrulP aldolase activity has been reported (Clarkson and Brohn, 1976, Misset and Opperdoes, 1984) but not the transphosphorylase activity. From digitonin permeabilized trypanosomes there was no significant glycerol and pyruvate production from FrulP in the presence of Gro3P and ADP as would be expected from scheme IVB. It has been shown that this pathway may not exist in the trypanosomes (Kiaira, 1983).

Opperdoes et al (1976) proposed another pathway (scheme IVC). They proposed the presence of a novel

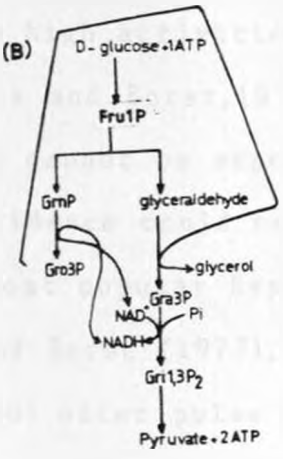
triosephosphate reductase so far not detected in the trypanosome. According to the hypothesis, the hexose-monophosphate would be cleaved to a triose sugar and a triosephosphate. The triosephosphate could be Gra3P which would then be oxidised by NAD^+ to Gri1,$3P_2$. The NADH generated would reduce the triose via triose reductase to glycerol. From digitonin permeabilized trypanosomes in the presence of SHAM, no detectable glycerol or pyruvate were produced in the presence of SHAM, Fru1P and ADP as substrates as would have been expected if the proposed novel enzyme were operating (Kiaira, 1983). In addition due to the absence of triose reductase activity, Clarkson and Brohn (1976) proposed yet another pathway (scheme IVD).

They proposed that Fru1,$6P_2$ could be cleaved to Gra3P and GrnP. The NADH generated due to oxidation of Gra3P would be reoxidised by GrnP to Gro3P. The Gro3P can transfer the phosphoryl group to a hexose to form a hexose monophosphate which is converted to Fru1,$6P_2$. The only novel enzyme proposed was Gro3P: hexosphosphate transphosphorylase. The enzyme activity of this transphosphorylase has not so far been demonstrated. Digitonin permeabilized trypanosomes incubated with D-glucose and ADP could not produce pyruvate and glycerol after the addition of Gro3P.

Scheme IVB-D: Schematic representation of alternate pathways for anaerobic catabolism of glucose by the bloodstream forms of **Trypanosoma b.brucei** (Clarkson and Brohn, 1976).


(C)
D-glucose + 1ATP
Fru1P
Triose
Triose P
Pi
NAD
NADH
Gri1,3P2
glycerol
Pyruvate + 2ATP

(D)
D-glucose +1ATP
glycerol
Fru1,6P2
GrnP
Gra3P
NAD
Pi
NADH
Gro3P
Gri1,3P2
Pyruvate + 2ATP


(B)
D-glucose +1ATP
Fru1P
GrnP
glyceraldehyde
Gro3P
glycerol
Gra3P
NAD
Pi
NADH
Gri1,3P2
Pyruvate + 2ATP

However there was production of Gro3P and pyruvate in equimolar amounts in contrast to the situation with FrulP where none of them was formed. It was proposed that this pathway does not also operate in T.brucei (Kiaira, 1983; Opperdoes and Borst,1977) However it is possible that digitonin might inhibit the transphosphorylase. Hammond et al (1980) observed that Fru6P and Glc6P do not accumulate during anaerobiosis. They interpreted this to show the absence of the proposed pathway. Due to the high activities of the glycolytic enzymes (Opperdoes and Borst,1977) these two glycolytic intermediates cannot be expected to accumulate and thus their evidence could not be taken as adequate. Currently the most popular hypothesis was proposed by Opperdoes and Borst (1977), and schematised by Visser et al (1980) after pulse labelling experiments. All the enzymes involved in this pathway are present in sufficient activities combatible with the observed rate of glycolysis(scheme IVA).

1.2.9.2.3.2 Glycerol production under various conditions by other organisms

Trichomonas vaginalis which is an aerotolerant anaerobic protozoan flagellate has no mitochondria and haeme proteins (Chapman et al, 1985). Instead they have an organelle called the hydrogenosome. This organelle contains enzymes that catalyse the oxidation of pyruvate to CO_2, acetate and molecular hydrogen with conservation of energy to ATP by substrate level phosphorylation. Under anaerobic conditions these organisms produce lactate acetate and glycerol as major products, (Chapman et al, 1985). The details on the exact mechanisms of glycerol production have not been elucidated. There is more detail on glycerol production from studies in **Phycomyces blakensteanus** spores.

After breaking of dormancy of **P.blakensteanus** spores by heat shock, glycerol production has been reported. Van Schaftingen and Van Laere (1984) reported that heat shock of the spores is followed by a brief rapid production of glycerol which is maximum after 5-10 min and ends at 20 min. Extracts of the spores contain Mg^{2+} dependent glycerol 3-phosphatase (Gro3Pase) which hydrolysis L-Gro3P and GrnP to glycerol and Grn respectively but with a higher affinity for L-Gro3P. The Gro3Pase from spores that did

not undergo heat shock is inhibited by physiological concentration of Pi which induces cooperativity with the substrate. Incubation of cell-free extract or partially purified Gro3pase in the presence of ATP-Mg and catalytic subunit of cAMP dependent protein kinase lead to no inhibition by Pi and apparently confers it with similar kinetic properties as the heat treatment of the spores.

The same authors above proposed three events following spore activation. The first is the rapid formation of cAMP. It is initiated by heat treatment and reinforced by the presence of glucose (Van Mulders, 1984). The second is the activation of cAMP-dependent protein kinase by the formed cAMP. This activation is followed by phosphorylation and subsequently activation of trehalase (Hixon and Krebs, 1980), 6-phosphofructo-2-kinase (Francois et al, 1984) and Gro3Pase (Van Schftingen and Van Laere, 1985). The third one is the formation of glucose from trehalose catalysed by trehalase. The glucose is converted to the triosephosphates in the presence of $Fru2,6P_2$ which stimulates 6-phosphofructo 1-kinase (Van Laere, 1983). The triosephosphates are converted to glycerol under the catalysis of the activated Gro3Pase. The biological significance of the formed glycerol is not known.

It is apparent that Gro3Pase exists in two interconvertible forms: the active phosphorylated and inactive nonphosphorylated forms. It is unlikely that T.b.brucei employs a Gro3Pase during anaerobiosis, as this would lead to no net ATP production from glucose catabolism. Opperdoes and Borst (1977) reported insignificant activity for this enzyme. Considering that the trypanosomes have no energy reserves, they cannot maintain the motility observed under anaerobic conditions if no net ATP production occurs (Opperdoes et al, 1976).

1.2.9.2.4 Effect of oligomycin on glycolysis

Oligomycin inhibits oxidative phosphorylation in intact mitochondria of most aerobic cells. It binds to the protein that links F_1ATPase to the membrane and presumably prevents the normal inward movement of protons that bring about ATP formation (Racker, 1970; Senior, 1973).

Miller and Klein (1980) reported that pyruvate production, O_2 and Ca^{2+} uptake can be inhibited by oligomycin during the catabolism of glucose by T.b.brucei leading to death of the trypanosomes. This was unexpected because the bloodstream trypomastigotes do not have cytochromes and a functional TCA cycle (Ryley, 1956; Fulton and Spooner, 1959). Uncouplers of oxida-

tive phosphorylation could not reverse the inhibition as would be expected if it was in mammalian cells. They also observed that in the presence of glycerol alone or together with glucose as substrates, glycolysis was slightly reduced but the trypanosomes remained motile. Miller (1981) proposed that oligomycin inhibits a mitochondrial Mg^{2+} ATPase thus restricting the production of ADP and Pi required for maintaining flux through the phosphoglycerate kinase and pyruvate kinase reactions. This proposal cannot explain why the catabolism of glycerol was affected to a lesser extent than that of glucose. The only apparent difference of catabolism is on the glycolytic enzymes up to and including fructose 1,6-bisphosphate aldolase involved in the catabolism of glucose. All other enzymes of glycolysis are the same for both substrates. So far it has not been established as to whether oligomycin inhibits glycolysis directly or otherwise. If it acts directly on the glycolytic pathway there may be an important enzyme or protein unique to the trypanosomes that may be exploited as a target for novel trypanocides.

1.2.9.2.5 **SOME KEY ENZYMES OF ENERGY METABOLISM IN T.B.BRUCEI.**

Table I shows some activities of key enzymes of energy metabolism in the bloodstream forms of T.b.brucei.

Table II: Native and subunit masses of glycolytic enzymes from **T.b.brucei**. The number of individual enzyme determinations are given in brackets (Misset **et al**, 1986). Units are in kDA.

	Molecular mass of:		
	Native enzyme.	subunit.	Subunits no.
Hexokinase	295 ± 12	50.3 ± 0.8(10)	6
Glc6p-dehydrogenase	105 ± 6	62.4 ± 1.6(8)	2
Phosphofructokinase	196 ± 15	50.3 ± 0.8(10)	4
Aldolase	157 ± 3	40.5 ± 0.5(9)	4
Triosephosphate-isomerase	55 ± 0	27.0 ± 0.5(8)	2
Gra3p-dehydrogenase	139	38.5 ± 0.5(10)	4
Phosphoglycerate kinase	48 ± 4	47.0 ± 0.7(11)	1
Gro3p dehydrogenase	66 ± 6	47.0 ± 0.7(9).	2
Glycerol kinase	82	52.5 ± 0.4(8)	2

The high activities of the glycolytic enzymes indicate the central role of glycolysis in energy production in these organisms. The low activity of the adenylate kinase, oligomycin sensitive ATPase and the lack of key TCA cycle enzymes indicate the absence of mitochondrial oxidative pathways in the bloodstream trypomastigotes. (Ryley, 1956; Fulton and Spooner, 1959)

1.2.9.2.5.1 Glycosomal glycolytic enzyme

They are all basic proteins with isoelectric pH values between 8.8-10.2. These are 1-4 units higher than in the mammalian cytosolic counterparts and 3-6 units higher than in other unicellular organisms (Misset et al, 1986).
With an exception of the hexokinase and phosphofructokinase, all other glycosomal enzymes appear to be related to the homologous counterparts from other organisms. They have similar subunit masses and identical subunit compositions (Table II).
Glucose 6-phosphate isomerase, glyceraldehyde phosphate dehydrogenase, glycerol 3-phosphate dehydrogenase, aldolase and the phosphoglycerate kinase are 1-5 kDA larger than their homologous counterparts except for the triose phosphate isomerase and glycerol kinase which have the same molecular weight as in

other isolated organisms. It has been proposed that the larger subunit size and the basic character of all T.brucei proteins are involved in the transport of enzymes from the site of biogenesis (cytosol) toward the glycosome (Misset et al, 1986, Swinkels et al, 1987). Using pulse field gradient electrophoresis, Gobson et al (1985) have shown that trypanosomes of the subgenus trypanozoon are diploid for the house keeping genes: phosphoglycerate kinase, triosephosphate isomerase, glyceraldehyde phosphate dehydrogenase and the tubulin cluster. They observed that the genes for the phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase are located in very large DNA molecules but the gene for the triosephosphate isomerase is located in another fraction different from that for the other two glycolytic enzymes. This led them to conclude that since all the 3 glycolytic enzymes are located in the glycosomes, the genes for the glycosomal enzymes are not clustered in chromosomal segment of the trypanosomal genome.

1.2.9.2.5.1.1 Hexokinase

It is hexameric and has a molecular mass of 295 kDa and unlike the vertebrate enzyme it is not inhibited by G1c6P. The vertebrate enzyme is monomeric and has molecular mass of 100 kDa(Ureta,1982;Nwagwu and Opperdoes,1982). The T. brucei hexokinase is not specific for ATP like

other hexokinases. It utilizes ATP(100%), Inosine 5' triphosphate (77%), Uridine 5' triphosphate (63%), Cytidine 5' triphosphate (38%) and Guanosine 5' triphospate (23%). It is therefore apparent that its different from the mammalian counterpart. The structure of the binding site for the phosphoryl group donating substrate is different from that of the vertebrate hexokinases (Misset et al, 1986). Nwagwu and Opperdoes (1982) reported that its Km for glucose is 17 micromolar. It exhibits a broad PH optimum with a maximum at pH 7.8. No regulatory role has been assigned to this enzyme (Nwagwu and Opperdoes, 1982).

1.2.9.2.5.1.2 The phosphofructokinase

It has some allosteric characteristic with respect to Fru6P. 5' AMP acts as an allosteric effector. ATP activates the enzyme in a hyperbolic manner and Mg^{2+} is needed for full activity (Nwagwu and Opperdoes, 1982). The enzyme has subunit mass of 51 KDa. and under non-dissociating conditions it forms a tetramer of 196 $\pm$ 15 kDa. It resembles the bacterial enzymes rather than mammalian enzymes in that it is not activated by either Fru1,6P_2 or Fructose 2,6-bisphosphate and is not inhibited by either citrate or Pi (Misset et al, 1986; Cronin and Tipton, 1985; Nwagwu and Opperdoes, 1982).

1.2.9.2.5.1.3 Fructose bisphosphate aldolase

Its molecular mass is about 40 kDa (Misset et al, 1984). Clayton (1985) used low stringency hybridizition with rabbit aldolase DNA to select clones of T.brucei encoding a protein of 41.027 kDa which had about 50% identity with rabbit aldolase A and slightly lower homology with B-type aldolases. The homologous mRNA was about 6-fold more abundant in the bloodstream than in the procyclic forms. From genomic mappings four copies of aldolase gene were found to be arranged as 2 copies of tandem repeat. The enzyme was found to have a short N terminal relative to other known aldolases. It was proposed that this terminal may be involved in glycosomal localization. Misset et al (1984) showed that the enzyme belongs to class I aldolases. It forms a Schiff base intermediate with the substrates, Frul,$6P_2$ and GrnP. It does not require divalent ions such as Zn^{2+}, Fe^{2+}, Mn^{2+} and Co^{2+}.

It cleaves FrulP to GrnP and glyceraldehyde at a rate of 1.9 units/mg pure protein. A cleavage ratio of Fru 1,$6P_2$ to FrulP by the enzyme was approximated to be 7.

1.2.9.2.5.1.4 Triosephosphate isomerase

Its molecular mass and that of the glycerol kinase is similar to that found in other organisms (Misset

et al,1986). It is found exclusively in the glycosome. It shows 50 per cent homology to triosephosphate isomerase from other organisms. It contains a marked excess of positive charges due to basic amino acids ,distributed in two or more clusters along the polypeptide chain. Modelling of the 3-dimensional stuctures using known coordinates of homologous enzymes from other organisms indicate it has two "hot spots" common to the aldolase and glyceraldehydephosphate dehydrogenase,about 40 Å apart which in themselves include a pair of basic amino acid residues separated by 7 Å (Wirenga et al, 1987). Wirenga et al (1987) proposed the possibility that the potency of the trypanocide suramin ,may be related to its symmetry and the fact that its two clusters of negative charges is separated by a distance of about 40 Å. The " hot spots" of the three enzymes mentioned above may interact with the clusters of negative charges in suramin resulting in inhibition of their catalytic activity.

1.2.9.2.5.1.5 Glyceraldehyde-3phosphate dehydrogenase

Michels et al(1986) showed two tandemly linked

identical genes that code for the glycosomal enzyme. They code for a protein of 38.9 kDa and amino acids The enzyme shows 52-57 per cent homology with known sequences of the enzyme from 10 other organisms, residues involved in NAD^+ binding and catalysis and subunits contacts are conserved in the the whole enzyme different sources. Misset et al(1987) reported that there are two isoenzymes in T. brucei.The glycosomal isoenzyme represents about 80 per cent of the total,whereas the cytosolic isoenzyme represents about 20 per cent. Both isoenzymes utilise NADH. The isoelectric points for the glycosomal and cytosolic isoenzymes are 9.1-9.5 and 7.8-8.0 respectively Both display maximal activity at pH 8.0. The homology between cytosolic isoenzyme from different sources varies from 48-80 per cent.The N-terminal of both cytosolic and glycosomal isoenzymes differ by 52 per cent.

1.2.9.2.5.1.6 Phosphoglycerate kinase

Like the other glycolytic enzymes,it is synthesised in the cytosol on free ribosomes(Opperdoes et al.1986).It has two isoenzymes :one is glycosomal and the other cytosolic cytosolic . Both display optimum activities at pH 6.0-9.0 .The isoelectric point for the

glycosomal and cytosolic enzymes is 9.3 and 6.3 respectively. The cytosolic isoenzyme is monomeric with a molecular mass of about 45 kDa. This is similar to that in other organisms but is about 2 kDa smaller than that of glycosomal phosphoglycerate Kinase. The isoenzymes do not display the broad specificity of purine and pyrimidine nucleoside triphosphates as is the case with yeast, and rabbit muscle enzyme. They show restricted specificity for ATP just as the enzyme from **Spirulina platensis.** There is very low activity with GTP, ITP, UTP and CTP. Like most glycolytic enzymes it is inhibited by suramin. The glycosomal enzyme is more sensitive to suramin than the cytosolic one. There is correlation between inhibitory effect of suramin and the isoelectric point of respective phosphoglycerate Kinases. The strongest inhibition is found for the glycosomal isoenzyme which has the highest isoelectric point. Glycosomal hexokinase, aldolase and the glycerol3-phosphate dehydrogenase are 10-20 fold more sensitive to inhibition by suramin than the mammalian or yeast enzymes which have lower isoelectric point. Glc6P isomerase has low isoelectric point and low affinity for suramin (Misset and Opperdoes, 1987). The genes for the glycosomal and cytosolic isoenzymes in **T.brucei** have been identified and sequenced. These are genes A, B

and C. Gene B codes for the cytosolic isoenzyme whereas gene C codes for the glycosomal isoenzyme. The role of gene A has not been clearly defined. There is 95% homology for the two phosphoglycerate kinase genes. The high homology is thought to be due to gene conversions, partially erasing the differences that might have arisen since the duplication of the ancestral gene. A few modifications are required to convert the cytosolic enzyme to a glycosomal one. There are two types of differences between the cytosolic and glycosomal isoenzyme: the glycosomal isoenzyme has C terminal extension with 20 amino acids and a striking higher net positive charge than the cytosolic enzyme, yeast horse and human (Osinga et al, 1985).

1.2.9.2.5.1.7 The Glycerol Kinase

It catalyses the reaction;

ATP + glycerol ⇌ ADP + Gro3P

The standard free energy change is +22 kJ/mol. It has been proposed that T.brucei glycerol kinase catalyses the formation of ATP and glycerol from ADP and Gro3p under cellular conditions (Hammond and Bowman, 1980; Opperdoes and Borst, 1977). From the standard free energy change, the equilibrium for the above reaction would lie far to the right.

Hammond and Bowman (1980) proposed that the enzyme

also catalyses the transphosphorylation between ADP and Gro3P. The transphosphorylation was assayed in a medium containing 55 mM glucose, 0.4 mM $NADP^+$, 0.01 to 1.0 mM ADP, 1 I.U/ml hexokinase and 1 I.U/ml Glc6P dehydrogenase. The reaction was started by the addition of 0.1 to 28 mM Gro3P. They asserted that the inclusion of glucose was to trap the ATP formed. The Michaelis constants for the solubilized enzyme are $Km^{Gro} = 0.12 \pm 0.05$ mM and $Km^{ATP} = 0.19 \pm 0.04$ mM; $Km^{ADP} = 0.12 \pm 0.04$ mM and $Km^{Gro3P} = 5.12 \pm 1.47$ mM. The ratio of the velocities of the forward (Vf) to reverse (Vr) reactions ranged between 21 to 171 after solubilization indicating substantive increase of Vf. The evidence to support that the glycerol kinase catalyses ATP formation from ADP and Gro3P in the trypanosome has been offered(Hammond and Bowman-,1980a) Firstly, both the glycerol kinase and the Gro3P : ADP transphosphorylase were inhibited 50% by 30 micromolar melarsen oxide. Secondly, it was observed that both enzyme activities decrease by the same magnitude with time when a partially purified enzyme was stored at 4°C. Thirdly, **Trypanosoma congolense** which has low activity of the glycerol kinase has also low ability to make glycerol during anaerobiosis. Fourthly, the activity for the Gro3P : ADP transphosphorylase of 78 nanomoles/min/mg protein is more

than is adequate to account for anaerobic rate of glycerol production of 40 nanomoles/min/mg protein at 25°C. The following objections may be raised to this evidence. These authors did not report whether inhibition by melarsen oxide is unique to the glycerol kinase or it also inhibits other sulfhydryl enzymes as expected (Webb,1966) from first explanation. If it inhibited other sulfhydryl enzymes at a similar magnitude, the first evidence may be open to question.

From the second statement,the decrease of glycerol kinase activity by storage at 4°C may haveoccurred just like with many other enzymes. It was not indicated whether the activity for other glycolytic enzymes was investigated. The low ability to form glycerol by **T. congolense** may not necessarily be due to the low activity of the glycerol kinase. Other enzymes of glycolysis should have been determined and compared with the glycerol kinase. The increase in the activity (Vf) of the glycerol kinase from 0.8 to 2.9 micromoles/min/mg protein and the decrease in Gro3P : ADP transphosphorylase (Vr) from 0.037 to 0.017 micromole/min/mg protein at 25°C after solubilization of a particulate fraction with Tritox X-100 indicate that these two might be distinct enzymes (Hammond and Bowman,1980b). The same authors above proposed that the decrease in Vr may be due to confo-

rmational change in glycerol kinase upon solubilization. Data in support of the idea that the glycerol kinase and ADP: Gro3p transphosphorylase are distinct enzymes has been published by Hammond et al (1985). Although no explanation was made as to the implication of their results, it is clear that the glycerol kinase in isolated glycosomes is 82% latent whereas the "reverse" glycerol kinase activity is not. The results indicate that the glycerol kinase and the Gro3P: ADP transphosphorylase may not reside in the same cellular compartment. It is worth noting that no one has so far shown that purified glycerol kinase from **T.brucei** catalyses glycerol formation from ADP and Gro3P. Results from purified form may shed more light to its role in anaerobic glycolysis. The glycerol kinase from **T.brucei** though quite unstable has been purified. It has a PI of 9.0 and a molecular mass of 52.100 - 52.400 kDA each. There is no significant difference in molecular mass of **T.brucei** and E. coli, glycerol kinase (Misset et al).The E.coli glycerol kinase has 4 identical subunits. It is subject to product inhibition by ADP, mainly competitive with respect to ATP-Mg (apparent Ki = 0.5 mM) and noncompetitive with respect to glycerol. Gro3P is a weak competitive inhibitor with respect to glycerol (apparent Ki = 2 mM). The pattern of inhi-

bition is consistent with ordered reaction with glycerol as the first substrate to bind to the enzyme (Thorner,1975) The C.mycoderma has been reported to catalyse a reversible reaction in vitro (Hammond and Bowman, 1980). Michaelis constants are Km^{ADP} = 0.57 ± 0.14 mM and Km^{Gro3P} = 0.11 ± 0.02 mM; Km^{Gro} = 0.09 ± 0.02 mM and Km^{ATP} = 0.06 ± 0.02 mM. The Vf/Vr for this enzyme is 531 indicating that the enzyme is more suited to catalyse Gro3p and ADP formation from glycerol and ATP than ATP formation from ADP and Gro3P (Hammond and Bowman,1980b)

1.2.9.2.5.1.8 Glycerol 3-phosphate dehydrogenase

Mclaughlin (1985) reported evidence that this NAD^+-linked glycerol 3-phosphate dehydrogenase is intraglycosomal. It has no membrane association but is bound to the glycosomal core by weak ionic forces. It has a molecular mass of 60.000 - 66.000 kDa and two subunits of molecular mass 36.500 - 37.900 kDa (Misset et al, 1986).

1.2.9.2.6 The pentosephosphate pathway

Some enzymes activities of this pathway have been reported. Penketh and Klein (1986) have reported activities at 25°C of glucose 6-phosphate dehydrogenase and phosphogluconate dehydrogene of 4-5 nanomoles/min/10^8 trypanosomes. These enzymes catalyse

reactions that generate reduced NADP. So far there is no evidence for the presence of the transaldolase, ribulose 3-phosphate epimerase and transketolase enzyme activities that catalyze the conversion of pentosephosphates to hexosephosphates and triosephosphates that can be catabolised through the glycolytic pathway. Fish et al (1982) reported that radiolabel from (U-^{14}C) glucose could be incorporated into added exogenous purine or pyrimidine bases. This is consistent with the low activities of the above NADP linked dehydrogenases (Penketh and Klein, 1986) and the absence of other enzymes catalysing other reactions of the pentosephosphate pathway. The carbon flow through this pathway can be taken as negligible when compared with the net flow through the glycolytic pathway whose activities are more than a hundred fold (Table I). However the pathway for NADPH utilization is of importance in these trypanosomes and will be discussed later in more detail.

1.2.9.2.6.1 Oxygen toxicity in the trypanosomes

In most biological systems toxic products such as $OH^{\cdot}$, $O_2^{\cdot}$, H_2O_2 are produced by partial reduction of oxygen. They are extremely reactive. They damage unsaturated lipids, proteins, nucleic acids and other cell components. This in effect can result to cell injury and eventually to death.

The cell usually removes the superoxide anion ($O_2^{\cdot -}$) through a reaction catalysed by superoxide dismutase (SOD).

$$2O_2^{\cdot -} + 4H^+ \longrightarrow 2H_2O + O_2$$

The breakdown of H_2O_2 is catalysed by glutathione peroxidase or catalase ;

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + 1/2\ O_2$$

$$2GSH + H_2O_2 \xrightarrow[\text{peroxidase}]{\text{Glutathione}} 2GSSG + 2H_2O$$

Glutathione peroxidase can also catalyse the reduction of organic hydroperoxides according to the equation:

$$ROOH + 2GSH \longrightarrow GSSG + ROH + H_2O$$

T.b.brucei contain SOD and lack significant activities of catalase and glutathione peroxidase (Opperdoes et al, 1977) Glutathione reductase is another important enzyme. It catalyses the reaction:

$$GSSG + NADPH \longrightarrow 2GSH + NADP^+$$

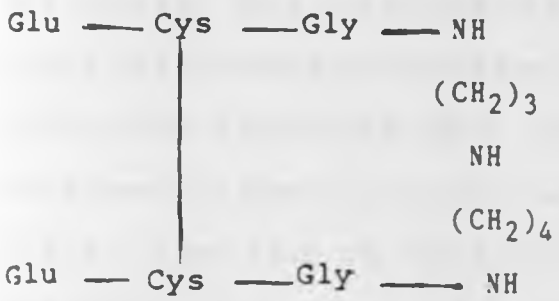
Reduced glutathione is important in maintaining the sulfhydryl groups of certain proteins in reduced state by reducing disulphide bonds.

T.b.brucei contain as much H_2O_2 as rat liver of 10^{-7} to 10^{-9} M (Penketh and Klein, 1986; Oshino et al, 1973). They consume H_2O_2 in significant amounts. The H_2O_2 produced appear to be generated from reactions

of the pentose phosphate pathway. The process involves NADPH and the newly discovered cofactor called trypanothione (Fairlamb, 1985; Penketh and Klein, 1986). The mechanism of H_2O_2 breakdown, involving trypanothione, appears to account for nearly all the H_2O_2 consumed by intact trypanosomes (Penketh and Klein, 1986). These investigators proposed that interruption of H_2O_2 degradation may inhibit glycolysis which is very sensitive to H_2O_2 and is the sole pathway for energy production.

1.2.9.2.6.2 <u>The trypanothione</u>

It is a unique spermidine containing a cyclic peptide. It consists of glutamate, cysteine, glycine and spermidine ;



It functions as a redox intermediate in the parasitic and free living trypanosomatids (Fairlamb and Cerami, 1985; Fairlamb et al , 1985). Trypanothione disulphide TS_2 is first enzymatically reduced in a reaction catalysed by a novel trypanothione reductase. It does not use GSSG as glutathione reductase

$TS_2 + NADPH + H^+ \longrightarrow T(SH)_2 + NADP^+$

Dihydrotrypanothione $T(SH)_2$ undergoes rapid nonenzymatic thiol exchange with intracellular disuphides such as GSSG and cystine.

$T(SH)_2 + GSSG \longrightarrow TS_2 + 2\ GSH$

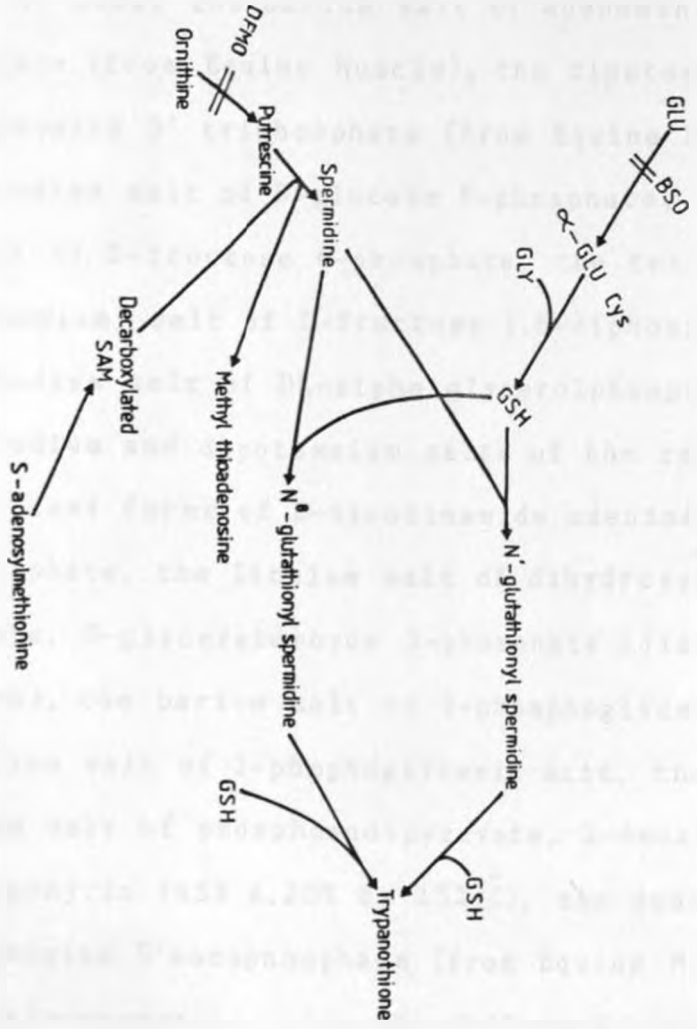
Due to the absence of the trypanothione or its equivalent in mammalian host, it is very important as a potential target for novel chemotherapentic agents.

Fairlamb et al (1986) have proposed a pathway for biosynthesis of trypanothione and N'-glutathionyl spermidine in the trypanosomatid **Crithidia fasciculata.** It is taken that a similar pathway occurs in **T.b.brucei.** They indicated that the experimental drugs difluromethyl ornithine (DFMO) and buthionine sulphoxime (BSO) inhibits ornithine decarboxylase and gamma-glutamylcysteine synthetase respectively resulting in inhibition of spermidine and glutathione biosynthesis (See Scheme V).The following are the structures of N'-glutathionyl spermidine and N^8-glutathionyl spermidine respectively:

Glu-Cys-Gly- $NH(CH_2)_3$-$NH(CH_2)_4$-NH_2 and

Glu-Cys-Gly- $NH(CH_2)_4$-$NH(CH_2)_3$-NH_2

Scheme V: The suggested pathway for the biosynthesis of trypanothione and N'-glutathionyl spermidine in the trypanosomatid **Crithidia fasciculata** (Fairlamb **et al**,1986).


DFMO
Ornithine
Putrescine
Spermidine
GLU
BSO
α-GLU CYS
GLY
GSH
Decarboxylated SAM
Methyl thioadenosine
S-adenosylmethionine
N⁸-glutathionyl spermidine
N'-glutathionyl spermidine
GSH
GSH
Trypanothione

CHAPTER II

MATERIALS AND METHODS

2.1 Fine chemicals and enzymes for metabolite and enzyme assays

The following were purchased from Sigma Chemical Co., St Louis, Mo, U.S.A : digitonin, salicylhydroxamic acid, the barium salt of adenosine 5' triphosphate (from Equine Muscle), the dipotassium salt of adenosine 5' triphosphate (from Equine Muscle), the disodium salt of D-glucose 6-phosphate, the barium salt of D-fructose 6-phosphate, the tetra(cyclohexylammonium) salt of D-fructose 1,6-diphosphate, the disodium salt of DL-alpha glycerolphosphate, the disodium and dipotassium salts of the reduced and oxidised forms of B-nicotinamide adenine dinucleotide phosphate, the lithium salt of dihydroxyacetone phosphate, D-glyceraldehyde 3-phosphate (diethylacetal form), the barium salt of 3-phosphoglyceric acid, the sodium salt of 2-phophoglyceric acid, the monopotassium salt of phosphoenolpyruvate, 2-deoxy D-glucose, oligomycin (65% A,20% B, 15% C), the sodium salt of adenosine 5'monophosphate (from Equine Muscle), and dihydroxyacetone; also the following enxymes; hexokinase (from rabbit muscle), phosphoglucose isomerase (Type III) phosphomannose isomerase (from Bakers yeast), aldolase (Type IV) from rabbit muscle, triosephosphate isomerase ,

pyruvate kinase (Type II) from rabbit muscle, enolase from bakers yeast, L-Lactic dehydrogenase (Type II) from rabbit muscle, glucose 6-phosphate dehydrogenase (Type III), glycerol kinase from Candida mycoderma, alpha-glycerolphosphate dehydrogenase (Type I) myokinase (Grade III) from rabbit muscles, and malic dehydrogenase from bovine heart. The concentration of L-Gro3p in the commercial DL-Gro3p was determined at 340 nm according to Hammond and Bowman (1980a) also described under section 2.5.7. All other chemicals used were of analytical grade.

2.2 Buffers:

The following buffers were routinely used in metabolic studies:

(i) Buffer A: 100 mM KCl, 5 mM $MgCl_2$, 2 mM K_2HPO_4 and 90 mM Tris-HCl. The pH was adjusted to 7.5 with 0.1M KOH and 0.1 M HCl. (Kiaira, 1983).

(ii) Tris-phosphate saline (TPS): 90 mM Tris-HCl, 51 mM NaCl, 40 mM Na_2HPO_4 and 4mM NaH_2PO_4.The pH was adjusted to 7.5 by 0.1 M NaOH and 0.1 M HCl.

(iii) Phosphate saline (PS): 0.3% (w/v) NaCl in 1 mM phosphate buffer, pH 7.5 (Njogu and Kiaira, 1982).

2.3 Organisms

2.3.1a Trypanosoma brucei EATRO 1969. It was isolated

from a female hyena in Tanzania in 1970. The parasites were inoculated into rats and mice, harvested and then frozen as stabilate. Early in 1978, the isolate was cloned at the International Centre for Insect Physiology and Ecology (ICIPE), Kenya, and proved to be infective to tsetse flies (Glossina morsitans) and rodents.

2.3.1b. **Trypanosoma brucei** S427.(Cunningham and Vickerman, 1962). It was originally isolated from sheep in 1962, and obtained as a stabilate from the International Laboratory for Research on Animal Dise ases (ILRAD). It is not transmissible by the tsetse fly (Brun et al, 1979).

2.3.2 In vivo growth and harvesting methods

Wistar or Sprague Dawley rats of body weight 200-300g were inoculated intraperitoneally with $1-5x10^6$ trypanosomes .The parasites were harvested after 72-96 hours when the parsitaemia was more than $1x10^8$/ml of blood. The rat was anaesthetised with diethyl ether, after which the thorax was opened to expose the heart. The heart was sprayed with 5% (w/v) trisodium citrate in TPS as anticoagulant. It was punctured with a needle of size 19G x 11/2 inches fitted to a 10 ml syringe, and as much blood as possible was withdrawn.

2.3.3 Separation of trypanosomes from the blood

The rat blood was centrifuged for 10 min at 1000xg at 4°C. The trypanosomes were isolated from the interface with a Pasteur pipette. Contaminating erythrocytes were removed by hypotonic shock as follows: The typanosome-rich layer was incubated for 3 min at 37°C in 100 volumes of 1 mM sodium phosphate buffer of pH 7.5 containing 0.3% (w/v) NaCl. Haemolysis was stopped by addition of 0.1 volumes of tenfold concentrated TPS buffer (section 2.2) containing 5-10 mM D-glucose. The suspension was centrifuged at 1000xg for 10 min at 4°C to obtain a trypanosome pellet, which was suspended in buffer A or TPS buffer containig an appropriate substrate depending on the purpose of the experiment.

2.3.5 Counting of the trypanosomes (Absher, 1973)

All the counting was done using a Neubauer haemocytometer. A coverslip was wetted on the edges and passed over the haemocytometer grid so that interference patterns appeared. The haemocytometer was filled up by touching the edge of the fixed coverslip with a pasteur pipette containing the trypanosome suspension. Trypanosomes in the centre large square and the four corner squares were counted. The trypanosome suspension was usually diluted so that the

number of parasites in each large square was 20-100. The number of trypanosomes per ml of undiluted supension was calculated from the equation $\frac{10^4 nD}{5}$

where n = number of trypanosomes counted in 5 large squares after a dilution of the original suspension D-fold.

2.4 Respiration experiments

Oxygen uptake by the trypanosomes was determined using a Clark type oxygen electrode (Yellow Springs, YS1 Model 53) polarised at -0.8V in a magnetically stirred cylindrical cuvette of 1.90 ml capacity. The rate of oxygen uptake was monitored on a Sargent Welch recorder coupled to the electrode. The electrode was always allowed to equilibrate with the buffer for more than 5 min at 25°C before the trypanosomes were introduced. The concentration of oxygen in the incubation medium was taken as 240 micromolar (Chance and Williams, 1956).

2.5 Metabolite assays

2.5.1 Preparation of samples for metabolite assays

For the determination of ATP, ADP, AMP and the glycolytic intermediates, $3\text{-}5 \times 10^8$ trypanosomes were

incubated in 25 ml Erlenmeyer flasks containing a final volume of 1 ml, under constant shaking in a Dubnoff Metabolic Shaking Incubator. For the end products of glycolysis, $1\text{-}2\times10^{8}$ trypanosomes per ml were incubated. Whenever SHAM was present, its concentration was 2 mM. Metabolism was stopped by addition of ice-cold perchloric acid to a final concentration of 7% (v/v). The deproteinised samples were centrifuged at 1000xg for 10 min and then neutralised with 6 M KOH. Aliquots of the protein free neutral extracts were used for metabolite assay. All the metabolites were assayed on a Unicum SP 1800 spectrophotometer coupled to a Pye Unicum recorder or a microcomputer controlled Perkin Elmer 550 UV/VIS spectrophotometer coupled to a model 561 recorder. The metabolites were determined according to Bergmeyer (1974) unless otherwise is indicated.

2.5.2 Calculations

An extinction coefficient of 6.22 and 3.4 cm^2 per micromole of NADH or NADPH used or produced was used in the calculation for metabolite concentration in all the assays done at 340nm or 366 nm respectively (Bergmeyer, 1974). The concentration (C) of the metabolites in micromoles per ml in each sample assayed was estimated according to the following equation:

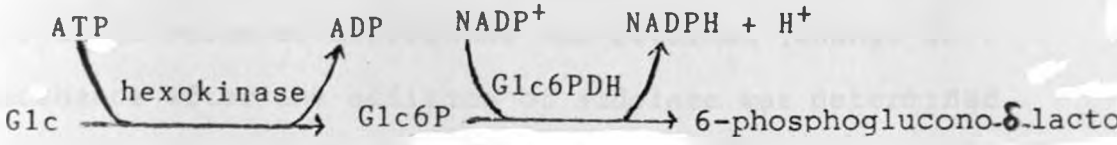
$$C = \frac{\text{change in absorbance} \times V}{6.22 \text{ or } 3.4 \times v}$$

V indicates total volume of assay mixture and v indicates the volume of sample used.

The results were finally expressed in micromoles or nanomoles per 10^8 trypanosomes.

2.5.3 ATP

ATP was assayed in a cuvette containing 50 mM KCl, 10 mM $MgSO_4$, 0.2 mM EDTA, 1.0 mM $NADP^+$, 20 mM glucose, 1.5 I.U hexokinase, 4.0 I.U. G1c6PDH in 50 mM Tris-HCl (pH 7.5). The reactions occurring during the assay are:



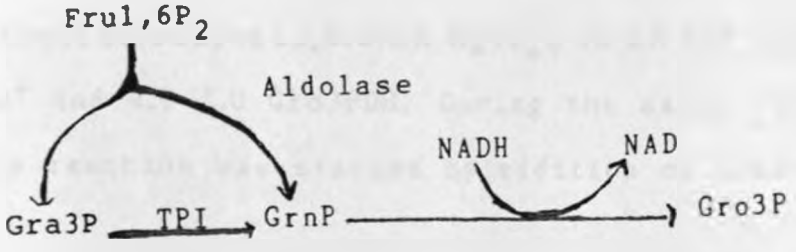
2.5.4 ADP and AMP

These were assayed in the same cuvette containing 71 mM triethanolamine buffer (pH 7.5), 0.40 mM PEPyr, 1.2 mM $MgSO_4$, 142 mM KCl and 0.2 mM NADH. Endogenous Pyruvate was eliminated by pre-incubation with 20 I.U lactate dehyrogenase. More NADH was added until there was no significant further change in absorbance. Assay for the ADP was started by addition of 7 I.U pyruvate kinase. When the absorbance was stable for about 5 min, change in absorbance was determined.

For AMP assay, ATP was added to make 1.2 mM in the cuvette. When there was no further change in absobance the reaction was started by addition of 10 I.U adenylate kinase. The net change in absorbance was determined when the absorbance reading was stable. The levels of ADP and AMP were calculated from the net change in absorbance after the addition of pyruvate kinase and adenylate kinase respectively. The sequence of reactions is as for the assay of PEPyr.

2.5.5 Frul,$6P_2$ aGrnP and Gra3P

The assay mixture consisted of 94 mM triethanolamine buffer (pH 7.6), and 0.2 mM NADH. GrnP assay was started by addition of 4.0 I.U Gro3PDH. Gra3P assay was started by addition of 7.0 I.U triosephosphate isomerase(TPI). To determine Frul,$6P_2$, 5 I.U aldolase were added. When a constant value of absorbance was obtained ,change in absorbance after the addition of aldolase was determined. The concentration of Frul,$6P_2$ was calculated from half the change in absorbance after the addition of aldolase . GrnP and Gra3P concentrations were calculated from the net changes in absorbance after the addition of Gro3PDH and TPI respectively. Reactions sequence is:



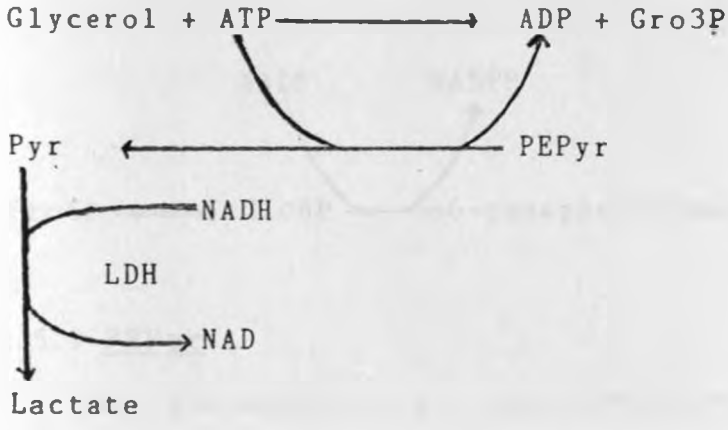
2.5.6 Glycerol

Glycerol was assayed as described by Fairlamb and Bowman (1980). Samples of volume 50 microlitres to 100 microlitres were added to a reaction mixture containing 50 mM KCl, 10 mM $MgSO_4$, 0.2 mM EDTA, 0.15 mM NADH, 4.0 mM ATP, 5.0 mM PEPyr, and 5.5 I.U Lactate dehydrogenase (LDH) in 50 mM Tris-HCl (pH 7.5). Endogenous ADP and pyruvate were eliminated from the deproteinised samples by pre-incubation with 4.0 I.U pyruvate kinase (PK) and LDH until a stable absorbance was obtained. More NADH was added to restore the concentration to 0.15 mM after which 2.0 I.U of glycerol kinase were added to start the reaction. The change in absorbance was read at 366 nm whenever SHAM was present in the incubation mixture.

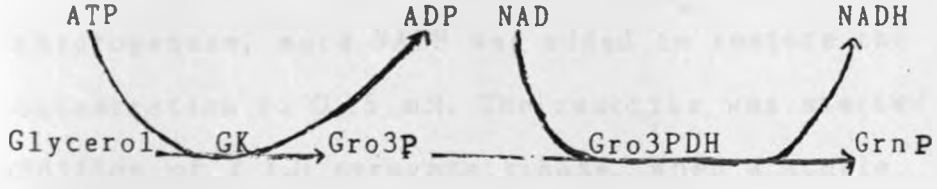
2.5.7 Glycerol and Gro3P.

They were sometimes assayed in the same sample at 366 nm. The assay mixture contained glycine-hydrazine buffer (PH 9.5) consisting of 0.10 M glycine, 0.69 M hydrazine sulphate, 5.0 mM $MgSO_4$, 40 mM ATP 2.3 mM NAD^+ and 4.0 I.U Gro3PDH. During the assay for Gro3P, the reaction was started by addition of Gro3PDH in

the absence of glycerol kinase. The tatal change in absorbance (ΔE_{Gro3P}) was obtained after 10-15 min. To determine the level of glycerol in the same sample 2.o I.U glycerol kinase was added until there was no further change in absorbance.The total change in absorbance after the addition of glycerol kinase was ΔE_{Gro}. The levels of Gro3P and glycerol were calculated from ΔE_{Gro3P} and ΔE_{Gro}. The sequence of reactions is:



and in 2.5.7 above it is

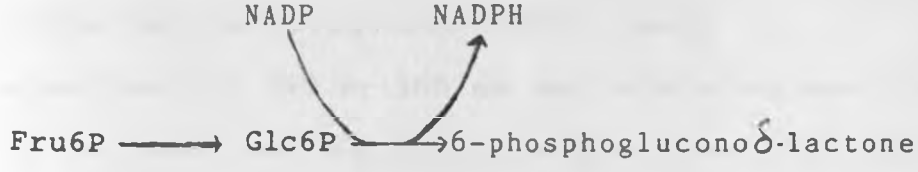


2.5.8 Glc6P and Fru6P

These were assayed in the same cuvette containing a reaction mixture similar to that for ATP determination but without the hexokinase and glucose. The

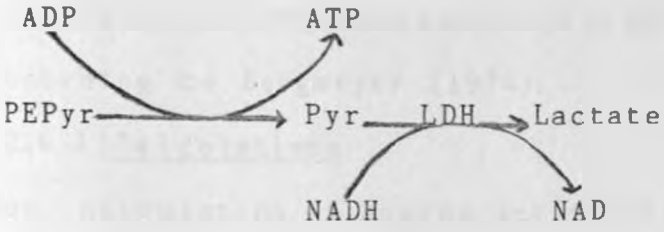
assay for Glc6P was started by addition of 4.I.U glucose6-phosphate dehydrogenase. When constant absorbance reading was observed for about 5 min, change in absorbance due to Glc6P was determined. Fru6P was assayed by addition of 3.5 I.U PGI. The changes of absorbance after the addition of Glc6P -dehydrogenase and PGI were used to calculate the concentration of Glc6P and Fru6P respectively.

The sequence of reactions during the assay was:



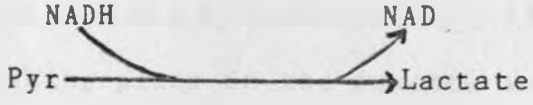
2.5.9 PEPyr

This was assayed in a reaction mixture similar to that described for ADP and AMP. PEPyr was excluded but 1.1 mM ADP was included. After elimination of endogenous pyruvate by incubation with 20 I.U lactate dehydrogenase, more NADH was added to restore the concentration to 0.15 mM. The reaction was started by addition of 7 I.U pyruvate kinase. When a stable absorbance was obtained, change in absorbance was determined and used to calculate PEPyr concentration. The sequence of reactions is:



2.5.10 Pyruvate

This was assayed immediately after neutralization of the deproteinised samples, in a reaction mixture containing 94.5 mM phosphate buffer (PH 7.0) and 0.15 mM NADH. The assay was started by addition of 5.5 I.U of lactate dehydrogenase (LDH). Change in absorbance at 340 or 366 nm was determined and used to estimate the concentration of pyruvate in each sample.



2.6 Enzyme assays

A suspension of 5-10x10^7 trypanosomes in 1 ml PS buffer was thawed after storage for about 14 days at -20^{o}C and lysed with 0.1% (w/v) Triton X-100. The lysate was kept in ice. Small aliquots were removed for the determination of the respective enzyme activities in a 1 ml quartz cuvette. All assays were performed at 340 nm on a microcomputer controlled Perkin Elmer 550 UV/VIS spectrophotometer. The change in absorbance was read after 15-60s for a period of 4-6 min. Unless otherwise

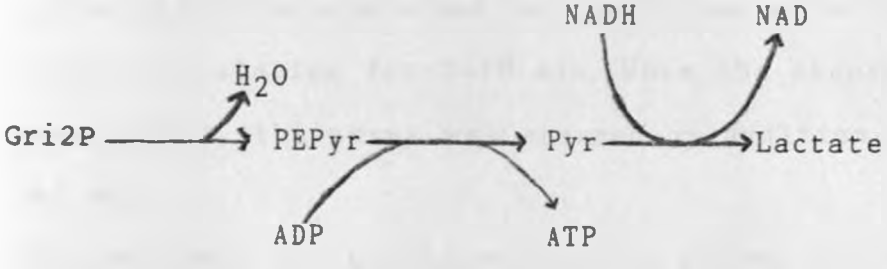
indicated, all the enzymes activities were determined according to Bergmeyer (1974).

2.6.1 Calculations

For calculation of enzyme activities, the equation in subsection 2.6.2, was used except that the term,' change in absorbance,' was replaced by,'change in absorbance per min'. The results were expressed as nanomoles/min/mg protein.

2.6.2 Enolase (E.C 4.2.1.11)

The assay mixture contained 83 mM triethanolamine buffer (pH 7.6) 3.3 mM $MgSO_4$, 0.20 mM NADH, 50 mM KCl, 1.1 mM ADP, 18 I.U lactate dehydrogenase, 2.7 I.U pyruvate kinase and 20-40 microlitres of sample. The reaction was started by addition of 1 mM Gri2P. Reactions taking place in the cuvette were;



2.6.3 Glycerol kinase (E.C 2.7.1.30)

The assay mixture consisted of glycine hydrazine buffer (pH 9.8) containing: 1.8 mM $MgCl_2$ 0.40 mM NAD^+. 3.0 mM glycerol, 4.0 I.U Gro3P dehydrogenase and 20-50 microlitres sample. The buffer consisted of

182 mM glycine and 3.78 M hydrazine. The assay was started by addition of 1.4 mM ATP. Reactions occurring are the same as those during the assay for glycerol.

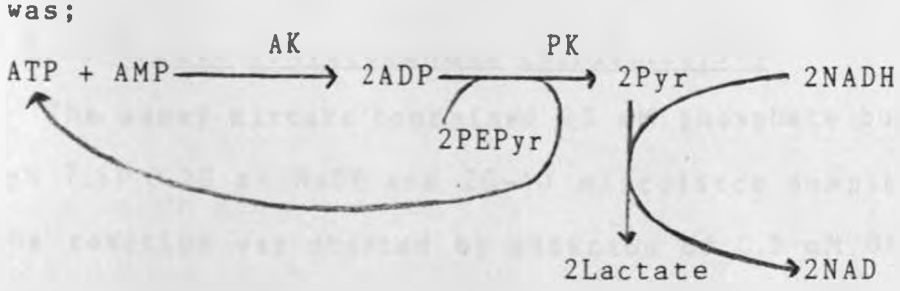
2.6.4 Pyruvate Kinase (EC.2.7.1.40)

The assay mixture was as for enolase except that ADP and pyruvate kinase were not added. The sample volume was 50-200 microlitres. When the endogenous pyruvate was depleted the assay was started by addition of 1.1 mM ADP. The sequence of reactions is as under Enolase.

2.6.5 Adenylate Kinase E.C 2.7.4.3

The assay mixture contained 71 mM triethanolamine buffer (pH 7.6), 1.2 mM ATP, 0.40 mM PEPyr, 1.2 mM $MgSO_4$, 142 mM KCl, 0.20 mM NADP, 7.0 I.U pyruvate Kinase, 20 I.U LDH and 20-40 microlitre sample. Endogenous pyruvate and other intermediates were depleted by pre-incubation for 5-10 min. When the absorbance was stable, the assay was started by addition of 1.4 mM AMP.

The sequence of reactions occuring during the assay was;



2.6.6 Gro3P dehydrogenase (E.C.1.1.1.8)

The assay was carried out in 290 mM triethanolamine buffer (pH 7.6) containing 0.35 mM GrnP, 0.2 mM NADH and 20-50 microlitre sample. To start the reaction GrnP was added. The sequence of reactions is as in the assay for Gro3P.

2.6.7 Phosphoglucose isomerase(E.C. 5.3.1.9)

The assay mixture consisted of 85 mM triethanolamine buffer (pH 7.6), 1.4 mM Fru6P , 7.0 mM $MgCl_2$,0.40 mM $NADP^+$, 10 I.U Glc6P dehydrogenase and 20-40 microlitres sample. The reaction was started by addition of Fru6P. The sequence of reactions is as for the assay of Fru6P.

2.6.9 Hexokinase(E.C. 2.7.1.1)

The assay was carried out in 40 mM triethanolamine buffer (pH 7.6) containing 222 mM D-glucose, 8.0 mM $MgCl_2$ 0.91 mM $NADP^+$, 0.60 I.U Glc6P dehydrogenase and 20-100 microlitres sample. The assay was started by addition of 0.60 mM ATP. The sequence of rections is as for the assay of ATP.

2.6.10 Malate dehydrogenase (E.C.1.1.1.37)

The assay mixture contained 95 mM phosphate buffer (pH 7.5) 0.20 mM NADH and 20-40 microlitre sample. The reaction was started by addition of 0.5 mM OAA.

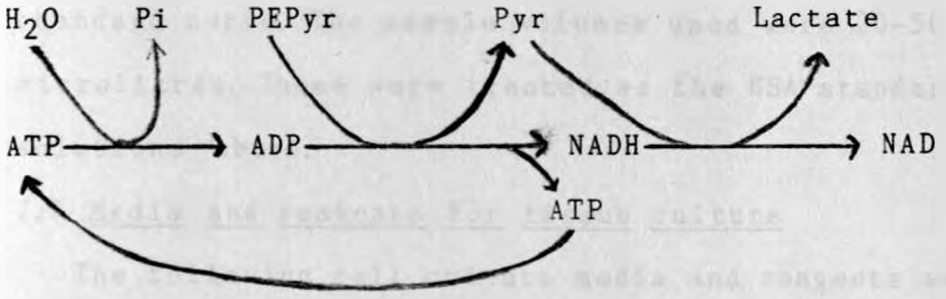
The reaction occurring in the cuvette was:

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

2.6.11 ATPase

This was assayed according to the method of Stigal **et al** (1978). In the assay mixture was 25 mM Tris acetate (pH 7.5), 25 mM K^+ acetate, 300 mM sucrose, 2 mM $MgCl_2$, 1 mM PEPyr, 0.20mM NADH, 3.0 I.U pyruvate kinase (PK) and 3.0 I.U LDH. The reaction was started by addition of 2 mM ATP.

The reaction sequence is:



2.6.12 Protein determination.

This was as described by Bergmeyer (1974). The following solutions were routinely used,

A. An aqueous solution of 20 mM $CuSO_4.5H_2O$ diluted 1:51 by another solution of 189 mM Na_2CO_3 and 0.67 mM $NaKC_4H_4O_6.4H_2O$ in 0.1 M NaOH.

B. Five volumes of **folin-ciocalteau** reagent diluted with nine parts of water.

C. Standard bovine serum albumin (BSA) of concen-

tration 1mg/ml in double distilled water.
To construct a standard curve 7.5-45 microlitres BSA was added to mixture A (1.15ml) and distilled water to make a volume of 1.3 mls. This was kept at room temperature for 10 min after slight shaking to ensure adequate mixing. Mixture B (0.11 ml) was added and after further mixing the solution was kept at room temperature for 30 min and the absorbance was read in a 1 ml cuvette at 578 nm. After plotting a curve of absorbance versus concentration of BSA, the concentration of protein in the experimental samples was determined at the corresponding absorbance in the standard curve. The sample volumes used were 20-50 microlitres. These were treated as the BSA standard solutions above.

2.7 Media and reagents for tissue culture

The following cell culture media and reagents were used routinely and were purchased from Sigma Chemical Company, St. Louis, MO U.S.A: RPMI 1640, MEM with Earle's salts, Medium 199, HEPES, MOPS, L-glutamine powder, lyophilised hypoxanthine/thymidine (HT), adenosine, L-alanine, p-aminobenzoic acid, L-arginine, d-biotin, cytidine, folic acid, D-glucose, guanosine, hypoxanthine, L-methionine, L-proline, L-phenylalanine, pyruvic acid, L-serine, sodium bicarbonate, L-threonine, thymidine, uracil, uridine, L-valine, and

Hanks balanced salt solution. All these media and reagents were tissue culture grade. The following were also used and were purchased from Flow Laboratories, U.K: Trypsin-EDTA, MEM non-essential amino acids (100x concentrated), MEM amino acids (50xconcentrated), penicillin streptomycin, kanamycin and gentamycin. Unless otherwise indicated all the cell culture technique are as outlined by Paul (1970).

2.8 Glassware for aseptic work

Glass pipettes and storage bottles of pyrex were used routinely. They were washed and rinsed three times with deionised distilled water. The media storage bottles were autoclaved for 20 min at 15 lb pressure. Clean pipettes were sterilized in a pipette holder by dry heat at 160°C for 90 minutes.

2.9 Sterile plastic ware

Polystyrene culture flasks with a cell growth surface area of 25 cm^2 (T-25) were routinely used. They were Falcon products, Bioquest; Coceykeyville, MD 21030 U.S.A or Costar; Cambridge, Mass 02139, U.S.A. Filter units were of 0.22 microns porosity and of capacity 115 ml, purchased from Nalgene Labware, Rochester, New York U.S.A.

2.10 Aseptic handling of culture material

Media preparation and handling of cultures was carried out in a vertical laminar flow cabinet. Incuba-

tions were performed in dry incubators set at 37 or 28°C.

2.11 Media

The following media were used for the initiation of primary cultures and maintenance of the fibroblast-like cells

2.11.1 Minimum essential medium (MEM)

Powdered MEM with glutamine but without sodium bicarbonate was dissolved in 900mls sterile distilled de-ionised water according to the instructions of the manufacturer (Sigma Chemical Co.) It was then supplemented with 20mM HEPES, 10mls MEM non-essential amino acids (100 times concentrated) and 10 mM sodium bicarbonate. The pH was adjusted to 7.2 using 4 N NaOH at 25°C. The volume was then made to 1000 mls. It was sterilized by filtration using a filtration unit fitted with a membrane of 0.22 microns porosity. The medium was stored in 500 ml sterile bottles at 5°C and used within one month.

2.11.2 Complete MEM

The MEM solution when supplemented with either 10 or 20% serum, together with 100 microgram streptomycin and 100 international units penicillin per ml was referred to as complete MEM. Complete MEM for the growth of the trypanosomes had 20% serum whereas for the growth of the fibroblast-like cells had only 10%

serum. The refrigerator life of complete MEM was 4 to 5 days.

2.11.3 RPMI 1640

This was as effective as MEM. It was prepared from a powdered preparation as described for MEM above. Complete RPMI 1640 for trypanosome or fibroblast-like cells was prepared from RPMI 1640 supplemented as described above for complete MEM.

2.12 Trypsin EDTA

A small portion of ten times concentrated trypsin/EDTA was diluted ten fold with Hanks balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. This was diluted 1:8 with the Ca^{2+} and Mg^{2+} free HBSS (PH 7.4) for subpassage of the fibroblastlike cells. It was prepared fresh each time. For initiation of cultures of embryonic cells the ten times diluted trypsin EDTA was further diluted 1:2 instead of 1:8 with the same HBSS.

2.13 Antibiotics

Stock solutions of penicillin/streptomycin of concentration 5000 micrograms streptomycin and 5000 international units penicillin per ml, kanamycin of concentration 50,000 micrograms per ml, and gentamycin of concentration 5000 micrograms per ml were used for primary cultures. Penicillin and streptomycin were replaced by 30 micrograms kanamycin per

ml complete medium or 20 micrograms gentamycin per ml complete medium.

2.14 Hanks balanced salt solution (HBSS)

This is a slight modification of the original formulation by Hanks (1949). The modification was made by the manufacturer (Flow Laboratories, U.K)
The ten times concentrated liquid stock HBSS without Ca^{2+} Mg^{2+} and $NaHCO_3$ was diluted tenfold with sterile distilled deionised water. It was sterilized by filtration as described above for culture media. The colour of the solution was adjusted to orange corresponding to pH 7.0 by adding drops of 7.5% (w/v) $NaHCO_3$. It was stored in a refrigerator and used within two weeks.

2.15 Initiation of fibroblast-like cell lines

The thymus, spleen, gluteal muscle, lungs or heart muscle were removed aseptically from a bovine foetus (3-4 months old) and placed on sterile petri dishes. They were washed with Ca^{2+} and Mg^{2+} free Hanks BSS. They were then chopped into fine pieces using crossed scalpels. The pieces were transferred to a flask containing 20 ml of 0.05% trypsin EDTA. The flasks were incubated in a metabolic shaking incubator at 37°C for 30 min. The pieces were allowed to settle. After this the supernatant was collected

and centrifuged for 10 minutes at about 200xg. The pellet was resuspended in HBSS and again centrifuged at 200xg for 10 minutes. It was then resuspended in 5 mls of complete MEM or RPMI 1640. Fresh trypsin/EDTA solution (0.05%) was added to the remaining tissue pieces in the flask. The flasks were incubated and the same procedure above was repeated for 2.5 hours. The viable cells were determined by trypan-blue exclusion haemocytometer count.

To each T-25 flask was added $1x10^6$ viable cells in a total volume of 5 mls. The flasks were then incubated at 37°C. The medium in each flask was replaced with fresh medium after 24 and 48 hours to remove debris and unattached cells. After 4-7 days thymus, gluteal muscle and lungs fibroblast-like cells were split 1:2 and later 1:3. The spleen and heart muscle fibroblasts were split 1:2 after 6-10 days.

2.16 Maintenance of fibroblast-like cells

The spent medium was replaced with fresh complete medium after 2-3 days. Each culture was split weekly after trypsinization.

2.16.1 Trypsinization

The medium was decanted and the cells were washed thrice with 2 mls Ca^{2+} and Mg^{2+} free HBSS to remove serum which inhibits the activity of trypsin. The

cells were then rinsed with 1 ml trypsin/EDTA (0.006%) in Ca^{2+} and Mg^{2+} free HBSS,after which 1 ml of 0.006% trypsin EDTA was added to the flasks containing the cells. The flasks were incubated at 37°C until the cells rounded up (5 to 15 minutes). The cells were knocked from the plastic substrate. Five mls of complete MEM or RPMI 1640 were added to each flask to inactivate the trypsin. Two or three mls of the cell suspension was added to three or two T-25 flasks containing appropriate volume of complete MEM or RPMI 1640. Each incubation was of total volume 5 ml.

2.17 Initiation of trypanosome culture

A rat or a mouse with rising parasitemia of $5x10^7$/ ml was used as a source of trypanosomes for in vitro culture. The animal was bled aseptically by cardiac puncture. The rat blood was collected using a 2 ml sterile syringe containing 500-1000 units heparin fitted with a needle of size 19 G x 1 1/2 inches, whereas mouse blood was collected using a 1 ml sterile syringe fitted with a needle of size 21G X 1 1/2 inches.

2.18 In vitro Stages of Trypanosoma b. brucei during cyclic transformation

Cyclic development of T.b.brucei was achieved basi-

cally according to Hirumi et al (1980). To increase the yield of the trypanosomes at each stage various conditions were investigated (Chapter III) and the following is a summary of the procedure that was adopted.

Tissue culture flasks of volume 50 ml and surface area for cell growth of 25 cm^2 (T-25) containing a near confluent monolayer of fibroblast-like cells derived from bovine embryonic spleen, thymus or gluteal muscle and blood stream forms of T.b.brucei (EATRO 1969 or S 427) in a total volume of 6 ml were incubated at 28°C. The cultures were fed every other day by removing 1 to 2 mls of the trypanosome suspension and exchanging it with fresh culture medium.

The midgut forms produced were further incubated for 7 - 14 days without feeding or opening the flask. They transformed to the apparently non dividing sluggish proventricular forms. Subcultures were made from the proventricular form cultures with $1x10^4$ trypanosomes/ml. The culture suspension was exchanged with 1-2 ml fresh medium when the trypanosomes reached a density of more than $1x10^6$/ml. Feeding was continued for 8-15 days after which a small population of the trypanosomes morphologically similar to the epimastigotes was observed. Infective metacyclic trypamastigotes were obtained by continued feeding of the

cultures by exchanging 1-2 ml of the culture suspension with fresh medium every other day.

2.19 Preparation of samples for biochemical studies

The trypanosome suspensions obtained during the time of feeding of the cultures were centrifged at 1000xg for 10 min. The pellet was washed 3-4 times at 4°C with 10 volumes phosphate saline buffer containing the appropriate substrate depending on the purpose of the experiment. The pellet was finally suspended in 1ml of the appropriate buffer and kept in ice for metabolic studies or frozen for enzyme assays.

2.20 Initiation of Metacyclic culture forms derived from tsetse fly salivary glands

This was done according to Nyindo et al (1979). Newly emerged tsetse flies of **Glossina morsitans morsitans** were allowed to feed on a rat infected with **T.b.brucei** EATRO 1969. The same tsetse flies were allowed to feed on a noninfected rabbit every other day. After 3-4 weeks the tsetse flies with salivary gland infection were immobilized by chilling for 5-10 minutes at 15-20°C. The wings were removed. These tsetse flies were surface sterilized by three washes with White's medium after which they were rinsed with complete RPMI 1640. They were dissected under a dissection microscope and the salivary glands were re-

moved and introduced into T-25 flasks containing 4 mls of complete RPMI 1640 and a confluent monolayer of fibroblast-like cells derived from bovine embryonic spleen, thymus or gluteal muscle. The cultures were incubated at 28°C and fed twice or thrice a week depending on the pH of the incubation medium, as judged by the colour of phenol red. For the first 2-3 weeks the increase in the number of parasites was very low. They were fed with fresh medium after centrifugation of the suspension in sterile 10 ml screw capped tubes. The supernatant was discarded. When the trypanosome density exceeded $1x10^5$ml, subcultures were repeatedly made until enough trypanosomes for biochemical experiments could be obtained. The trypanosome density was maintained at less than $3x10^6$/ml suspension because higher densities resulted in rapid death and loss of the cultures.

CHAPTER III

IN VITRO PROPAGATION OF THE INSECT FORMS OF T.B.BRUCEI AND THEIR METABOLISM OF GLYCEROL AND GLUCOSE

3.1 Results

3.1.2 The search for better conditions for the propagation of large numbers of various trypanosome stages during cyclic transformation

Conditions that favour the propagation of sufficient quantities of the different insect stages of T.b.brucei for metabolic studies were investigated. Four T-25 flasks containing a near confluent monolayer of fibroblast-like cells and $2x10^6$/ml S427 bloodstream trypomastigotes of T.b.brucei were incubated at 28°C. The effect of feeding the cultures after various time intervals on trypanosome multiplication was investigated. Table III shows the days of feeding the trypanosomes by exchange of 1 or 2 ml culture suspension with fresh complete MEM. The trypanosomes in flask A were fed on days 1,3 and 5 with 1 ml complete MEM. These intervals were to allow the parasites to increase. On day 5 the trypanosomes in this flask were dividing very rapidly. The dividing forms were morphologically similar to the mid-gut forms. The flask was incubated at 28°C for 14 days without opening. The midgut forms predominated in the

Table III: Days of feeding transforming trypanosomes incubated at 28°C. Each culture was initiated with $2x10^6$ trypanosomes /ml. Feeding was by exchanging 1 or 2 ml suspension with fresh complete MEM. * indicates the experiment was discontinued.

Day	Volume(ml) exchanged			
	Flask A	Flask B	Flask C	Flask D
1	1.0	0	0	0
3	1.0	0	0	0
5	1.0	0	0	0
11	0	2	0	0
14	0	0	1.0	0
15	0	0	1.0	0
17	0	0	1.0	*

cultures 3-4 days after the last feeding.

In flask B the trypanosomes were not fed until day 11. By day 5 many trypanosomes had died after which the surviving 5-10% started to increase at a very slow rate. Due to the poor health of the trypanosomes as judged by phase contrast microscopy, 2 ml of the culture suspension was exchanged with fresh medium after 11 days. This led to increased multiplication. Midgut forms were observed just as in flask A. The flask was incubated without feeding or opening for 14 days.

In flask C there was no feeding of the trypanosomes until day 14. Most of the trypanosomes died. Judging from phase contrast microscopy, less than 5% of the trypanosomes were still motile on day 14. Due to this observed loss of the trypanosomes and the apparent lack of multiplication, those that remained alive were fed with 1 ml complete medium on days 14,15 and 17. This feeding led to increased trypanosome multiplication. The flask was then kept closed for 14 days. In flask D the trypanosomes were not fed at all. By day 17 all were dead and the experiment was discontinued.

It was concluded that feeding by an exchange of 1 ml of the trypanosome suspension every other day with the same volume of fresh medium within the first

Table IV: Effect of initial density of cultured proventricular trypomastigotes on their transformation to the midgut-like forms.

Subculture	Trypanosome density
I	$5x10^5$/ml
II	$1x10^5$/ml
III	$5x10^4$/ml
IV	$1x10^4$/ml
V	$5x10^3$/ml

5 days leads to a faster trypanosome multiplication.It appears that the dead trypanosomes which could not transform released substances (possibly lysosomal enzymes) which are toxic to the live trypanosomes and must be decreased by removal of 2 or 3 ml, and by addition of fresh medium. Within the 14 days the flasks were closed, the midgut forms transformed to slender but longer forms similar to those described by Hirumi et al(1979),as proventricular forms. The total number of trypanosomes in each flask was 2-$10x^7$/ml although the majority were barely moving.The phenol red in the medium was almost colourless indicating a pH less than 7.0.

Five subcultures were made in duplicate with different trypanosome numbers to determine whether the initial trypanosome density affects in vitro transformation. Table IV shows the various inoculi of proventricular trypomastigotes into each subculture.

Exchange of 2 ml culture suspension with the same volume of complete fresh medium evey other day in subcultures I and II resulted in the production of trypanosomes similar to the midgut forms. These trypomastigotes were very active and dominated the subcultures. They reached a density of 0.5-$1.8x10^7$/ml every other day. There was a small number of trypanosomes

(5-10%) similar to the proventricular forms.

In subculture III, feeding was required after 7 days, judging from pH decrease and increase in trypanosome density. Feeding was continued just as with subcultures I and II. After 8-13 days, a few short forms with a blunt posterior end and a free flagellum (epimastigotes) were observed. In subcultures IV and V no feeding was required before epimastigotes were observed. In these last two subcultures, the proventricular forms enlarged to give rise to short midgut-like forms within 10-15 days of subculture. The midgut-like forms did not increase as fast as in subcultures I and II although they also gave rise to the epimastigote forms. The highest trypanosome density in subcultures IV and V was $1x10^6$/ml.

The subcultures containing epimastigotes were tested for infecivity by inoculating 10^6 to Balb c mice intraperitoneally. None of the mice was infected. After at least 2 days since the appearance of epimastigotes, inoculation of 1000 to 1 million trypanosomes to Balb/c mice resulted in infection with a prepatent period of 4-10 days. These infective cultures contained trypanosomes smaller than the midgut-like forms with a free flagellum, a sharp posterior end, and undulating membrane. These were taken to be the metacyclic trypanosomes. The highest percentage

of these forms was about 1-2.5 of the total population. It was based on morphological similarity to the metacyclic forms observed in tsetse salivary glands under phase contrast microscopy and by intraperitoneal inoculation of various trypanosome numbers ranging from 100 to 1 million into Balb/c mice. Attempts to separate the metacyclic trypomastigotes from the other forms lacking the surface coat, by passing them through DEAE-Cellulose column according to Lanham (1968), were unsuccessful and the trypanosomes remained on the column.

It was possible to obtain up to about 5% metacyclic trypanosomes from the fly transmissible T.brucei EATRO 1969. This was higher than that reported by Hirumi et al (1979) of up to 1%. Midgut forms derived from EATRO 1969 did not transform to a significant number of proventricular forms when incubated for a long time without feeding or opening the flasks. The cultures could not survive for more than 10 days without feeding. It was interesting to note that although few of the proventricular forms were observed after 7-10 days incubation at 28°C without feeding, epimastigotes and metacyclic trypanosomes were obtained later just as with S 427. It was concluded that a biochemical transformation,possibly at the gene level, occurs in the midgut forms when they are

incubated for some time without feeding or opening the flask. This transformation apparently does not have to be expressed by morphological changes such as transformation to the proventricular forms. Notwithstanding the difficulty of separating the metacyclic trypanosomes from the other forms, a maximum of 6x 10^5 per T-25 flask could be obtained with EATRO 1969. It was estimated that for each biochemical experiment requiring live trypanosomes, at least 2x10^5 trypanosomes are required. This would consequently require maintaining 300 T-25 flasks. Considering the costs and the difficulty of handling the large numbers of feeder layers and the large number of culture flasks, this method was found unsuitable for the propagation of the metacyclic trypomastigotes for biochemical experiments. However, it was the only suitable method for the propagation of the midgut forms,midgut-like forms, and the proventricular forms, used later in biochemical investigation.
Propagation of the metacyclic forms according to the method of Nyindo et al (1979) was also investigated.

3.1.3 Metacylic trypanosomes derived from infected tsetse salivary glands

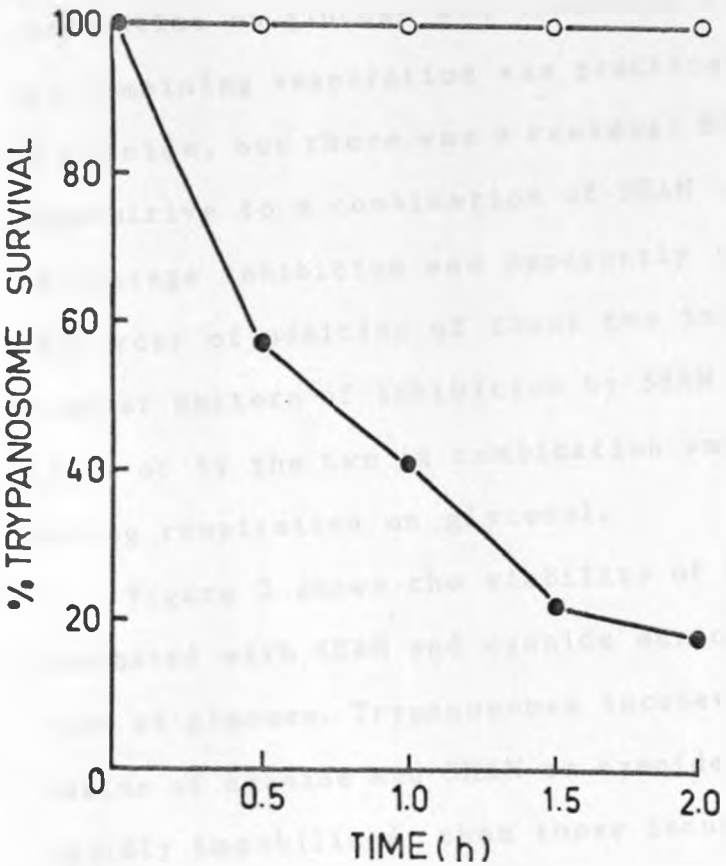
The trypanosomes found in the cultures consisted mainly of two populations: the short stout and the long slender forms similar to those photographed by

Nyindo et al (1978,1979). The long slender trypomastigotes with an undulating membrane, morphologically similar to the tsetse salivary gland metacyclic forms constituted about 80% whereas the short stout forms constituted about 20% of the total trypanosomes.These trypanosomes were observed to be dividing. No epimastigotes were observed in these cultures. The highest trypanosome density in each culture was $1\text{-}3x10^6$/ml. The trypanosomes were very sensitive to pH less than 7.0. Trypanosome growth was best in feeder layers that did not continue to grow after the growth surface was confluent. Such feeder layers were derived from whole rat embryos or bovine embryonic spleen. All the cultures were infective to Balb c mice after intraperitoneal inoculation of 10 to 100 trypanosomes. Infectivity by lower inocular was not investigated.

3.1.4 Metabolism of glycerol,glucose, and proline by the in vitro propagated insect forms of T.b.brucei

Figure I shows a time course of the survival of the midgut trypanosomes when incubated with glucose or glycerol. About 80% of the trypanosomes incubated with glucose as substrate died within 2 hours whereas all those incubated with glycerol survived for a longer period. They survived on proline as with glycerol. All immobile trypanosomes were taken as dead

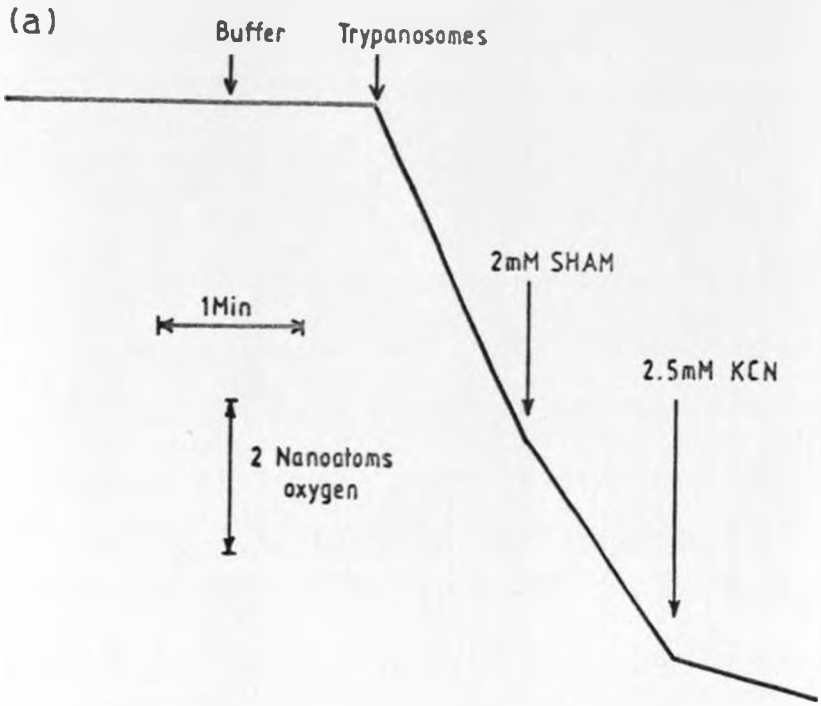
Figure 1: A time course of the survival of the midgut forms of **T.b.brucei** incubated with glucose ●-● and glycerol O-O or proline. The incubation medium was PS buffer containing either 10 mM glucose, glycerol or proline. The number of trypanosomes corresponding to 100% survival was $5x10^6$/ml .


100
80
60
40
20
0
% TRYPANOSOME SURVIVAL
0.5
1.0
1.5
2.0
TIME (h)

and were not counted. The surviving trypanosomes respired on both glucose and glycerol at rates of 45 and 60 nanoatoms oxygen/min/10^8 trypanosomes respectively.Figure 2 shows that only about 31% of the respiration on glucose was inhibited by SHAM alone. The remaining respiration was practically abolished by cyanide, but there was a residual 8% which was insensitive to a combination of SHAM and cyanide. The percentage inhibition was apparently independent of the order of addition of these two inhibitors. A similar pattern of inhibition by SHAM or cyanide alone or by the two in combination was obtained during respiration on glycerol.

Figure 3 shows the viability of the trypanosomes incubated with SHAM and cyanide during the utilization of glucose. Trypanosomes incubated with a combination of cyanide and SHAM or cyanide alone were more rapidly immobilised than those incubated with SHAM alone. To determine whether these inhibitors could simulate anaerobiosis in these trypanosomes similar to that one in the bloodstream trypomastigotes, glycerol production was investigated in the presence of both inhibitors. There was no detectable increase in glycerol in the incubation medium over a period of two hours. This was despite the presence of a high activity of glycerol kinase in these organisms

Figure 2: Effect of SHAM and cyanide on the respiration by the **in vitro** propagated midgut forms of T.b.brucei catabolising glucose. (a) shows inhibition of respiration by SHAM before cyanide was added and (b) shows inhibition of respiration by cyanide before SHAM was added.
Each incubation contained $1x10^7$ trypanosomes. The buffer used was PS. It contained 10 mM glucose.


(a)
Buffer
Trypanosomes
2mM SHAM
1Min
2.5mM KCN
2 Nanoatoms
oxygen

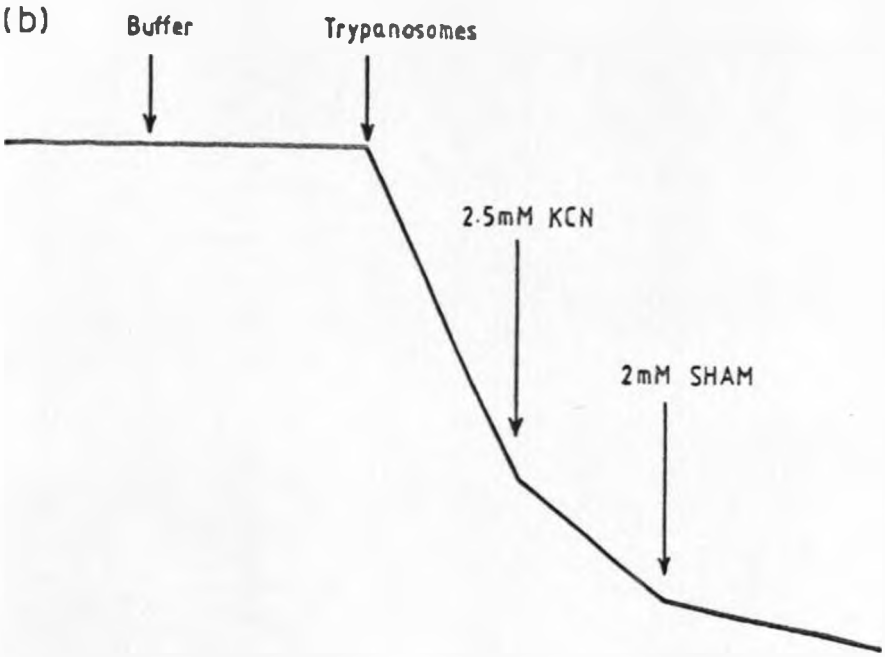

(b)
Buffer
Trypanosomes
2.5mM KCN
2mM SHAM

Figure 3: Effect of SHAM and cyanide on the survival of the midgut forms incubated with glucose as substrate. The symbols O-O ,●-●, Δ Δ, ▲-▲ indicate survival on glucose with no inhibitor, SHAM alone alone , cyanide alone, and with a combination of SHAM and cyanide respectively. The concentration of SHAM and KCN was 2.0 and 2.5 mM respectively. Trypanosome density corresponding to 100% survival was $4x10^6$/ml.

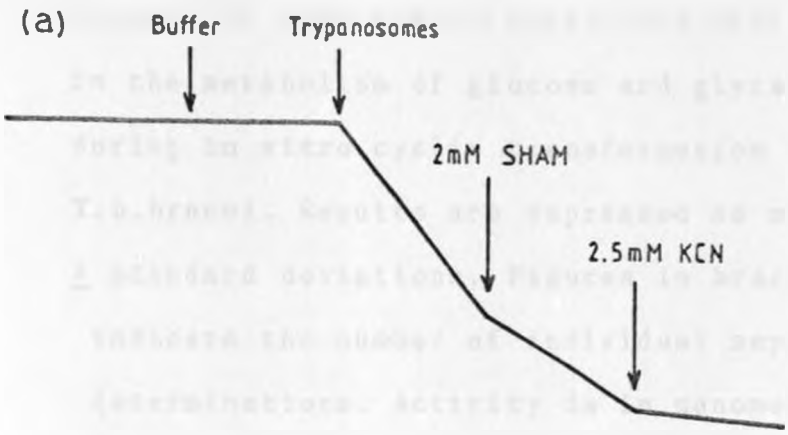
% TRYPANOSOME SURVIVAL
0
20
40
60
80
100
TIME (min)
0
10
20
30
40
50
60

(Table V).

Table V column A shows the activities of glycerol kinase and glycerol 3-phosphate dehydrogenase, which catalyse the initial stages of glycerol catabolism were higher than those of hexokinase and phosphoglucose isomerase which catalyse the initial stages of glucose catabolism. This may account for the better survival of these organisms on glycerol than on glucose as shown in Figure I. Due to the low yield of motile proventricular forms, respiration and viability on glucose or glycerol were not determined. Table V (column B) shows some glycolytic enzymes that catabolise glucose or glycerol. The activities were 2-7 fold less than in midgut forms; pyruvate kinase activity was consistently very low between zero and 5 nanomoles /min/mg protein. It is possible that these low levels of enzyme activities are due to decreased use of the oxidative pathways, considering that the trypanosomes were incubated without opening the flasks for entry of O_2 or release of CO_2.

The midgut-like forms also respired on glucose and glycerol. They could survive on these substrates for 2 hours without death unlike the midgut forms which could survive for a similar period with glycerol but not glucose. Figures 4a and 4b show that the rates of respiration glucose and glycerol were 61 and

Figure 4: Effect of SHAM and cyanide during the respiration of the midgut-like of **T.b.brucei** utilizing (a) glucose and (b) glycerol. Each incubation contained 10 mM glucose or glycerol in PS buffer. The number of trypanosomes in each incubation was $1x10^7$.


(a)
Buffer
Trypanosomes
2mM SHAM
2.5mM KCN

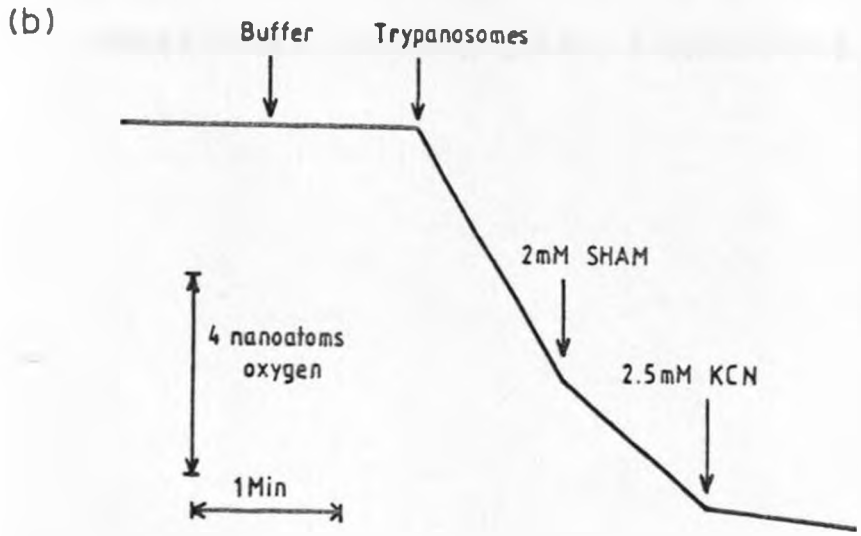
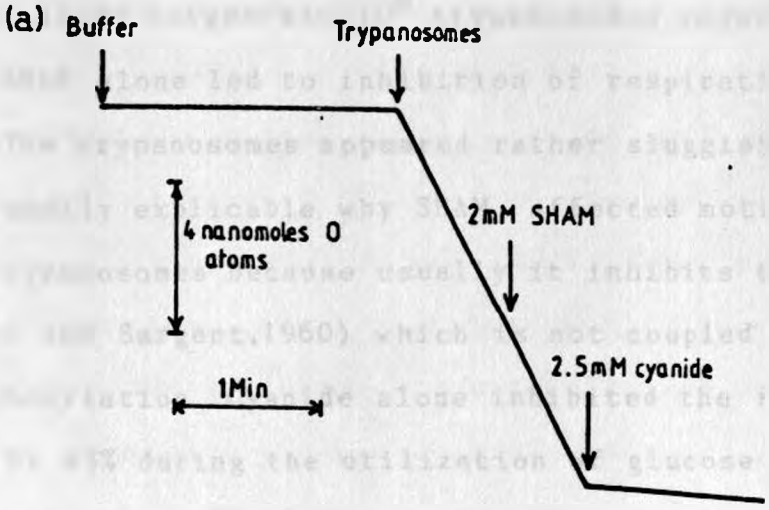
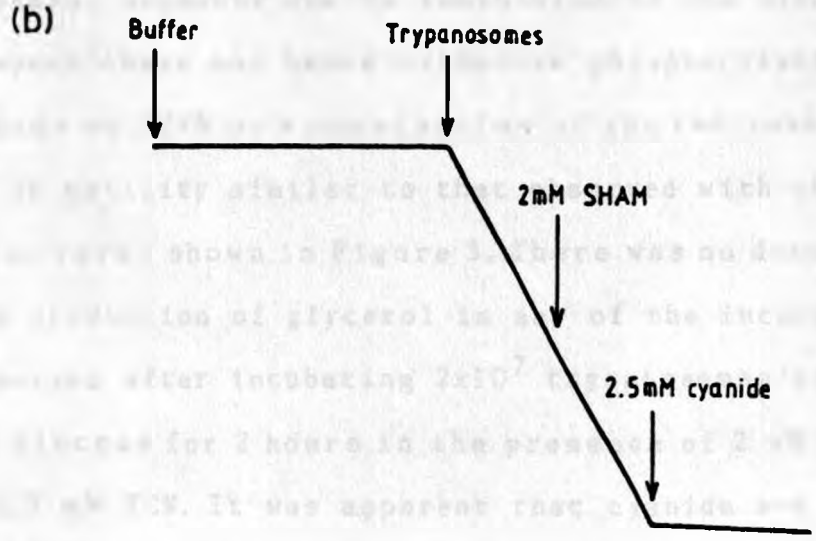

(b)
Buffer
Trypanosomes
2mM SHAM
2.5mM KCN
4 nanoatoms
oxygen
1Min

Table V: Changes in some enzyme activities involved in the metabolism of glucose and glycerol during **in vitro** cyclic transformation of **T.b.brucei**. Results are expressed as means ± standard deviations. Figures in brackets indicate the number of individual separate determinations. Activity is in nanomoles/ min/mg protein. Columns A,B,C and D indicates activities from the midgut forms, proventricular forms, midgut-like forms, and the cultured metacyclic forms from tsetse flies salivary glands respectively.

Enzyme	A	B	C	D
Hexokinase	180 ± 20(4)	70 ± 25(3)	620 ± 50(4)	474 ± 98(4)
Phosphoglucose isomerase	68 ± 15(4)	20 ± 10(3)	160 ± 28(4)	400 ± 45(4)
Glycerol kinase	750 ± 50(5)	100 ± 25(4)	700 ± 81(4)	592 ± 60(4)
Gro3p dehydrogenase	680 ± 48(4)	150 ± 18(3)	650 ± 50(4)	358 ± 32(4)
Pyruvate kinase	10 ± 3(3)	3 ± 2.1(4)	20 ± 6(4)	33.7 ± 5.1(4)
Enolase	145 ± 32(4)	22 ± 5(3)	28 ± 3(3)	40.7 ± 1.2(4)
Malate dehydrogenase	200 ± 22(4)	122 ± 16(3)	680 ± 40(4)	210 ± (4)
Adenylate kinase	720 ± 41(4)	310 ± 28(3)	232 ± 24(3)	879±70(4)
Total ATPase	145 ± 10(3)	80 ± 14(3)	583 ± 66(3)	358 ± 31(4)
Oligomycin sensitive ATPase	90 ± 26(3)	66 ± 13(3)	435 ± 20(4)	142 ± 20(4)

Figure 5: Effect of SHAM and cyanide on the respiration on proline by the midgut-like forms. (a) shows respiration by the midgut forms and (b) respiration by the midgut-like forms. The buffer used was PS containing 10 mM L-proline as substrate. Each incubation contained $1x10^7$ trypanosomes.


(a)
Buffer
Trypanosomes
4 nanomoles O atoms
2mM SHAM
2.5mM cyanide
1Min


(b)
Buffer
Trypanosomes
2mM SHAM
2.5mM cyanide

80 nanoatoms oxygen/min/10^8 trypanosomes respectively. SHAM alone led to inhibition of respiration by 50%. The trypanosomes appeared rather sluggish. It was not readily explicable why SHAM affected motility of the trypanosomes because usually it inhibits the GPO (Grant and Sargent,1960) which is not coupled to ADP phosphorylation. Cyanide alone inhibited the respiration by 45% during the utilization of glucose or glycerol. About 5% of the respiration was insensitive to both SHAM and cyanide. Cyanide alone led to a rapid decrease of trypanosome motility and subsequently death, probably due to inhibition of the electron transport chain and hence oxidative phosphorylation. Cyanide or SHAM or a combination of the two caused loss of motility similar to that observed with the midgut forms shown in Figure 3. There was no detectable production of glycerol in any of the incubations even after incubating $2x10^7$ trypanosomes/ml with glucose for 2 hours in the presence of 2 mM SHAM and 2.5 mM KCN. It was apparent that cyanide and SHAM together could not adequately simulate anaerobiosis because of the 5% respiration resistant to both inhibitors. However even if the respiration was fully eliminated by these inhibitors,the trypanosomes could not have been surviving anaerobically like the bloodstream forms (Brohn and Clarkson,1978) because 80% of

them appeared immobilised within 10 min, unlike the bloodstream forms which remained motile for more than 2 hours. Table V column C shows that the activities of the enzymes catalysing the initial reactions of glucose and glycerol metabolism : hexoki-nase, phosphoglucose isomerase, glycerol kinase and glycerol 3-phosphate dehydrogenase are sufficient to explain the high rate of the respiration and the survival of the midgut-like trypanosomes on these substrates.

The activities of the total ATPase, oligomycin sensitive ATPase and malate dehydrogenase were higher in midgut-like forms than in any of the trypanosome stages (Table V). This could suggest a greater use of the oxidative pathways in energy production than in other trypanosome stages.

Since it was not possible to separate the infective metacyclic forms from the midgut like and epimastigote forms, activities of key enzymes of glucose and glycerol metabolism were determined in the trypanosomes cultured. Results similar to those for the midgut forms were obtained (Table V column C). This was probably due to the low per cent of the non midgut forms. The metacyclics comprised up to 5% of the total population whereas the epimastigotes accounted for about 1% judging from morphological characteris-

tics under the phase contrast microscopy.
The midgut, the proventricular and midgut-like forms survived on proline for at least 2 hours ,as well as on glycerol (Figure I), and respired on this substrate. Figure 5 shows respiration of the midgut and midgut like forms on proline, and the effects of adding SHAM and cyanide. SHAM had no effect on respiration indicating that electrons derived from proline do not reduce molecular oxygen via GPO. Rates of respiration on proline in the midgut and midgut like forms were 78 and 72 nanoatoms oxygen/min/10^8 trypanosomes respectively. Respiration of the proventricular forms was not studied due to lack of enough viable trypanosomes. Respiration on glucose or glycerol was not determined in the in vitro propagated metacyclic forms derived from tsetse salivary glands. This was due to inability to obtain sufficient cultures, because a supposedly suitable batch of serum from Flow Laboratories turned out to be toxic to the trypanosomes. The cultures were discontinued after 4 months. The trypanosomes from the few cultures that had been maintained before the new batch of serum was used, could survive on 10 mM glucose or glycerol in PS buffer for at least one hour. The activities of hexokinase,phosphoglucose isomerase, glycerol kinase,and glycerol-3-phosphate dehydrogenase(Table V

column D) are consistent with the ability of these trypanosomes to use both glucose and glycerol for energy production. The activities of enolase and pyruvate kinase were fairly low, however this could not be explained because it has been reported that such trypanosomes produce large amounts of pyruvate and glycerol (Njogu and Nyindo,1981).

3.2 Discussion

3.2.1 Cyclic transformation of T.b.brucei

The sequence of transformation of T.b.brucei is: short stumpy bloodstream forms ⟶ midgut forms ⟶ proventricular forms ⟶ epimastigote forms ⟶ metacyclic forms ⟶ long slender bloodstream forms ⟶ short stumpy bloodstream forms (Hoare,1972). The mitochondria of the midgut, proventricular and epimastigote forms are more enlarged than in the bloodstream forms (Bohringer and Hecker,1975., Hoare,1972) probably due to the development of the respiratory chain. According to Hirumi et al (1979), the sequence of in vitro transformation is: bloodstream forms ⟶ midgut forms ⟶ proventricular forms ⟶ midgut-like forms ⟶ epimastigote forms ⟶ metacyclic forms ⟶ bloodstream forms.

The in vitro propagated midgut forms possess all or part of the respiratory chain because respiration on either glucose or glycerol was inhibited by

cyanide. Due to the presence of identical looking organelles in both the midgut and proventricular forms, it is assumed that these forms are biochemically identical (Hoare,1972). The midgut like trypanosomes observed in the **in vitro** cultures have not been reported in the insect vector although it is possible that they might have been mistaken for the midgut forms and thus escaped notice. They were first observed by Hirumi et al(1979), and then in this investigation. From the enzyme activities and respiration on both glucose and glycerol, it can be inferred that the midgut like forms are distinct trypanosome stages. From comparison of enzyme activities in Tables I and V, it can be concluded that these trypanosomes are different from the procyclic trypomastigotes. Since the midgut forms could not transform directly to the proventricular forms it could be proposed that some genes are activated by the high H^+ and CO_2 concentration achieved when the culture flasks containing the midgut forms were closed for 7 days. It could be that the expression of genes for most catabolic pathways are partially repressed due to low O_2 tension and accumulation of CO_2. It is also possible that due to lack of or adequate concentrations of appropriate substrate such as amino

acids and accumulation of acidic products, protein biosynthesis was decreased hence the absence of noticeable trypanosome multiplication. Considering that after 7 days, the proventricular forms could transform to the midgut like forms, it is proposed that a biochemical transformation must have occurred in a significant number of the parasites. The subculture of such parasites in fresh medium appears to trigger off the expression of the genes for the oxidative pathways including the GPO. Cultures containing the metacyclic trypanosomes had also some epimastigotes. This is consistent with the situation in the tsetse salivary glands (Steiger,1973) although no trypanosomes have been reported in the salivary gland similar to the midgut like forms observed in the **in vitro** cultures.

Cultures containing the metacyclic forms derived from tsetse salivary glands apparently contained no epimastigote forms, although it is these which are believed to transform to metacyclic forms.

Instead the metacyclic forms were found to be dividing just as reported by Nyindo **et al** (1978,1979). This is contrary to the suggestion by Vickerman (1969) that the metacyclic forms do not divide in the tsetse salivary glands.

3.2.2 Glycerol metabolism by the in vitro propagated metacyclic trypomastigotes derived from tsetse salivary glands

Njogu and Nyindo (1981) reported that the cultured metacyclic forms derived from infected tsetse salivary glands could not respire on proline or succinate but metabolised glucose to produce pyruvate and glycerol in the molar ratio of 3:1. In the presence of SHAM, respiration was completely inhibited and equimolar amounts of pyruvate and glycerol were formed. The rate of glycerol production was increased by 60% within the first one hour of incubation with SHAM but declined to almost zero in the following two hours. The rate of pyruvate production decreased by a similar magnitude. Cyanide had no effect on respiration. This is consistent with the utilization of the glycolytic pathway as the major source of ATP and GPO as the only major terminal oxidase,just as in the bloodstream forms. The apparent difference between the metacyclic and the bloodstream forms lies in the ability of the former to produce both glycerol and pyruvate during aerobiosis. It appeared that under aerobic conditions there are two competing pathways: one for the production of glycerol and the other for pyruvate. One would therefore expect significant activities of all the enzymes of glycolysis in

these organisms.

The results of this investigation show that the metacyclic forms derived from tsetse salivary glands had high enough activities of hexokinase, phosphoglucoisomerase, glycerol kinase and glycerol 3-phosphate dehydrogenase to account for the aerobic rate of pyruvate production of 0.580 micromoles/h/10^7 trypanosomes (Njogu and Nyindo, 1981). Activities of enolase and pyruvate kinase were apparently low relative to the others. It is not possible to oxidise Gra3P or GrnP to pyruvate without following the sequence;

Gri1,3P_2 ⟶ Gri3P ⟶ Gri2P ⟶ PEPyr ⟶ Pyr, catalysed by phosphoglycerate kinase, phosphoglycerate mutase, enolase and the pyruvate kinase respectively. It is likely therefore that the low measured activities of enolase and pyruvate kinase was the result of storage of the trypanosomes at -20°C for about 14 days to pool enough material for the enzyme assays; they may also have been due to the presence of endogenous inhibitors in the trypanosome lysates released after the disruption of the trypanosome membranes, dilution of the concentrations of some activators such as Fru2,6P_2, (Cronin and Tipton,1985) or absence of that part of the pathway involving these enzymes. In addition, taking 10^8 trypanosomes as equivalent to 1 mg protein (Danforth,

1967) and from the activities of enolase and pyruvate kinase (Table V), the maximum rate of pyruvate production would be 2 micromoles/h/mg protein. This is less than that calculated from the report of Njogu and Nyindo (1981) during aerobiosis, which was about 5.8 micromoles pyruvate/h/mg protein.

The ability of the metacyclic forms derived from tsetse salivary glands to produce glycerol under aerobic conditions and the low activities of enolase and pyruvate kinase may be explained from these results as follows:

Since the metacyclic forms appear to rely on glycolysis like the bloodstream forms, glucose is catabolised according to the equation;

Glc + ATP + Pi $\longrightarrow$ Gri3P + Gro3P + ADP.

This is likely to occur at a rate consistent with the activities of the hexokinase up to and including the glycerol3-phosphate dehydrogenase, and the phosphoglycerate kinase. If the low activities of enolase and pyruvate kinase were not due to storage at -20^{o}C and effect of solubilization proposed above glucose cannot be converted to pyruvate using the same pathway as in the bloodstream forms. I suggest two competing pathways for the utilization of Gro3P: the oxidation of Gro3P via the GPO to pyruvate and dephosphorylation to glycerol according to the equation; Gro3p + X

⟶ glycerol + X-P, where X is the phosphoryl group acceptor.

The oxidation of Gro3P via the GPO would occur at a rate consistent with the rate of conversion of Gri3P to pyruvate. The molar ratio of pyruvate to glycerol during aerobiosis may be dependent on the relative activities of the enzymes catalysing the conversion of Gri3P to pyruvate and Gro3P to glycerol among other factors.

During anaerobiosis or when the GPO is inhibited by SHAM (Njogu and Nyindo, 1981) there was net ATP production for at least one hour because there was a significant production of pyruvate and glycerol. The initial rate of glycerol and pyruvate production was 3.5 micromoles/h/mg protein which declined to almost zero after 2 hours. This shows that the pathway of glycerol production can be stimulated by inhibition of the GPO just as in the bloodstream forms (Fulton and Spooner, 1959). Apparently these parasites also maintain a constant rate of glycolysis when GPO is inhibited as reported in the bloodstream forms (Brohn and Clarkson, 1978). The enzyme that catalyses the conversion of Gro3P to glycerol in the metacyclic. forms has not been established. It would presumably be the same enzyme as that in the bloodstream forms, usually taken as glycerol kinase (Aman and Wang,

1986; Opperdoes and Borst, 1977; Visser et al, 1981). The presence of high activities of glycerol kinase in trypanosome stages that do not produce glycerol suggests that its main role may be in free glycerol utilisation:

ATP + glycerol $\longrightarrow$ ADP + Gro3P

In trypanosomes other than the bloodstream and the metacyclic forms, Gro3P may be oxidised to pyruvate in conjunction with either the electron transport chain or GPO with concomitant ATP production (Scheme III). This is consistent with the role of glycerol kinase in other organisms (Cancedo et al 1968). Whether glycerol kinase has a dual function in the bloodstream and the metacyclic forms, needs to be established unequivocally.

It is stressed that the presence of glycerol kinase in a number of trypanosome stages seems to be unrelated to glycerol formation. Considering that glycerol kinase, glycerol 3-phosphate dehydrogenase, and GPO were present in all the stages, studied, it is tempting to propose that the genes coding for these enzymes are regulated by the same inducer. Hayashi and Iin (1965) have shown that Gro3p is the inducer for the expression of the glycerol kinase and the GPO in Escherichia coli.

3.3 Conclusions

From this investigation the following conclusions may be drawn.

(a) It is more economical to obtain the metacyclic trypomasigotes of T.b.brucei for biochemical experiments by continous propagation of those found in the tsetse salivary glands than by **in vitro** cyclic transformation of the bloodstream forms.

(b) High activities of the glycerol kinase are not the only criteria for glycerol production during anaerobiosis in the different stages of T.b.brucei.

(c) Both SHAM and cyanide in combination are lethal to most of the in vitro propagated trypanosomes although they do not adequately simulate anaerobiosis as is the case in the bloodstream forms in the presence of SHAM alone.

(d) Although the cultured insect forms survive on glycerol under aerobic conditions, they donot survive on glucose as substrate when respiration is inhibited 92-95 per cent by a combination of cyanide and SHAM. Survival of these trypanosomes on glucose and concomitant glycerol production would be expected if the presence of the glycerol kinase was the only criterion for glycerol production.

CHAPTER IV

Catabolism of glycolytic intermediates by digitonin-permeabilized T.b.brucei

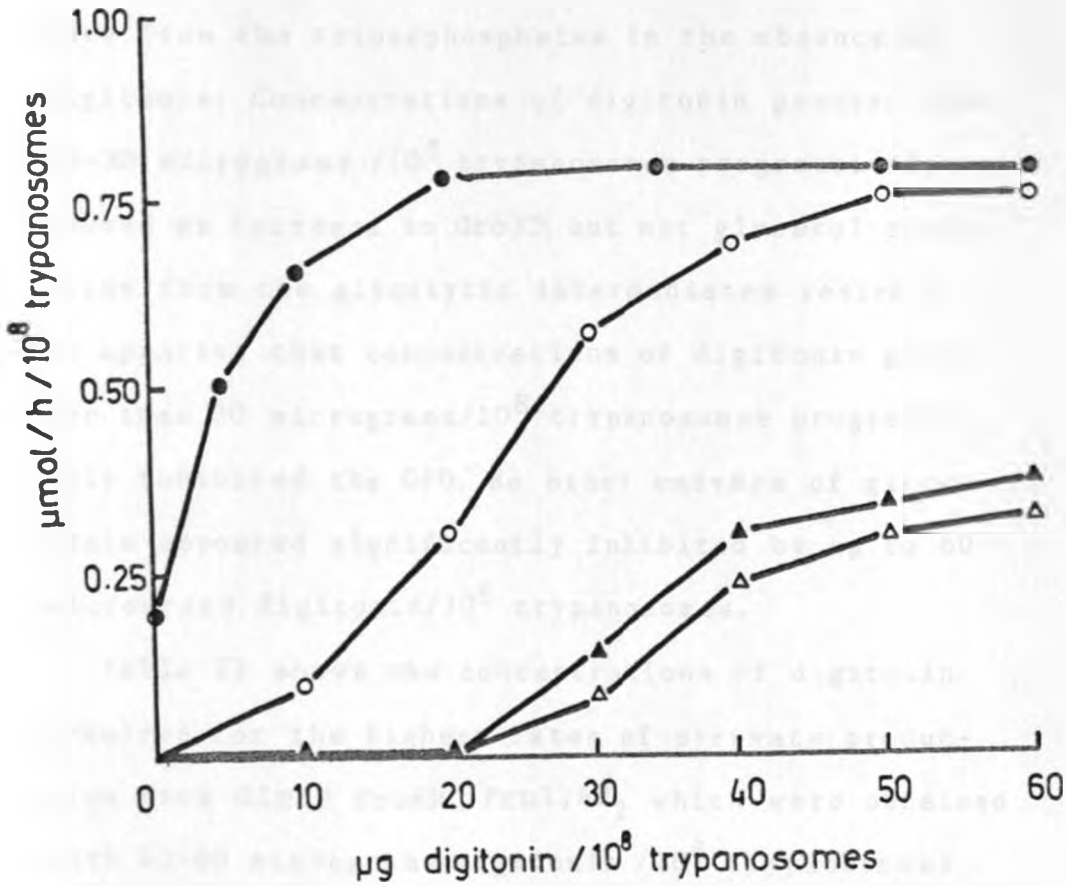
4.1 Results

4.1.1 Aerobic pyruvate production from glycolytic intermediates by bloodstream forms of T.b.brucei: stimulation by permeabilization with digitonin.

It has been shown that **T.b.brucei** can respire on glycolytic intermediates in the presence of the appropriate concentration of digitonin (Kiaira and Njogu,1983). In order to show that the glycolytic intermediates can be catabolised also via the whole span of the glycolytic pathway in trypanosomes permeabilised to different extents, pyruvate was determined after incubation with Glc6p, **Glc6P**, **Frul,6P$_2$**, **Gra3P**, and Grnp at various concentrations of digitonin. For glycolytic intermediates that have to be phosphorylated prior to catabolism, 2.5 mM ATP was included ; to those that do not require further phosphorylation, 1.5 mM ADP was added (Kiaira,1983).

There was no significant pyruvate production from **Glc6P**, **Fru6P** or **Frul,6P$_2$** without digitonin. Increasing concentrations of digitonin increased pyruvate production from the glycolytic intermediates tested. Mg^{2+} was required in all cases involving digitonin permeabilization (Kiaira,1983),

Figure 6: A comparison of the concentrations of digitonin required for maximum rate of pyruvate production from Glc6P and GrnP. The incubations contained 10 mM Glc6P or 20 mM GrnP and 2.5 mM ATP. O-O and Δ-Δ indicates pyruvate and Gro3P production respectively from Glc6P. ●-● and ▲-▲ indicates pyruvate and Gro3P production respectively from Grnp. A similar pattern of results was obtained when Glc6P was replaced by Fru6P or $Fru1,6P_2$.


1.00
0.75
0.50
0.25
µmol / h / 10⁸ trypanosomes
0
10
20
30
40
50
60
µg digitonin / 10⁸ trypanosomes

otherwise the rate of pyruvate production was very low. Figure 6 shows pyruvate and Gro3P production from Glc6P and Grnp at various concentrations of digitonin. The pattern of results obtained with Fru6P and $Frul,6P_2$ was similar to that for Glc6P whereas that for the Gra3P was similar to that for Grnp. There was little production of pyruvate from the triosephosphates in the absence of digitonin. Concentrations of digitonin greater than 25-30 micrograms /10^8 trypanosomes progressively caused an increase in Gro3P, but not glycerol production from the glycolytic intermediates tested.It is apparent that concentrations of digitonin greater than 30 micrograms/10^8 trypanosomes progressively inhibited the GPO. No other enzymes of glycolysis appeared significantly inhibited by up to 60 micrograms digitonin/10^8 trypanosomes.

Table VI shows the concentrations of digitonin required for the highest rates of pyruvate production from Glc6P Fru6P , $Frul,6P_2$ which were obtained with 40-60 micrograms digitonin /10^8 trypanosomes whereas with Gro3P GrnP, Gra3P, Gri2P , Gri3P and PEPyr as substrates 20-30 micrograms digitonin/10^8 trypanosomes were required. Adding a digitonin concentration greater than 60 micrograms/10^8 trypanosomes was not exceeded because it inhibits respi-

Figure 7: Effects of increasing the concentration of digitonin on pyruvate and Gro3P production by **T.b.brucei** incubated with glucose in the presence and absence of 2.5 mM ATP. Glucose concentration was 10 mM. Δ-Δ and ▲-▲ indicates pyruvate and Gro3P respectively in the presence of ATP. O-O indicates pyruvate production without ATP.

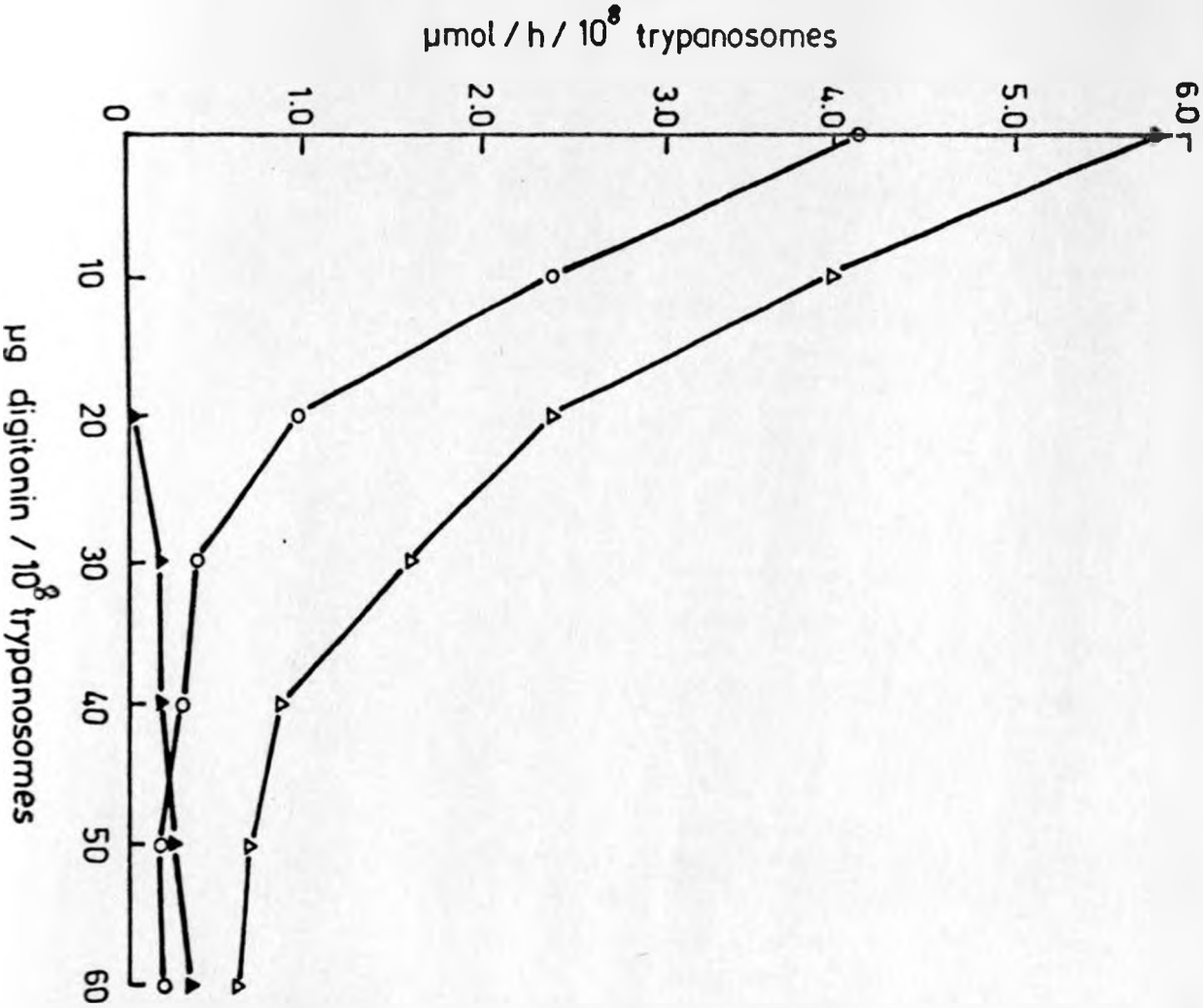

µmol / h / 10⁸ trypanosomes
0
1.0
2.0
3.0
4.0
5.0
6.0
µg digitonin / 10⁸ trypanosomes
10
20
30
40
50
60

Table VI: Concentrations of digitonin required for maximum pyruvate production from phosphorylated glycolytic intermediates. The incubations contained saturating concentrations of glycolytic intermediates, 2.5 mM ATP or ADP and various concentratios of digitonin. The concentration of digitonin (B) was in micrograms/10^8 trypanosomes. The rate of pyruvate production (C) was in micromoles/h/10^8 trypanosomes. Substrate concentration (A) was in mM.

Substrate	A	B	C
Glucose	10	-	3.6 ± 0.8(4)
Glc6P	10	50 ± 8(4)	0.74 ± 0.11(4)
Fru6P	30	52 ± 8(4)	1.53 ± 0.32(4)
Frul,$6P_2$	15	50 ± 7(4)	0.73 ± 0.14(4)
Gra3P	20	24 ± 8(3)	0.70 ± 0.10(3)
GrnP	20	25 ± 8(3)	2.40 ± 0.40(3)
Gri3P	10	25 ± 3(3)	2.40 ± 0.40(3)
Gri2P	10	24 ± 4(3)	3.40 ± 0.5(3)
PEPyr	10	24 ± 4(3)	3.50 ± 0.6(3)

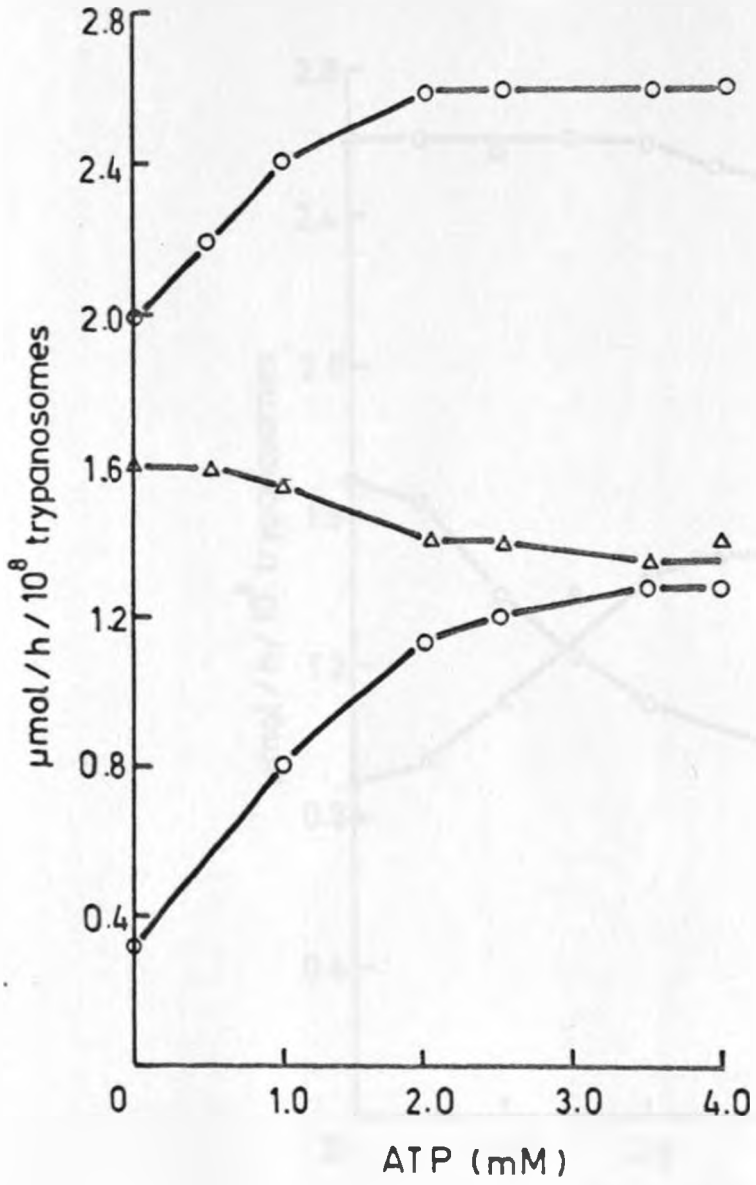
ration (Kiaira and Njogu, 1983). Figure 7 shows pyruvate production from glucose at increasing concentrations of digitonin in the presence and absence of ATP. Digitonin progressively caused a decrease in pyruvate production.There was slightly more pyruvate produced at various concentrations of digitonin in the presence of ATP than in its absence. In addition there was a progressive increase in Gro3P levels when the digitonin concentration was gradually increased above 20 micrograms/10^8 trypanosomes.

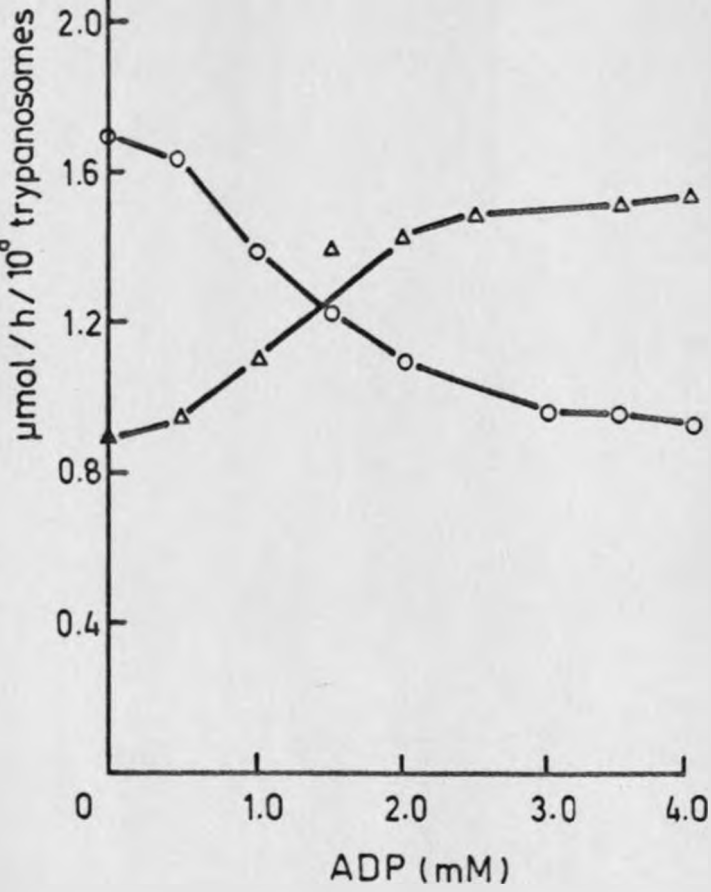
4.1.2 Products of glucose catabolism in intact T.b.brucei under simulated anaerobic condition:effects of exogenous ATP and ADP

Equimolar amounts of pyruvate and glycerol are formed by T.b.brucei incubated with glucose and SHAM (Brohn and Clarkson,1978). Transport of exogenous ATP, ADP and AMP in T.b.brucei has been demonstrated(Sanchez et al ,1976). The effects of ADP and ATP on the products of glucose catabolism in the presence of SHAM has been investigated. Figure 8a shows that as the concentration of ATP was increased from zero to 4 mM in the presence of 2.5mM ADP, pyruvate production was increased by 30% and Gro3P was increased from very small amounts to

Figure 8: Effect of increasing the concentration of ATP or ADP on the production of pyruvate ●-●, Gro3P O-O, and glycerol Δ-Δ. The incubations initially contained 2.5 mM SHAM, 10 mM glucose and either (a) 2.5 mM ATP or (b) 2.5 mM ADP

(a)




2.0
1.6
1.2
0.8
0.4
µmol / h / 10⁸ trypanosomes
0
1.0
2.0
3.0
4.0
ADP (mM)

(b)

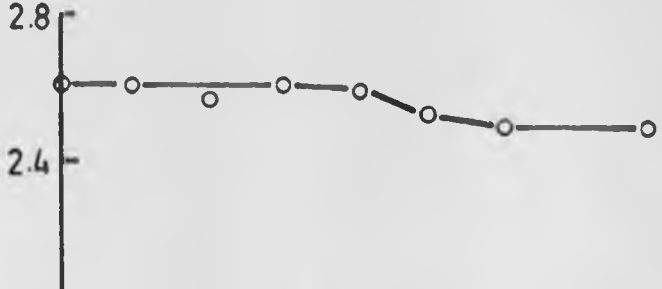
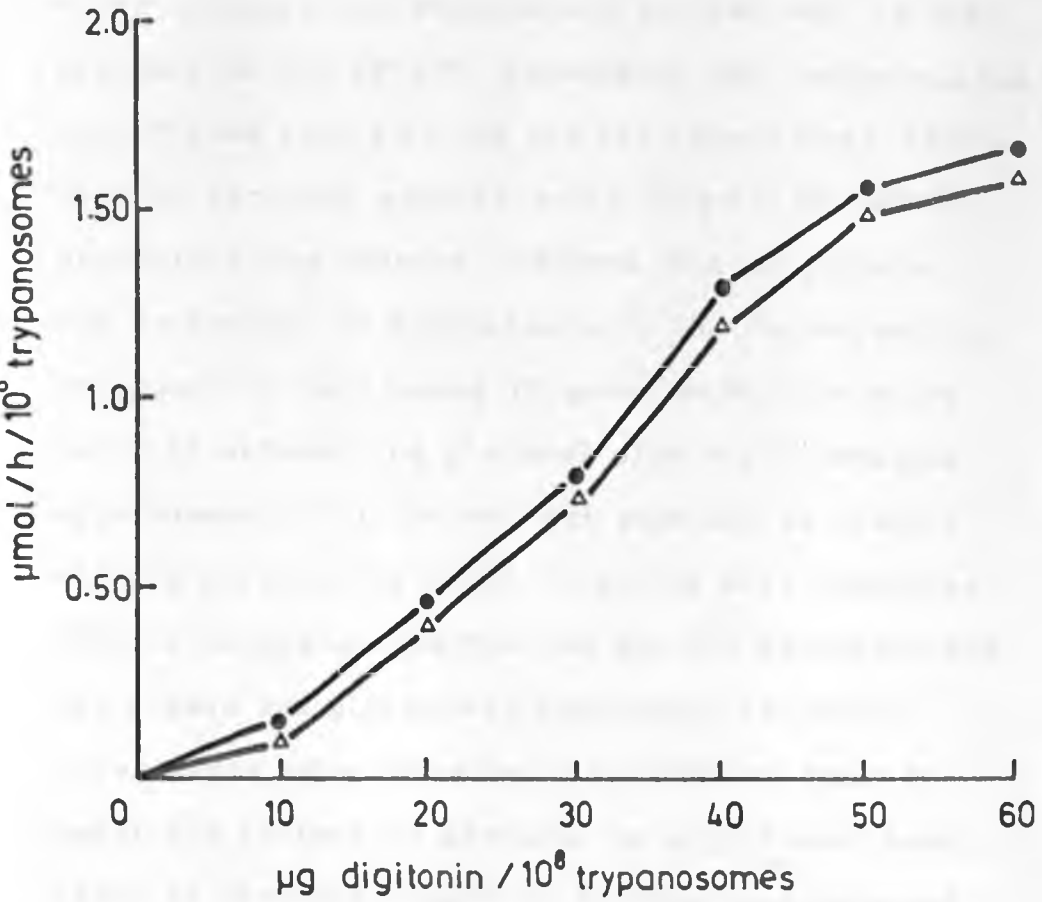


Figure 9: Increasing the catabolism of Glc6P by addition of digitonin in the presence of SHAM. The incubations contained 10 mM Glc6P, 2 mM SHAM and 1.5 mM ADP. ●-● indicates pyruvate and Δ-Δ Gro3P produced.


2.0
1.50
1.0
0.50
0
10
20
30
40
50
60
µmol / h / 10⁸ trypanosomes
µg digitonin / 10⁸ trypanosomes

about four fold whereas glycerol was slightly reduced. In parallel experiments carried out in the presence of 2.5 mM ATP, increasing the concentration of ADP from zero to 4 mM did not affect the production of pyruvate significantly (Figure 8b). Gro3P production was reduced whereas that of glycerol was increased by approximatetly the corresponding decrease. In both cases (Figures 8a,8b) the molar ratio of pyruvate to glycerol plus Gro3P remained approximately 1:1. It was not possible to convert all the glycerol to Gro3P by adding more exogenous ATP. To determine whether ADP and ATP permeabilized the plasma and glycosomal membranes, the above experiments were repeated with Glc6P or Fru6P as substrate instead of glucose. No significant production of pyruvate, Gro3P or glycerol was detected and the trypanosomes were immobilized within 5 minutes. This suggests that although exogenous ATP and ADP have access to the glycolytic enzymes,the hexosephosphates cannot replace glucose as substrate in intact trypanosomes due to permeability barrier. Gro3P appeared able to leave the parasites (Figures 8a and 8b).

4.1.3 Products of catabolism of glycolytic intermediates by digitonin-permeabilized T.b.brucei under simulated anaerobiosis

Figure 10: Increasing the catabolism of Fru6P in T.b.brucei by addition of digitonin in the presence of SHAM.The incubations contained 1.5 mM ADP, 2 mM SHAM and 30 mM Fru6P. O-O indicates pyruvate and Δ-Δ Gro3P produced.

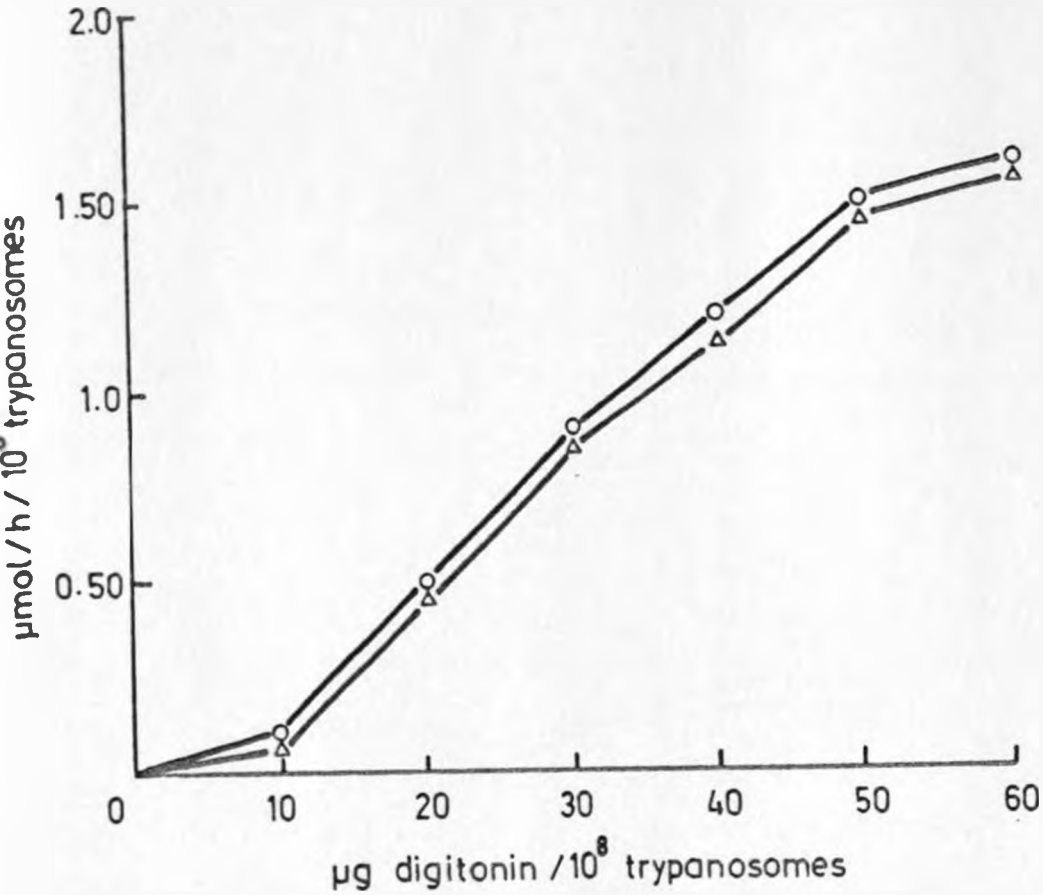
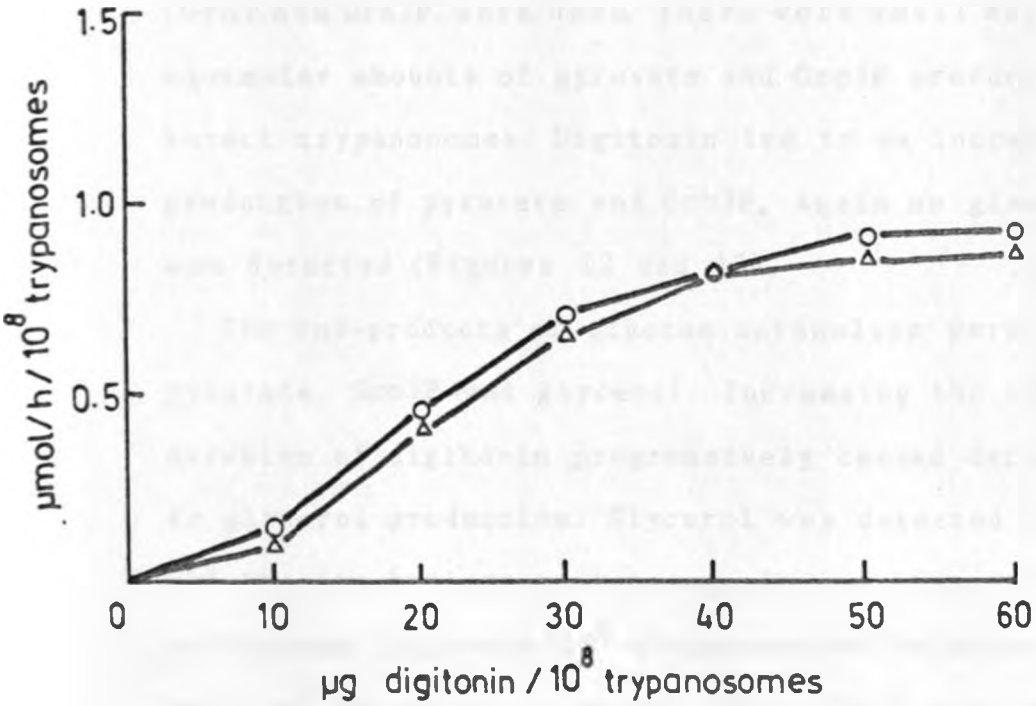

2.0
1.50
1.0
0.50
0
10
20
30
40
50
60
µmol / h / 10⁸ trypanosomes
µg digitonin / 10⁸ trypanosomes

Figure 11: Increasing Frul,$6P_2$ catabolism by addition of digitonin in the presence of SHAM. Each incubation contained 1.5 mM ADP, 15 mM Frul,$6P_2$ and 2 mM SHAM. O-O indicates pyruvate and Δ-Δ Gro3P produced.


1.5
1.0
0.5
µmol/h/10⁸ trypanosomes
0
10
20
30
40
50
60
µg digitonin / 10⁸ trypanosomes

The possibility that digitonin permeabilized T.b.brucei might form pyruvate and glycerol anaerobically from glycolytic intermediates was investigated. A parallel control experiment was always run with D-glucose as substrate under similar conditions. Figures 9, 10, and 11 show that increasing concentrations of digitonin resulted in production of equimolar amounts of Gro3P and pyruvate from Glc6P, Fru6P and Frul,$6P_2$. Glycerol was not detected in any of the incubations containing the hexosephasphates as substrates. When the triosephosphates; Grn P and Gra3P were used, there were small but equimolar amounts of pyruvate and Gro3P produced by intact trypanosomes. Digitonin led to an increase in production of pyruvate and Gro3P. Again no glycerol was detected (Figures 12 and 13).

The end-products of glucose catabolism were pyruvate Gro3P and glycerol. Increasing the concentration of digitonin progressively caused decrease in glycerol production. Glycerol was detected in all the incubations containing glucose even at 60 micrograms digitonin/10^8 trypanosomes.The molar ratio of pyruvate to glycerol plus Gro3P was always approximately 1:1(Figure 14).

Incubation of the trypanosomes with the glycolytic intermediates: Gri3P,Gri2P and PEPyr which do not

Figure 12: Increasing the catabolism of GrnP by addition of digitonin in the presence of SHAM. The respective incubations contained 2 mM SHAM, 20 mM GrnP and 1.5 mM ADP. O-O indicates pyruvate and Δ-Δ Gro3P produced.

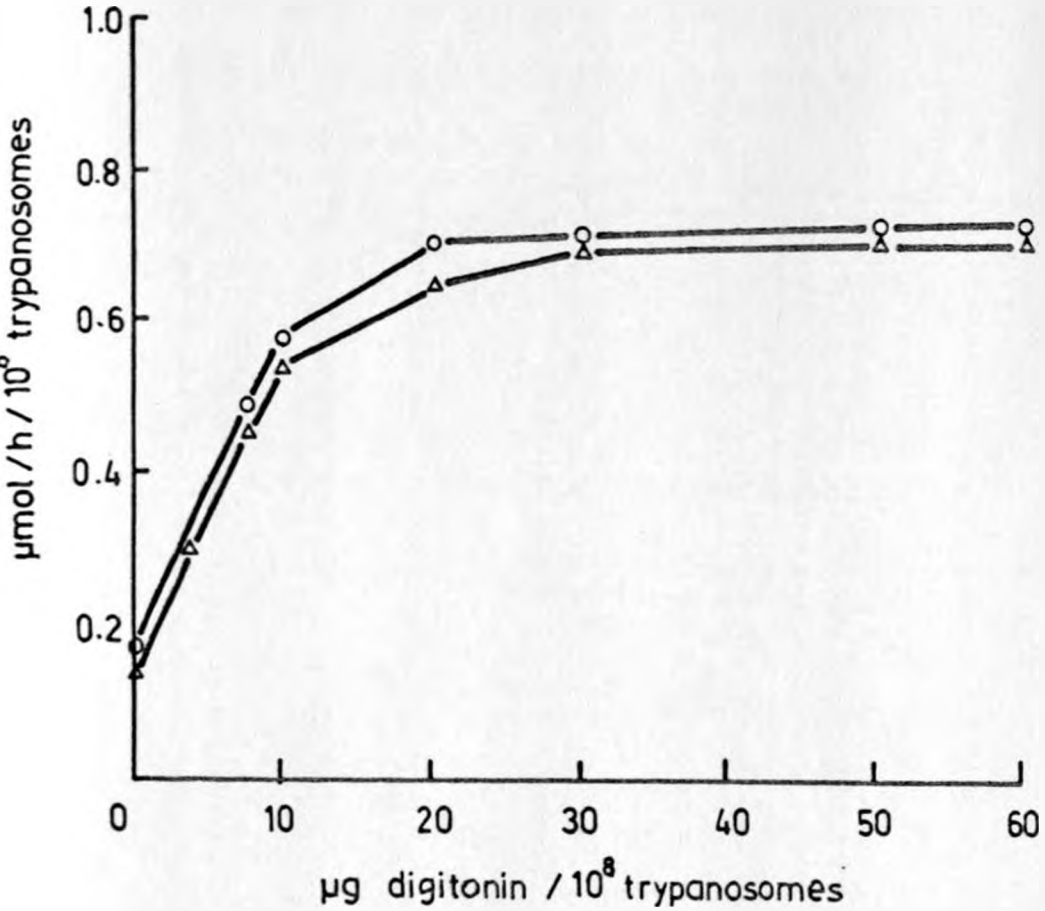

1.0
0.8
0.6
0.4
0.2
µmol / h / 10⁸ trypanosomes
0
10
20
30
40
50
60
µg digitonin / 10⁸ trypanosomes

Figure 13: Increasing the catabolism of Gra3P by addition of digitonin in the presence of SHAM. Ineach incubation mixture was 2 mM SHAM, 1.5 mM ADP and 20 mM Gra3P. O-O indicates pyruvate and Δ-Δ Gro3P produced.

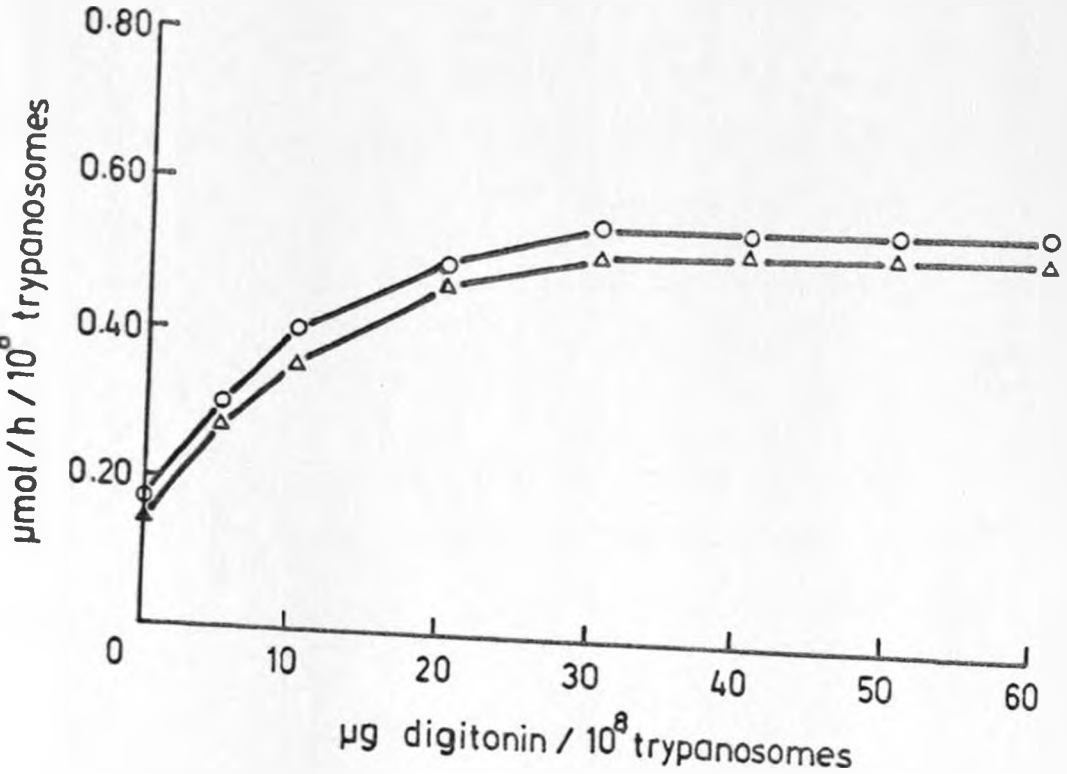

0·80
0.60
0.40
0.20
0
10
20
30
40
50
60
µmol / h / 10⁸ trypanosomes
µg digitonin / 10⁸ trypanosomes

Figure 14: Effect of increasing the concentration of digitonin on pyruvate O-O, glycerol ▲-▲ and Δ-Δ Gro3P from glucose. The trypanosomes were incubated with 1.5 mM ADP, 2 mM SHAM and 10 mM glucose.

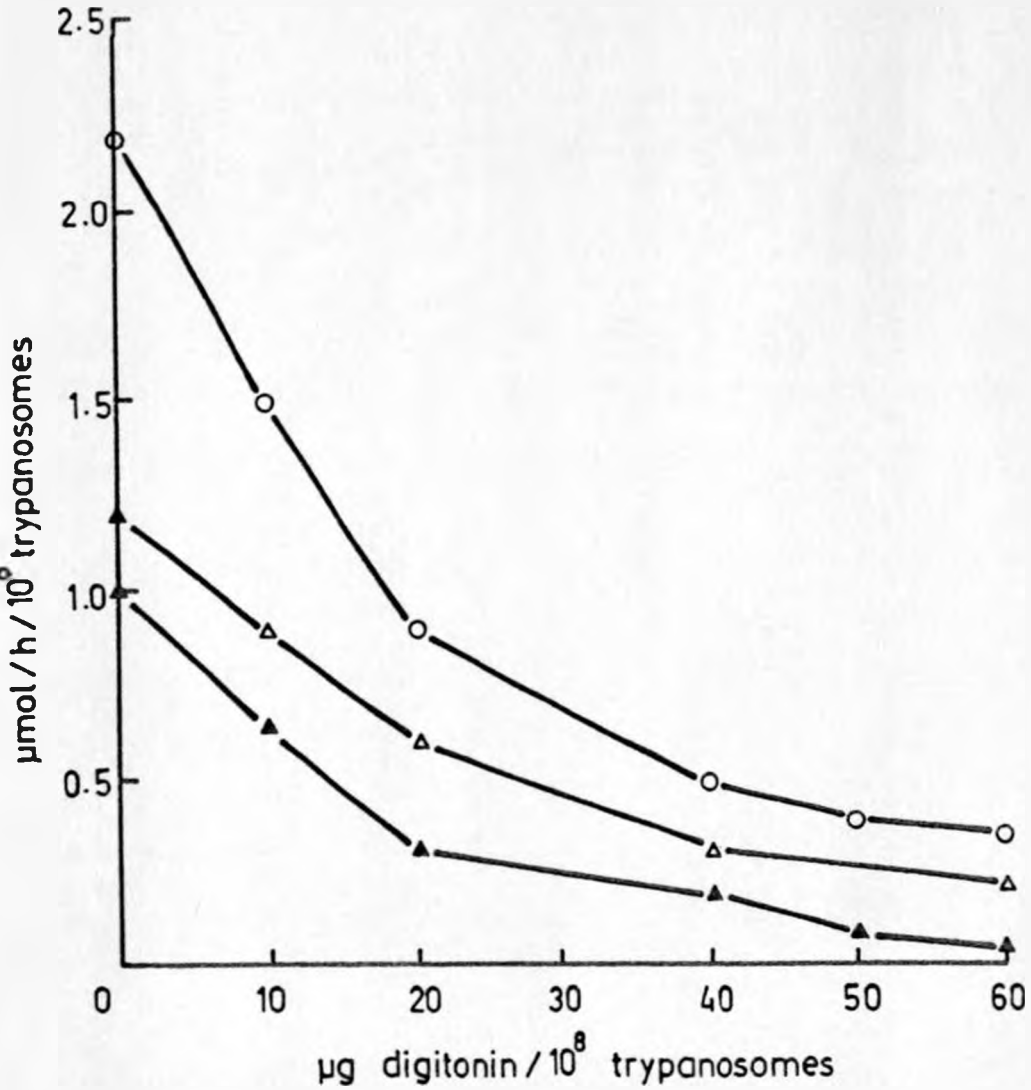
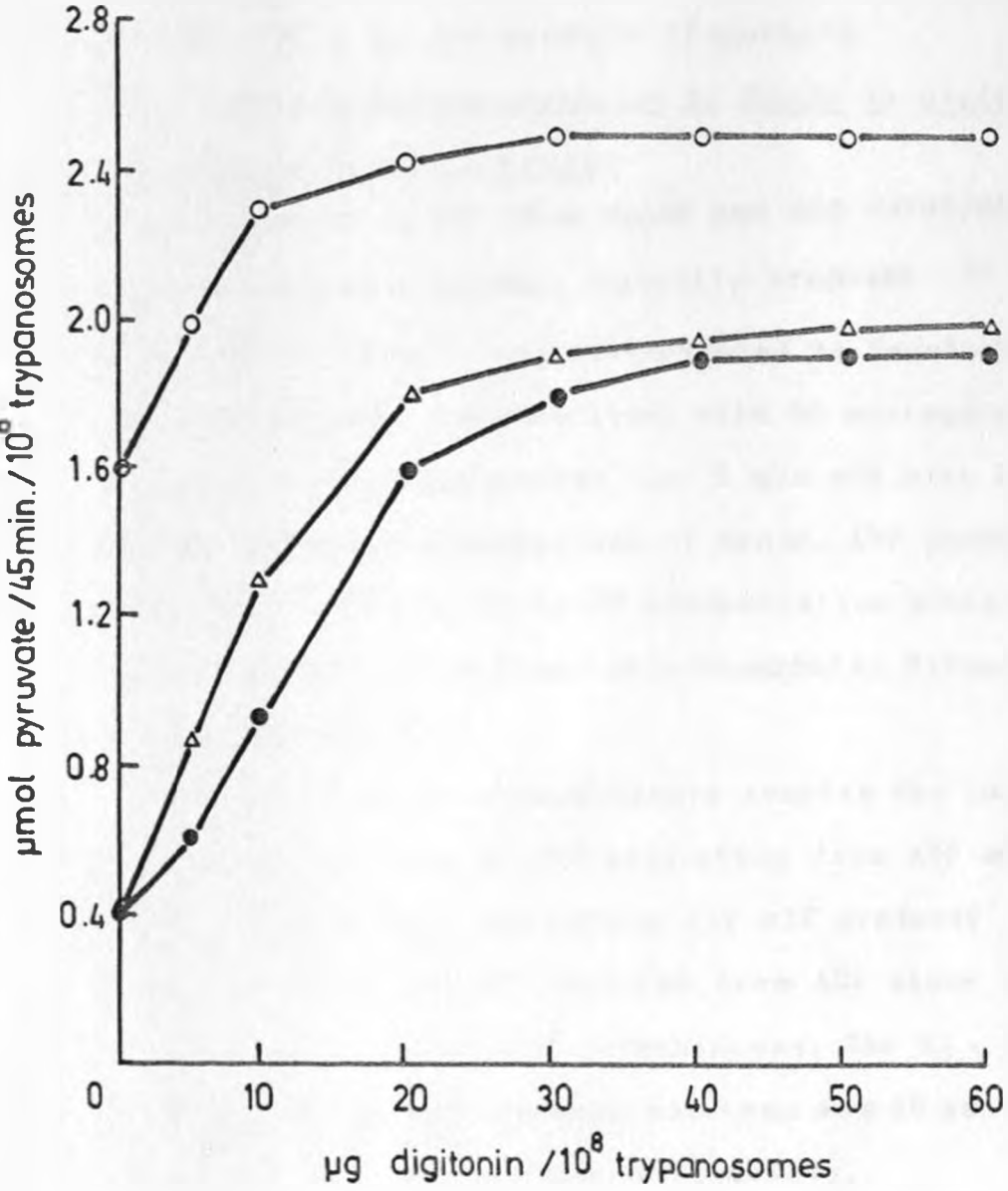

2.5
2.0
1.5
1.0
0.5
µmol / h / 10⁸ trypanosomes
0
10
20
30
40
50
60
µg digitonin / 10⁸ trypanosomes

Figure 15: Increasing the conversion of Gri3P, Gri2P, and PEPyr to pyruvate by permeabilization with digitonin. In each incubation was 2 mM SHAM, 1.5 mM ADP and either 10 mM Gri3P, Gri2P or PEPyr. O-O, Δ-Δ and ●-● indicates pyruvate production from PEPyr, Gri3P, and Gri2P respectively.


2.8
2.4
2.0
1.6
1.2
0.8
0.4
µmol pyruvate /45min./10⁸ trypanosomes
0
10
20
30
40
50
60
µg digitonin /10⁸ trypanosomes

lead to reduction of NAD^+ led to production of pyruvate only as end-products (Figure15).

4.1.4 ADP transphosphorylation by Gro3p in digitonin permeabilized T.b.brucei

The production of ATP from Gro3P and ADP catalysed by the reverse glycerol kinase, initially proposed by Opperdoes and Borst(1977) was investigated by incubating the trypanosomes permeabilized with 60 micrograms digitonin /10^8 trypanosomes for 5 min and with 5 mM ADP at various concentrations of Gro3P. ATP production was a function of Gro3P concentration greater than 10 mM but did not follow a hyperbolic Michaelis-Menten pattern.

Figure 16 shows representative results for the saturation kinetics of ATP production from ADP and Gro3P after making corrections for ATP produced from ADP alone. The ATP produced from ADP alone was 20-28 nanomoles /min/10^8 trypanosomes. The $S_{0.5}$ for Gro3P was 30 mM and the apparent Vmax was 36 nanomoles/min/10^8 trypanosomes respectively.

Figure 17 shows plots of ATP production at various concentrations of ADP ranging from zero to 10 mM in the presence of 100 mM Gro3P . ATP production in the presence of this concentration of Gro3p increased with increase in ADP concentration. The increase did not follow saturation kinetics similar

Figure 16: ATP production at various Gro3P concentrations in digitonin-permeabilized **T.b.** brucei. The trypanosomes were permeabilized with 60 micrograms digitonin/10^8 trypanosomes, 2 mM SHAM and 5 mM ADP for 5 min. Apparent Vmax was 36 nanomoles/min/10^8 trypanosomes. $S_{0.5}$ was 30 mM Gro3P.The Vmax and $S_{0.5}$ from 5 separate experiments was 40 $\pm$10 nanomoles/min/10^8 trypanosomes and 33.2 $\pm$5.8 mM respectively.

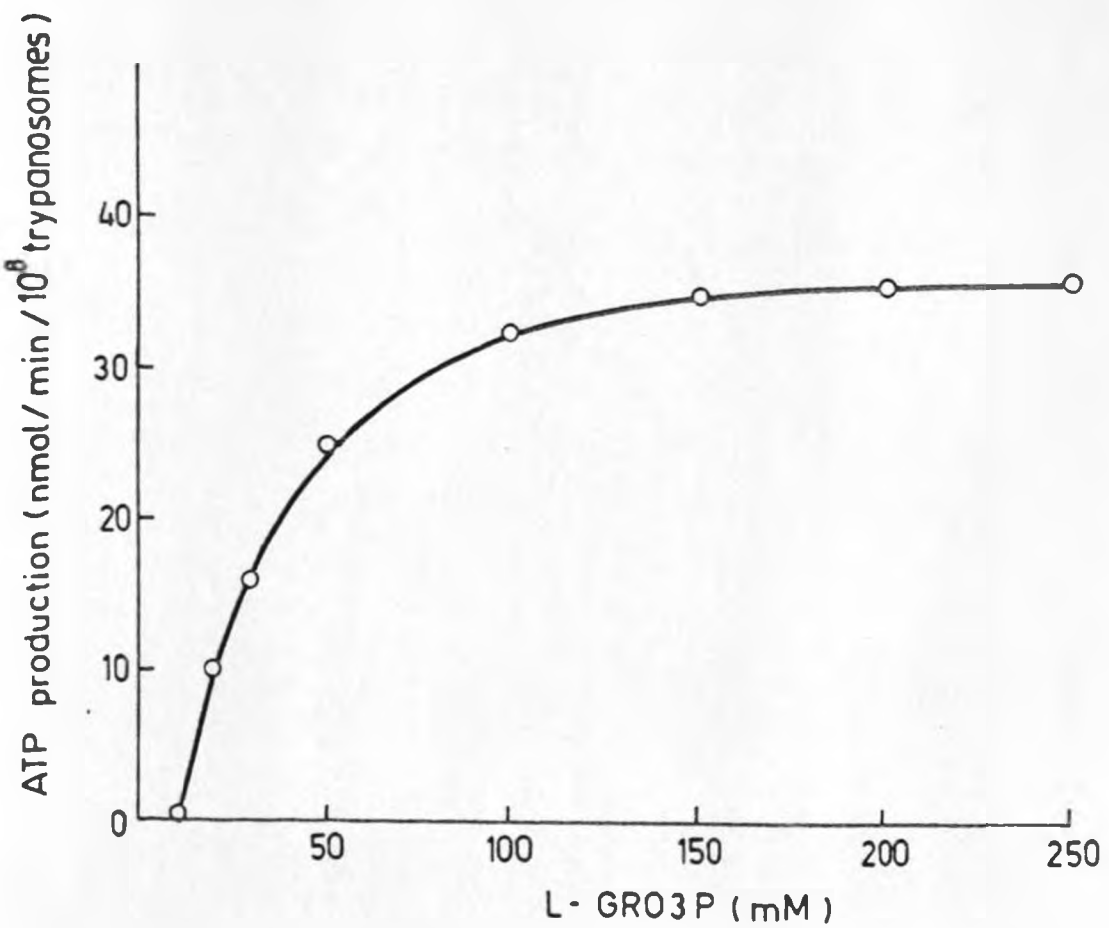
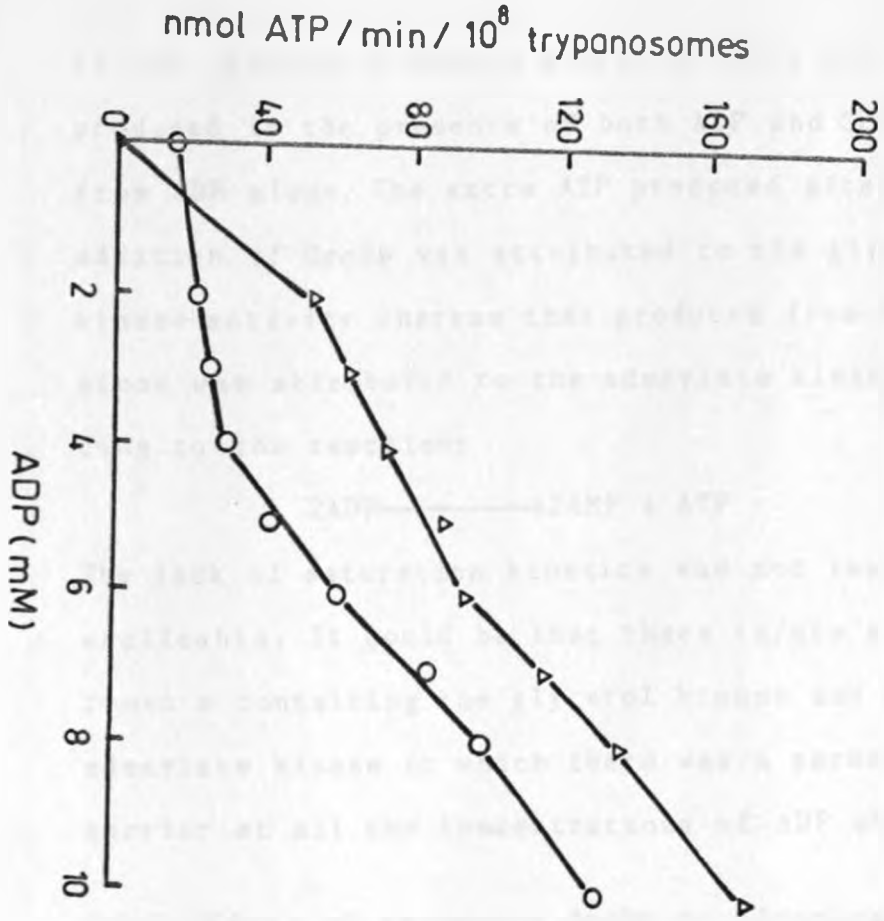

ATP production (nmol / min / 10^8 trypanosomes)
0
10
20
30
40
50
100
150
200
250
L - GRO3P (mM)

Figure 17: Increasing the rate of ATP production by various concentrations of ADP in digitonin permeabilized T.b.brucei incubated with Gro3P . The parasites were permeabilized with 60 micrograms digitonin/10^8 trypanosomes and incubated with 100 mM L-Gro3P and the concentrations of ADP shown for 5 min. Δ Δ and O-O indicates ATP production with and without Gro3P respectively.


nmol ATP / min / 10^8 tryponosomes
0
40
80
120
160
200
2
4
6
8
10
ADP (mM)

to the Michaelis-Menten kinetics. More ATP was produced in the presence of both ADP and Gro3P than from ADP alone. The extra ATP produced after the addition of Gro3P was attributed to the glycerol kinase activity whereas that produced from ADP alone was attributed to the adenylate kinase according to the reaction;

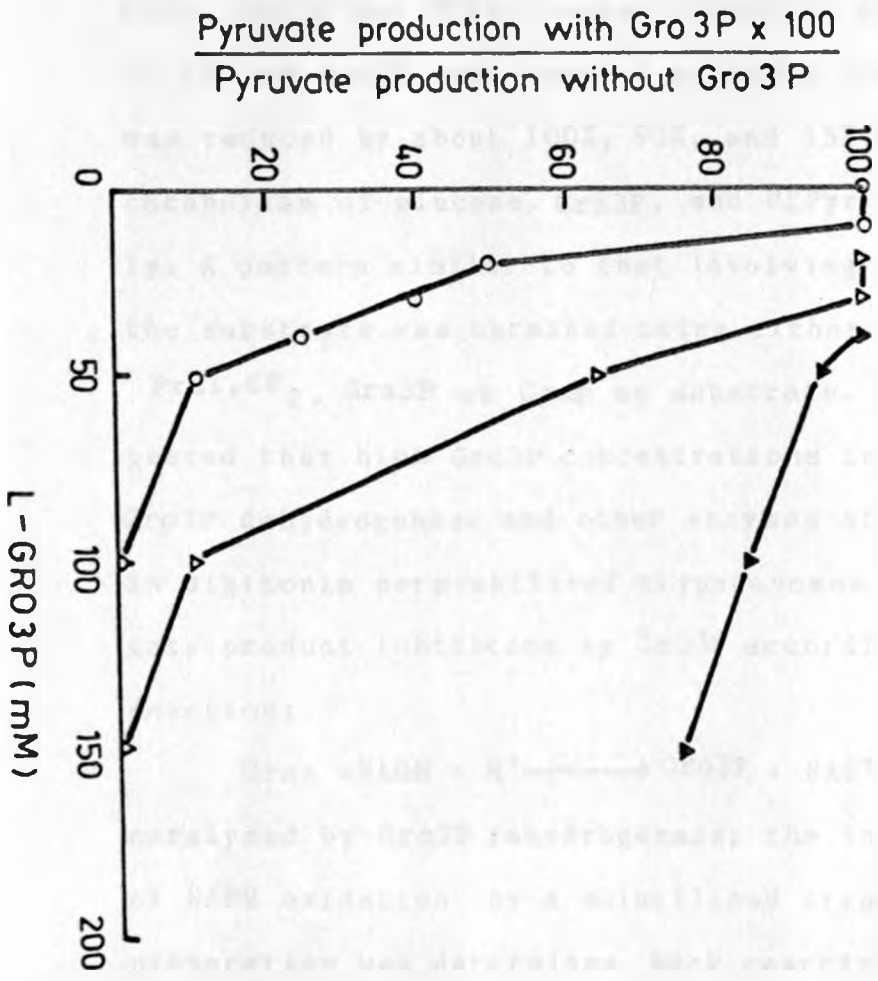
$$2ADP \longrightarrow 2AMP + ATP$$

The lack of saturation kinetics was not readily explicable. It could be that there is/are microenviroments containing the glycerol kinase and the adenylate kinase in which there was a permeability barrier at all the concentrations of ADP shown.

4.1.5 Effect of exogenous Gro3P on glycolysis in digitonin-permeabilized trypanosomes

Large concentrations of Gro3P in the glycosome are likely to inhibit glycolysis by inhibiting the reoxidation of NADH. The range of Gro3P concentration at which glycolysis is not inhibited was determined by assaying the rate of pyruvate production at various concentrations of exogenous Gro3P in digitonin-permeabilized T.b.brucei incubated with 5 mM ADP and 2 mM SHAM and with substrates that generate NADH such as Glc6P, Fru6P , $Fru1,6P_2$, Gra3P and GrnP. Compounds that do not generate NADH such

Figure 18: Inhibition of pyruvate production from glucose, Gri3P and PEPyr by Gro3P in digitonin permeabilised T.b.brucei. All the incubations contained 2 mM SHAM, 2.5 mM ADP and 60 micrograms digitonin/10^8 trypanosomes. O-O , Δ-Δ and ▲-▲ shows pyruvate production from 10 mM glucose, Gri3P and PEPyr respectively. Hundred per cent pyruvate production was 0.58,3.0, and 4.0 micromoles /h/10^8 trypanosomes during the catabolism of glucose, Gri3P and PEPyr respectively.


Pyruvate production with Gro 3 P x 100
Pyruvate production without Gro 3 P
0
20
40
60
80
100
L-GRO3P (mM)
0
50
100
150
200

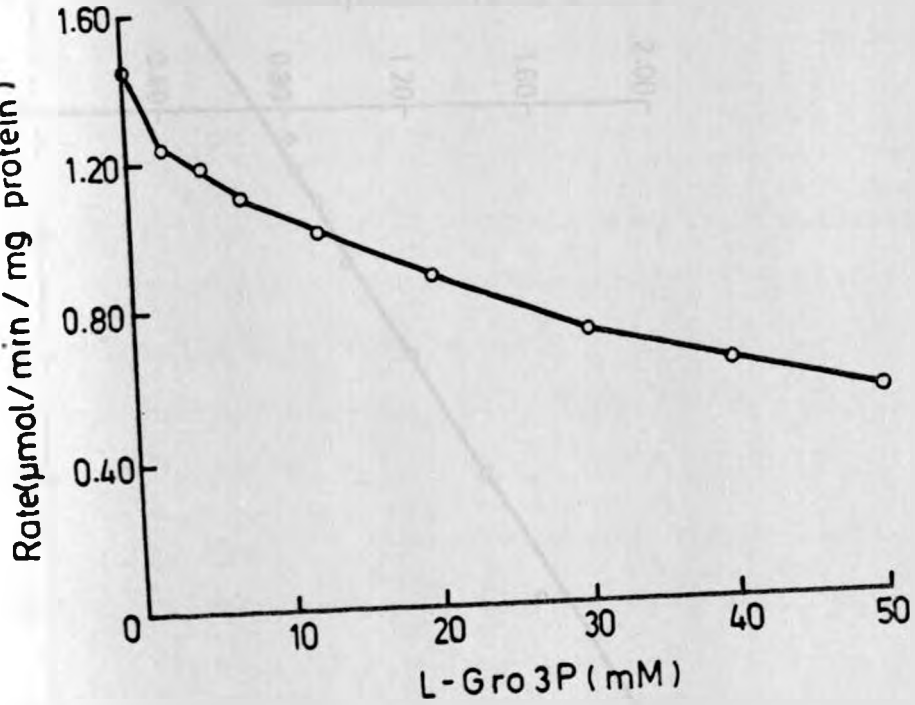
as Gri3P and PEPyr were included in parallel experiments. Figure 18 shows representative results for the inhibition of pyruvate production from glucose,Gri3P and PEPyr by Gro3P. Concentrations of Gro3P of up to 10 mM, 30 mM, and 40 mM respectively did not affect pyruvate production from glucose, Gri3P and PEPyr respectively. In the presence of 100 mM Gro3P, the rate of pyruvate production was reduced by about 100%, 90%, and 15% during he catabolism of glucose, Gri3P, and PEPyr respectively. A pattern similar to that involving glucose as the substrate was obtained using either Fru6P, Fru6P Fru1,$6P_2$, Gra3P or Grnp as substrate. It was suggested that high Gro3P concentrations inhibits Gro3P dehydrogenase and other enzymes of glycolysis in digitonin permeabilized trypanosomes. To investigate product inhibition by Gro3P according to the reaction;

$$\text{Grnp} + \text{NADH} + H^+ \longrightarrow \text{Gro3P} + NAD^+$$

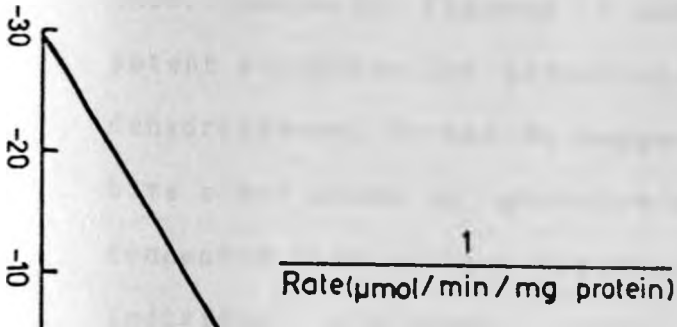
catalysed by Gro3P dehydrogenase; the initial rate of NADH oxidation by a solubilised trypanosome preparation was determined. Back reaction was considered unlikely because no exogenous NAD^+ was added. The endogenous NAD^+ concentration was not sufficient to cause back reaction. Figure 19a shows that various concentrations of Gro3P inhibited the

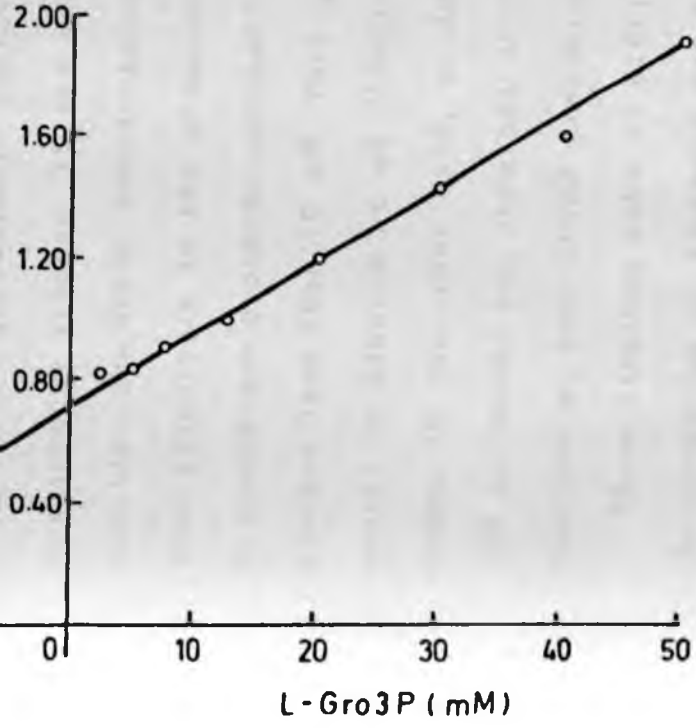
Figure 19: Inhibition by Gro3P of the initial rate of NADH oxidation by GrnP catalysed by T.b. brucei Gro3P dehydrogenase. The trypanosomes were lysed with 0.1% (w/v) Triton X-100. The assay system is described under section 2.6.6. Plots of the rate of NADH oxidation against L-Gro3P concentration are shown in (a) whereas Dixon plots of the same data are shown in (b).

(a)


1.60
1.20
0.80
0.40
0
10
20
30
40
50
Rate(μmol/min/mg protein)
L-Gro3P(mM)

(b)




2.00
1.60
1.20
0.80
0.40
0
10
20
30
40
50
L-Gro3P (mM)

reduction of GrnP by NADH. Figure 19b shows Dixon plots of the same data to give an apparent Ki of 29.5 mM. It is apparent from Figure 19a that various concentrations of Gro3P inhibit the reduction of NADH. Comparing Figures 18 and 19, Gro3P was a more potent inhibitor for glycolysis than of Gro3P dehydrogenase. It may be suggested that Gro3P inhibits other sites of glycolysis. Similar Gro3P concentrations without digitonin had no effect on individual glycosomal enzymes of glycolysis in trypanosome lysates. It was concluded that Gro3P inhibits the Gro3P dehydrogenase among others and that glycolysis in the presence of SHAM in intact trypanosomes proceeds optimally at Gro3P concentration less than 10 mM. This agrees with the report by Hammond et al (1985) with isolated glycosomes. On the other hand, no detectable amount of ATP is formed via reversal of the glycerol kinase reaction at such Gro3P concentration (Figure 16).

These findings make it difficult to explain how the formation of glycerol and ATP occurs during anaerobiosis in intact trypanosomes .

4.2 Discussion

4.2.1 Localization of glycolytic enzymes in T.b.brucei

The glycolytic enzymes in **T.b.brucei** are distributed in the glycosome and cytosol (Opperdoes and Borst,1977). This is supported by results from latency experiments, differential centrifugation and pulse labelling experiments (Visser and Opperdoes, 1980.,Oduro et al, 1980a,b.,Visser et al,1981). An attempt to investigate the extent of permeabilization of the **T.b.brucei** plasma and glycosomal membranes assessed by how much the exogenous non-permeant glycolytic intermediates gain access to the glycolytic enzymes has been made. This was quantified in terms of the formation of their end-products.The utilization of the triosephosphates required 20-30 micrograms digitonin/10^8 trypanosomes whereas that of the larger hexosephosphates required 40-60 micrograms digitonin/10^8 trypanosomes. Table VI shows that the differences in the concentration of digitonin required for the highest rate of pyruvate production might be due to the molecular size of the intermediates. They may also point to two compartments in the parasite; one made accessible to the triosephosphates by 20-30 micrograms digitonin/10^8 trypanosomes and the other made accessible to hexosephosphates by 40-60 micrograms digitonin/10^8 trypanosomes. They can also be interpreted to be in support of the localization of some

glycolytic enzymes in the glycosome and others in the cytosol(Visser and Opperdoes,1980).

4.2.2 Effect of exogenous ATP and ADP on anaerobic glycolysis

The glycosome is a single membrane bounded organelle which contains enzymes that catalyse the conversion of glucose to Gri3P and Gro3P in **T.b.brucei**(Opperdoes and Borst,1977.,Oduro **et al**, 1980a,b). A permeability barrier for the glycolytic intermediates, ATP, ADP, AMP, NAD and inorganic phosphate has been proposed (Opperdoes and Borst,1977) but has been demonstrated for only the hexosephosphates using T.b.brucei isolated glycosomes(Opperdoes and Nwagwu,1980.,Hammond **et al**, 1985). The transport of exogenous ATP,ADP,AMP and cyclic AMP in intact **T.brucei** has been demonstrated (Sanchez **et al**,1976). The maximum rates of uptake were 21.88;23.81;149.82,and 29 micromoles/h /10^8 trypanosomes respectively. No such studies at the glycosomal membrane have been possible because of the difficulty of isolating pure, absolutely intact glycosomes. In this investigation a supposedly glycosomal reaction, catalysed by the glycerol kinase. could be manipulated by exogenous ATP and ADP in intact trypanosomes incubated with SHAM.This

observation was unexpected because the glycosome was thought to be impermeable to ATP and ADP (Opperdoes and Borst,1977). The glycosomal and plasma membranes could not have been permeabilized because Glc6P or Fru6P which are partly catabolised in this organelle (Visser et al,1981) could not produce pyruvate without the addition of digitonin. This suggests that in the presence of ATP, there is still a permeability barrier(s) for exogenous glycolytic intermediates such as Glc6P and Fru6P. A carrier for ATP and ADP at the glycosomal membrane may explain these results since increasing exogenous ADP concentrations in incubations at a fixed ATP concentration stimulated glycerol production, and inhibited that of GrO3P It is proposed that the cellular levels of glycerol and Gro3P are controlled by the glycosomal molar ratio of ATP/ADP.Under anaerobic conditions this ratio is low resulting in more glycerol formation. The increase of Gro3P due to a high ATP/ADP molar ratio in the presence of SHAM, suggests either phosphorylation of the preformed glycerol or inhibition of Gro3P dephosphorylation.

4.2.3.Production of glycerol and net ATP in T.b.brucei during anaerobic catabolism of various intermediates

The lack of glycerol production from glycolytic

intermediates by digitonin permeabilized trypanosomes in the presence of SHAM is not readily explicable. From the hypothesis of Opperdoes and Borst (1977), it can be argued that since digitonin permeabilizes the glycosomal membrane, it would be impossible to raise the concentration of Gro3P and ADP high enough in the glycosome to reverse the glycerol kinase reaction. In line with this arguement it was anticipated that optimum rates of both pyruvate and ATP production would be found in digitonin permeabilized trypanosomes in the presence of saturating ADP and Gro3P concentrations.The reverse activity of glycerol kinase in digitonin permeabilized trypanosomes at 5 mM exogenous ADP gave an $S_{0.5}$ value for Gro3P of 33.2$\pm$ 5.8 mM and an apparent Vmax of 40$\pm$10 nanomoles /min/10^8 trypanosomes (Figure 16). The apparent Vmax indicates that the rate of ATP and glycerol formation via a reversal of the glycerol kinase in these permeabilized trypanosomes is comparable to that in intact trypanosomes of 37 nanomoles /min/10^8 (Figure 14). This may be interpreted to suggest that glycerol kinase catalyses glycerol production during anaerobiosis in vivo.However, concentrations of Gro3P greater than 10 mM inhibited pyruvate production at the Gro3P dehydrogenase reaction.When

the glycolytic rate was maximum (Figure 18) the reverse glycerol kinase activity was undetectable (Figure 16). This suggests that Gro3P should inhibit its own formation before its concentration is enough to force the formation of glycerol and ATP. Apparently the trypanosomes cannot attain optimum rates of glycolysis and both ATP and glycerol formation via the glycerol kinase reaction. These results therefore suggest that the formation of ATP from ADP and Gro3P as shown in Figure 16 may be a mere laboratory phenomenon and is not a reflection of **in vivo** situation. Hammond et al,(1985) reported that whereas glycerol kinase activity in isolated glycosomes is 82% latent the apparent reverse glycerol kinase activity is not latent at all.This suggests the possibility of the presence of another enzyme that **resembles** the reverse glycerol kinase activity. If it exists, it should require a lower concentration of Gro3P ranging from 5 to 10 mM for maximum activity suggested by Hammond et al,(1985) and also shown in Figure 16, whereas the reverse glycerol kinase **in situ** would require at least 27.4 to 33.8 mM for half maximum activity. The presence of another enzyme that plays a role related to that of the glycerol kinase is also suggested by the observation that permeabilization of trypano-

somes incubated with SHAM and glycolytic intermediates results in the formation of equimolar Gro3P and pyruvate only whereas glycerol and Gro3P are formed from glucose under similar conditions (Figures 9,10,11,12,and 13).If the reverse glycerol kinase catalyses glycerol formation in vivo then glycerol should have been detected from the glycolytic intermediates just as from glucose.

4.3 Conclusions

The following conclusions may be drawn from this study.

(a) High concentrations of Gro3P inhibit the Gro3P dehydrogenase and glycolysis in the presence of SHAM in intact trypanosomes presumably proceeds optimally at Gro3P concentrations less than 10 mM.

(b) Glucose can be metabolised to glycerol in digitonin-permeabilized **T.b.brucei** incubated with SHAM whereas glycolytic intermediates cannot under similar conditions.

(c) The trypanosomes may not attain sufficient concentrations of Gro3P to drive the production of ATP and glycerol via glycerol kinase according to the equation;

$$ADP + Gro3P \longrightarrow ATP + glycerol.$$

(d) The enzyme catalysing glycerol production under anaerobic conditions requires intact membrane(s).

(e) Exogenous ATP and ADP have access to the glycosomal glycolytic enzymes whereas glycolytic intermediates donot.

CHAPTER V

ANAEROBIC CATABOLISM OF HEXOSE SUGARS AND GLYCERONE BY T.B.BRUCEI : PATHWAYS OF GLYCEROL PRODUCTION

5.1 Results

From the results of Chapter 1V it was suggested that if the glycerol kinase catalyses glycerol formation under cellular conditions according to the equation:

Gro3P + ADP $\longrightarrow$ glycerol + ATP

as shown in Scheme IVA then the initial rate of pyruvate production from glucose, fructose and mannose should be approximately half of that observed aerobically after simulation of anaerobiosis by the addition of SHAM. Such a decrease has been shown by other workers during the catabolism of glucose (Brohn and Clarkson, 1978) but not with fructose or mannose. Experiments were therefore carried out to determine the catabolism of mannose, fructose and glycerone in the absence and presence of SHAM.

5.1.1 Aerobic catabolism of glucose, mannose,fructose and glycerone

Pyruvate production from mannose, fructose and glycerone was determined after various time intervals (Figure 20a,b). A parallel control experiment with glucose as substrate was included. Pyruvate production from the two hexoses was linear for approximately 90 minutes just as from glucose. The production of pyruvate from

Figure 20a: A time course of pyruvate production by **T.b.brucei** incubated with fructose glucose and mannose. O-O , ▲-▲ , and Δ-Δ indicates pyruvate production from fructose, glucose, and mannose respectively. The concentration of each substrate was 10 mM.

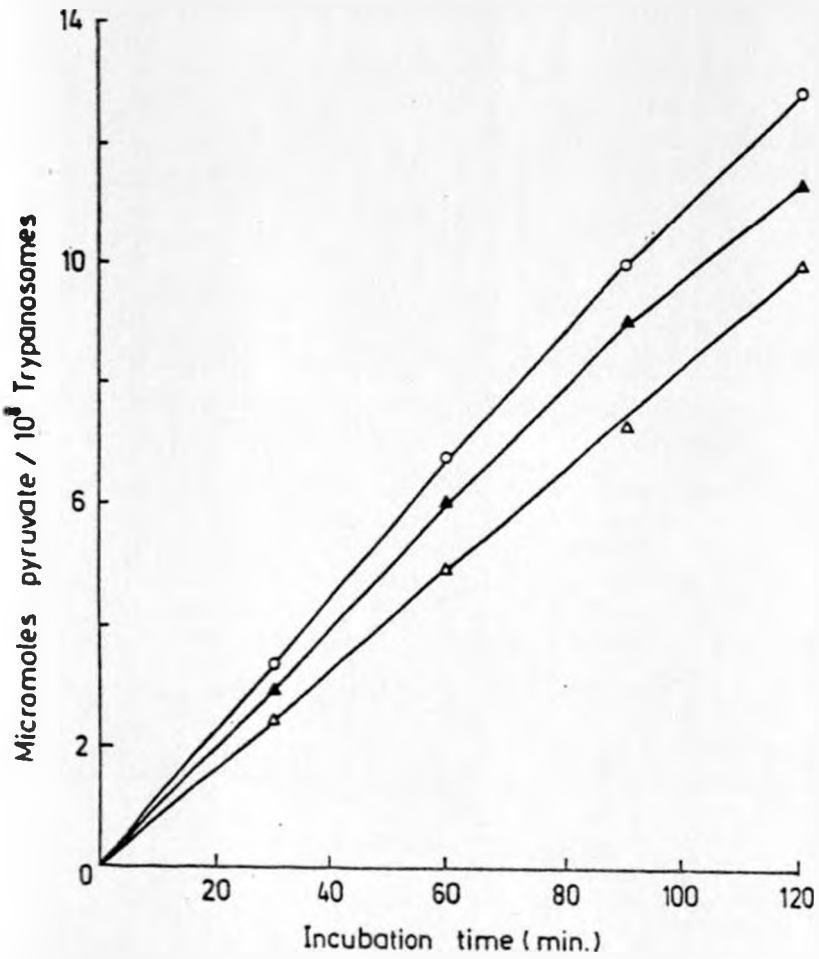
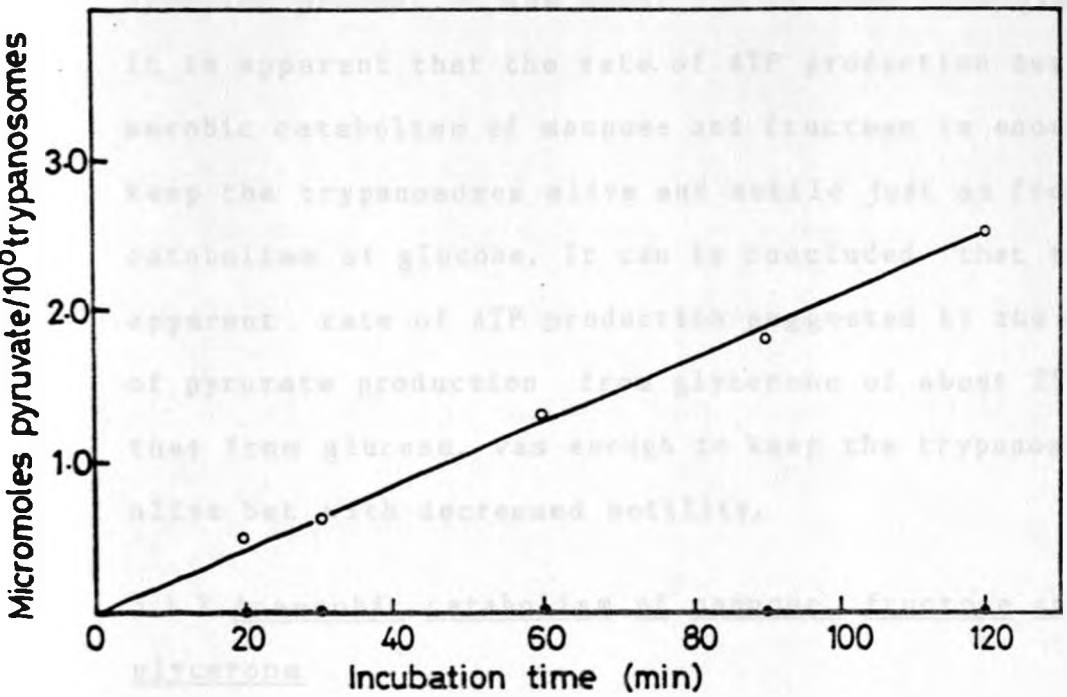

14
10
6
2
0
20
40
60
80
100
120
Micromoles pyruvate / 10^8 Trypanosomes
Incubation time (min.)

Figure 20b: A time course of aerobic and anaerobic pyruvate production from glycerone catabolism in T.b.brucei. O-O and ●-● indicates aerobic and anaerobic pyruvate production respectively. Glycerone concentration was 10 mM.

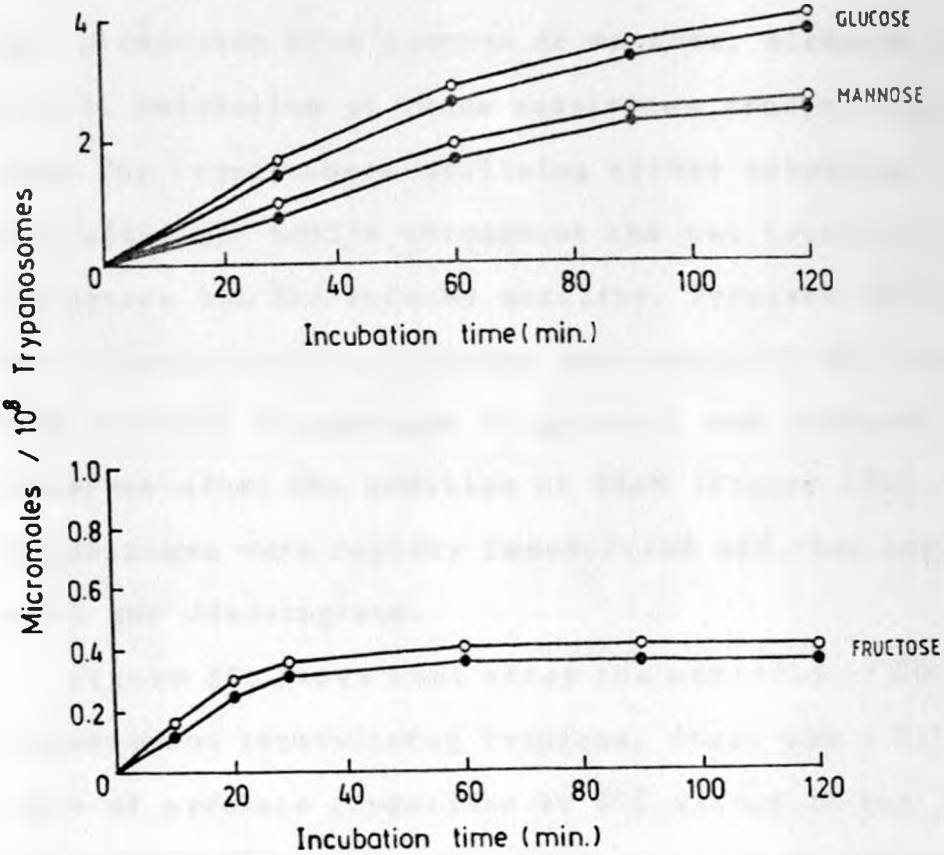

Micromoles pyruvate/10^8 trypanosomes
3·0
2·0
1·0
0
20
40
60
80
100
120
Incubation time (min)

fructose and mannose was about 110% and 90% respectively compared to that from glucose. The trypanosomes in each case remained alive and motile throughout the two hours of incubation. The trypanosomes incubated with glycerone also survived throughout the two hours of incubation (Figure 20b) but appeared rather sluggish. Pyruvate production was about 25% of that from glucose. It is apparent that the rate of ATP production during aerobic catabolism of mannose and fructose is enough to keep the trypanosomes alive and motile just as from the catabolism of glucose. It can be concluded that the apparent rate of ATP production suggested by the rate of pyruvate production from glycerone of about 25% of that from glucose, was enough to keep the trypanosomes alive but with decreased motility.

5.1.2 Anaerobic catabolism of mannose, fructose and glycerone

Pyruvate and glycerol were determined after various time intervals in parallel experiments to those reported in Figure 20 but in the presence of SHAM. Pyruvate and glycerol produced from the catabolism of mannose and fructose were equimolar, just as from glucose. Figure 21a shows that the pattern of pyruvate production from mannose was similar to that from glucose. Comparing the results shown in Figures 20 and 21a, addition of SHAM caused initially about 50-60% decrease of pyru-

Figure 21: A time course of pyruvate and glycerol production by **T.b.brucei** incubated with glucose, fructose and mannose. ○○ and ●● indicates pyruvate and glycerol production respectively from an incubation containing 10 mM substrate and 2 mM SHAM.

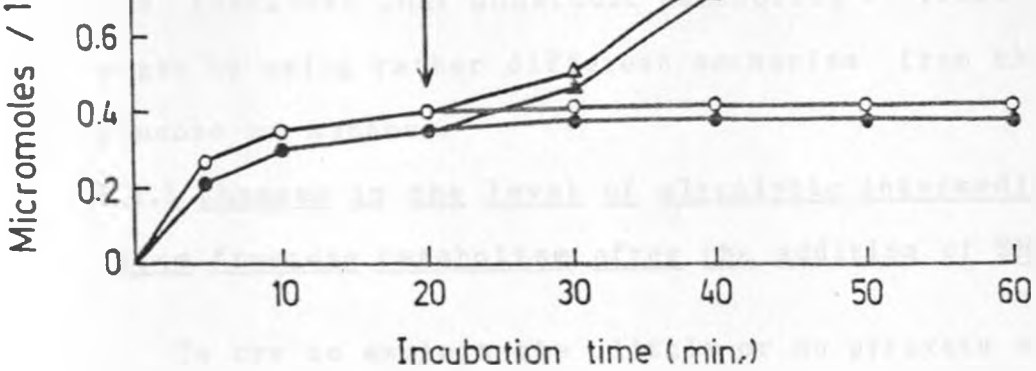

GLUCOSE
MANNOSE
FRUCTOSE
Micromoles / 10^8 Trypanosomes
Incubation time (min.)
Incubation time (min.)
0
2
4
0.2
0.4
0.6
0.8
1.0
20
40
60
80
100
120

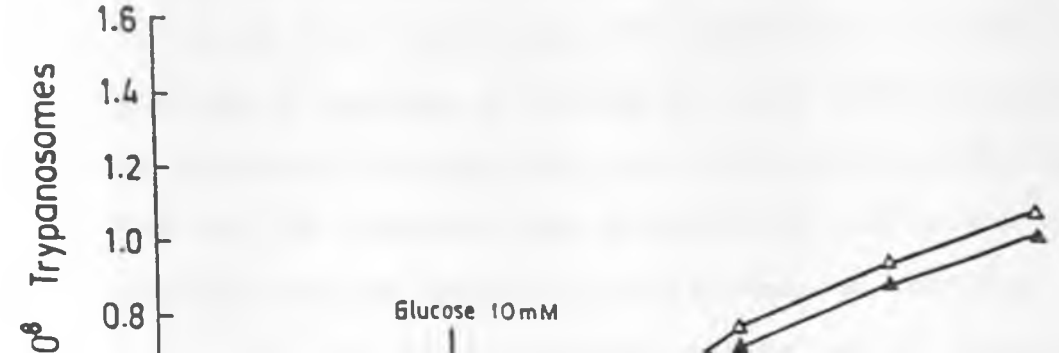
vate production from glucose or mannose, although the overall catabolism of these substrates remained the same. The trypanosomes utilising either substrate were alive and motile throughout the two hours of incubation but had reduced motility. Pyruvate and glycerol production from mannose was initially 60% that from glucose. No pyruvate or glycerol was produced from glycerone after the addition of SHAM (Figure 20b). The trypanosomes were rapidly immobolised and then began to swell and disintegrate.

Figure 21b shows that after the addition of SHAM to trypanosomes catabolising fructose, there was a decrease of pyruvate production by 85% within 20 min and 100% within 60 min. Morphological examination revealed that the trypanosomes were sluggish 10 minutes after the addition of SHAM and motionless after 30 minutes. Routine checks under phase contrast microscopy revealed that addition of glucose within 10-30 minutes of incubation with SHAM revived them to full activity after 3-5 minutes. Figures 21 and 22 show that most of the pyruvate and glycerol from fructose alone was produced within 10 minutes. Addition of glucose after 20 minutes of incubation with SHAM and fructose caused a rapid resumption of production of equimolar amounts of pyruvate and glycerol. Pyruvate production rapidly increased from a negligible rate to 1.16

Figure 22: A time course of pyruvate and glycerol production by **T.b.brucei** pre-incubated with SHAM and fructose before the addition of glucose. The trypanosomes were incubated for 20 min with 10 mM fructose and 2 mM SHAM after which 10 mM glucose was added.

O-O and ●-● indicate pyruvate and glycerol production from fructose and Δ-Δ and ▲-▲ pyruvate and glycerol from a combination of fructose and glucose.


Micromoles / 1
0.6
0.4
0.2
0
10
20
30
40
50
60
Incubation time (min.)


1.6
1.4
1.2
1.0
0.8
10^8 Trypanosomes
Glucose 10mM

micromoles/h/10^8 trypanosomes.

It was concluded that although most of the trypanosomes incubated with SHAM and fructose were immobilized within 10 min, they were alive for about 30 minutes. It is likely that little net ATP production occurred within the 30 minutes of incubation with SHAM and fructose as evidenced by the slow rate of pyruvate production. The net ATP produced was probably not sufficient to sustain optimum metabolic activities and motility. It was concluded that anaerobic catabolism of fructose might be using rather different mechanism from that of glucose or mannose.

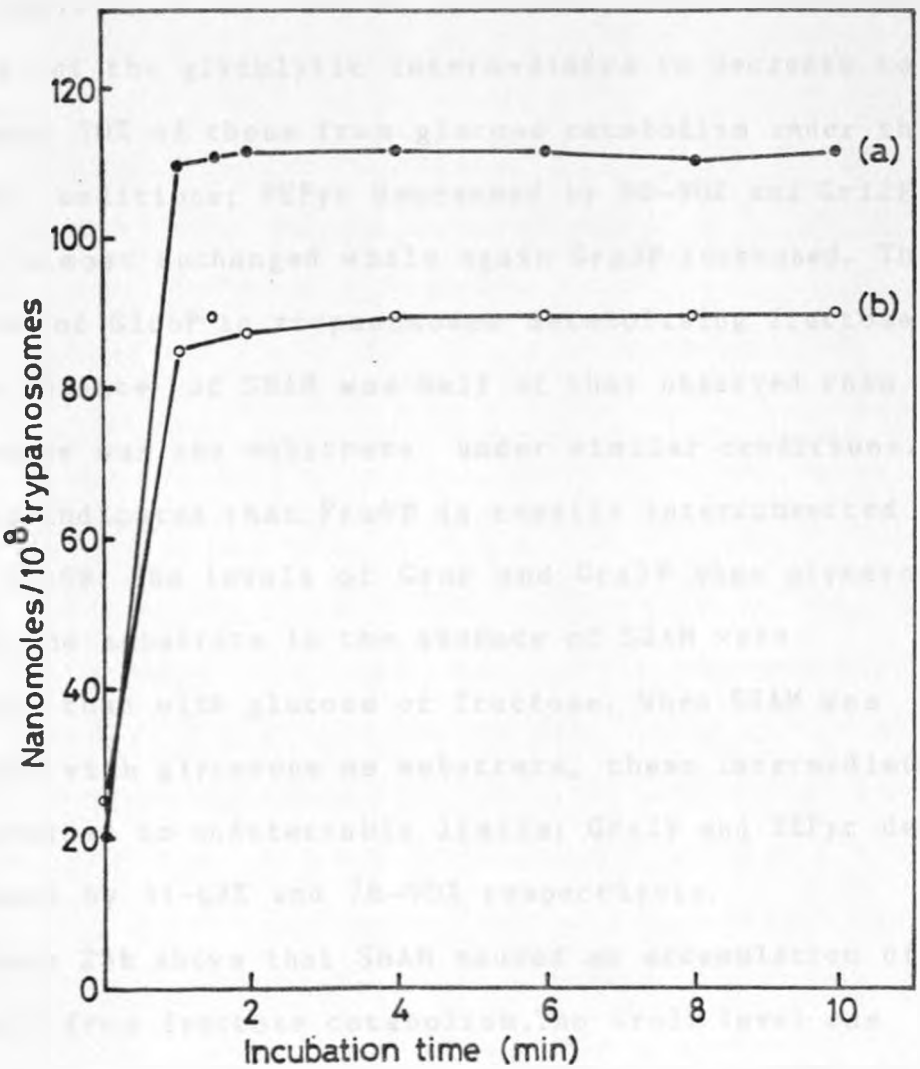
5.1.3 Changes in the level of glycolytic intermediates from fructose catabolism after the addition of SHAM.

To try to explain the little or no pyruvate and glycerol production when trypanosomes were incubated with fructose or glycerone in the presence of SHAM, a time course of the level of glycolytic intermediates was carried out after the addition of SHAM to incubations containing glucose (control), fructose or glycerone as substrate. With all the three substrates,there was a rapid decrease in levels of the glycolytic intermediates to a minimum within 2 min except for Gro3P (Table VII). With glucose as substrate, the concentration of glycolytic intermediates; Glc6P , Fru6P,

Table VII: Levels of some glycolytic intermediates in the presence and absence of SHAM during the utilization of glucose, frucose and glycerone. The trypanosome were incubated with 10 mM substrate and SHAM for 5 min. Results are expressed as means± standard deviation from 4 different experiments . The units are nanomoles/10^8 trypanosomes . ND indicates 'not detected'.

Metabolite	Glucose -SHAM	Glucose +SHAM	Fructose -SHAM	Fructose +SHAM	Glycerone -SHAM	Glycerone +SHAM
Glc6P	14.0±2.0	1.8±0.3	7.0±1.1	1.0±0.3	ND	ND
Fru6P	4.0±0.3	1.0±1.0	5.1±1.0	0.6±0.2	ND	ND
Fru1,6P_2	8.8±1.6	2.3±0.4	6.0±1.5	1.0±0.2	ND	ND
Gri3P	1.5±0.4	0.7±0.2	2.5±0.3	0.4±0.1	0.3±0.2	ND
GrnP	1.3±0.6	0.6±0.1	7.0±0.5	1.0±0.3	0.8±0.2	0.3±0.1
Gri2P	170±8	5.2±0.6	168±20	4.9±0.4	45.5±5.0	19.0±0.6
PEPyr	45±10	24.0±0.6	49±4	3.4±0.5	9±2	1.4±0.3

Figure 23: A time course of the changes in the levels of Gro3P from (a) glucose and (b) fructose catabolism in T.b.brucei after the addition of SHAM. The concentration of either glucose or fructose was 10 mM.


120
100
80
60
40
20
0
2
4
6
8
10
(a)
(b)
Nanomoles/10^8 trypanosomes
Incubation time (min)

Fru1,6P_2, GrnP, Gra3P, and Gri2P decreased by 60-97% ; PEPyr decreased by 30-57%; only Gro3P increased. These results are comparable to those of Hammond and Bowman (1980a). With fructose as substrate, SHAM caused the level of the glycolytic intermediates to decrease to almost 50% of those from glucose catabolism under the same conditions; PEPyr decreased by 80-90% and Gri2P was almost unchanged while again Gro3P increased. The level of Glc6P in trypanosomes metabolising fructose in the absence of SHAM was half of that observed when glucose was the substrate under similar conditions. This indicates that Fru6P is readily interconverted to Glc6P. The levels of GrnP and Gra3P when glycerone was the substrate in the absence of SHAM were lower than with glucose or fructose. When SHAM was added with glycerone as substrate, these intermediates decreased to undetectable limits; Gri2P and PEPyr decreased by 51-63% and 76-90% respectively.

Figure 23b shows that SHAM caused an accumulation of Gro3P from fructose catabolism.The Gro3P level was maximum after 45 seconds of incubation with SHAM. In a separate experiment it was observed that the level of Gro3P did not subsequently change even after 60 minutes incubation with SHAM although all the trypanosomes were dead. It was concluded that PEPyr production from anaerobic fructose catabolism in the steady state was probably

not sufficient to sustain ATP production as it was the case of anaerobic glucose catabolism. However, these observations alone were not sufficient to explain the poor survival of the trypanosomes on fructose or glycerone in the presence of SHAM.

5.1.4 Concentrations of adenine nucleotides after the addition of SHAM during the catabolism of fructose, glucose, mannose and glycerone

Table VIII shows that in the absence of SHAM, trypanosomes incubated with glucose or fructose produced approximately the same amount of ATP. With mannose as substrate, the ATP level decreased slightly than with glucose. The level of ADP or AMP during the catabolism of glucose, fructose or mannose were approximately the same. On the other hand the level of ATP in trypanosomes catabolising glycerone was much lower than the one from trypanosomes utilising the three hexoses. The level of ADP in trypanosomes incubated with glycerone without SHAM was nearly four fold higher than in those incubated with glucose; they also contained about 5 fold more AMP. These results suggest that during aerobiosis glucose, fructose and mannose are catabolised at approximately the same high rates producing a high steady state level of ATP and low ADP and AMP levels. These results indicate that glycerone is catabolised slowly (Figures 20,a,b); despite

Table VIII: Adenine nucleotides levels in T.b.brucei catabolising glucose, fructose mannose and glycerone in the presence and absence of SHAM. The trypanosomes were incubated in buffer A for 10 min with 10 mM of the substrates shown. Results are in nano-moles/10^8 trypanosomes and are expressed as means$\pm$ standard deviations from 4 separate experiments.

Substrate	SHAM	Adenine nucleotide levels		
		ATP	ADP	AMP
Glucose	-	13.7±1.8(4)	3.3±0.1(4)	1.8±0.2(4)
Glucose	+	7.3±0.4(3)	6.9±0.5(4)	8.5±0.4(4)
Fructose	-	14.5±1.0(3)	3.6±0.3(4)	1.8±0.1(4)
Fructose	+	2.8±0.8(4)	8.6±0.2(40)	9.9±0.2(4)
Mannose	-	11.7±1.7(4)	4.0±0.3(4)	2.1±0.1(3)
Mannose	+	5.5±0.9(4)	10.0±0.2(4)	8.8±0.3(4)
Glycerone	-	2.6±0.3(4)	12.1±0.5(4)	9.5±0.6(4)
Glycerone	+	0.8±0.2(4)	14.1±1.3(4)	11.0±0.5(4)

Figure 24a: A time course of the changes in adenine nucleotide levels in **T.b.brucei** during the utilization of gluose after the addition of SHAM. Trypanosomes were incubated in buffer A containing 10 mM glucose. Timing was started after the addition of SHAM. Aliquots were removed at the times shown and processed for the assays.

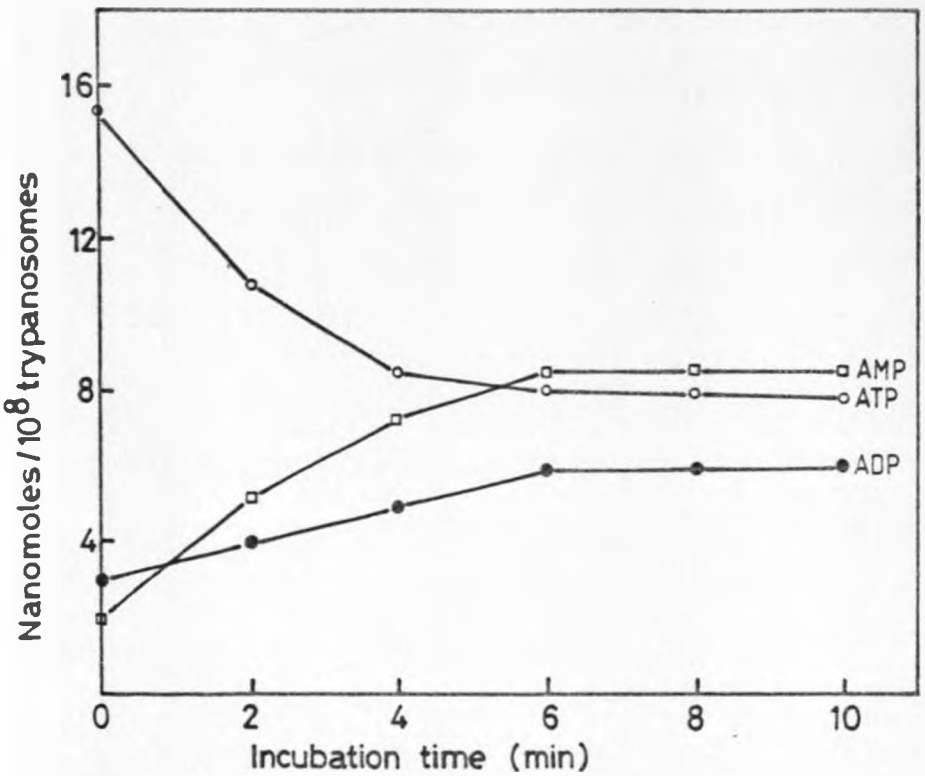
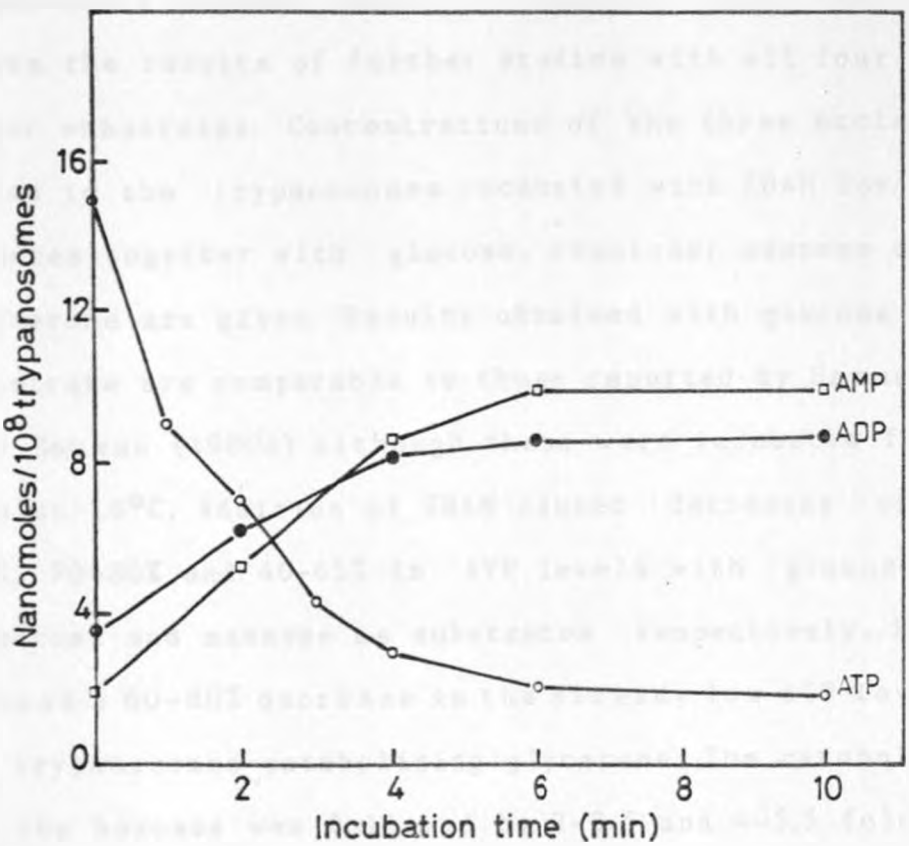

16
12
8
4
0
2
4
6
8
10
Nanomoles / 10^8 trypanosomes
Incubation time (min)
AMP
ATP
ADP

Figure 24b: A time course of the changes in adenine nucleotides levels in **T.b.brucei** during the utlization of fructose after the addition of SHAM. Experimental procedure was as in Figure 24(a).


Nanomoles/10^8 trypanosomes
16
12
8
4
0
2
4
6
8
10
Incubation time (min)
AMP
ADP
ATP

the high levels of of ADP and AMP in the absence of SHAM due to inadequate ADP phosphorylation. Time course studies with glucose and fructose as substrates showed a rapid decrease of ATP and rapid increase in ADP and AMP on addition of SHAM (Figure 24), with a steady state being reached within 4-6 minutes. Table VIII shows the results of further studies with all four sugar substrates. Concentrations of the three nucleotides in the trypanosomes incubated with SHAM for 5 minutes together with glucose, fructose, mannose or glycerone are given. Results obtained with glucose as substrate are comparable to those reported by Hammond and Bowman (1980a) although these were incubated for 7.5 min at 18°C. Addition of SHAM caused decreases of 40-50%, 70-80% and 40-65% in ATP levels with glucose, fructose and mannose as substrates respectively. SHAM caused a 60-80% decrease in the already low ATP level in trypanosomes catabolising glycerone. The catabolism of the hexoses was followed by 2-2.5 and 4-5.5 fold increases in the levels of ADP and AMP respectively. SHAM caused complete inhibition of pyruvate production from glycerone indicating, that glycolysis had completely stopped. Death of the trypanosomes was attributed to the rapid decrease in ATP production. The decreased rate of pyruvate production from fructose after addition of SHAM and the very pronounced fall in ATP level

(Table VIII) suggest that SHAM is a more potent inhibitor of ATP production in the trypanosomes when fructose was the substrate than from either glucose or mannose.

5.1.5 Levels of Gro3p in T.b.brucei catabolising fructose, mannose and glycerone after the addition of SHAM.

Table IX shows that the level of Gro3p in T.b. brucei utilising glucose (control), fructose and mannose ranged from 20-28 nanomoles/10^8 trypanosomes. The level in trypanosomes utilising glycerone was only about 50% of this. Addition of SHAM lead to a 2.5-4 fold increase in Gro3P with all the four sugar substrates. Gro3P level during mannose catabolism in the presence of SHAM was less than from either glucose or fructose catabolism. Although fructose was a poor substrate for ATP and glycerol synthesis during anaerobiosis, the Gro3P level was in the same range as with glucose. The level of Gro3P from glycerone catabolism was approximately half of that from glucose catabolism. The decreased rate of glycolysis during the catabolism of fructose may not be attributed to differences in the level of Gro3P because the Gro3P level was in the same range as from the catabolism of glucose.

Table IX: Levels of Gro3P in T.b.brucei catabolising glucose, fructose, mannose and glycerone in the presence and absence of SHAM. The trypanosomes were incubated in buffer A for 5 min with 10 mM of the given substrates. Results are expressed as means± standard deviations. Figures in brackets indicate the number of separate determinations from different experiments.

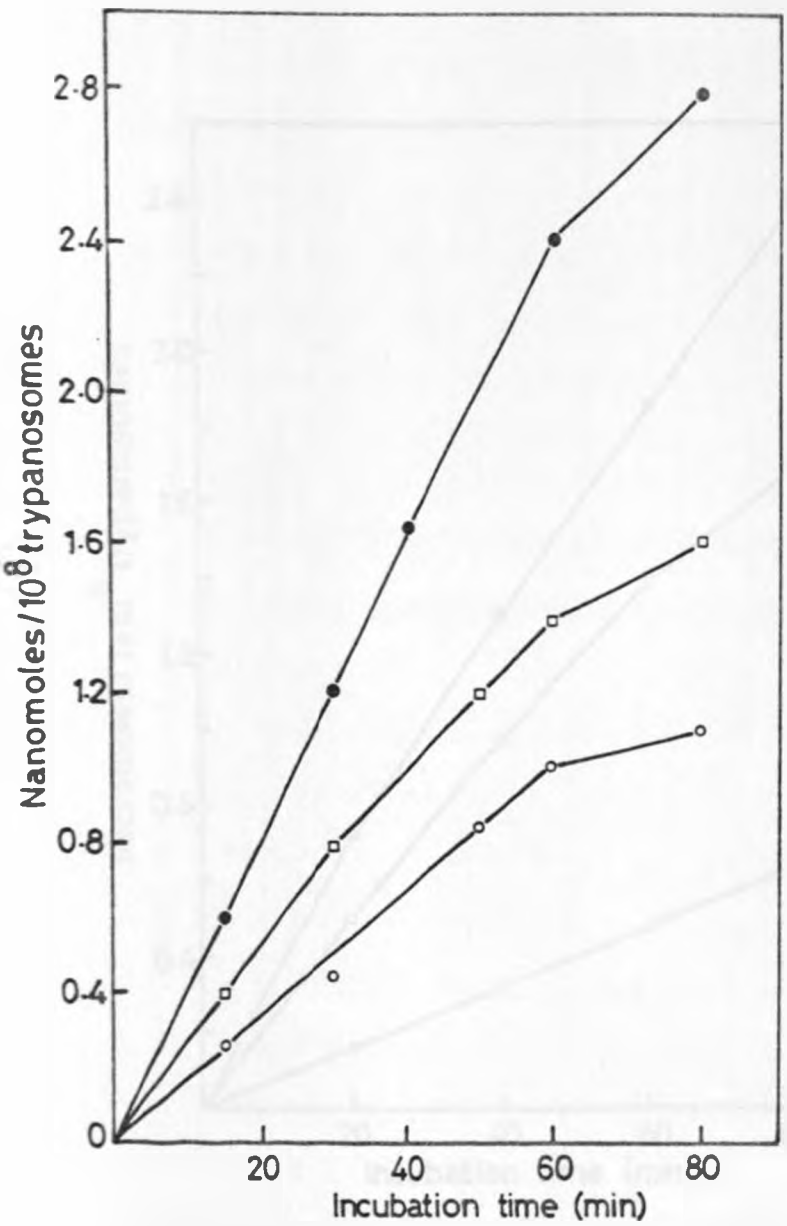
Substrate	SHAM	Gro3P levels
Glucose	-	27.4±8.2(5)
Glucose	+	90.3±15.3(4)
Fructose	-	28.4±10.2(5)
Fructose	+	75.1±16.3(4)
Mannose	-	20.2±5.3(4)
Mannose	+	60.1±8.2(5)
Glycerone	-	12.0±1.1(4)
Glycerone	+	46.3±5.1(4)

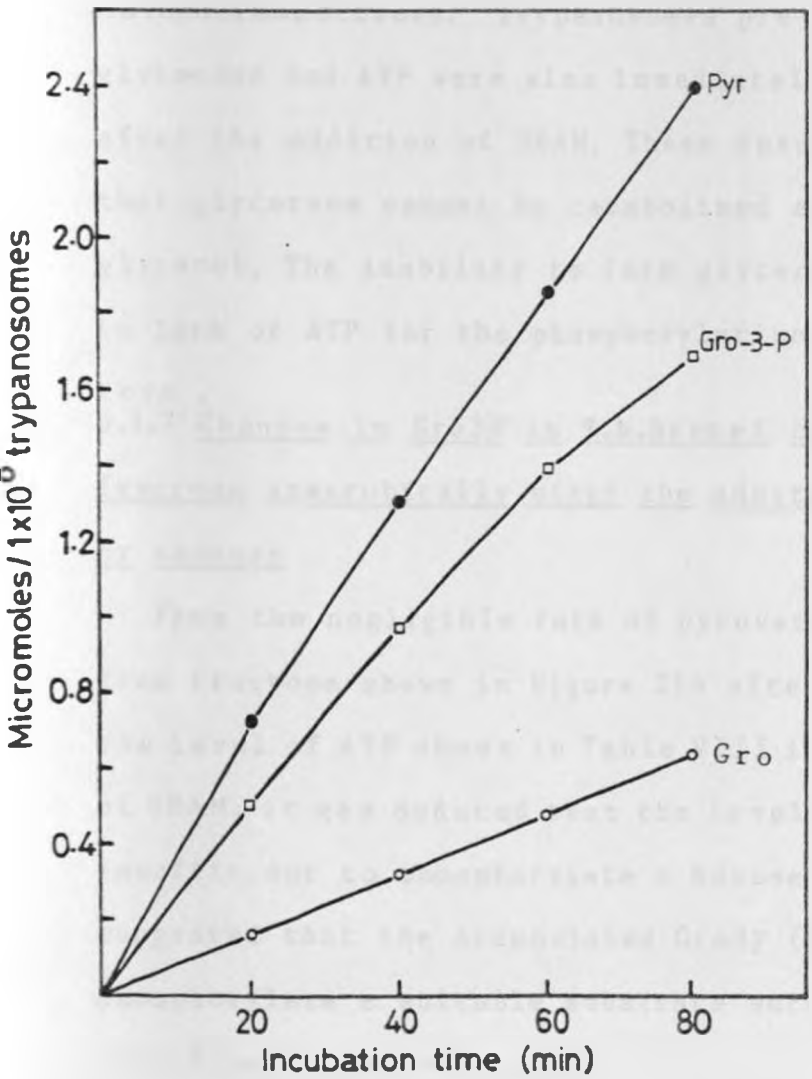
5.1.6 Effect of exogenous ATP on anaerobic fructose and glycerone catabolism.

It has been shown that T.b.brucei can utilise exogenous ATP (Chapter IV). Figure 25a shows a time course of pyruvate, glycerol and Gro3P production from glucose by T.b.brucei incubated with SHAM and 2.5 mM ATP. Equimolar amounts of pyruvate and glycerol plus Gro3P were formed. The initial molar ratio for Gro3P to glycerol was 1:1.5. The trypanosomes remained alive and motile throughout the period the experiments were carried out. Figure 25b shows pyruvate, glycerol and Gro3P production from fructose in the presence of SHAM. The trypanosomes were alive and motile for more than 80 min unlike those incubated without ATP which apparently survived with significant rate of glycolysis for 10 min. The rate of pyruvate production was 2 fold that obtained initially in the absence of ATP (Figure 21b). Glycerol rproduction was linear through out the 80 min incubation period. The rate of glycerol prodution was approximately similar to that observed initially in the absence of ATP. This shows that ATP caused increased Gro3P formation from fructose, and its excretion but apparently not its dephosphorylation.

These results suggest that the anaerobic catabolism of fructose was limited by the lack of availability of ATP for the phosphorylation of fructose

Figure 25: A time course of pyruvate, glycerol, and Gro3P production from (a) glucose or (b) fructose in T.b.brucei incubated with SHAM and ATP. The concentrations of ATP and glucose were 2.5 mM and 10 mM respectively. ●-● , □ □ , and 0-0 indicates pyruvate, Gro3p and glycerol respectively.


2·8
2·4
2·0
1·6
1·2
0·8
0·4
0
20
40
60
80
Incubation time (min)
Nanomoles/10^8 trypanosomes


Micromoles / 1x10^8 trypanosomes
2·4
2·0
1·6
1·2
0·8
0·4
20
40
60
80
Incubation time (min)
Pyr
Gro-3-P
Gro

.Addition of ATP to trypanosomes incubated with glycerone and SHAM did not increase pyruvate, glycerol or Gro3P production. Instead the trypanosomes remained immobilized. Trypanosomes pre-incubated with glycerone and ATP were also immediately immobilized after the addition of SHAM. These results suggest that glycerone cannot be catabolised anaerobically to glycerol. The inability to form glycerol is not due to lack of ATP for the phosphorylation of the glycerone .

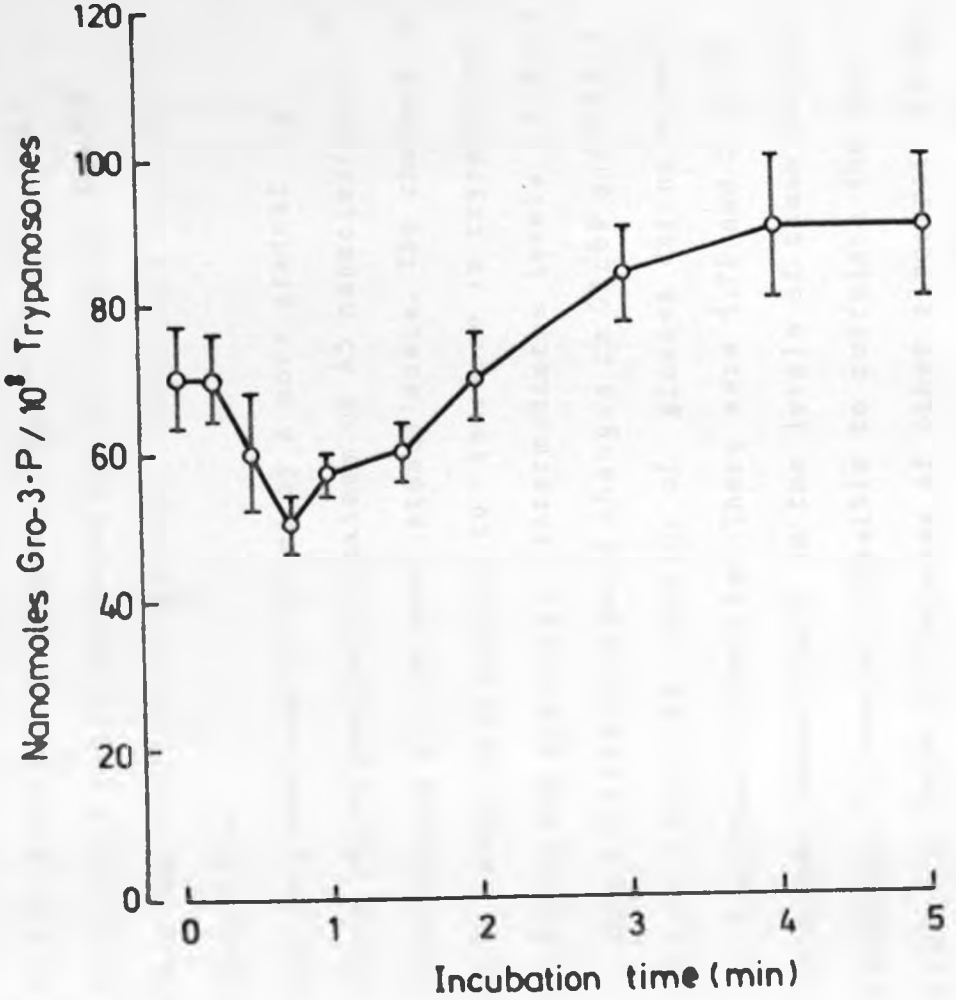
5.1.7 Changes in Gro3P in T.b.brucei catabolising fructose anaerobically after the addition of glucose or mannose

From the negligible rate of pyruvate production from fructose shown in Figure 21b after 30 min, and the level of ATP shown in Table VIII in the presence of SHAM, it was deduced that the level of ATP was insufficient to phosphorylate a hexose sugar. It was suggested that the accumulated Gro3P (Table IX) may phosphorylate a suitable substrate such as glucose according to the equation:

Glucose + Gro3p ⟶ Glc6P + glycerol

This possibility was further investigated by determining changes in the level of Gro3P in trypanosomes first immobilized by pre-incubation with SHAM and fructose for the first 30 min and then restored

Figure 26: A time course of the changes in Gro3P levels as a result of addition of glucose to T.b.brucei pre-incubated wih fructose and SHAM. The trypanosomes were incubated for 20 min with 10 mM SHAM after which 10 mM glucose was added. Samples were removed at the times shown for metabolite assays. Vertical bar lines indicate ± standard errors of mean from 4 different experiments.


120
100
80
60
40
20
0
Nanomoles Gro-3-P / 10^8 Trypanosomes
0
1
2
3
4
5
Incubation time (min)

by the addition of glucose. Figure 26 shows that there was a rapid decrease in the level of Gro3P after the addition of glucose, reaching a minimum after 45 s.

This decrease was followed by a more gradual increase which reached a maximum of 45 nanmoles/min/10^8 trypanosomes in the new steady state. The changes in Gro3P level corresponded to increase in trypanosome motility and glycolytic intermediates levels. Figure 27 shows a time course of changes in Glc6P and PEPyr levels after the addition of glucose during anaerobic fructose catabolism. There were 1.70 and 5 fold increases respectively in the levels of these intermediates. It was not possible to correlate the decrease in Gro3P to increase of other glycolytic intermediates due to the rapid glycolytic flux. Visser et al (1981) reported that end-products of glucose catabolism are excreted within 15 s.

In another experiment, mannose was added to trypanosomes pre-incubated with fructose and SHAM for 30 min instead of glucose. Figure 28 shows the changes in Gro3P after the addition of mannose. A decrease in Gro3P level was observed reaching a minimum after one minute after which it increased rather slowly. The decrease in Gro3P level was less rapid than when glucose was added. The trypanosomes were revived but had

Figure 27: A time course of the changes in the levels of Glc6P and PEPyr in **T.b.brucei** pre-incubated with fructose and SHAM after the addition of glucose. The trypanosomes were incubated in buffer A containing 10 mM fructose and 2 mM SHAM for 20 min. Glucose (20 mM) was added and samples were removed after the times shown and processed for metabolite assays.

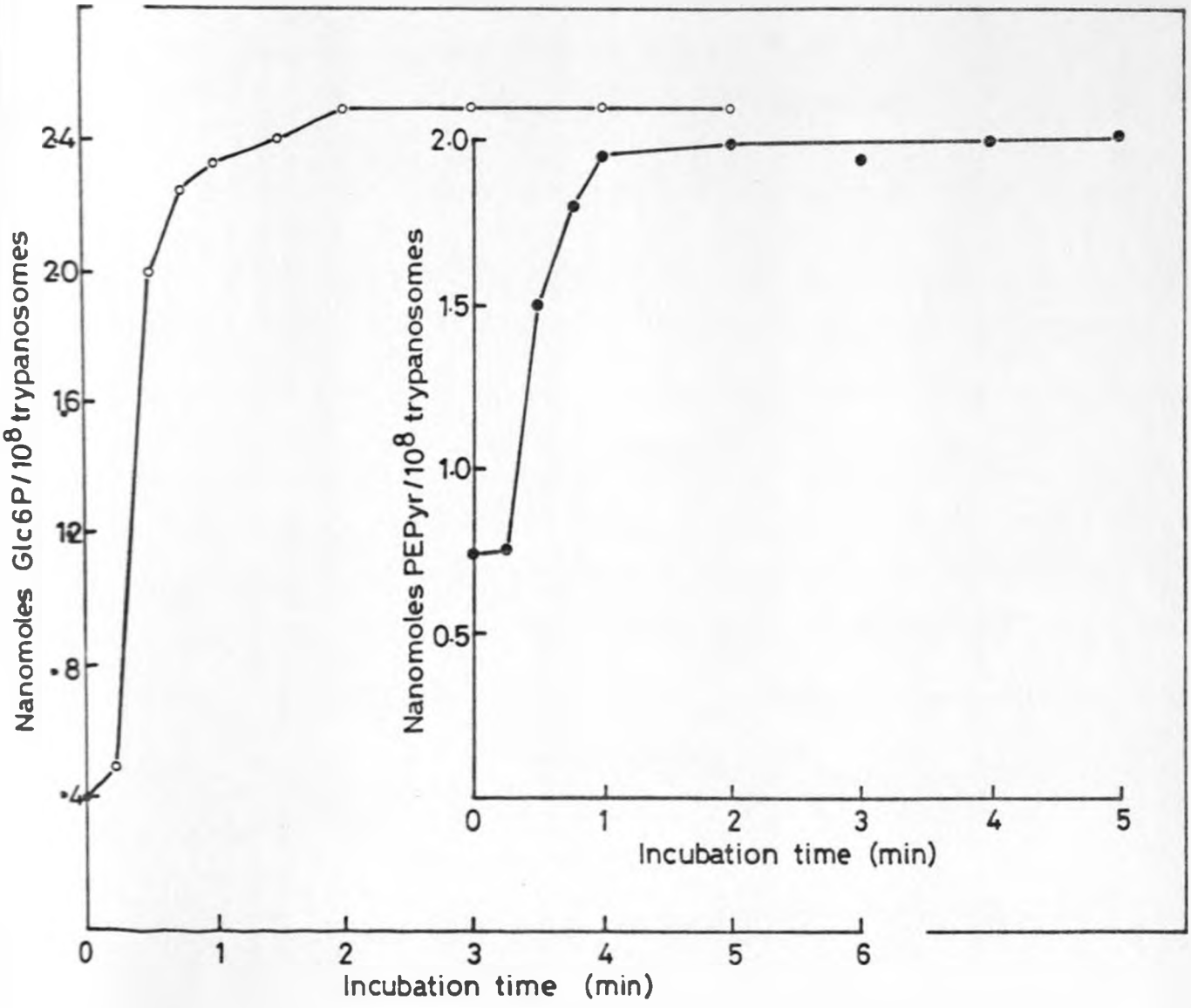
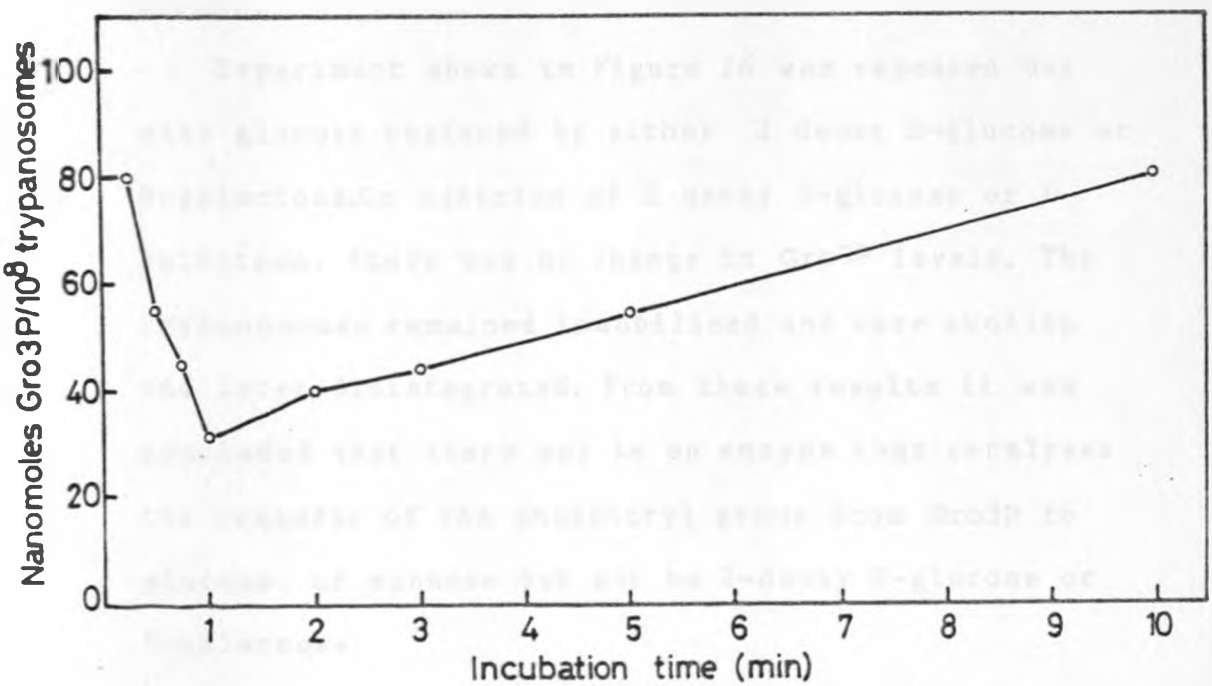

Nanomoles Glc6P/10^8 trypanosomes
2·4
2·0
1·6
1·2
·8
·4
0
1
2
3
4
5
6
Incubation time (min)
Nanomoles PEPyr/10^8 trypanosomes
2·0
1·5
1·0
0·5
0
1
2
3
4
5
Incubation time (min)

Figure 28: Changes in the level of Gro3P in T.b.brucei pre-incubated with fructose and SHAM after the addition of mannose. The trypanosomes were pre-incubated with 10 mM fructose and SHAM for 20 min after which 10 mM mannose was added. Samples were removed for Gro3P assay after the the times shown.


Nanomoles Gro3P/10^8 trypanosomes
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Incubation time (min)

less motility than in incubations where glucose was added.

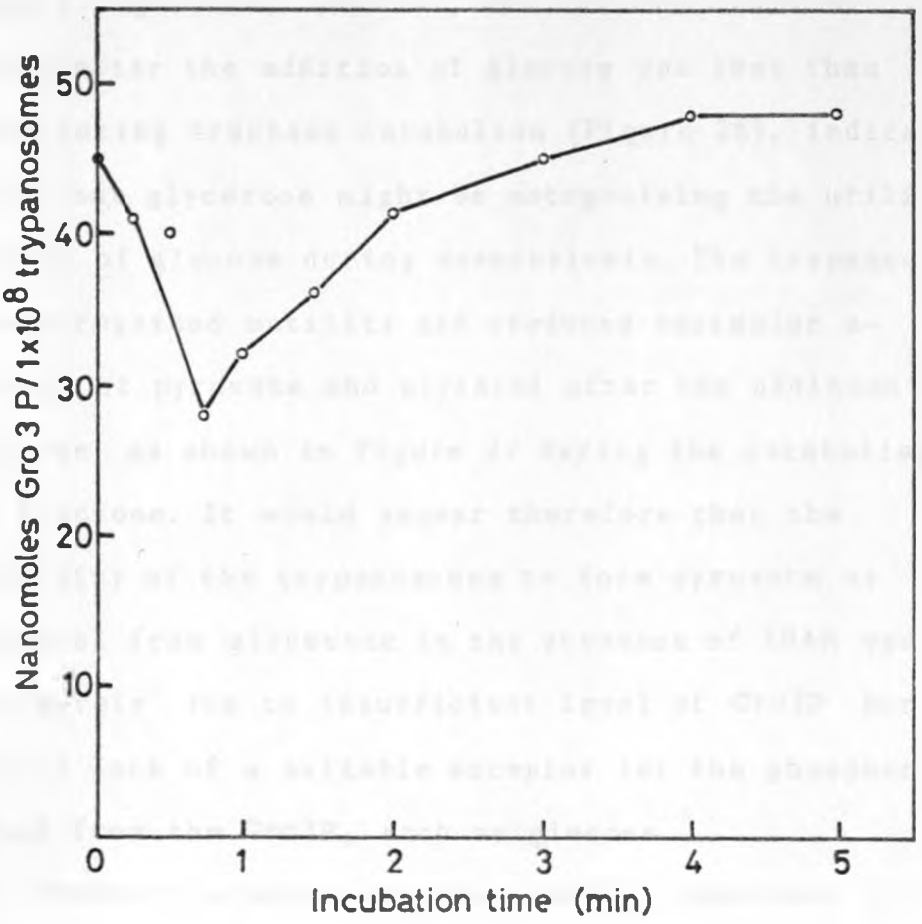
The fact that Gro3P did not return to the original level as rapidly with mannose as with glucose may reflect a relatively slow conversion of mannose 6-phosphate to Fru6P, because Hammond et al (1985) have reported low activities of phosphomannose isomerase.

Experiment shown in Figure 26 was repeated but with glucose replaced by either 2 deoxy D-glucose or D-galactose.On addition of 2 deoxy D-glucose or D-galactose, there was no change in Gro3P levels. The trypanosomes remained immobilized and were swollen and later disintegrated. From these results it was concluded that there may be an enzyme that catalyses the transfer of the phosphoryl group from Gro3P to glucose, or mannose but not to 2-deoxy D-glucose or D-galactose.

5.1.8 Changes in the level of Gro3P in T.b.brucei pre-incubated with glycerone and SHAM after the addition of glucose or fructose

Glucose was added to trypanosomes immobilized and almost depleted of ATP and glycolytic intermediates by pre-incubation for 5 min with SHAM and glycerone to determine whether the level of Gro3P shown in the table IX is sufficient for optimum rate of Glc6P and

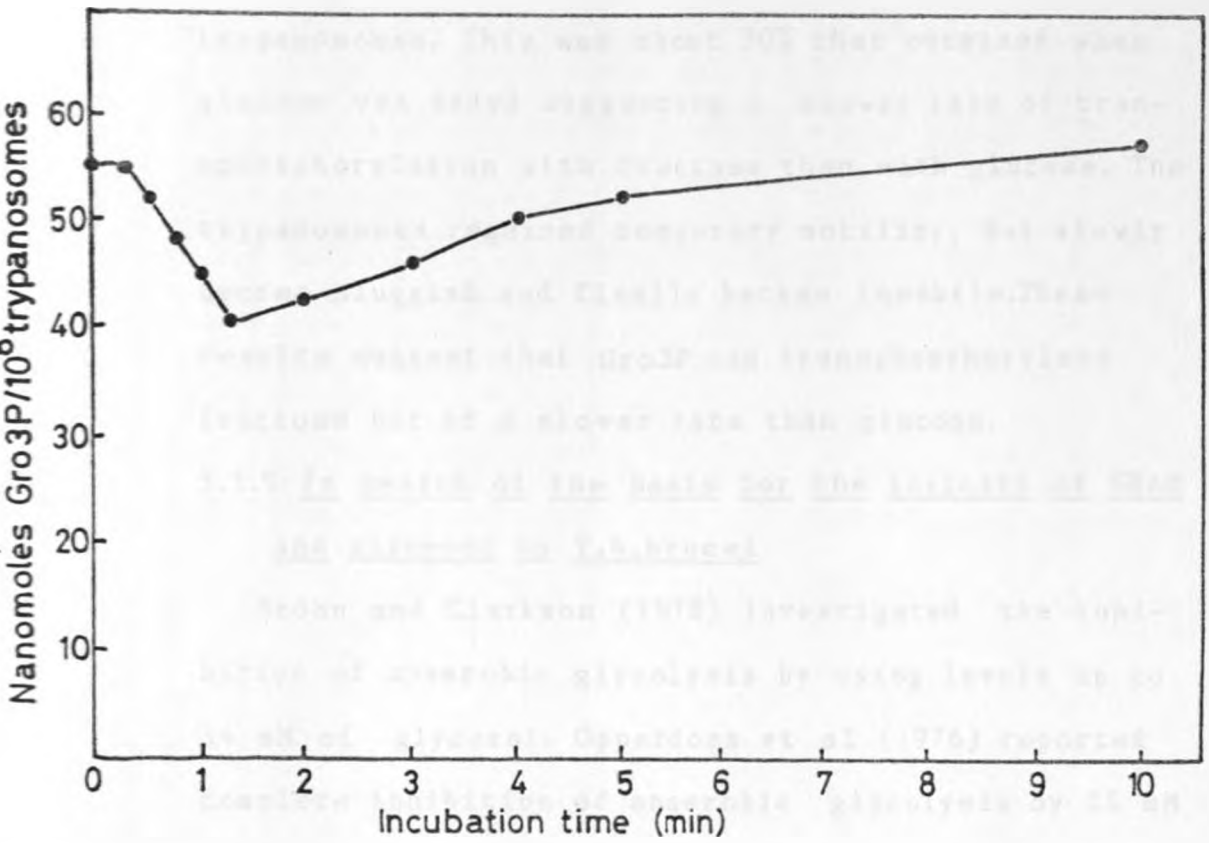
Figure 29: A time course of the changes in the levels of Gro3P in **T.b.brucei** pre-incubated with glycerone and SHAM after the addition of glucose. Glycerone concentration was 10 mM. Glucose (10 mM) was added 5 min after incubation with SHAM. Samples were removed at the times shown for metabolite assays.


Nanomoles Gro 3 P / 1x10^8 trypanosomes
50
40
30
20
10
0
1
2
3
4
5
Incubation time (min)

glycerol formation. Figure 29 shows that addition of glucose led to a decrease in Gro3P at a rate of 26 nanomoles/min/10^8 trypanosomes. This was followed by a rapid increase in Gro3P just as when the trypanosomes were pre-incubated for 30 min with fructose and SHAM (Figure 26). However, the maximum level of Gro3P after the addition of glucose was less than that during fructose catabolism (Figure 26), indicating that glycerone might be antagonising the utilization of glucose during anaerobiosis. The trypanosomes regained motility and produced equimolar amounts of pyruvate and glycerol after the addition of glucose as shown in Figure 22 during the catabolism of fructose. It would appear therefore that the inability of the trypanosomes to form pyruvate or glycerol from glycerone in the presence of SHAM was not merely due to insufficient level of Gro3P but due to lack of a suitable acceptor for the phosphoryl group from the Gro3P, such as glucose.

To determine whether the slow rate of anaerobic glycolysis with fructose as substrate (Figure 21b) was due to the lack or low activity of phosphorylation step using Gro3P , trypanosomes were first incubated with glycerone and SHAM for 5 min, and then fructose was added. Figure 30 shows that addition of fructose led to a slow decrease in Gro3P which reached a

Figure 30: A time course of the changes in the levels of Gro3P in **T.b.brucei** pre-incubated with glycerone and SHAM after the addition of fructose. Experimental procedure was as described in the legend of Figure 29, except that 10 mM fructose was added instead of glucose.


Nanomoles Gro3P/10^8 trypanosomes
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
Incubation time (min)

minimum after 75 seconds and then increased slowly. The rate of Gro3P. decrease was 13 nanomoles/min/10^8 trypanosomes. This was about 30% that obtained when glucose was added suggesting a slower rate of transphosphorylation with fructose than with glucose. The trypanosomes regained temporary motility, but slowly became sluggish and finally became immobile.These results suggest that Gro3P can transphosphorylate fructose but at a slower rate than glucose.

5.1.9 In search of the basis for the toxicity of SHAM and glycerol to T.b.brucei

Brohn and Clarkson (1978) investigated the inhibition of anaerobic glycolysis by using levels up to 34 mM of glycerol. Opperdoes et al (1976) reported complete inhibition of anaerobic glycolysis by 55 mM glycerol. No systematic investigation as to how glycerol inhibits anaerobic glycolysis has so far been reported. Pyruvate production from glucose and fructose has been determined at various levels of glycerol, and in the presence of SHAM. Figures(31a and 31b) show that increasing concentrations of glycerol progressively inhibited pyruvate production ; inhibition was complete at 4 mM and 7 mM from the catabolism of glucose and fructose respectively. At these concentrations, the trypanosomes were rapidly immobilized and were dead 5 minutes after the addi-

Figure 31: Inhibition of pyruvate production from glucose or fructose in the presence of SHAM by various concentrations of glycerol. Trypanosomes were incubated with fructose (10 mM) or glucose (10 mM) and SHAM for 5 min before glycerol was added.

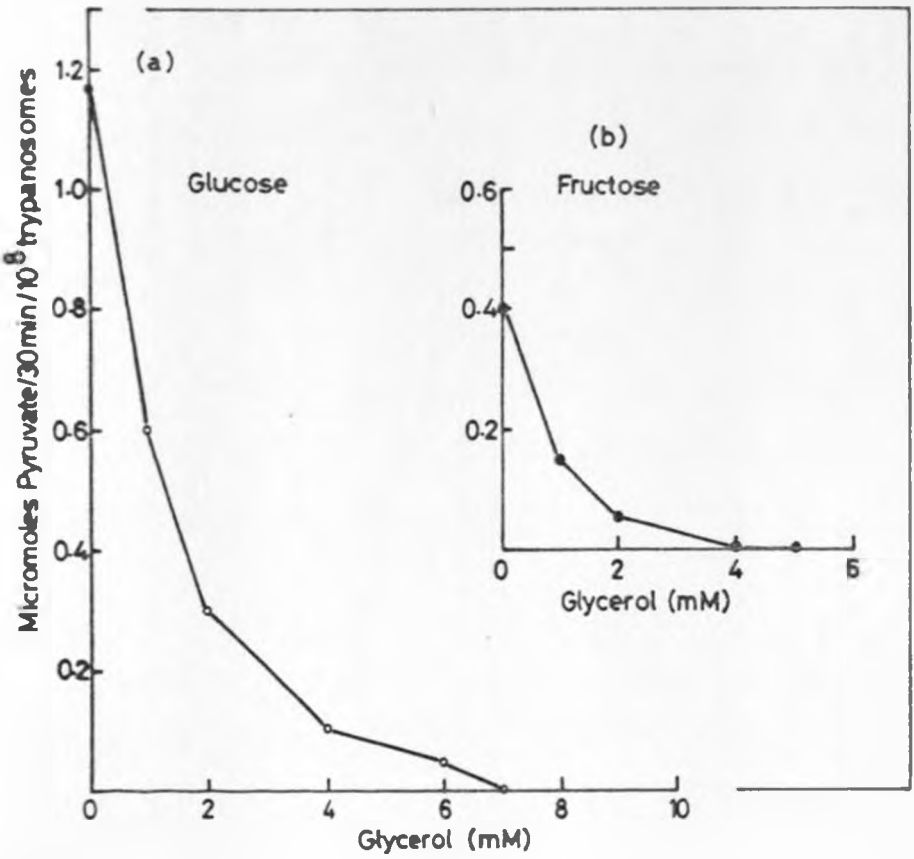

(a)
Glucose
(b)
Fructose
Micromoles Pyruvate/30min/10^8 trypanosomes
1.2
1.0
0.8
0.6
0.4
0.2
0
2
4
6
8
10
Glycerol (mM)
0.6
0.4
0.2
0
2
4
6
Glycerol (mM)

Figure 32a: Changes in the levels of Gro3P in **T.b.brucei** with time after successive addition of SHAM or glycerol in the presence of ATP during glucose catabolism. The concentration of glucose in buffer A was 10 mM. SHAM or glycerol were added to make 2 and 10 mM respectively.

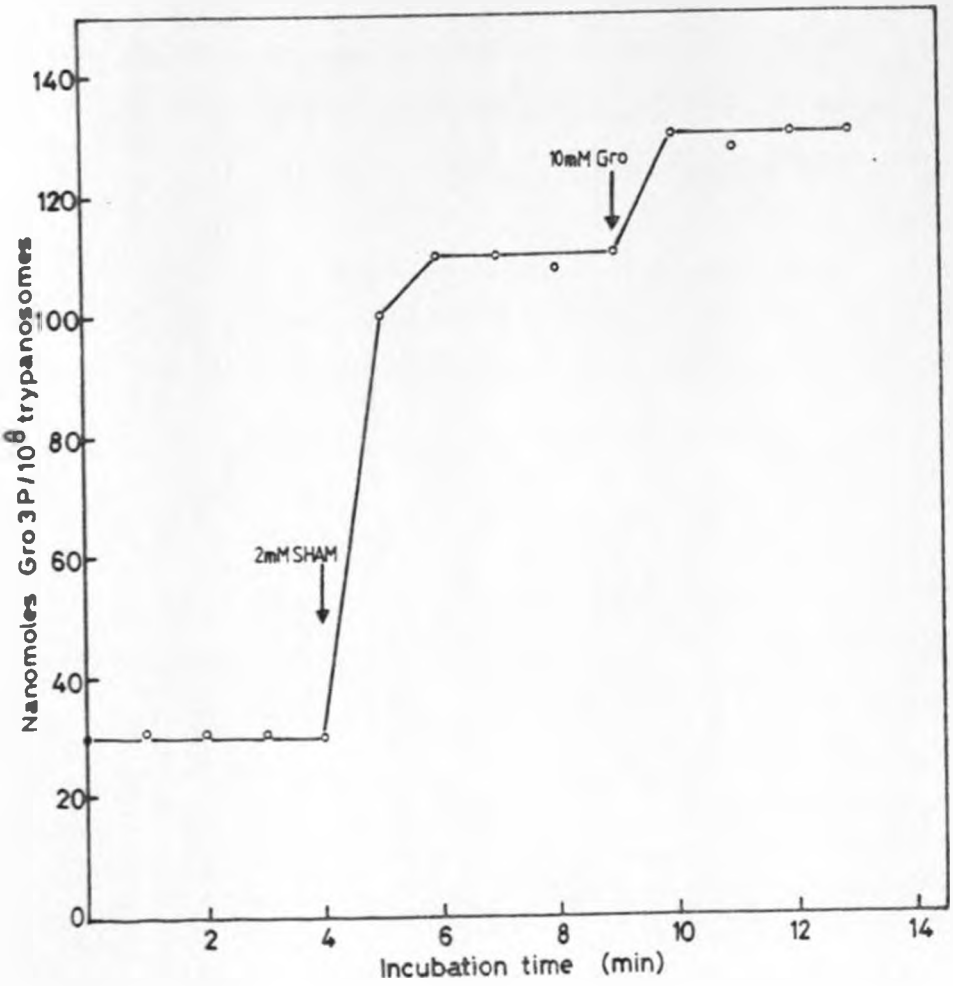
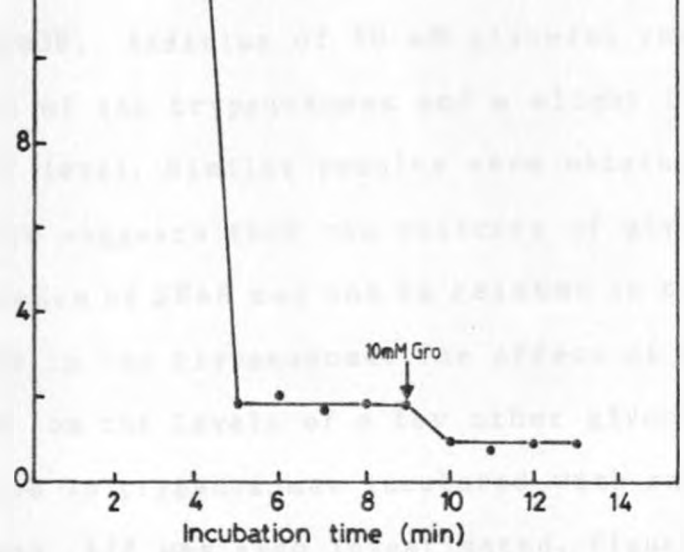
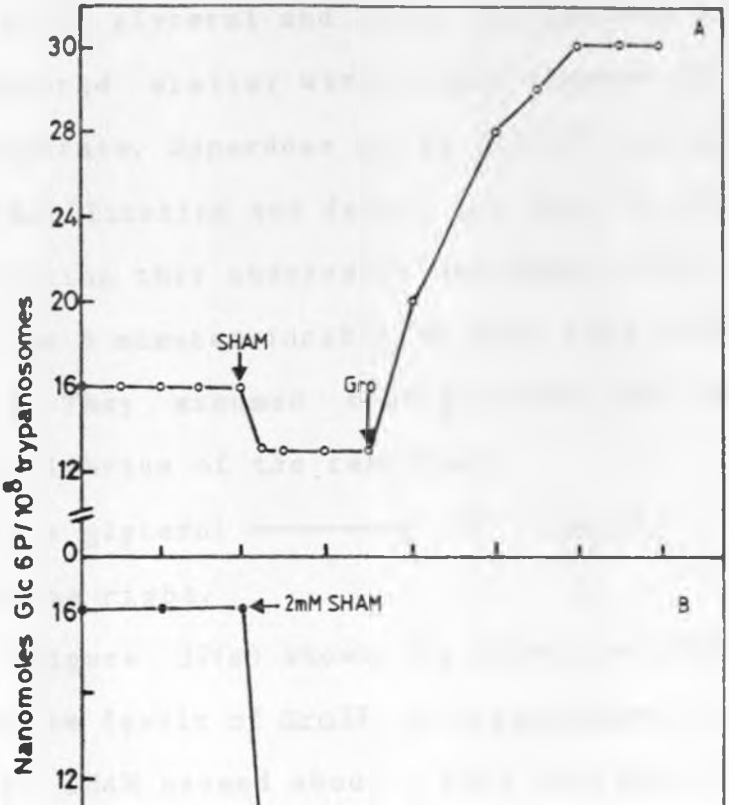

140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
10mM Gro
2mM SHAM
Nanomoles Gro 3P / 10^8 trypanosomes
Incubation time (min)

Figure 32b: Changes in the levels of Glc6P in **T.b.brucei** after successive addition of SHAM and glycerol in the (A) presence and (B) absence of ATP during the catabolism of glucose (10 mM). Experimental procedure is as described in the legend of Figure 32a except that 2.5 mM ATP was was added as indicated.


8
4
0
10mM Gro
2
4
6
8
10
12
14
Incubation time (min)


A
30
28
24
20
16
12
0
SHAM
Gro
B
16
12
2mM SHAM
Nanomoles Glc 6P/10⁸ trypanosomes

tion of glycerol and SHAM. The pattern of inhibition appeared similar with either glucose or fructose as substrate. Opperdoes et al (1976) also observed rapid immobilization and death, but with 55 mM glycerol. In addition they observed a decrease of 92% in ATP level after 5 minutes incubation with both SHAM and glycerol. They assumed that glycerol was driving the equilibrium of the reaction;

ATP + glycerol $\longrightarrow$ ADP + Gro3P

to the right.

Figure 32(a) shows the effect of SHAM and glycerol on the levels of Gro3P in trypanosomes incubated with ATP. SHAM caused about 4 fold increase in the levels of Gro3P. Addition of 10 mM glycerol resulted in death of the trypanosomes and a slight increase in Gro3P level. Similar results were obtained without ATP.

This suggests that the toxicity of glycerol in the presence of SHAM may not be related to the level of Gro3P in the trypanosome. The effect of glycerol and SHAM on the levels of a few other glycolytic intermediates in trypanosomes incubated with and without exogenous ATP was also investigated. Figure 32b shows the effect SHAM and glycerol on the levels of Glc6P. Addition of SHAM after 4 min pre-incubation with ATP resulted in a 19% decrease in Glc6P level. When 10 mM glycerol was added 3 min after pre-

Table X: Levels of some glycolytic intermediates from glucose catabolism by **T.b.brucei** incubated with SHAM after the addition of glycerol alone or in combination wih ATP. Glycerol, ATP, and SHAM were added to make 10, 2.5, and 2.0 mM respectively. The results are in nanomoles/10^8 trypanosomes and are expressed as means $\pm$ standard deviation from 4 different experiments.

Additions	Glycolytic intermediates					
	Glc6P	Frul,6P_2	Gra3P	GrnP	Gri2P	PEPyr
None	14.0±2.1	8.8±1.6	1.5±0.4	1.3±0.6	174± 8	45±10
SHAM	1.8±0.3	2.3±0.4	0.7±0.2	0.6±0.1	17±0.8	24.0±0.7
SHAM + Gro	13.0±1.6	7.0±2.1	1.1±0.3	1.0±0.2	1.0±0.3	0.9±0.4
SHAM + ATP	38.5±6.2	16.9±2.4	11.5±1.6	10.9±1.8	60.1±17.1	200±17
SHAM + ATP+ Gro	32.7±4.3	12.8±1.9	8.6±1.3	9.8±0.7	1.5±0.3	1.8±0.4

incubation with SHAM and ATP, there was a 130% increase in Glc6P level. On the other hand, addition of SHAM without ATP resulted in 90% decrease in Glc6P level; only a slight further decrease occurred after the addition of glycerol.

Table X shows the levels of afew other glycolytic intermediates in trypanosomes incubated with ATP after the addition of SHAM and glycerol. Addition of SHAM followed by 10 mM glycerol resulted in slight decrease in the level of Glc6P, Frul,$6P_2$, GrnP and Gra3P but a decrease of 96-99% Gri2P and PEPyr. It was suggested that glycerol inhibits the dephosphorylation of Gro3P resulting in accumulation of its precursors; Gra3P GrnP and the hexose phosphates. Considering that Gro3P could apparently escape from the trypanosomes incubated with SHAM and ATP during the utilization of glucose (Chapter IV), it was proposed that inhibition of anaerobic glycolysis by glycerol in the presence of ATP may not occur, because the trypanosomes can excrete Gro3P without dephosphorylation (Scheme IVA). Inhibition of pyruvate formation shown in Figure 31 was obtained in the presence of ATP and various concentrations of glycerol. There was no significant increase in Gro3P, in the presence of exogenous glycerol contrary to where the experiment was done without glycerol. It is appa-

rent that exogenous glycerol may also inhibit the transport of the glycerol formed in the trypanosomes.

5.2 Discussion

5.2.1 Aerobic catabolism of the hexoses and glycerone

The bloodstream forms of **T.b.brucei** can utilize fructose, glycerol, glucose and mannose in vitro. The low serum concentrations of fructose, less than 0.1 mM (Vrana and Fabry, 1983); and glycerol, 0.1 mM (Lin, 1977); and the much higher concentrations of glucose 5 mM, and mannose, 1.5 mM (Marier, et al, 1982) indicates that glucose and mannose would be the main substrates in the mammalian host. Southworth and Read (1969) reported rapid uptake of glucose, mannose, fructose, glycerol and the nonmetabolizable 2-deoxy D-glucose by **T.b. gambiense.** The Km and Vmax values for uptake were: glucose, 1.35 mM and 47.9; fructose, 1.58 mM and 20.1; mannose, 0.84 mM and 48.3; glycerol 0.60 mM and 39.7; 2-deoxy D-glucose, 0.16 mM and 30.4. The Vmax was in micromoles per gram dry weight per 2 minutes. These authors proposed a glucose transport at the plasma membrane site through which glucose and mannose are transported, and a separate specific transport site for fructose. Gruenberg et al (1980b) have shown that the transport site for glycerol at the plasma membrane in **T.b. brucei** is distinct from that of glucose.

Seed et al (1969) showed that **T.b.gambiense** and **T.b.rhodesiense** respire on fructose and mannose. No one

has so far reported a detailed study of the utilization of these sugars by the species T.brucei. Hammond et al (1985) showed that lysed T.b.brucei glycosomes can catabolise mannose and fructose in the presence of ATP to GrnP which then oxidises NADH. It has been shown for the first time during this investigation that these organisms can catabolise fructose and mannose to pyruvate. The high rate of aerobic pyruvate production from fructose, mannose and glucose (Figure 20) is a function of the uptake across the plasma membrane and probably across the glycosomal membrane, in addition to the activities of the glycolytic enzymes. The catabolism of these hexoses differs in that fructose is phosphorylated to Fru6P whereas glucose and mannose are phosphorylated to Glc6P and Man6P respectively which have to be converted to Fru6P . Risby and Seed (1969) reported that the rates of phosphorylation of glucose, fructose and mannose by partially purified hexokinase were in the ratio 1:0.82:0.64. From the high rates of pyruvate production from the hexoses catabolism, glycolytic intermediates and ATP (Figure 20, Table VII) , I propose that the following overall equation summarises the utilization of glucose, fructose and mannose in aerobic glycolysis.

$$\text{Hexose} + 2\text{ADP} + 2\text{Pi} + O_2 \longrightarrow 2\text{Pyr} + 2\text{ATP} + 4H_2O$$

Since T.b.brucei can catabolise glycerol aerobically

(Opperdoes et al, 1976), it was hoped that glycerone which is a more oxidised precursor of glycerol, could also be catabolised to pyruvate after phosphorylation in the same pathway as GrnP (Scheme IVA). Glycerol kinase catalyses the phosphorylation of Grn (Thorner,1975) according to the equation:

$$Grn + ATP \longrightarrow ADP + GrnP$$

The activity of glycerol kinase using glycerone as substrate at 25°C was 0.82 micromoles/min/mg protein. This was 18% of the activity with glycerol as substrate. The low rate of pyruvate production observed from glycerone was perhaps due to the slow rate of its phosphorylation. The following overall equation summarises aerobic catabolism of Grn to pyruvate

$$Grn + ADP + Pi + 0.5\ O_2 \longrightarrow Pyr + ATP + H_2O\ .$$

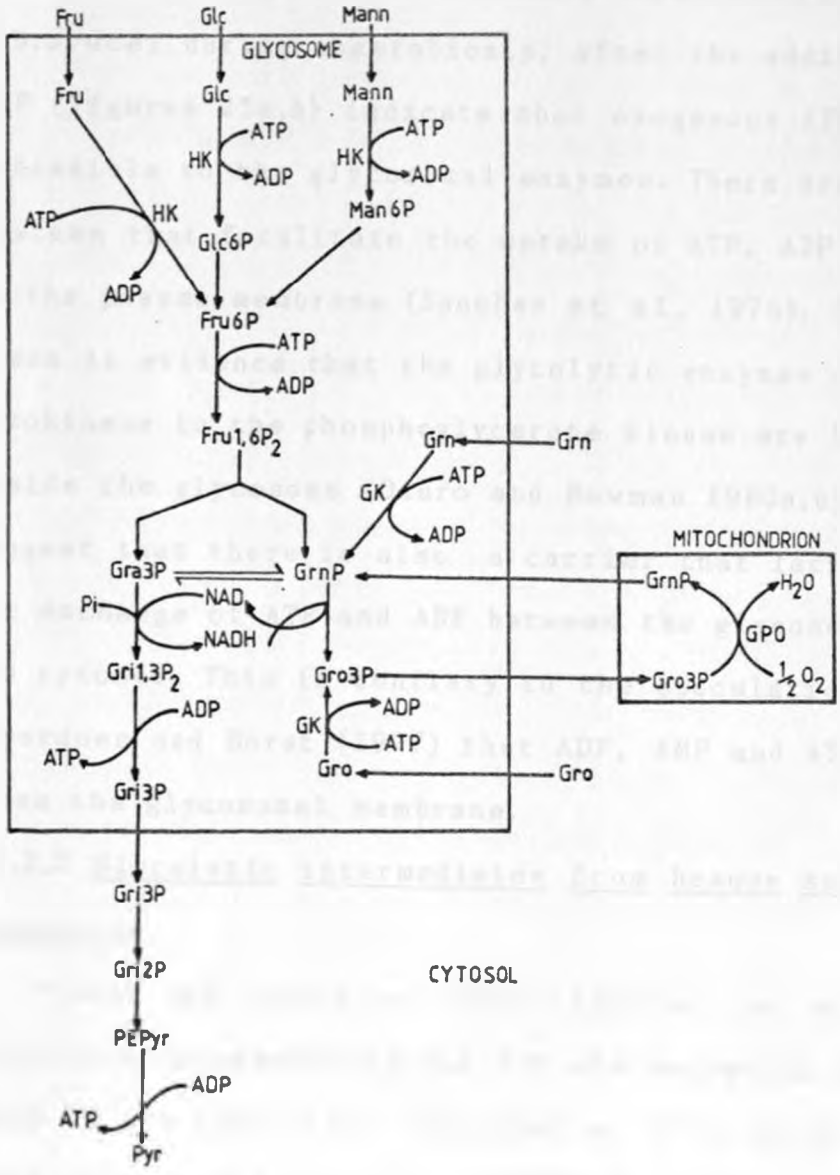
The high levels of ADP and AMP (Table VIII) suggest that there were insufficient levels of $Gri1,3P_2$ and PEPyr among other triosephosphates resulting in slow rate of substrate level phosphorylation.

Scheme VI summarises the proposed sequence of reactions of aerobic glycolysis during the utilization of the hexoses and the trioses during aerobiosis. This is a modification of scheme IVA.

5.2.2 Anaerobic glycolysis

5.2.2.1 Regulation of the end-products of glucose catabolism by exogenous ATP and ADP.

Scheme VI: Proposed pathway for aerobic catabolism of hexose sugars, glycerone and glycerol in the bloodstream forms of T.b.brucei. It is a modification of scheme IVA from which more detail can be obtained. HK and GK indicate hexokinase and glycerol kinase respectively.


Fru
Glc
Mann
GLYCOSOME
Fru
Glc
Mann
ATP
HK
ADP
ATP
HK
ADP
ATP
HK
Man6P
Glc6P
ADP
Fru6P
ATP
ADP
Fru1,6P2
Grn
Grn
ATP
GK
ADP
MITOCHONDRION
Gra3P
GrnP
GrnP
H2O
Pi
NAD
NADH
GPO
Gri1,3P2
Gro3P
Gro3P
½ O2
ADP
ATP
GK
ADP
ATP
Gro
Gro
Gri3P
Gri3P
Gri2P
CYTOSOL
PEPyr
ADP
ATP
Pyr

The increased production of Gro3P, glycerol and pyruvate from glucose or fructose catabolism by intact T.b.brucei during anaerobiosis, after the addition of ATP (Figures 25a,b) indicate that exogenous ATP is accessible to the glycosomal enzymes. There are carrier systems that facilitate the uptake of ATP, ADP and AMP at the plasma membrane (Sanchez et al, 1976). Since there is evidence that the glycolytic enzymes from the hexokinase to the phosphoglycerate kinase are located inside the glycosome (Oduro and Bowman 1980a,b), I suggest that there is also a carrier that facilitates the exchange of ATP and ADP between the glycosome and the cytosol. This is contrary to the speculation of Opperdoes and Borst (1977) that ADP, AMP and ATP cannot cross the glycosomal membrane.

5.2.2.2 Glycolytic intermediates from hexose sugars catabolism

Visser and Opperdoes (1980) reported that most glycolytic intermediates and ATP are decreased by half after 10 min incubation with SHAM at 37°C; Gri3P was found to decrease 4 fold but Gro3P by contrast increased 4 fold. These authors used Krebs/Ringer/bicarbonate buffer supplemented with 55 mM glucose and 15% rat plasma. Their results were different from those of Hammond and Bowman (1980a), who incubated trypanosomes with SHAM in phosphate saline glucose buffer without

the rat plasma for 7.5 min at 18°C and found that various glycolytic intermediates decreased several fold after the addition of SHAM. The differences may be attributed to the presence of rat plasma. In this study I have made a systematic investigation of the changes in glycolytic intermediates after the addition of SHAM at 25°C. Addition of SHAM led to a rapid decrease of intermediates which reached a minimum within 2 min; only Gro3P increased. The levels of glycolytic intermediates, ATP, ADP and AMP when glucose was substrate are comparable with those found by Hammond and Bowman (1980a). When Fructose was the substrate, the levels of the glycolytic intermediates; Glc6P, Fru6P, Fru1,6P_2, Grnp, Gra3P Gri3P, Gri2P and PEPyr decreased more on addition of SHAM than was the case when glucose was the substrate (Table VII) again within 2 min and only Gro3P increased. This suggested that the phosphorylation of fructose was depressed more than that of glucose by addition of SHAM.

5.2.2.2.1 Compartmentation of glycolytic intermediates

Does the decrease in the levels of glycolytic intermediates shown in Table VII reflect changes in the glycosome, cytosol or both compartments? From pulse labelling kinetics Visser et al (1981) identified two pools of glycolytic intermediates in T.b.brucei. These are glycosomal and cytosolic pools, constituting 20-30%

and 70-80% respectively of total cellular glycolytic intermediates. The second pool appeared not to be directly involved in glycolysis as it equilibrated rather slowly with the glycosomal pool. The cytosolic counterpart of the glycolytic pathway could constitute a significant percentage of the glycolytic flux because 8-30% of the glycosomal glycolytic enzyme activities are not latent (Visser and Opperdoes, 1980). The glycosomal glycolytic enzymes may have cytosolic isoenzymes except for the triosephosphate isomerase, phosphoglycerate kinase and glyceraldehyde 3-phosphate dehydrogenase (Misset and Opperdoes, 1984; Swinkels et al, 1986; Osinga et al, 1985, Misset et al, 1986; Miset et al, 1987; Opperdoes et al, 1986). So far there is no definite evidence for cytosolic isoenzymes of the hexokinase, phosphoglucose isomerase, glycerol kinase and glycerol 3-phosphate dehydrogenase although latency studies suggest that they may exist (Visser and Opperdoes, 1980). The absence of cytosolic triosephosphate isomerase could suggest that the cytosolic glycolytic pathway lacks of interconversion of Grnp to Gra3p . There is however no information so far on whether one or both the glycosomal and cytosolic pools of glycolytic intermediates are decreased by addition of SHAM. From this investigation, addition of SHAM caused more than 70% decrease of most glycolytic intermediates

during the utilization of glucose and fructose. This suggests that the decrease includes the cytosolic pool because the glycosomal pool constitutes only 20-30% of the glycolytic intermediates. The cytosolic pool has a high turnover rate because the decrease was maximum after about 2 min incubation with SHAM. This is not easily reconciled with the view that the glycosomal membrane is impermeable to glycolytic intermediates. The finding that the glycosome might have carriers for ATP and ADP (Figures 25a,b) indicates that there may be equilibration of the glycosomal and cytosolic ATP and ADP. Therefore during anaerobiosis the decrease in ATP level is a reflection of the cellular status rather than of one cellular compartment. This would suggest that the glycolytic intermediates would probably decrease by a similar magnitude in the two compartments due to decrease in the phosphorylation of the hexoses. Therefore the level of the glycolytic intermediates and adenine nucleotides shown in Tables VII and VIII is probably a reasonable reflection of the level in both the glycosomal and cytosolic compartments.

5.2.2.2.2 The pathway of glycerol formation

Scheme IVA proposed by Visser et al, (1981)has so far sufficed to explain the production of glycerol from glucose under anaerobic conditions. The following equation summarises anaerobic pyruvate and glycerol produ-

ction from glucose and mannose shown in Figure 21.

Glucose + ADP + Pi ⟶ 1 Pyruvate + 1 glycerol + 1 ATP

It is assumed in the scheme that glycerol and net ATP production occurs according to the equation ;

ADP + Gro3P ⟶ ATP + Glycerol.

It was hoped that fructose could also be a suitable substrate for anaerobic glycolysis, as it was for aerobic glycolysis (Figure 20) whereby it maintained the trypanosomes alive for more than 90 min. The rapid suppression of fructose catabolism (Figure 21b) by SHAM could not be readily explained by a block in the phosphorylation of ADP by Gro3P because there was an accumulation of both Gro3P and ADP comparable to that observed when glucose or mannose were the substrates. It can be concluded that high Gro3P and ADP levels are not the only requirements for glycerol formation. It was noted that the phosphorylation of fructose is not rate limiting during aerobiosis as shown by the results of this investigation (Figure 20). Since all the other enzymes for Fru6P catabolism are common to both glucose and fructose catabolism, then the rate of pyruvate production from fructose in the presence of SHAM should have been decreased by only about 50% as was the case with glucose and mannose (Figure 21a). Considering that SHAM caused a remarkable suppression

of glycolysis, it is concluded that some compound, other than ADP or fructose must be phosphorylated by Gro3P during anaerobiosis under cellular conditions.

This possibility was first tested by incubating the trypanosomes with a nonhexose, glycerone, which could produce large amounts of Gro3P and ADP in the presence of SHAM. It was envisaged that ATP, pyruvate and glycerol production would occur according to the following overall equation if the reverse glycerol kinase activity could catalyse ATP and glycerol formation.

2Grn + ADP + Pi $\longrightarrow$ 1 pyruvate + 1 glycerol + 1 ATP

Glycerone was not metabolised at any noticeable rate, however, and the already very low level of glycolytic intermediates (apart from Gro3P) decreased further with SHAM, whilst a slight increase of ADP and AMP occurred. Since despite the accumulation of ADP and Gro3P, no glycerol or ATP was apparently formed, the assay described in the legend of figure 26 was devised to determine whether anaerobic glycolysis depends on the presence of glucose or mannose which are found at reasonable amounts in serum (Marier et al, 1982). The initial decrease of cellular Gro3P and the increase of glycolytic intermediate levels which resulted from the addition of glucose to trypanosomes pre-incubated with fructose and SHAM suggests that it was used in the

production of crucial glycolytic intermediates. Since glucose metabolism differs from that of fructose only in that Glc6P precedes Fru6P, I propose that a glucose transphosphorylation system is operative, in which Gro3P can phosphorylate glucose directly much more efficiently than it can phosphorylate fructose. The initial rate of Gro3P decrease after the addition of glucose may be taken to represent the rate at which it is constantly being removed from the glycolytic mainstream under anaerobic conditions during the catabolism of glucose. After this, there is a steady state at which the rate at which it is formed is equivalent to the rate it is removed. The calculated initial rate of Gro3P removal was 2.40 micromoles/h/10^8 trypanosomes after addition of glucose. This probably corresponds to its rate of removal at steady state. This initial rate of Gro3P utilization is comparable to the initial rate of glycerol production during glucose catabolism of 2.6 micromoles/h/10^8 trypanosomes (Figure 21a). A value close to this rate was obtained when glycerone was used as the source of Gro3P in the presence of SHAM. Although there was less Gro3P from glycerone than from fructose, glucose or mannose, its initial rate of decrease after the addition of glucose was as fast as when it was produced from fructose. Mannose could also cause decrease of Gro3P after the addition of glucose

to trypanosomes pre-incubated with frucose and SHAM. The initial rate of Gro3P decrease of about 2.70 micromoles/h/10^8 trypanosomes should correspond to its rate of utilization at steady state. This rate is is also comparable to the initial rate of glycerol formation from mannose in the presence of SHAM (Figure 21a) of 2.4 micromoles/h/10^8 trypanosomes. Gro3P could apparently also transphophorylate fructose at an initial rate of 13 nanomoles/min/10^8 trypanosomes which corresponds to an initial rate of 0.78 micromoles/h/10^8trypanosomes. This is comparable to the initial rate of Gro3P decrease after the addition of fructose. The calculated initial rate of the Gro3P decrease was 0.72 micromoles/h/10^8. This rate was less than when either glucose or mannose was the substrate in similar experiments. I propose that the transphosphorylation of these hexoses is catalysed by a phosphotransferase system. Considering that the highest rate of transphosphorylation was with glucose, which is the physiological substrate, I propose that the enzyme or enzyme complex should be named as Gro3P: glucose phosphotransferase. From the above characteristic decreases of Gro3P and the corresponding rates of anaerobic glycolysis, I suggest that transphosphorylation activity is more with glucose than with mannose and least with fructose. The enzyme cannot apparently use galactose

or 2-deoxy D-glucose as subsrates. It may be concluded from this investigation that Gro3P: glucose phosphotransferase activity sufficient to keep the trypanosomes alive for more than an hour, is realised only with aldose sugars that have a configuration of hydroxyl groups and hydrogen atoms about C3, C4, C5 and C6 similar to that of glucose. In addition, it appears that the same configuration of hydroxyl groups must be kept if the sugar is a ketose.

Transphosphorylation of hexose sugars in T.b.brucei was first proposed by Clarkson and Brohn (1976). They suggested that Gro3P may transphosphorylate a hexose, resulting in reduction in the ATP input necessary for glycolysis, and thus yielding net ATP production under anaerobic conditions. This suggestion has never been investigated in detail until this study. Transphosphorylation of a hexose has been taken as rather unlikely because so far no such an activity has been obtained or characterised . Hammond and Bowman (1980a) argued that the lack of accumulation of Glc6P and Fru6P from glucose in the presence of SHAM could be used as evidence against transphosphorylation of a hexose by Gro3P. They ignored the fact that Glc6P and Fru6P cannot accumulate due to the high activities of the glycolytic enzymes catalysing their utilization (Opperdoes and Borst, 1977).

The calculated standard free energy change for the Gro3P: glucose phosphotransferase reaction is +4.6 kJ/mol. This is significantly less than for the glycerol kinase assayed in the direction of ATP formation, which is +22.4 kJ/mol (Hammond and Bowman, 1980). The lower standard free energy change for the novel enzyme favours its suitability over the glycerol kinase. It appears more thermodynamically economical for the cell to reduce ATP input in glycolysis by utilizing Gro3P to phosphorylate glucose than to form extra ATP from ADP and Gro3P. Formation of ATP via the reverse glycerol kinase activity appears thermodynamically unlikely under cellular conditions.

What is the cellular localization of the proposed Gro3P : glucose phosphotransferase?. Hammond **et al**, (1985) reported no latency for the glycerol kinase, assayed in the direction of ATP formation whereas, the forward glycerol kinase assayed in the direction of Gro3P formation was 82% latent in isolated glycosomes. This observation indicates the presence of two enzymes; one in the glycosome and the other at the glycosomal membrane. The enzyme exhibiting no latency was assayed in an incubation system containing Gro3P, ADP, glucose, $NADP^+$, Gro3P dehydrogenase, endogenous hexokinase, glucose 6-phosphate dehydrogenase and following the reduction of $NADP^+$. From the assay mixture either

or both of the following two enzymes could have been assayed, because the same substrates can form Glc6P.

(i) The reverse glycerol kinase assay reaction sequence is

$$\text{Gro3P} + \text{ADP} \longrightarrow \text{Gro} + \text{ATP}$$

$$\text{Glc} + \text{ATP} \longrightarrow \text{Glc6P} + \text{ADP}$$

$$\text{Glc6P} + \text{NADP}^+ \longrightarrow \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

(ii) The Gro3P : Glucose phosphotransferase reaction sequence is:

$$\text{Gro3P} + \text{Glucose} \longrightarrow \text{Glc6P} + \text{glycerol}$$

$$\text{Glc6P} + \text{NADP}^+ \longrightarrow \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$$

The overall equation for both assays is:

$$\text{Glucose} + \text{Gro3P} + \text{NADP}^+ \longrightarrow \text{6-phosphogluconate} + \text{glycerol} + \text{NADPH} + \text{H}^+$$

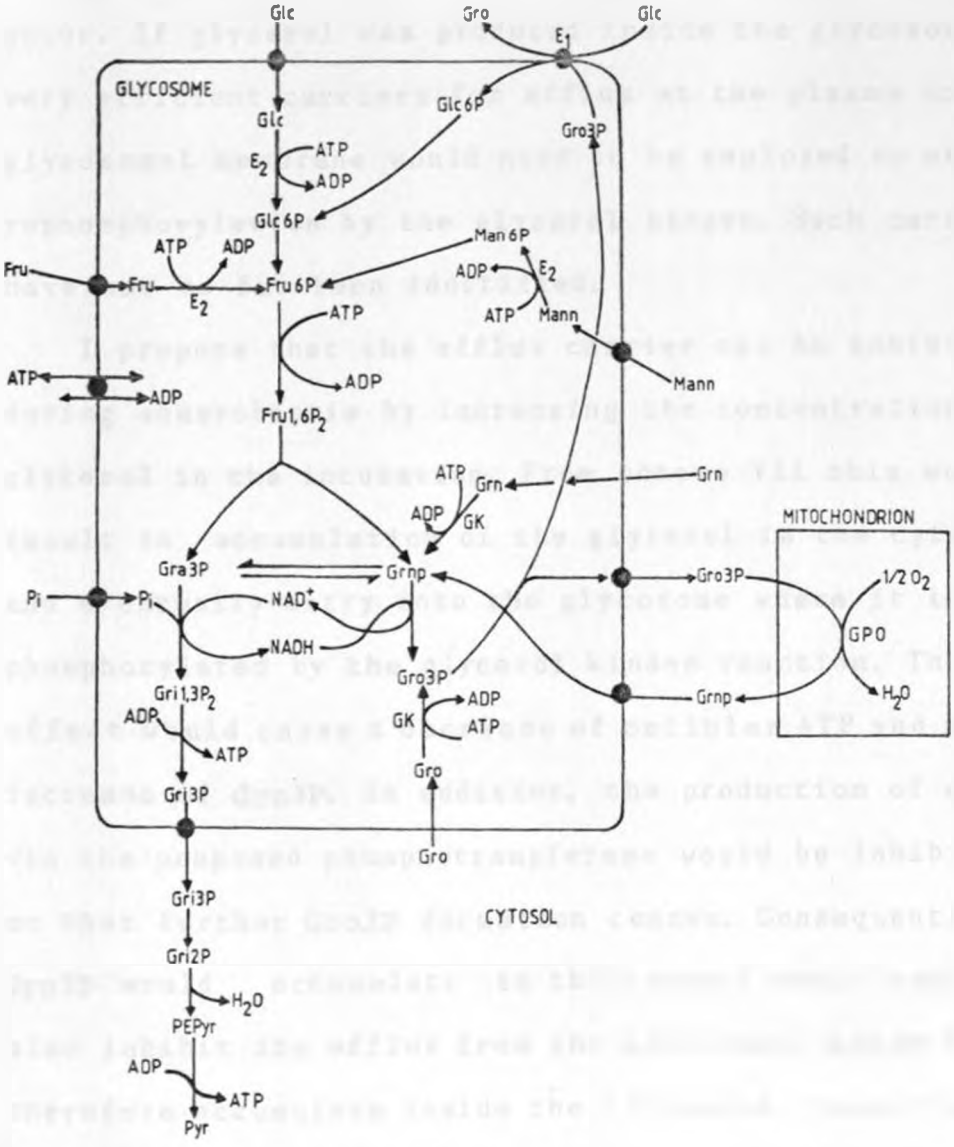
It seems likely that Gro3P: glucose phosphotransferase was assayed instead of the glycerol kinase. In addition, Hammond and Bowman (1980b) provided additional evidence in support of the above idea using the same assay mixture. They reported that solubilization of partially purified glycosomes with Triton X-100 resulted in a decrease of the presumed reverse glycerol kinase activity from 0.037 to 0.017 micromoles

/min/mg protein and an increase of the forward glycerol kinase activity from 0.8 to 2.9 micromoles/min/mg protein. This observation implies that the glycerol kinase (forward) is a distinct enzyme from that catalysing Gro3P, dephosphorylation. It is unlikely that a change in conformation of glycerol kinase as suggested by Hammond and Bowman (1980b) could occur during solubilization which could confer it with such unusual characteristics, such that reverse and forward activities apparently have separate binding sites for Gro3P and ADP as is implied by the changes in the activity after solubilization with Triton X-100. I propose that these authors assayed Gro3P:glucose phosphotransferase rather than reverse glycerol kinase activity. In addition I suggest that the Gro3P: glucose phosphotransferase activity is located at the glycosomal membrane as reported for the purported reverse glycerol kinase (Hammond and Bowman 1980b). The Gro3P: glucose phosphotransferase appears to be an integral membrane protein requiring some lipid component for full activity, as evidenced by decrease in glycerol formation which results from permeabilization of the trypanosome membranes with digitonin (see Chapter IV). Scheme VII shows a possible compartmentation of anaerobic glycolysis in **T.b. brucei**. It involves the assumption of separate carriers for Gri3P, GrnP and Gro3P. During

anaerobiosis both Gri3P and Gro3P leave the glycosome. Gro3P transphosphorylates glucose to give Glc6P and glycerol whereas Gri3P is catabolised to pyruvate. Glc6P is catabolised to Gri3P and Gro3P. The overall effect is that one net mole ATP is formed per mole of glucose without involving a novel enzyme in substrate level phosphorylation. It would appear that the transport of Gro3P to the cytosol does not necessarily depend on the availability of GrnP because it has been shown that Gro3P could leave the trypanosome and accumulate in the incubation medium even during anaerobic conditions. (The passage of Gro3P across the plasma membrane to the incubation medium was not investigated).

From scheme IVA, glycerone alone could not form glycerol in the presence of SHAM because the Gro3P has to transphosphorylate glucose or mannose. I suggest that the glycerol kinase and Gro3P· glucose phosphotransferase do not share the same microenvironment so that the glycerol formed is not rephosphorylated. Gruenberg et al (1980b) showed that the efflux of glycerol from the trypanosome is more favourable than influx and that neither of the two processes is energy dependent. Both are concentration dependent. They also found the Km for efflux is lower than that for influx. This shows that

Scheme VII: Envisaged compartmentation of anaerobic glycolysis during the catabolism of hexose sugars and glycerol together with the location of the novel Gro3P: glucose phosphotransferase (E_1). E_2 indicates the hexokinase and the GK indicates the glycerol kinase. The substrates for E_1 are glucose, mannose, or frutose. O indicates envisaged transport proteins for the substrates and metabolites shown. For more details of this scheme see scheme IVA.


Glc
Gro
Glc
E1
GLYCOSOME
Glc
Glc6P
Gro3P
E2
ATP
ADP
Glc6P
Man6P
ATP
ADP
Fru
Fru
Fru6P
E2
ADP
E2
ATP
Mann
ATP
ADP
Mann
ATP
ADP
Fru1,6P2
ATP
Grn
Grn
ADP
GK
MITOCHONDRION
Gra3P
Grnp
Gro3P
1/2 O2
Pi
Pi
NAD+
NADH
GPO
H2O
Gro3P
Grnp
Gri1,3P2
ADP
ATP
GK
ADP
ATP
Gro
Gri3P
Gro
CYTOSOL
Gri3P
Gri2P
H2O
PEPyr
ADP
ATP
Pyr

glycerol has to be removed from the trypanosome to fairly low concentration relative to the concentration in the incubation medium before any net uptake can occur. If glycerol was produced inside the glycosome, very efficient carriers for efflux at the plasma and glycosomal membrane would need to be employed to avoid rephosphorylation by the glycerol kinase. Such carriers have not so far been identified.

I propose that the efflux carrier can be inhibited during anaerobiosis by increasing the concentration of glycerol in the incubation. From scheme VII this would result in accumulation of the glycerol in the cytosol, and eventually entry into the glycosome where it is phosphorylated by the glycerol kinase reaction. This in effect would cause a decrease of cellular ATP and an increase of Gro3P. In addition, the production of Glc6P via the proposed phosphotransferase would be inhibited so that further Gro3P formation ceases. Consequently Gro3P would accumulate in the cytosol which could also inhibit its efflux from the glycosome. Gro3P would therefore accumulate inside the glycosome resulting in inhibition of the Gro3P dehydrogenase reaction,and as a result Glc6P , Fru6P, Fru1,6P_2 , Grn P and Gro3P would also be expected to accumulate. This was observed in this investigation (Table X). In the presence of ATP these intermediates tended to increase several fold

indicating increased phosphorylation of glucose but without catabolism because pyuvate production was rapidly inhibited and Gri2P and PEPyr levels decreased remarkably. This is in support of the idea that high levels of Gro3P inhibits Gro3P dehydrogenase.

From the results shown in Figure 31, it was suggested that exogenous glycerol also inhibits the efflux of Gro3P at the plasma membrane because there was no increase in Gro3P in the medium in the presence of ATP after the addition of glycerol. Glycerol could not have inhibited the transport of Gro3P from the glycosome, because if it did the trypanosomes should not have catabolised glycerol to pyruvate under aerobic conditions. It may be tentatively concluded that during anaerobiosis, glycerol inhibits the transphosphorylation of the metabolisable hexoses by mass action effect. In addition it may also inhibit the efflux of cytosolic glycerol at the plasma membrane. This could be the curative basis, of intravenous administration of SHAM and glycerol (Brohn and Clarkson, 1976),in mice infected by **T.b.brucei.**

5.3 Conclusions

The following conclusions may be made from this investigation.

(a) The rate of ATP production from aerobic catabolism of fructose and mannose is sufficient to keep the

trypanosomes alive for at least 2 h just as when they are incubated with their physiological substrate, glucose.

(b) Unlike glucose and mannose, fructose and glycerone are poor substrates for anaerobic glycolysis.

(c) Gro3P is utilised very rapidly to phosphorylate glucose or mannose and not galactose or 2-deoxy D-glucose during anaerobiosis and that probably Gro3P: glucose phosphotransferase exists in the trypanosomes catalysing the reaction,

Gro3P + glucose $\longrightarrow$ glycerol + Glc6P.

CHAPTER VI

FURTHER CATABOLISM OF THE HEXOSES AND GLYCEROL: EFFECT OF OLIGOMYCIN

6.1 RESULTS

6.1.2 Effect of oligomycin on the formation of end-products of aerobic catabolism of some hexose sugars and glycerol by the bloodstream forms of T.b.brucei.

It was confirmed that oligomycin inhibits the production of pyruvate from glucose by **T.b.brucei** as initially reported by Miller and Klein (1980). The inhibition was dependent on the number of trypanosomes in the incubation vessel. Therefore the concentration of oligomycin was expressed as micrograms/10^8 trypanosomes instead of micrograms/ml. For reproducibility of the results the trypanosomes in each incubation were kept within the range $2.5\text{-}3.0\times10^8$.

Figure 33 shows the effect of various concentrations of oligomycin on pyruvate production from glucose, fructose, mannose and glycerol under aerobic conditions. It is apparent that 4 micrograms oligomycin/10^8 trypanosomes were required to inhibit pyruvate production by 70-80% whereas addition of another 4 micrograms oligomycin/10^8 trypanosomes caused slight inhibition. The maximum inhibition of pyruvate production was achieved by 8 to 10 micrograms

Figure 33: Effect of various concentrations of oligomycin on pyruvate production from fructose, glucose, mannose and glycerol. The incubations contained 10 mM of the same substrates. ■-■ , ○-○ , □-□ , and ●-● indicates pyruvate production from glycerol, fructose, glucose, and mannose respectively.

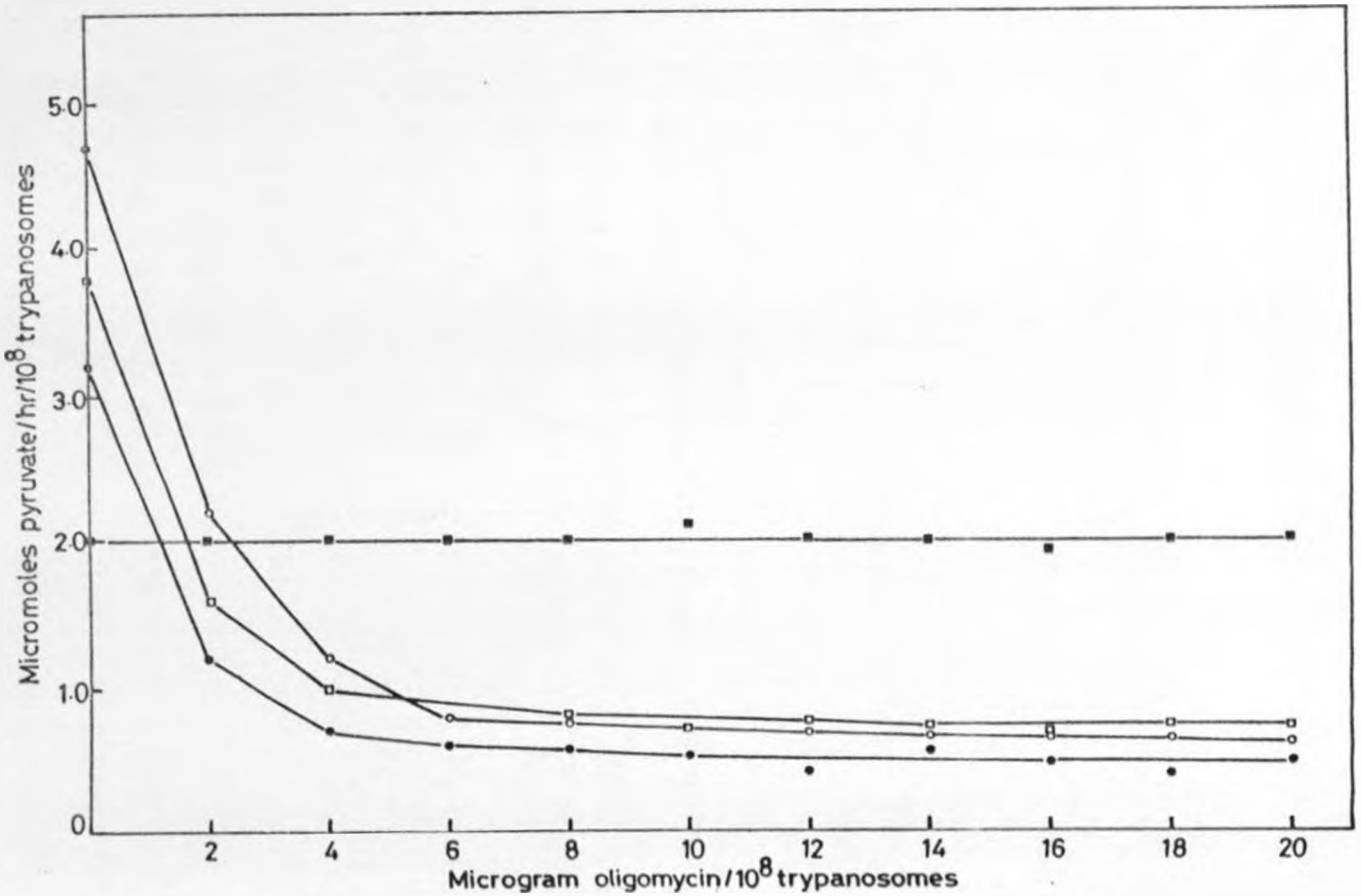

Micromoles pyruvate/hr/10^8 trypanosomes
5·0
4·0
3·0
2·0
1·0
0
2
4
6
8
10
12
14
16
18
20
Microgram oligomycin/10^8 trypanosomes

Figure 34: A time course of pyruvate production from glucose by **T.b.brucei** incubated with and without 10 micrograms oligomycin/10^8 trypanosomes and 10 mM glucose. O-O and ●-● indicates pyruvate production in the absence and presence of oligomycin respectively.

Figure 35: A time course of pyruvate production from glycerol in **T.b.brucei** incubated with and without oligomycin. O-O and ●-● indicates pyruvate production from glycerol in the presence and and absence of 10 micrograms/10^8 trypanosomes. Initial glycerol concentration was 10 mM.

Figure 34

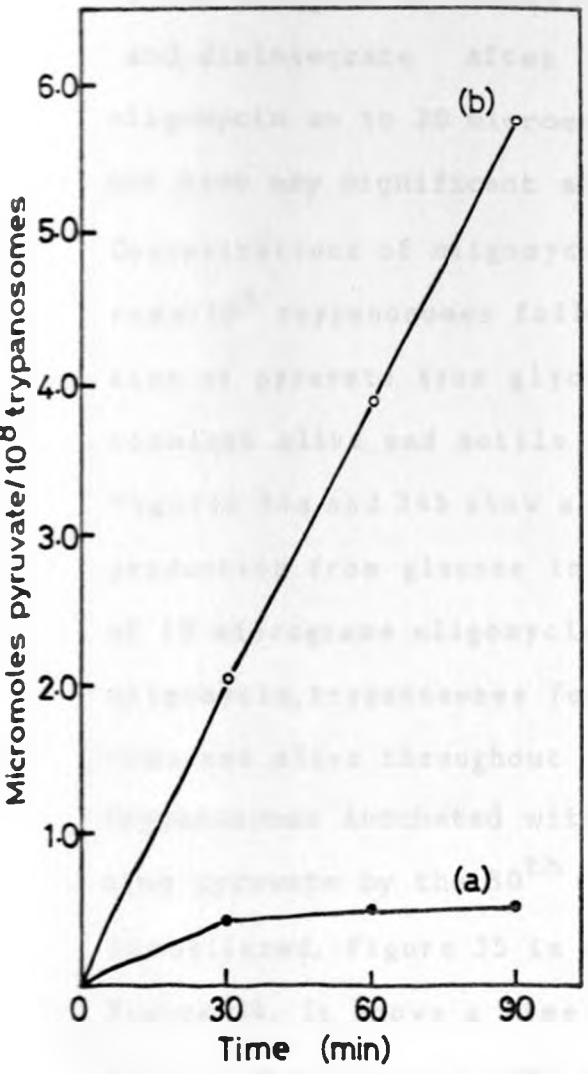
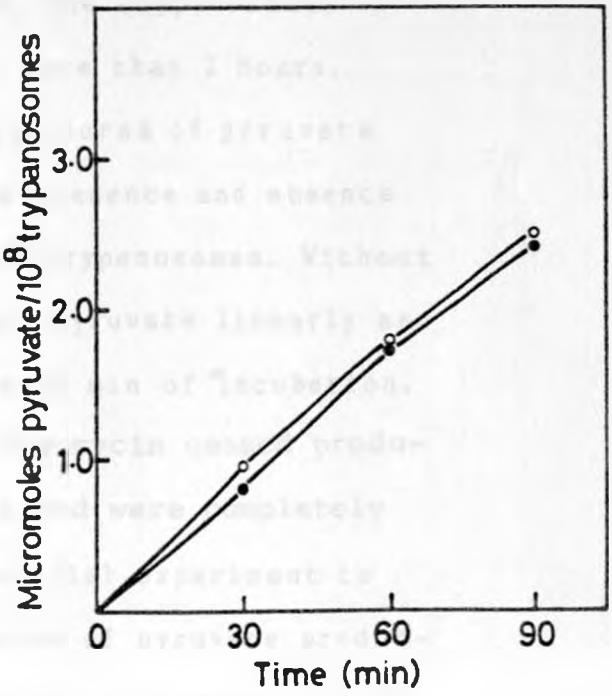
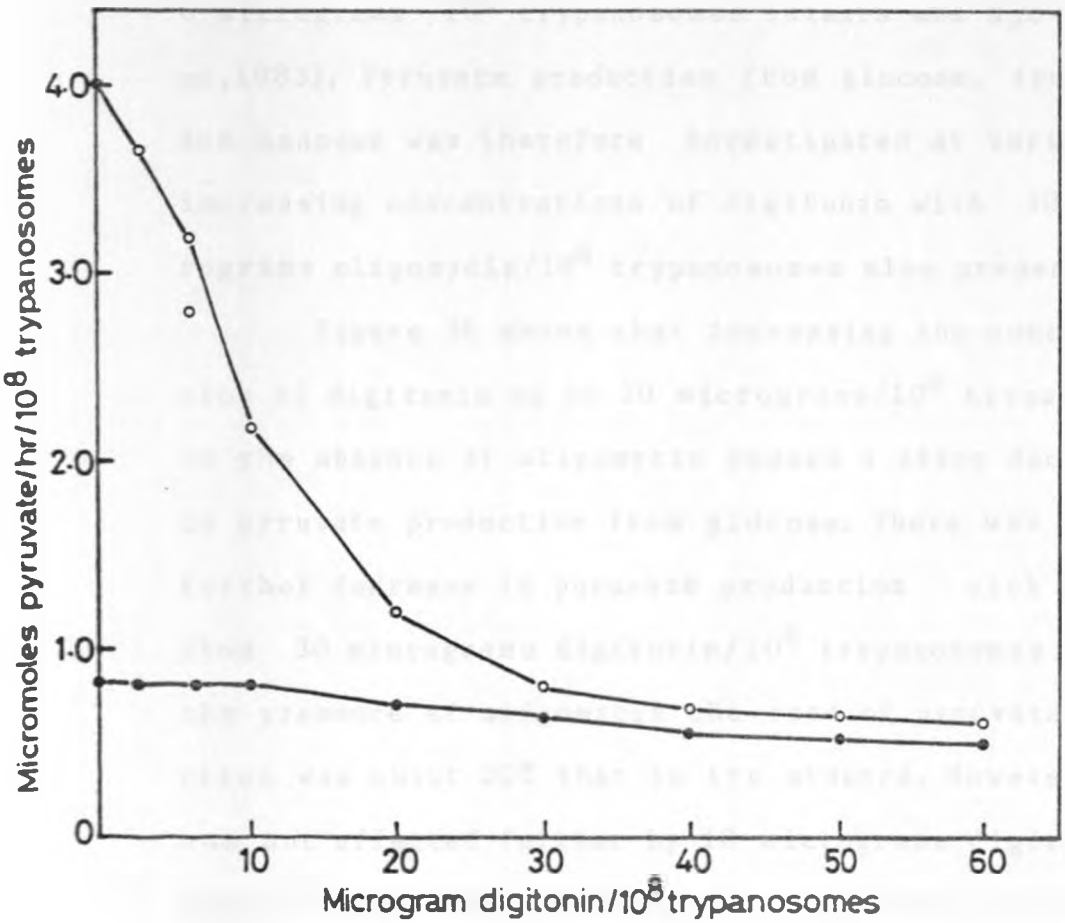


Figure 35



oligomycin/10^8 trypanosomes. The trypanosomes incubated with this concentration of oligomycin appeared sluggish or immobilized. They started to swell and disintegrate after 30 min. Addition of more oligomycin up to 20 micrograms/10^8 trypanosomes did not have any significant additional effect. Concentrations of oligomycin as high as 20 micrograms/10^8 trypanosomes failed to inhibit the production of pyruvate from glycerol. The trypanosomes remained alive and motile for more than 2 hours. Figures 34a and 34b show a time course of pyruvate production from glucose in the presence and absence of 10 micrograms oligomycin/10^8 trypanosomes. Without oligomycin, trypanosomes formed pyruvate linearly and remained alive throughout the 90 min of incubation. Trypanosomes incubated with oligomycin ceased producing pyruvate by the 30^{th} min and were completely immobilized. Figure 35 is a parallel experiment to Figure 34. It shows a time course of pyruvate production from glycerol in the presence and absence of 10 micrograms oligomycin/10^8 trypanosomes. In this case oligomycin had no effect and the trypanosomes were alive and motile throughout 90 min of incubation in its presence and absence. These results suggest that oligomycin inhibits a common step in the pathways of metabolism of glucose, fructose and mannose but not

Figure 36: Effect of increasing the concentration of digitonin on pyruvate production from glucose by **T.b.brucei** in the presence and absence of oligomycin. O-O and ●-● indicates pyruvate production without and with oligomycin respectively. Each incubation contained 10 mM glucose and 10 micrograms oligomycin/10^8 trypanosomes.

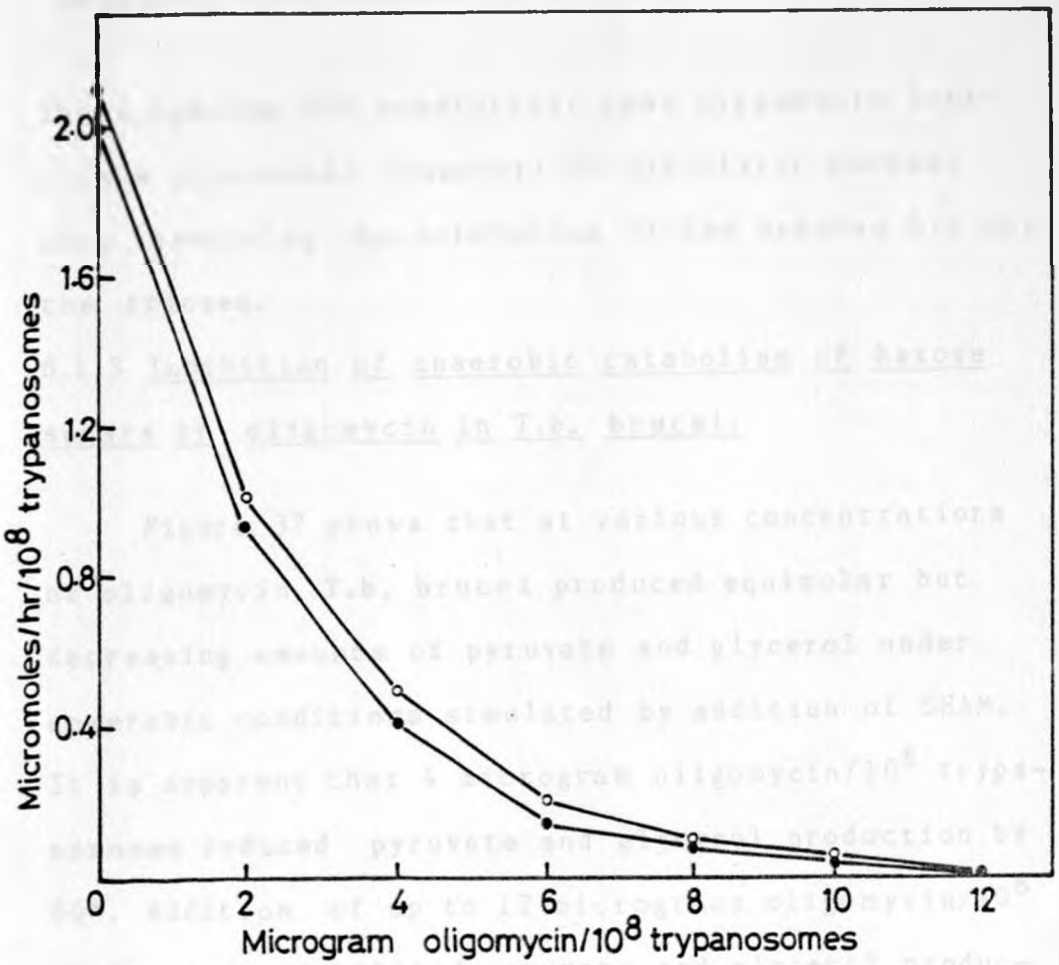

Micromoles pyruvate/hr/10^8 trypanosomes
4·0
3·0
2·0
1·0
0
10
20
30
40
50
60
Microgram digitonin/10^8 trypanosomes

glycerol. It was at first considered that this step might be the transport system(s) for the hexose sugars at the plasma membrane.

The glucose permeability barrier in **T.b. brucei** can be abolished by digitonin at a concentration of 6 micrograms /10^8 trypanosomes (Kiaira and Njogu,1983). Pyruvate production from glucose, fructose, and mannose was therefore investigated at various increasing concentrations of digitonin with 10 micrograms oligomycin/10^8 trypanosomes also present.

Figure 36 shows that increasing the concentration of digitonin up to 20 micrograms/10^8 trypanosomes in the absence of oligomycin caused a steep decrease in pyruvate production from glucose. There was no further decrease in puruvate production with more than 30 micrograms digitonin/10^8 trypanosomes . In the presence of oligomycin the rate of pyruvate production was about 20% that in its absence. However, this was not affected further by 10 micrograms digitonin/10^8 trypanosomes. This was in contrast to incubations without oligomycin where the same concentration of digitonin caused about 80% decrease in pyruvate production. Similar results to these were obtained with fructose or mannose as substrates instead of glucose. These results do not support the idea that oligomycin inhibits the transport of glucose, fructose,

Figure 37: Effect of various concentrations of oligomycin on pyruvate and glycerol production from glucose catabolism inthe presence of SHAM. Trypanosomes were incubated with 10 mM glucose and 2 mM SHAM at the concentrations of oligomycin shown. O-O and ●-● indicates pyruvate and glycerol production respectively.


Micromoles/hr/10^8 trypanosomes
2.0
1.6
1.2
0.8
0.4
0
2
4
6
8
10
12
Microgram oligomycin/10^8 trypanosomes

or mannose across the plasma membrane. If it did, the rates of production of pyruvate from these substrates in the presence and absence of oligomycin should be similar after the addition of sufficient digitonin that permeabilize the plasma membrane.

There remains the possibility that oligomycin inhibits a glycosomal transport or glycolytic pathway step involving the catabolism of the hexoses but not the trioses.

6.1.3 Inhibition of anaerobic catabolism of hexose sugars by oligomycin in T.b. brucei.

Figure 37 shows that at various concentrations of oligomycin, T.b. brucei produced equimolar but decreasing amounts of pyruvate and glycerol under anaerobic conditions simulated by addition of SHAM. It is apparent that 4 microgram oligomycin/10^8 trypanosomes reduced pyruvate and glycerol production by 80%. Addition of up to 12 micrograms oligomycin/10^8 trypanosomes inhibited pyruvate and glycerol production completely. Similar results were obtained with mannose as substrate but when the experiment was repeated with fructose, pyruvate and glycerol production were completely inhibited by 8 micrograms oligomycin/10^8 trypanosomes (Figure 38). All the trypano-

Figure 38: Effect of various concentrations of oligomycin on pyruvate and glycerol production from fructose catabolism in the presence of SHAM. The trypanosomes were incubated with 10 mM fructose and 2 mM SHAM in buffer A at the concentrations of oligomycin shown. O-O indicates pyruvate and ●-● glycerol prodution.

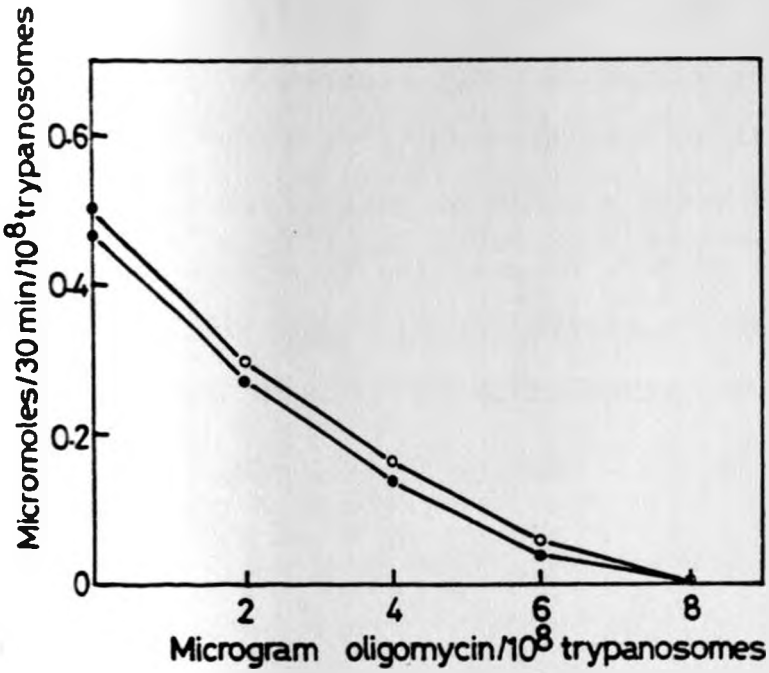
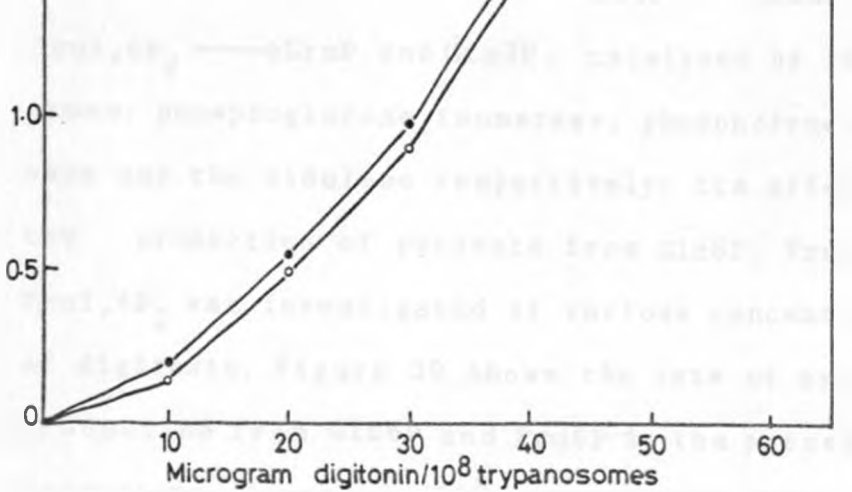
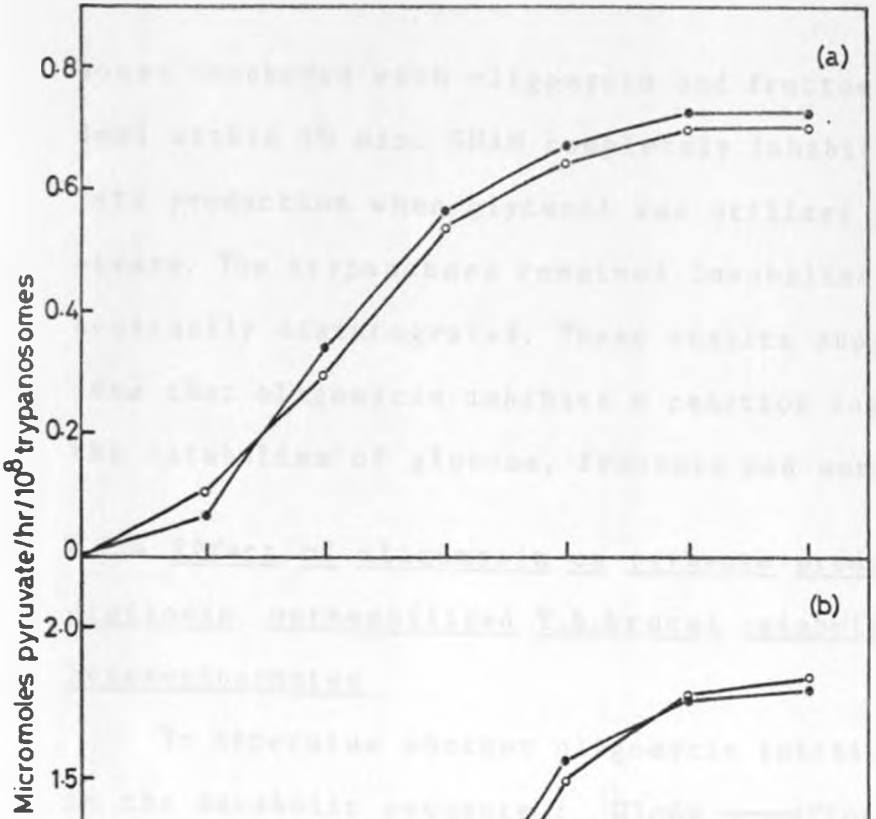

Micromoles/30 min/10^8 trypanosomes
0.6
0.4
0.2
0
2
4
6
8
Microgram oligomycin/10^8 trypanosomes

Figure 39: Pyruvate production from Glc6P and Fru6P at various concentrations of digitonin in T.b.brucei in the presence and absence of oligomycin. The concentrations of Glc6P, Fru6P and ATP were 10, 30, and 2.5 mM respectively. Oligomycin was present at 10 micrograms/10^8 trypanosomes. O-O and ●-● shows pyruvate production in the presence and absence of oligomycin. Figure 39a shows Glc6P utilization and Figure 39b shows Fru6P utilization.


1·0
0·5
0
10
20
30
40
50
60
Microgram digitonin/10^8 trypanosomes


(a)
(b)
0·8
0·6
0·4
0·2
0
2·0
1·5
Micromoles pyruvate/hr/10⁸ trypanosomes

somes incubated with oligomycin and fructose were dead within 10 min. SHAM completely inhibited pyruvate production when glycerol was utilized as substrate. The trypanosmes remained immobilised and eventually disintegrated. These results support the idea that oligomycin inhibits a reaction common in the catabolism of glucose, fructose and mannose.

6.1.4 Effect of oligomycin on pyruvate production in digitonin permeabilized T.b.brucei catabolising the hexosephosphates

To determine whether oligomycin inhibits a step in the metabolic sequence : Glc6P ⟶ Fru6P ⟶ Fru1,6P_2 ⟶ GrnP and Gra3P, catalysed by the enzymes; phosphoglucose isomerase, phosphofructokinase and the aldolase respectively; its effect on the production of pyruvate from Glc6P, Fru6P and Fru1,6P_2 was investigated at various concentrations of digitonin. Figure 39 shows the rate of pyruvate production from Glc6P and Fru6P in the presence of 10 micrograms oligomycin/10^8 trypanosomes and at various concentrations of digitonin. Pyruvate production in the presence of oligomycin was approximately the same as in its absence, at all the concentations of digitonin tested. Similar results were obtained with Fru1,6P_2 as substrate. It was concluded that oligomycin does not directly inhibit any of the three

Figure 40: A time course of the changes in the levels of Glc6P and Fru6P from glucose catabolism after the addition of oligomycin. Trypanosomes were incubated with 10 mM glucose in several 25 ml Erlenmeyer flasks. The experiment was started by rapid addition of 10 micrograms oligomycin/10^8 trypanosomes after immediate shaking. Incubations were terminated at the times shown.

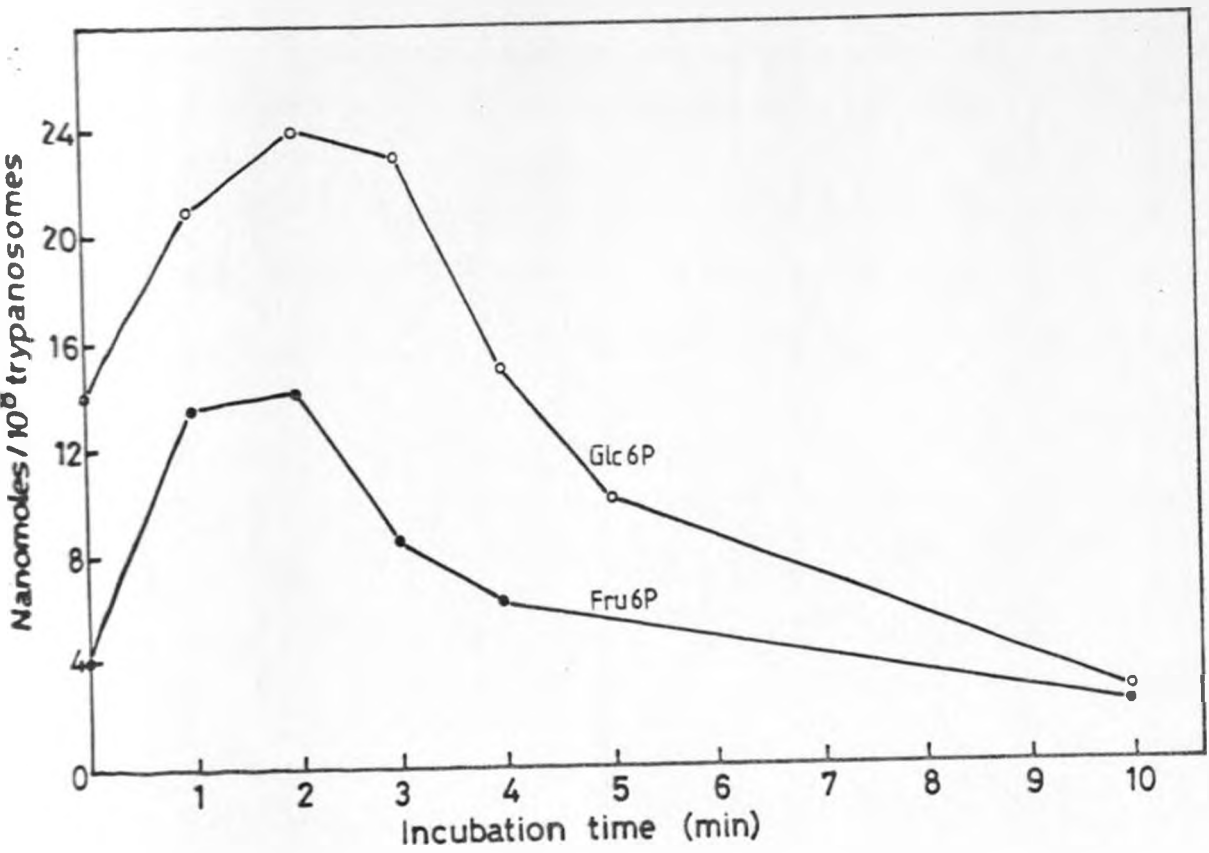
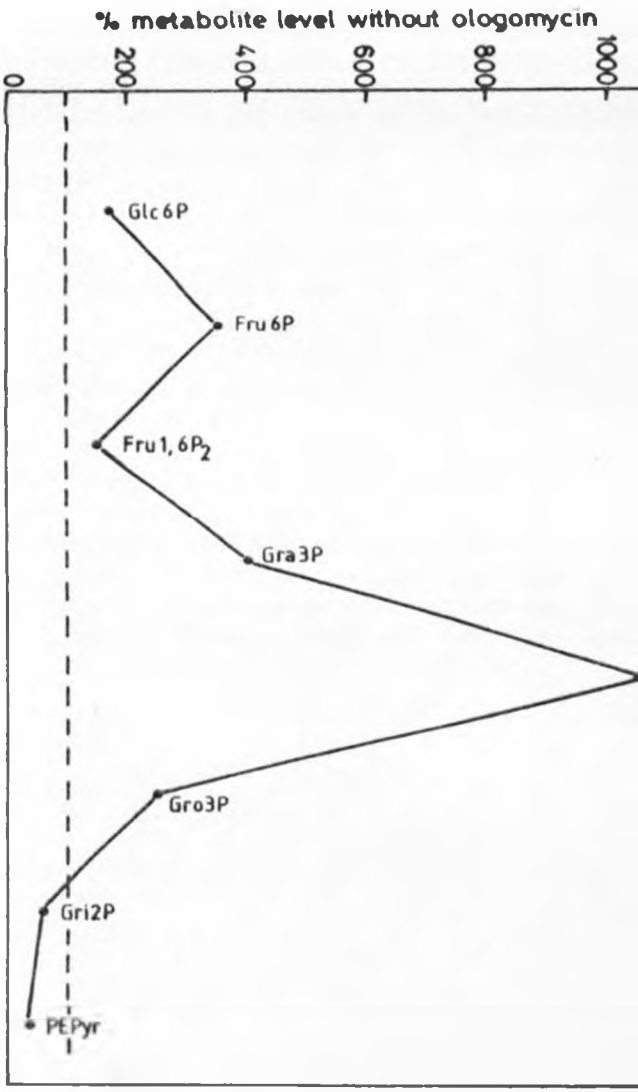

Nanomoles / 10^8 trypanosomes
24
20
16
12
8
4
0
1
2
3
4
5
6
7
8
9
10
Incubation time (min)
Glc 6P
Fru 6P

Figure 41: Cross-over plot for glycolytic intermediates in **T.b.brucei** incubated with oligomycin. Experimental procedure was as described in the legend of figure 40 except that the incubation time was 2 min. The levels of intermediates in the presence of oligomycin are expressed as percentage of those values in its absence.


% metabolite level without ologomycin
0
200
400
600
800
1000
Glc6P
Fru6P
Fru1,6P2
Gra3P
Gro3P
Gri2P
PEPyr

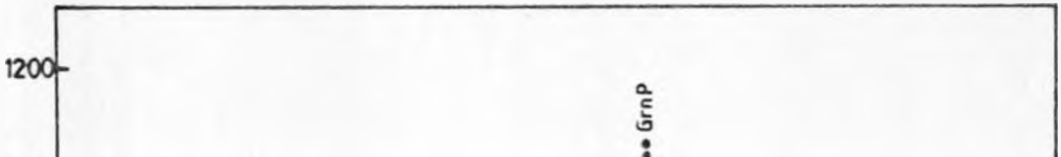

1200
GrnP

Table XI: Effect of oligomycin on the levels of some glycolytic intermediates in **T.b.brucei** catabolising glycerol. The concentration of oligomycin was 10 micrograms/10^8 trypanosomes whereas that of glycerol was 10 mM. The incubation time was 2 min. Results are in nanomoles/10^8 trypanosomes and are expressed as mean $\pm$ standard deviation from 4 separate experiments. ND indicates 'not detected'.

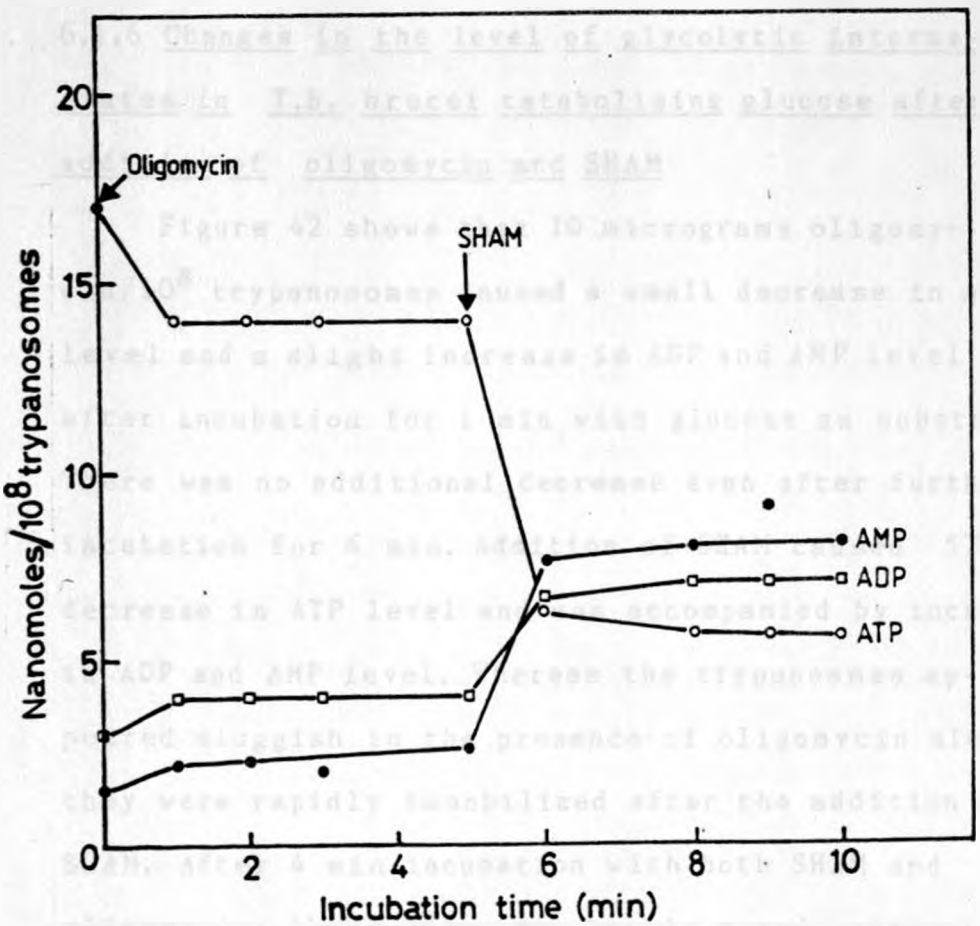
Metabolite	No oligomycin	plus oligomycin
Glc6P	ND	ND
Fru6P	ND	ND
Fru1,6P_2	0.3±0.1	0.2±0.11
Gra3P	0.7±0.2	0.76±0.19
GrnP	1.0±0.1	1.2±0.1
Gri2P	110±15	114±14
PEPyr	27±6	29±5

enzyme reactions mentioned above.

6.1.5 Aerobic glycolysis : changes of the cellular concentrations of glycolytic intermediates in T.b.brucei after the addition of oligomycin.

Figure 40 shows a time course of the levels of Glc6P and Fru6P in **T.b. brucei** catabolising glucose after the addition of oligomycin. The level of Fru1,6P_2, Gra3P, GrnP and Gro3P also increased rapidly to a maximum within 2-3 minutes and then decreased gradually as observed with Glc6p and Fru6P. Gri2P and PEPyr levels decreased after the addition of oligomycin. Figure 41 shows the cross-over plots for the glycolytic intermediates in **T.b.brucei** after incubation for 2 min with oligomycin.It shows a cross-over in the sequence of reactions between Gro3P and Gri2P formation. This suggests that oligomycin inhibits a glycolytic step occurring after the formation of Gro3p from the hexoses catabolism. Table XI shows that that levels of GrnP, Gra3P, Gro3P, Gri2P, PEPyr in **T.b.brucei** catabolising glycerol aerobically, and incubated with and without oligomycin were almost identical in contrast to the results with glucose as substrate. The trypanosomes could respire also on glycerol even in the presence of oligomycin. These results from glycerol catabolism indicate that although oligomycin inhibits respiration on

Figure 42: Changes in the level of adenine nucleotides in **T.b.brucei** after the addition of oligomycin followed by SHAM. The trypanosomes were incubated in buffer A containing 10 mM glucose and 10 micrograms oligomycin/10^8 trypanosomes during the first 5 min after which 2 mM SHAM was added.

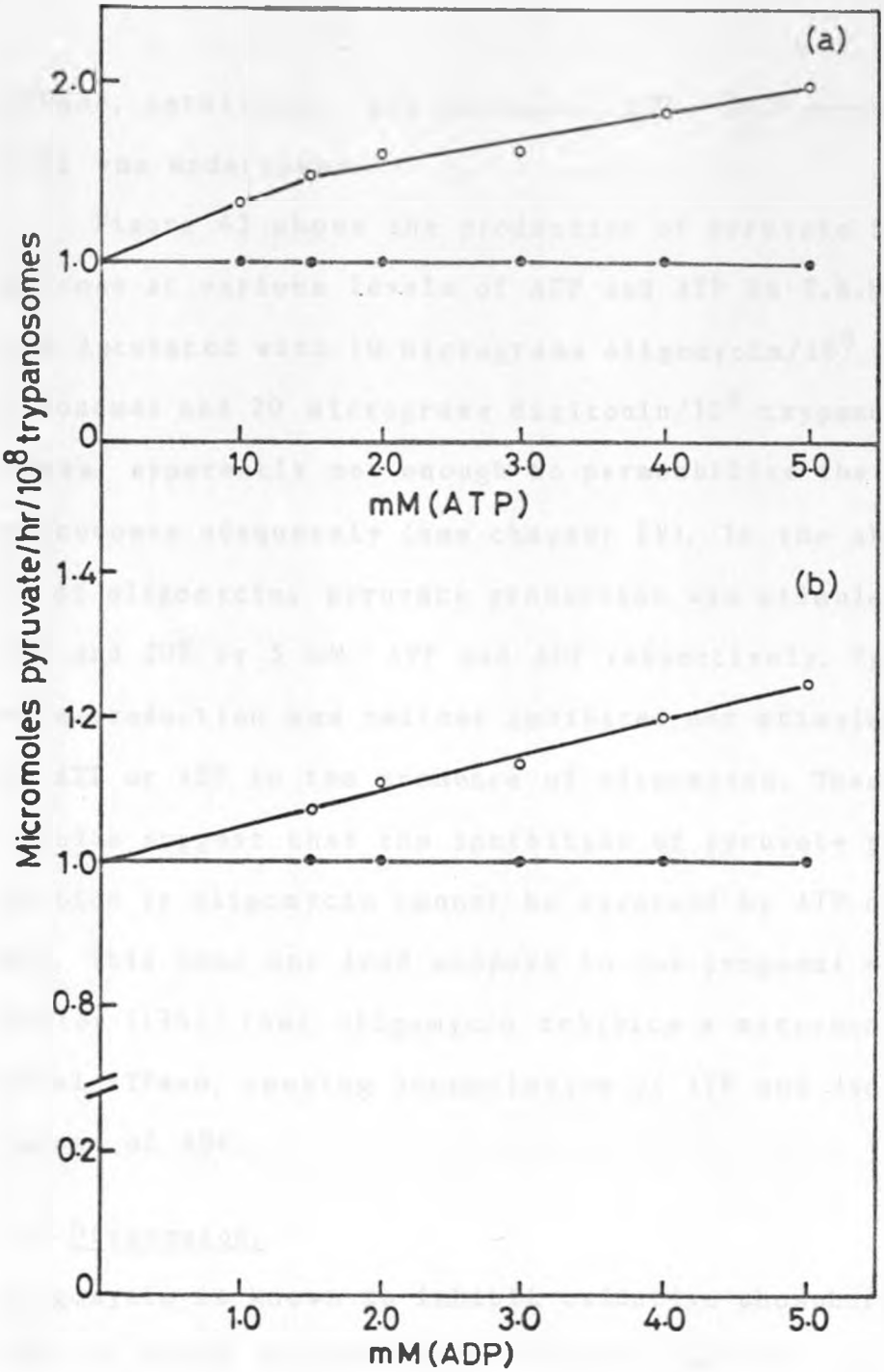

Oligomycin
SHAM
AMP
ADP
ATP
Nanomoles/10^8 trypanosomes
Incubation time (min)
0
5
10
15
20
2
4
6
8
10

glucose (Miller and Klein, 1980), it does not inhibit GPO, glycerol 3-phosphate dehydrogenase, triosephosphate isomerase, glyceraldehye 3-phosphate dehydrogenase, phosphoglycerate kinase, enolase, phosphoglyceromutase and pyruvate kinase.

6.1.6 Changes in the level of glycolytic intermediates in T.b. brucei catabolising glucose after the addition of oligomycin and SHAM

Figure 42 shows that 10 micrograms oligomycin/10^8 trypanosomes caused a small decrease in ATP level and a slight increase in ADP and AMP level after incubation for 1 min with glucose as substrate. There was no additional decrease even after further incubation for 4 min. Addition of SHAM caused 57% decrease in ATP level and was accompanied by increase in ADP and AMP level. Whereas the trypanosmes appeared sluggish in the presence of oligomycin alone, they were rapidly immobilized after the addition of SHAM. After 4 min incubation with both SHAM and oligomycin, these organisms barely moved indicating that SHAM augmented the toxicity of oligomycin. It could be suggested that in the presence of oligomycin, there was slow ATP synthesis not sufficient to sustain trypanosome motility for prolonged period. An indirect search into the possibility that oligomycin restricts ADP availability by inhibiting a Mg-

Figure 43: Effect of ATP and ADP on pyruvate production in **T.b.brucei** partially permeabilized by digitonin in the presence of oligomycin. O-O indicates pyruvate production at various concentrations of ATP or ADP in the absence of oligomycin. ●-● indicates pyruvate production at various concentrations of ATP or ADP in the presence of oligomycin. The concentrations of digitonin and oligomycin were 20 and 10 micrograms/10^8 trypanosomes. Glucose concentration in buffer A was 10 mM.


(a)
2·0
1·0
0
1·0
2·0
3·0
4·0
5·0
mM (ATP)
(b)
1·4
1·2
1·0
0·8
0·2
0
1·0
2·0
3·0
4·0
5·0
mM (ADP)
Micromoles pyruvate/hr/10⁸ trypanosomes

ATPase, catalising the reaction; ATP + H_2O ⟶ ADP + Pi was undertaken.

Figure 43 shows the production of pyruvate from glucose at various levels of ADP and ATP in T.b.brucei incubated with 10 micrograms oligomycin/10^8 trypanosomes and 20 micrograms digitonin/10^8 trypanosomes apparently not enough to permeabilize the glycosomes adequately (see chapter IV). In the absence of oligomycin, pyruvate production was stimulated 100 and 20% by 5 mM ATP and ADP respectively. Pyruvate production was neither inhibited nor stimulated by ATP or ADP in the presence of oligomycin. These results suggest that the inhibition of pyruvate production by oligomycin cannot be reversed by ATP or ADP. This does not lend support to the proposal of Miller (1981) that oligomycin inhibits a mitochondrial ATPase, causing accumulation of ATP and decrease of ADP.

6.2 Discussion.

Oligomycin is known to inhibit oxidative phosphorylation in intact mitochondria from most aerobic cells. It binds to the protein that links F_1 ATPase to the membrane and presumably prevents the normal inward movement of protons to the mitochondrial matrix that result in ATP formation at the inner membrane (Rac-

ker, 1970; Senior, 1973). Opperdoes et al (1976) showed the presence of an oligomycin sensitive ATPase in the bloodstream forms of **T.b.brucei**. They argued that this enzyme may be linked with fly transmissibility because **T.evansi**, not transmitted by the tsetse vector, had negligible activity of the enzyme. So far no other enzyme has been shown unequivocally to be sensitive to oligomycin.

The observation by Miller and Klein (1980) that oligomycin could inhibit pyruvate production and the uptake of O_2 and Ca^{2+} by the bloodstream trypomastigotes of **T.b.brucei** was rather surprising because these organisms do not have cytochromes or a functional TCA cycle (Ryley, 1956; Fulton and Spooner, 1959).

From figures 33, 34 and 35, it can be concluded that oligomycin inhibits a reaction that is common to the pathways of metabolism of glucose,fructose and mannose, but not glycerol. Phosphoglucose isomerase and phosphomannose isomerase activities are unlikely to be affected because the catabolism of fructose, which by-passes the involvement of these enzymes was also inhibited. The inhibition of hexose catabolism was not complete; this contrasts with the findings of Miller and Klein (1980) that the production of pyruvate from glucose and from glycerol was inhibited

100% and about 50% respectively. It may be that these workers used a dying population of trypanosomes, for the slower rate of pyruvate production they observed was only 30% of that found in the present study. The results shown in Figure 39 indicate that oligomycin does not inhibit hexokinase, phosphoglucose isomerase, 6-phosphofructokinase, and aldolase. Considering that pyruvate production from glycerol in intact trypanosomes was not inhibited, it is concluded that none of the enzymes for catabolism of the triose phosphates could have been inhibited by oligomycin (See scheme IVA). It can also be concluded that oligomycin does not inhibit enzymes of the Embden-Meyerhof pathway in **T.b.brucei**. Miller (1981) proposed that oligomycin inhibits the ATPase resulting in restriction of ADP and Pi production. This consequently implied that an accumulation of ATP and PEPyr would occur in the glycosome. The accumulated PEPyr and ATP would inhibit 6-phosphofructokinase, thus blocking glycolysis. This appeared able to explain why glycolysis could be inhibited to a larger extent during glucose catabolism (Miller and Klein, 1980). In this investigation it was found however that there was an increase of Frul,$6P_2$, Gra3P, GrnP, and Gro3P (Figure 41) which should have decreased according to the explanation of Miller (1980). In

addition oligomycin caused a decrease of ATP (Figure 42), PEPyr and Gri2P (Figure 41) and an increase of ADP. Also there was no increase in glycolytic rate in the presence of oligomycin after the addition of ADP, which has been shown in this study to have access to the glycolytic enzymes (Chapter IV). This finding indicates that the observed toxicity of oligomycin was not due to lack of ADP but due to inhibition of another/other site(s) of metabolism. It appears unlikely that the inhibition of glycolysis is due to a block of the oligomycin sensitive ATPase alone. If it was, pyruvate production should have been equally inhibited during the catabolism of glycerol as of glucose. It is possible that oligomycin may inhibit the mitochondrial ATPase and Ca^{2+} uptake in the mitochondrion as reported by Miller and Klein (1980), but this inhibition is not lethal to the trypanosomes because they survived for several hours on glycerol without decrease in motility. Oligomycin must therefore inhibit more strongly at another metabolic step which is associated with hexose sugar but not with the triose sugar catabolism.

Oligomycin caused an increase of Glc6P, Fru6P, Fru1,6P_2, Gra3P, GrnP and Gro3P level during catabolism of glucose(Figure 41) whereas it did not affect the level of the same intermediates during glycerol

catabolism (Table XI). This observation suggests that oligomycin inhibits Gro3P utilization only during the catabolism of the hexoses. Considering that oligomycin does not inhibit Gro3P dehydrogenase or GPO, as discussed above; the results of this study may be explained as follows.

Firstly, oligomycin could not have inhibited the transport of Pi across the glycosomal membrane (scheme IVA); if it did the levels of the glycolytic intermediates; Gra3P, GrnP, Frul,$6P_2$, Fru6P, Glc6P, should have decreased but not Gro3P, during both glucose and glycerol catabolism, On the contrary these intermediates and Gro3P increased during glucose catabolism. The triosephosphates from glycerol catabolism should have decreased except Gro3P, GrnP and Gra3P which should have increased (Table XI). It is apparent from these results that oligomycin does not inhibit the transport of Pi across the glycosomal membrane.

Secondly, oligomycin could not have inhibited the transport of Gri3P from the glycosome (scheme IVA). We would expect the levels of Gro3P, Grnp and Gra3P together with Gri3P and Gril,$3P_2$ to have increased from the catabolism of either glucose or glycerol. Since the levels of these intermediates from glycerol catabolism were not affected by the addition

of oligomycin, a carrier to facilitate an exchange of Gri3P with glycerol might appear operational but considering that glycerol is not required in glucose utilization Gri3P is rapidly produced and metabolised to pyruvate, the inhibition of Gri3P transport by oligomcin appears unlikely.

Thirdly, it could also be suggested that glycolysis occurs in both the glycosome and cytosol, and that oligomycin inhibits glycosomal glycolysis. This suggestion requires , sufficient activity of the enzymes ; glycerol kinase, Gro3P dehydrogenase, triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, and pyruvate kinase to be cytosolic. All the activity of phosphoglycerate mutase, enolase, and pyruvate kinase is cytosolic (Oduro et al,1980a,b). There is no evidence for cytosolic activity of glycerol kinase, and Gro3P dehydrogenase that could explain glycerol utilization.Infact the gene coding for cytosolic triosephosphate isomerase is absent in the trypanosomes (Swinkels et al; Osinga et al,1985), and there is no significant activity for the cytosolic isoenzymes of glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase (Misset et al ,1987). This apparent lack of significant activities of cytosolic enzymes that could

catalyse glycerol utilization does not support that glycerol catabolism occurs exclusively in the cytosol.

In addition the above suggestion does not explain why oligomycin caused the accumulation of glycolytic intermediates from hexose sugar catabolism (Figure 41).

Fourthly, it could be suggested that the novel Gro3P: glucose phosphotransferase proposed in chapter V (scheme VII) is a multienzyme complex and it catalyses the transport of hexoses and not glycerol into the glycosome. Glycerol might cross the membrane by passive diffusion. This would explain the inhibition of both aerobic and anaerobic catabolism of the hexoses to puruvate and lack of it during aerobic glycerol catabolism (Figures 33 and 35). The lack of glycerol formation despite the increased level of Gro3P after the addition of oligomycin (Figure 41) could be due to insufficient levels of Gro3P (Figure 41), ADP, and AMP (Figure 42). It could be that glycerol formation by the suggested novel enzyme, Gro3P: glucose phosphotransferase is regulated by the levels of ADP, ATP, and AMP. Whereas the above suggestion appears able to explain some observations made in this study, it does not account for the increase in Glc6P , Fru6P , Frul,$6P_2$, Grnp, Gra3P and Gro3P and the decrease in Gri2P and PEPyr as a result

of addition of oligomycin. The levels of these intermediates should have decreased if oligomycin inhibited the transport of hexose sugars. It appears from the glycolytic intermediates and the inhibition of both aerobic and anaerobic glycolysis that the novel Gro3P: glucose phosphotransferase is neither inhibited by oligomycin nor involved in hexose sugar transport.

Fifthly, oligomycin could inhibit the transport of Gro3P from the glycosome. I suggest the presence of a carrier sensitive to oligomycin that would transfer Gro3P from the glycosome to the cytosol. The carrier is envisaged to have a substrate binding site (S) and a regulatory site (R). Oligomycin could bind to (R). This would inhibit the binding of Gro3P at (S) by steric hindrance. Consequently Gro3P efflux would be inhibited. Glycerol could therefore relieve the inhibition of Gro3P efflux by displacing oligomycin at (R). The binding of glycerol at (R) would therefore appear to be competitive with respect to oligomycin. This idea could account for the lack of inhibition of pyruvate production by oligomycin from glycerol catabolism. The suggested carrier is distinct from the GrnP/Gro3P shuttle system speculated by Opperdoes and Borst(1977) and shown in scheme IVA. However, under aerobic conditions without oli-

gomycin , the traffic at the glycosomal membrane due to the GrnP and Gro3P carriers should be identical to GrnP/Gro3P shuttle system shown in scheme IVA. The Gro3P carrier was also suggested from other evidence in chapter V. From the above proposal it can be deduced that when the Gro3P carrier is inhibited , all the glycolytic intermediates that are precursors of Gro3P should accumulate inside the glycosome whereas other triose intermediates, Gril,$^{3P}_{2}$, Gri3P , Gri2P and PEPyr should decrease. This deduction is consistent with the results shown in Figure 41. The decrease of Gro3P precursors after the initial increase (Figure 40) is probably due to incomplete inhibition of the carrier by oligomycin.

The fifth suggestion appears able to account more adequately for the lack of inhibition by oligomycin during glycerol catabolism and the inhibitions by oligomycin during the catabolism of the hexose sugars. However, details of he mechanism of inhibition and the characteristics of the protein/enzyme inhibited remain to be established.

6.3 Conclusions

The following conclusions may be made from this study.

(a) Inhibition of the Mg-ATPase by oligomycin during glycerol catabolism is not lethal to the trypano-

somes. Therefore oligomycin must inhibit another site of metabolism.

(b) Although oligomycin inhibits the catabolism of glucose, fructose and mannose, it does not inhibit their uptake.

(c) Oligomycin sensitivity of the glycolytic pathway is associated with trypanosome membrane(s) integrity.

(d) Oligomycin does not specifically inhibit the the novel enzyme Gro3P: glucose phosphotransferase suggested in chapter V, because it inhibited both aerobic pyruvate production and the anaerobic glycerol and pyruvate production from the catabolism of glucose, fructose, and mannose.

(e) Oligomycin probably inhibits a carrier for metabolite transport across the glycosomal membrane.

CHAPTER VII

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