"AMINO ACID METABOLISM IN BLOODSTREAM

TRYPANOSOMA BRUCEI BRUCEI"

By

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I, Jacques Muturi Kabaru, hereby declare that this thesis is my original work and has not been presented for a degree in any other University.

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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Prof. R.M. Njogu my first supervisor for introducing me into scientific research and for his constant encouragement and help during the study. I owe my interest in scientific research to him.

I wish also to thank Professor G. Maina my second supervisor for his encouragement during my period of study.

I am indebted to the following: Mr. J.K. Kiairu for his constructive criticism during the study and for the help offered during the preparation of this thesis, Dr. L. Mutharia and Mr. V.N. Konji for correcting my draft copy, Mr. Lavi of Nuclear Center, University of Nairobi for providing me with liquid nitrogen, my fellow postgraduate students for their useful discussions, all the technical staff of the Biochemistry Department who contributed in many ways to my work and to Ms. G.W. Gikonyo for neatly typing the manuscript.

This thesis is dedicated to my parents, brothers and sisters whose patience and support have constantly inspired me throughout the course of this study.

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ABBREVIATIONS

ADP	Adenosine 5' diphosphate
ATP	Adenosine 5' triphosphate
ADH	Alanine dehydrogenase
NH_3	Ammonia
CoASH	Coenzyme A
DEAE	Diethylaminoethyl cellulose
DHAP	Dihydroxyacetone phosphate
K_2HPO_4	Dipotassium hydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate
DTT	Dithiothreitol
EATRO	East African Trypanosomiasis Research Organization
EC	Enzyme Commission
EDTA	Ethylene diamintetra acetic aci
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GLDH	Glutamate dehydrogenase
GOT	Glutamate oxaloacetate transaminase
GPT	Glutamate pyruvate transaminase
α-GPDH	α-Glycerol phosphate dehydrogenase
HK	Hexokinase
HCl	Hydrochloric acid
ICIPE	International Centre for Insect Physiology and Ecology
LDH	Lactate dehydrogenase

$MgCl_2$	Magnesium chloride
MDH	Malate dehydrogenase
M.E	Malic enzyme
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
OAA	Oxaloacetic acid
PCA	Perchloric acid
PS	Phosphate buffer
PSG	Phosphate buffer containing glucose
PEP	Phosphenol pyruvate
KCl	Potassium chloride
PK	Pyruvate kinase
NaCl	Sodium chloride
NaOH	Sodium hydroxide
Tris	Tris (hydroxymethyl) aminomethane

UNIT ABBREVIATIONS

cm	Centimetre
gm	Gram
g	Gravity
hr	Hour
I.U	International Units in micromoles/minute
M	Molar
mA	Milliampere
mg	Milligram
min	Min

ml	Millilitre
mM	Millimolar
nm	Nanometre
pH	-Log H^+ concentration
sec	Second
μg	Microgram
μl	Microlitre
μmole	Micromole
V	Volt
v/v	Volume/volume
w/v	Weight/volume

PREFIXES

Multiple	Prefix	Abbreviation
10^{-3}	Milli	m
10^{-6}	Micro	μ
10^{-9}	Nano	n

SUMMARY

The main purpose of this study was to investigate key enzymes involved in amino acid metabolism in bloodstream Trypanosoma brucei brucei and the leakage of some of these enzymes into the plasma of infected rats.

Using glutamate pyruvate transaminase (GPT) (EC 2.6.1.2) as a cytosolic marker and α-glycerolphosphate dehydrogenase (α-GPDH) (EC 1.1.1.8) as a glycosomal marker, the intracellular location of glutamate oxaloacetate transaminase (GOT) (EC 2.6.1.1), NAD-linked malate dehydrogenase (NAD-linked MDH) (EC 1.1.1.37), NADP-linked malic enzyme (NADP-linked ME) (EC 1.1.1.40) and glucose-6-phosphate dehydrogenase (G6PDH) (EC 1.1.1.49) in bloodstream T. b. brucei was investigated. This was done by determining the release of these enzymes from isolated bloodstream trypanosomes by increasing cycles of freeze-thawing or increasing concentrations of Triton X-100 and Digitonin.

In one set of experiments, the release of GPT, GOT, NAD-linked MDH and α-GPDH was studied with 10^8 trypanosomes/ml of PSG. To release maximal activity of GPT, GOT, and NAD^+-linked MDH, 2 cycles of freeze-thawing, 0.04% Triton X-100 or 140 μg Digitonin/10^8 trypano-

somes was required. Six freeze-thawing cycles or 0.07% Triton X-100 released maximal activity of ∝-GPDH. The maximum concentration of Digitonin used (280 µg/10^8 trypanosomes) did not release maximal activity of ∝-GPDH from the trypanosomes. It was concluded that GOT and NAD-linked MDH were cytosolic since they were released in a pattern similar to that of the cytosolic marker GPT.

In another set of experiments, the release of GPT, G6PDH, NADP-linked ME and ∝-GPDH was studied with 5 x 10^8 trypanosomes/ml PSG. To release maximal activity of GPT and G6PDH, 2 cycles of freeze-thawing, 0.04% Triton X-100 or 170 µg Digitonin/5 x 10^8 trypanosomes was required. To release the maximal activity of NADP-linked ME, 2 cycles of freeze-thawing, 0.04% Triton X-100 or 226 µg Digitonin/5 x 10^8 trypanosomes was required. The maximum concentration of Digitonin used (280 µg/ 5 x 10^8 trypanosomes) did not release maximal activity of ∝-GPDH from the trypanosomes. It was concluded that G6PDH was cytosolic since it was released in a pattern similar to that of the cytosolic marker GPT. The localisation of NADP-linked ME could not be established conclusively.

The plasma levels of GPT, GOT, Pyruvate Kinase (PK) (EC 2.7.1.40) ∝-GPDH and lactate dehydrogenase

(LDH) (EC 1.1.1.27) were measured in healthy and T. b. brucei infected rats. At a parasitemia of approximately 10^8 parasites/ml blood, the plasma level of GPT was elevated 4 - 5 fold; GOT, 2 - 3 fold; PK, 3 fold; ∝GPDH, 2 fold but the level of LDH was not altered. It was proposed that the additional enzyme activity in plasma during T.b. brucei was of trypanosomal origin. To confirm the origin of additional enzyme activities in plasma of T.b. brucei -infected rats, starch gel electrophoresis of healthy and infected rat serum was done. Parallel experiments were set up with parasite lysate. In addition to normal rat serum GPT and GOT, infected rat serum contained trypanosomal GPT and GOT. It was concluded that the elevated levels of these enzymes in serum of rats during infection was due to leakage from trypanosomes.

The activity of GPT in bloodstream T.b. brucei was 0.52 umoles/min/mg protein. The role of this enzyme in trypanosomes was investigated. Isolated trypanosomes were incubated in PSG containing 10 mM L-glutamate at 25°C. Alanine and ∝-ketoglutarate were produced in equimolar quantities and the amount of alanine produced was a function of glutamate concentration in the incubation medium. Paper chromatography analysis of post-incubation medium showed alanine to be the only amino acid produced

during the incubations. It was proposed that the role of GPT in bloodstream T. b. brucei is to convert some of the pyruvate generated in glycolysis to alanine.

The role of GOT in bloodstream T. b. brucei was also investigated. Trypanosomal GOT activity was 0.075 µmole/min/mg protein, NAD-linked MDH was 0.140 µmoles/min/mg protein and that of NADP-linked ME was 0.012 µmoles/min/mg protein. Trypanosomal lysates in P. incubated at $25^{o}C$ with 10 mM L-aspartate, 10 mM α-ketoglutarate and 5 mM NADH produced significant amounts of L-malate (0.51 ± 0.005 µmoles L-malate/hr/10^8 trypanosomes). It was speculated that GOT generates oxaloacetate from aspartate and α-ketoglutarate as shown below;

$$\text{Aspartate} + \alpha\text{-Ketoglutarate} \xrightleftharpoons{\text{GOT}} \text{Glutamate} + \text{Oxaloacetate}$$

The oxaloacetate then acts as a substrate for NAD-MDH generating malate by reduction consuming NADH as shown below;

$$\text{Oxaloacetate} + \text{NADH} \xrightleftharpoons{\text{NAD-linked MDH}} \text{NAD}^{+} + \text{Malate}$$

The malate so produced is oxidatively decarboxylated to pyruvate by NADP-linked ME generating NADPH as

shown below;

$$\text{Malate} + \text{NADP}^+ \underset{}{\overset{\text{NADP-linked ME}}{\rightleftarrows}} \text{Pyruvate} + \text{NADPH} + CO_2$$

It was further speculated that the most likely role of GOT in bloodstream T. b. brucei is involvement in a series of coupled reactions leading to transhydrogenation of NADH with $NADP^+$. The NADPH generated in this transhydrogenation would supplement that generated by G6PDH. The activity of G6PDH in bloodstream T. b. brucei was 0.012 µmoles/min/mg protein.

The presence of other trypanosomal aminotransferases was investigated using trypanosome lysates in the presence of 10 mM L-amino acid and 10 mM ∝-ketoglutarate at 25°C. The transamination activities obtained with various L-amino acids expressed as µmoles L-glutamate/hr/mg protein were; alanine, 1.44 ± 0.041; aspartate, 0.67 ± 0.012; glutamine, 0.33 ± 0.007; isoleucine, 1.1 ± 0.015; leucine, 1.02 ± 0.028, methionine, 0.65 ± 0.012; phenylalanine, 1.13 ± 0.022; tryptophan, 0.39 ± 0.014; tyrosine, 0.79 ± 0.014 and valine, 1.13 ± 0.013. Two speculations were derived from these results. Firstly was that in addition to the reported trypanosomal transaminases, there are novel non-specific transaminases acting on 10 amino acids with ∝-ketoglutarate

as the amino group acceptor. Secondly, that in addition to the reported trypanosomal transaminases there are at least 5 novel specific transaminases acting on glutamine, isoleucine, leucine, valine and methionine with α-ketoglutarate as the amino group acceptor.

In mammals the transamination and catabolism of methionine may lead to production of very toxic intermediates; methanethiol and hydrogen sulphide. The location of the methionine transaminating enzyme activity in the trypanosomes was investigated. Using GPT as a cytosolic marker, the release of the methionine transaminating enzyme from parasites by freeze-thawing was determined. Maximal activities of both GPT and the methionine transaminating enzyme were released by 2 freeze-thawing cycles. It was concluded that the methionine transaminating enzyme is cytosolic and may leak into the plasma of infected animals contributing to the pathogenicity of trypanosomes.

CHAPTER 1

INTRODUCTION

1.1. THE TRYPANOSOME

Trypanosomes are flagellated protozoa of the order kinetoplastidae and the family *Trypanosomatidae* (Hoare, 1972; Vickerman, 1976). *Trypanosoma brucei brucei*, *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are important causative agents of African trypanosomiasis. They are morphologically similar but differ in host range and virulence (Hoare, 1972). These organisms are parasites which inhabit bloodstream and lymphatic systems of mammals (Ssyenyonga *et al*, 1975).

It is believed that *T. b. gambiense* and *T.b. rhodesiense* are subspecies of *T. b. brucei*. These trypanosomes comprise the *T. brucei* sub-genera (Vickerman, 1965; Hoare, 1972). *Trypanosoma b. gambiense* and *T.b. rhodesiense* cause sleeping sickness in humans and trypanosomiasis in livestock and wild animals. *Trypanosoma b. brucei* causes trypanosomiasis in livestock and wild animals but it is non infective to humans (Hoare, 1972).

Trypanosomes belonging to the *T. brucei* sub-genera are transmitted by the tsetsefly of the genus *GLossina* within which they undergo a series of developmental stages (Vickerman, 1976). The tsetse-fly ingests parasites when taking a blood meal from an infected animal. In the mid-gut of the tsetsefly stumpy forms (which are non-dividing in the mammalian host) differentiate into procyclic forms which have lost the surface coat and are non-infective to the mammalian host (Barry and Vickerman, 1979). After about three weeks, the procyclic forms migrate to the insect salivary gland where they develop into metacyclic forms which have a surface coat and are infective to the vertebrate host (Vickerman, 1969; Le Ray *et al*, 1978). The infective metacyclics in the saliva are injected by the feeding tsetsefly into the bloodstream of the host where they quickly develop into long slender bloodstream forms which divide rapidly by binary fission, alter their metabolic pathways and undergo the characteristic antigenic variation (McNeillage *et al*, 1969; Barry and Hajduk, 1978). The bloodstream trypanosomes can be transmitted mechanically to animals by syringe passage.

A population of homogeneous slender forms free from stumpy forms can be obtained from the rapid passage of a trypanosome clone in laboratory animals (Clarkson and Brohn, 1976). These clones provide a pure population for biochemical studies.

1.2 ENERGY METABOLISM IN TRYPANOSOMES

The bloodstream trypanosomes depend entirely on glycolysis for their energy production (Ryley, 1956; Foulton and Spooner, 1959) and the glycolytic enzymes are located in a microbody-like organelle called the glycosome (Opperdoes and Borst, 1977; Opperdoes *et al*, 1984). These bloodstream forms have a tubular mitochondrion without cristae and they do not possess a functional tricarboxylic acid (TCA) cycle (Ryley, 1962). Reoxidation of NADH under aerobic conditions is effected by an α-glycerophosphate oxidase (αGPO) system which consists of an α-glycerophosphate dehydrogenase and a terminal oxidase (Bowman and Flynn, 1976; Opperdoes *et al*, 1976). The oxidase reduces molecular oxygen to water and it is not coupled to adenosine diphosphate (ADP) phosphorylation (Grant and Sargent, 1960). In bloodstream *T. b. brucei* an ATPase is present but it's function is not clear. It cannot be involved in the coupling of electron transport to adenosine triphosphate (ATP) synthesis since a respiratory chain is absent in these organisms (Opperdoes *et al*, 1977a).

Oxidation of 1 mole of glucose produces approximately 2 moles of pyruvate and a net 2 moles of ATP. There is some evidence that some pyruvate may

be converted to alanine (Grant and Fulton, 1957; Chapell et al, 1972). However the transamination of pyruvate to alanine and the stoichiometry of the products have not been investigated in detail.

During anaerobiosis or in the presence of salicylhydroxamic acid (SHAM) an inhibitor of the terminal oxidase, the amount of ATP and pyruvate produced is halved. Pyruvate and glycerol are produced in approximately equimolar quantities (Evans and Brown, 1972).

1.3 AMINO ACID METABOLISM

Amino acids are used by living organisms for the biosynthesis of proteins and other biomolecules. They may also serve as a source of energy (Lehninger, 1975). The amino acids in the organism are obtained either by synthesis or from the environment. Amino acids that can be synthesized by an organism from other compounds or other amino acids are termed non-essential. Those amino acids that cannot be synthesized by an organism are termed essential. In mammals the essential amino acid requirement is known (Lehninger, 1975). In trypanosomes, the essential amino acid requirement has not been determined. However, studies have been carried out on the metabolism of some amino acids in these

organisms as discussed below.

1.3.1 Amino acid transport in trypanosomes

There are reports of amino acid transport in bloodstream trypanosomes. The amino acid uptake mechanisms in *T.b. gambiense* and *T.b. brucei* are summarized in Table I.

1.3.2 Amino acid catabolism in bloodstream trypanosomes

1.3.2.1 Catabolism of threonine

In bloodstream forms of *T.b. brucei*, threonine catabolism leads to the formation of acetate and glycine in equimolar quantities. Some of the intermediates and enzymes of the pathway of threonine catabolism have been identified (Linstead *et al*, 1977).

Linstead *et al*, 1977 recovered labelled amino acetone, acetate and glycine from bloodstream *T.b. brucei* incubated with [$U^{14}C$] L-threonine. Production of acetate and glycine was stimulated by NAD^+ and coenzyme A. The proposed pathway of threonine catabolism in bloodstream *T.b. brucei* is

TABLE I

AMINO ACID UPTAKE IN TRYPANOSOMES

Species	Type of uptake	Amino acid	Reference
T. b. gambiense	Mediated transport	Leucine, Glycine Alanine	Southworth and Read, (1972)
		Threonine	Hansen and Read, (1973)
	Mediated transport and diffusion	Lysine Arginine, Phenylalanine Glutamate, Methionine, Lysine	Southworth and Read, (1972) Hansen and Read, (1973)
	Diffusion	Ornithine, Aspartate	Southworth and Read, (1972)
T.b. brucei	Mediated transport	Glycine, Alanine Serine, Threonine Valine, Leucine Proline, Methionine, Glutamate, Lysine	Voorheis, (1971)

summarized in Scheme I.

Threonine dehydrogenase and amino acetone synthase activities are present in bloodstream *T.b. brucei* (Linstead *et al*, 1977) as well as carnitine acetyl transferase (Gilbert and Klein, 1982). Threonine dehydrogenase and carnitine acetyltransferase are probably mitochondrial matrix enzymes (Opperdoes *et al*, 1981). In the proposed pathway of threonine catabolism in bloodstream form *T.b. brucei*, threonine dehydrogenase oxidizes threonine to 2-amino-3-oxobutyrate which spontaneously decarboxylates to aminoacetone (Laver, 1959; Klein *et al*, 1976). Amino acetone then reacts with CO_2 and coenzyme A under the catalysis of aminoacetone synthase producing glycine and acetylCoA. The acetyl-CoA then reacts with carnitine in a reaction catalysed by carnitine acetyl transferase producing acetyl carnitine which is probably hydrolysed by acetylcarnitine hydrolase to acetate. Klein and co-workers in 1976 reported carnitine-stimulated deacylation of acetylCoA by bloodstream form *T.b. brucei*.

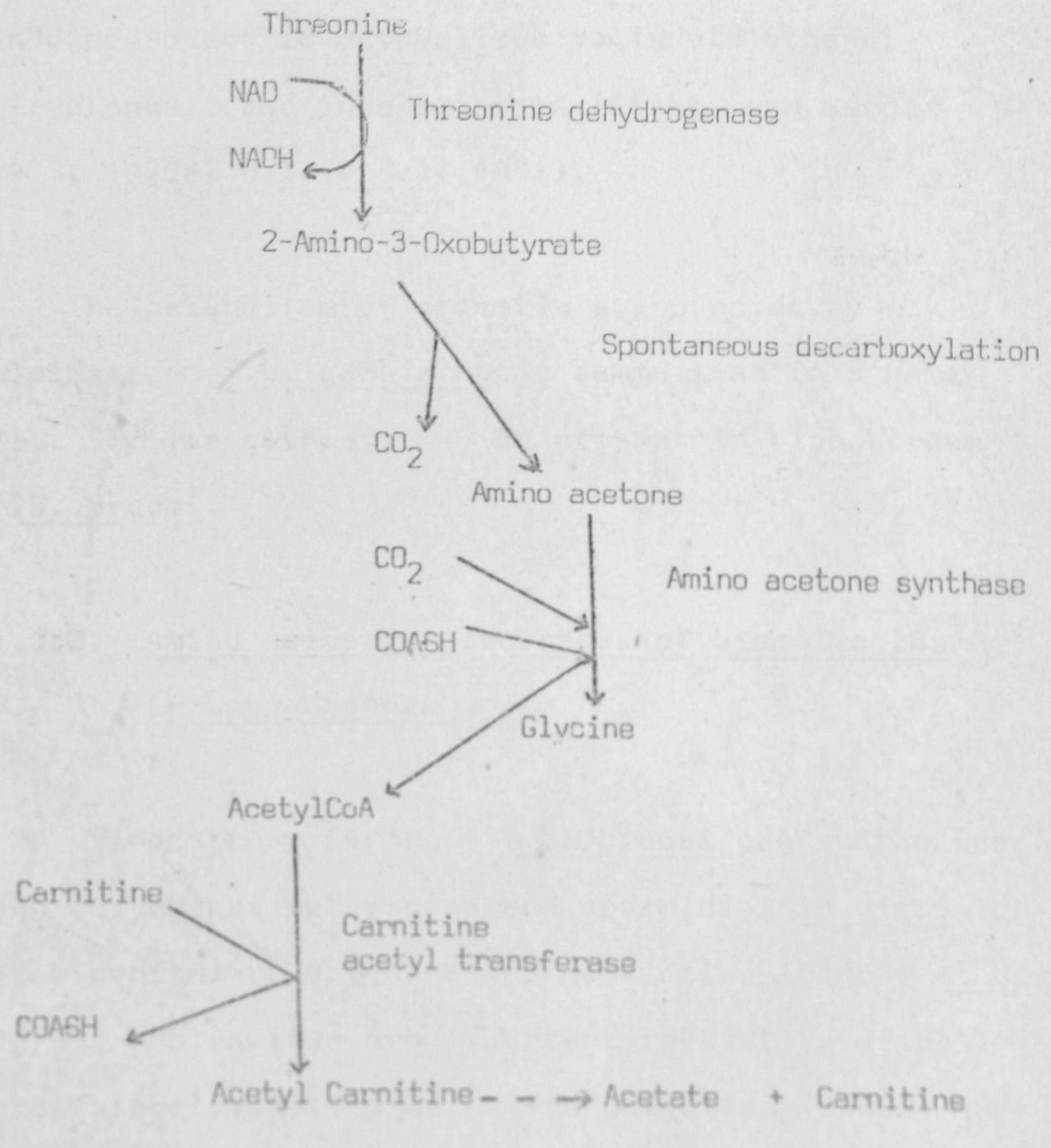
1.3.2.2. Catabolism of aromatic amino acids

The catabolism of aromatic amino acids has been studied in bloodstream form *T.b. gambiense*.

SCHEME I

PROPOSED PATHWAY OF THREONINE CATABOLISM IN BLOODSTREAM T. B. BRUCEI

Linstead *et al*, (1977); Gilbert *et al*,(1983)



In these organisms, phenylalanine is transaminated to phenylpyruvate and tyrosine is transaminated to p-hydroxyphenylpyruvate which may be reduced to p-hydroxyphenyllactate in the presence of NADH (Stibbs and Seed, 1975b). Tryptophan is transaminated to indolepyruvate which in the presence of NADH is reduced to indolelactate. In the absence of NADH, indolepyruvate is metabolized to indole ethanol (Tryptophol) and indoleacetate (Stibbs and Seed, 1973; 1975a; Hall *et al*, 1981).

The catabolism of aromatic amino acids by bloodstream *T.b. gambiense* is summarised in Scheme II. Similar pathways may be present in bloodstream *T.b. brucei*.

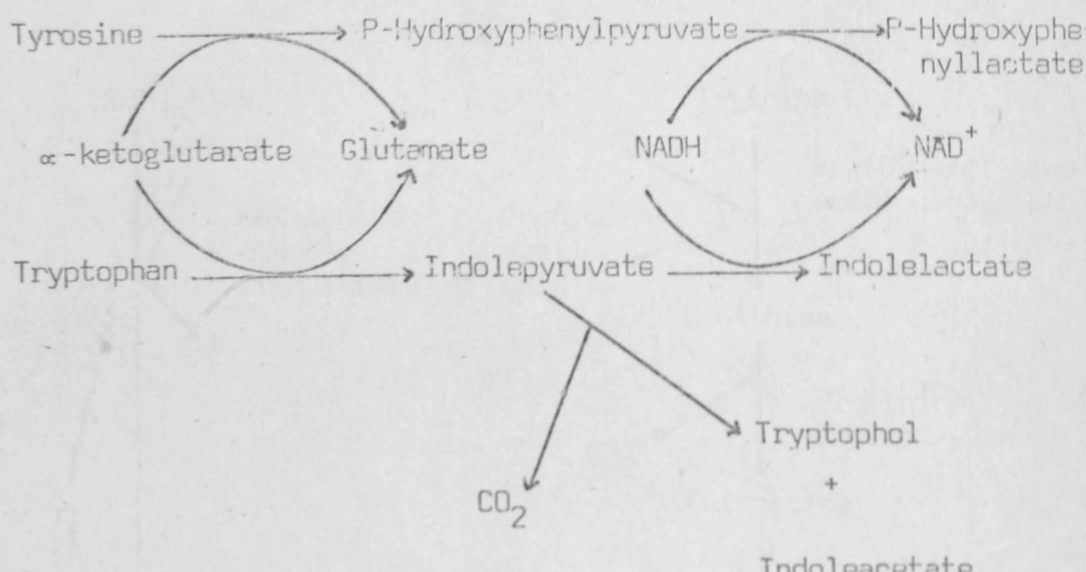
1.3.3. Amino acids as precursors of biomolecules in trypanosomes

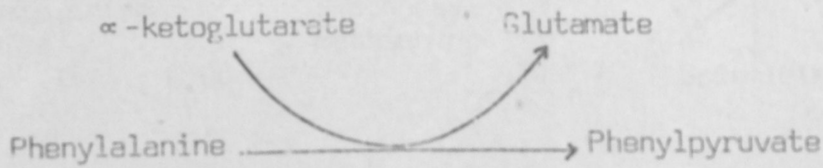
Bloodstream forms of *T.b. brucei* can synthesize the polyamines putrescine and spermidine *in vitro* from ornithine, arginine and methionine (Bacchi *et al*, 1979). Polyamines are important regulators of cell growth, proliferation and differentiation (Janne, 1978). The proposed pathway of polyamine synthesis in bloodstream *T.b. brucei* is summarized in Scheme III. Ornithine decarboxylase, the enzyme which catalyses the decarboxylation of ornithine to putrescine is

SCHEME II

CATABOLISM OF AROMATIC AMINO ACIDS BY BLOODSTREAM T.B. GAMBIENSE

(Gutteridge and Coombs, 1977)

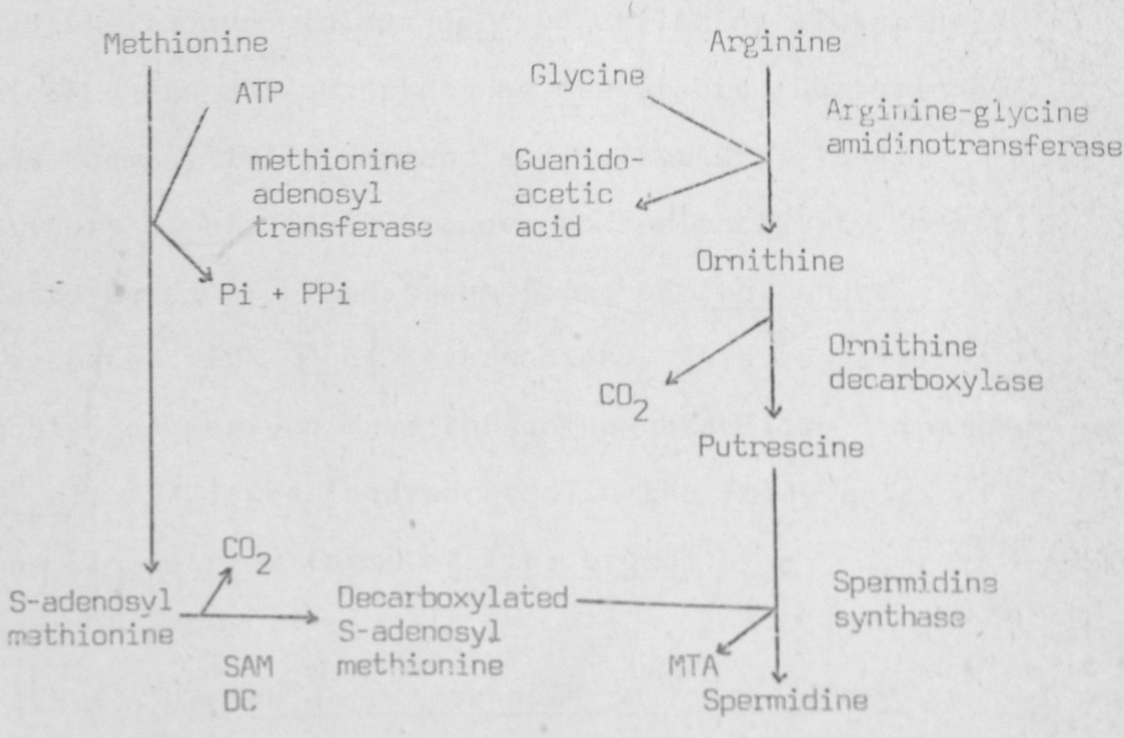




SCHEME III

POLYAMINE SYNTHESIS IN BLOODSTREAM T. BRUCEI

(Bacchi *et al*, 1979)



SAM, DC = S-adenosylmethionine decarboxylase

MTA = Methylthioadenosine.

strongly inhibited by α-difluoro methyl ornithine (DFMO) (Bacchi *et al*, 1980) and α-monoflucromethyl dehydro-ornithine methyl ester (Bitonti *et al*, 1985). *Trypanosoma b. brucei* - infected mice showed complete clearance of parasite and cure after treatment with these compounds.

There is evidence that trypanosomes are able to synthesize pyrimidines *de novo* utilizing glutamine, bicarbonate and aspartate as precursors (Gutteridge and Coombs, 1977; Hammond and Gutteridge, 1984). Gilbert *et al*, (1983) recovered radioactivity in the fatty acids of bloodstream forms of *T.b. brucei* incubated with $[^{14}C]$ L-threonine. This suggests that acetylCoA derived from threonine catabolism (Linstead *et al*, 1977) was incorporated in the fatty acids of the bloodstream forms of *T.b. brucei*.

1.3.4. Amino acid synthesis in trypanosomes

Chappel *et al*, (1972) studied amino acid synthesis in isolated bloodstream forms of *T.b. gambiense* using ^{14}C labelled substrates. They showed that trypanosomes incubated with glucose synthesized alanine. Incubation of the trypanosomes with alanine produced cysteine and taurine while incubations with aspartate produced serine. Furthermore, incubation with glutamate produced cysteine

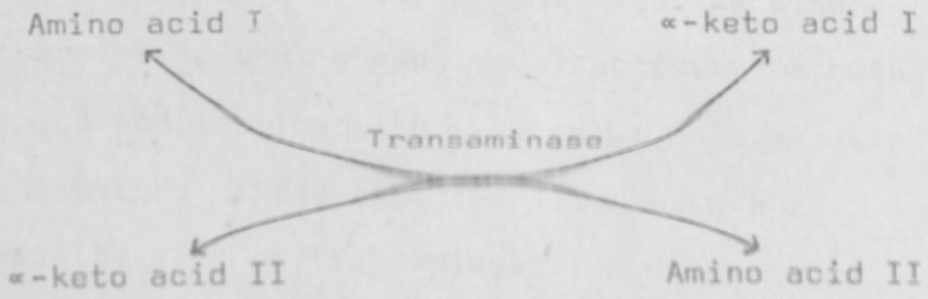
and taurine while incubations with arginine produced taurine, proline and glutamate. Thus bloodstream *T.b. gambiense* trypamastigotes are capable of interconverting or synthesizing some amino acids *in vitro*.

1.4. TRANSAMINASES

Transaminase activity is widespread in living organisms (Rudman and Meister, 1953; Stenmark *et al*, 1974; Tachiki and Tochikura, 1975; Patel *et al*, 1977; Paris and Magasanik, 1981). Approximately 60 transaminases are known (Christen and Metzler, 1985). The characteristic transamination reaction is summarised in Scheme IV.

SCHEME IV

Transamination



An orgamism may contain several transaminases with broad and overlapping specificity so that it

is difficult to assess the precise functional roles for each of the enzymes. For example in E.coli, Rudman and Meister, (1953) have reported transamisases designated A, B and C. "A" transaminates leucine, phenylalanine and tyrosine, "B" transaminates isoleucine, valine, leucine, phenylalanine and tyrosine with α-ketoglutarate as the second substrate. "C" transaminates valine with pyruvate as second substrate.

Ichihara, (1975) reported three branched-chain amino transferases I, II and III in rat tissues. Supernatant fractions of all rat tissues examined contained enzyme I while brain, ovary and placenta also contained enzyme III. Enzyme I and III acted equally on valine, leucine and isoleucine. The best amino group acceptor for all three enzymes was α-ketoglutarate, but keto analogues of the branched-chain amino acids were good acceptors. Ikeda et al, (1976) reported a rat liver enzyme II or leucine aminotransferase that showed equal activity on both methionine and leucine with α-ketoglutarate as amino group acceptor. These examples illustrate the nonspecifity of some transaminases.

1.4.4. Transaminases in trypanosomes

Several transaminases have been reported in

bloodstream trypanosomes. There is evidence that bloodstream form of *T.b. gambiense* possess tyrosine aminotransferase, phenylalanine aminotransferase (Stibbs and Seed, 1975b) and tryptophan aminotransferase (Stibbs and Seed, 1973; 1975a; Hall *et al*, 1981). The presence of similar transaminases in bloodstream form *T.b. brucei* has not been reported.

The presence of GPT and GOT in trypanosomes has been widely reported. Glutamate pyruvate transaminase was reported in *T.b. brucei* (Visser and Opperdoes, 1980; Steiger *et al*, 1980; Godfrey and Kilgour, 1973; 1976), *T.b. gambiense* and *T.b. rhodesiense* (Godfrey and Kilgour, 1976). Glutamate oxaloacetate transaminase was reported in *T.b. brucei* (Opperdoes *et al*, 1977a,b; Kilgour and Godfrey, 1973; Godfrey and Kilgour, 1973, 1976), *T.b. gambiense* and *T.b. rhodesiense* (Godfrey and Kilgour, 1976). The presence of other transaminases in the *T. brucei* sub-genera has not been investigated.

1.4.2. Role of transaminases in trypanosomes

Proposals have been made on the roles of some transaminases in trypanosomes. Stibbs and Seed, (1973, 1975a) suggested that the catabolism of tryptophan via transamination in bloodstream form

T.b. gambiense was linked to carbohydrate metabolism and was involved in the removal of pyruvate formed from glycolysis by converting it to alanine. Reynolds, (1975) has proposed that GPT and GOT in bloodstream *T. evansi* - a parasite of camels could be involved in coupled series of reactions leading to a transhydrogenation of NADH with $NADP^+$. Both NAD-linked MDH and NADP-linked ME participate in the reactions as shown below;

$$\text{Pyruvate} + \text{Glutamate} \xrightleftharpoons{\text{GPT}} \alpha\text{-ketoglutarate} + \text{Alanine}$$

$$\alpha\text{-Ketoglutarate} + \text{Aspartate} \xrightleftharpoons{\text{GOT}} \text{Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightleftharpoons{\text{NAD-linked MDH}} \text{Malate} + NAD^+$$

$$\text{Malate} + NADP^+ \xrightleftharpoons{\text{NADP-linked ME}} \text{Pyruvate} + \text{NADPH} + H^+ + CO_2$$

The overall reaction would be as follows;

$$\text{Aspartate} + \text{NADH} + NADP^+ \rightleftharpoons \text{Alanine} + CO_2 + NAD^+ + \text{NADPH}$$

The NADPH generated by these reactions in the cytosol of the trypanosome may be used in fatty acid synthesis. There is evidence that bloodstream trypanosomes can interconvert and desaturate fatty acids. Dixon *et al*, (1971) reported that bloodstream forms of *T.b. rhodesi-*

ense can interconvert and desaturate C_{16} and C_{18} fatty acids.

Other reactions capable of producing NADPH in trypanosomes have been studied. In addition to NADP-linked ME, Ryley, (1962) reported low activities of glucose-6-phosphate dehydrogenase and NADP-linked isocitrate dehydrogenase in bloodstream form T.b. rhodesiense. Reynolds, (1975) reported low activities of glucose-6-phosphate dehydrogenase, NADP-linked isocitrate dehydrogenase as well as NADP-linked ME in bloodstream form T. evansi. In both cases the reported activity of NADP-linked ME was comparatively higher than that of glucose-6-phosphate dehydrogenase and NADP-linked isocitrate dehydrogenase.

A transhydrogenation reaction as proposed by Reynolds, (1975) in T. evansi may be present in bloodstream form T.b. brucei although conclusive studies have not been reported. In bloodstream form T.b. brucei, transhydrogenation may be an important source of NADPH in view of the low activities of other NADPH generating reactions. Mutharia, (1977) reported low activity of G6PDH in bloodstream form T.b. brucei. The location of this enzyme in bloodstream form T.b. brucei has not been investigated. Opperdoes, et al, (1977b) reported low activity of NADP-linked isocitrate dehydrogenase in the mitochon-

dria of bloodstream form T.b. brucei. A cytosolic NAD-linked MDH has been reported in bloodstream form T.b. brucei (Opperdoes et al, 1981; Falk et al, 1980). Kilgour, (1980) and Klein et al, (1975) reported the presence of NADP-linked ME in bloodstream form T.b brucei. The localisation of the enzyme in the bloodstream trypomastigotes has not been reported.

1.5 SERUM ENZYMES IN TRYPANOSOMAL INFECTIONS

Various studies have reported elevated levels of transaminase activity in the serum of animals infected with trypanosomes. Moon et al, (1968) reported elevated serum GPT and GOT in mice infected with T.b. rhodesiense. Stibbs and Seed, (1975) found elevated tyrosine aminotransferase in the serum of Microtus montanus infected with T.b. gambiense. These authors proposed that the additional serum transaminases were from the trypanosomes. Boid, et al, (1980) found elevated levels of GPT and GOT in the serum of camels infected with T. evansi and suggested that the additional GOT activity was both of trypanosomal and host origin whereas the GPT was of trypanosomal origin.

Goodwin and Guy, (1973) suggested that the rise in serum GOT in rabbits infected with T.b. brucei was due to host cell necrosis. Lippi and Sebastiani,

(1958) (quoted by Gray, 1963) proposed the increase in serum GPT and GOT levels in T.b. brucei - infected guinea pigs was due to lesion of the liver, myocardium, adrenal glands and nervous tissue. There is no concensus on the explanation as to the origin of additional serum transaminase activity during trypanosomal infections in animals.

1.6 PLASMA AMINO ACID LEVELS DURING TRYPANOSOMAL INFECTIONS

The levels of free plasma amino acids are altered during trypanosomal infections. Newport et al, (1977) reported alteration of free plasma amino acids in *Microtus montanus* infected with *T.b. gambiense*. In that study the plasma levels of threonine, serine, glutamate, glycine, valine, isoleucine, leucine, tyrosine and tryptophan were depressed whereas levels of proline and alanine were elevated. Isoun and Isoun, (1978) reported depressed levels of methionine, isoleucine, leucine, tyrosine, tryptophan and phenylalanine and elevated levels of serine, glutamate, glutamine, glycine, alanine and proline in the plasma of rats infected with *T.b. brucei*. The highest elevation was of the amino acid alanine and the authors proposed that this was due to the transamination of pyruvate

- an end product of glucose metabolism in the bloodstream forms of T.b. brucei.

1.7 PATHOGENICITY OF TRYPANOSOMES RELATED TO AMINO ACID METABOLISM

1.7.1. Depression of host amino acid levels

Tyrosine is an important precursor of the catecholamines; dopamine, adrenaline and norepinephrine. Reduction of serum tyrosine during trypanosomal infections leads to decreased levels of catecholamines in the infected animal (Newton, 1978). Lowered catecholamine levels in infected animals results in changes in activity patterns, glycogen, lipid metabolism and mental depression (Jouvet, 1969). The reported reduction in the levels of tryptophan in the plasma of trypanosome infected animals (Newport et al, 1977; Isoun and Isoun, 1978) could lead to decreased synthesis of niacin and serotonin by the host leading to a pellagra-like syndrome, changes in sleep patterns and depression (Stibbs and Seed, 1975a).

1.7.2 End products and intermediates of amino acid metabolism

One of the end products of tryptophan catabolism

in trypanosomes is indoleethanol (tryptophol) (Stibbs and Seed, 1973; 1975a). There is evidence that tryptophol can cause convulsions and death by respiratory depression when injected into mice, rats and cats (Sabelli et al, 1969). Tryptophol has also been reported to cause immunodepression in laboratory rodents (Ackerman and Seed, 1976). Seed and co-workers (1978) showed that tryptophol rapidly lyses red blood cells, and a similar action on synaptic membranes may cause changes in the transmission of nerve impulses.

Elevated levels of ∝-keto acids are reported in urine and blood of trypanosome-infected animals. Grant and Fulton (1957) reported elevated levels of pyruvate in the blood of rats infected with T.b. rhodesiense. Hall and Seed, (1981) reported increased excretion of aromatic ∝-keto acids, phenylpyruvate, p-hydroxyphenylpyruvate, indole-3-pyruvate, indole-3-lactate and indole-3-acetate in the urine of Microtus montanus infected with T.b. gambiense.

The catabolism of methionine by bloodstream form trypanosomes has not been investigated. Studies have shown that methionine is one of the most toxic amino acids. When in excess in the diet of mammals methionine caused growth depression and tissue damage (Harper et al, 1970; Benevenga, 1974). Two

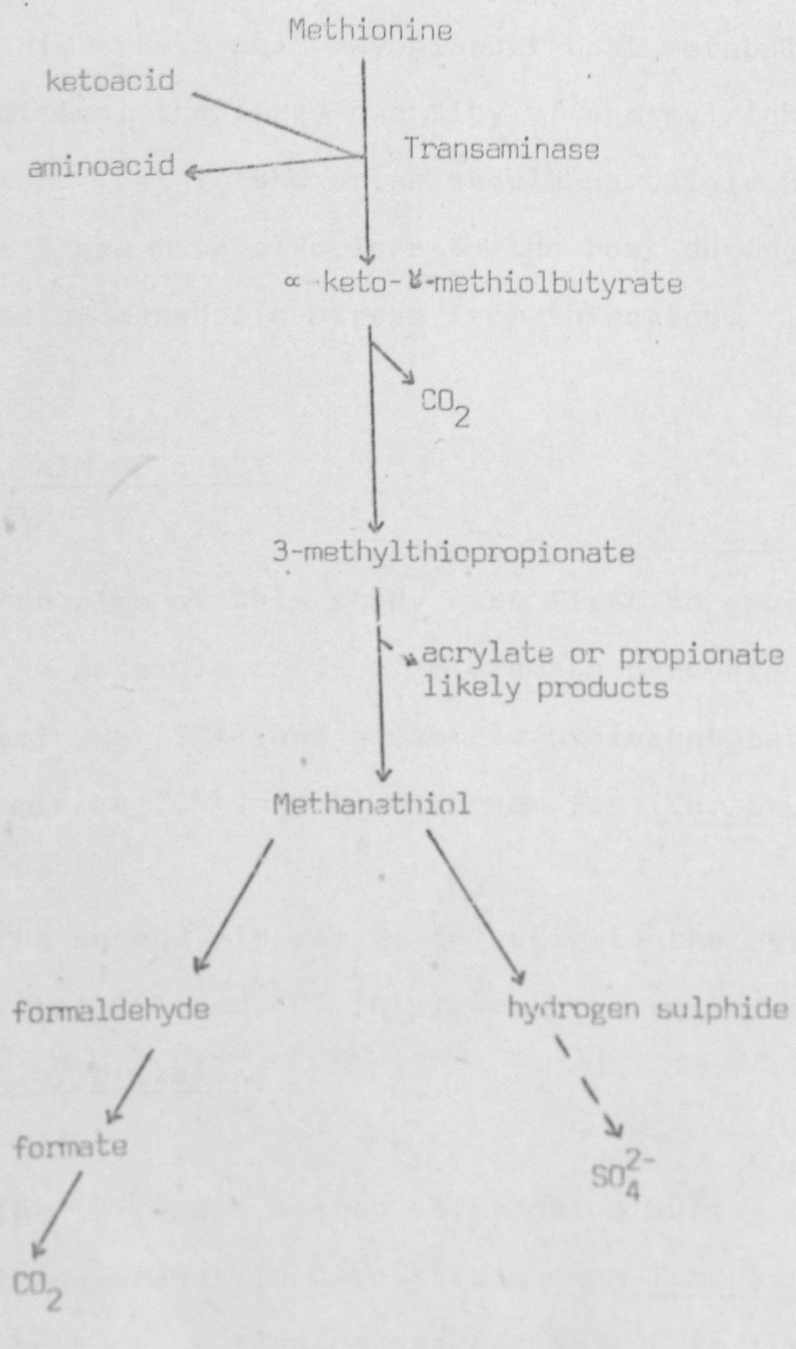
pathways are known for the catabolism of methionine in mammals. The first is the transsulfuration pathway (Gaull and Tallan, 1974; Steele and Benevenga, 1978; Case and Benevenga, 1976) which leads to the formation of homocysteine, cystathionine and cysteine. The second is the transamination pathway (Case and Benevenga, 1976; Mitchell and Benevenga, 1978; Steele et al, 1979; Benevenga, et al; 1976; Benevenga and Steel, 1979; Steele and Benevenga, 1978) which leads to the formation of among other intermediates 3-methylthiopropionate, methanethiol, formaldehyde and hydrogen sulphide (Scheme IV). Steele et al, (1979) observed that 3-methylthiopropionate caused as much growth depression in rats as methionine. Zieve et al, (1974) reported that in rats, 0.5 µM methanethiol in blood is sufficient to cause coma. Hydrogen sulphide is toxic to animals in small amounts by interfering with the cytochrome oxidase (Shy, 1978).

In host blood the substrates for the transamination of methionine are available and accessible for metabolism by the infecting trypanosomes. Enhanced catabolism of methionine via the transamination pathway in trypanosomal infections could lead to complications in host similar to those observed in methionine poisoning.

SCHEME V

TRANSAMINATION PATHWAY OF METHIONINE CATABOLISM

(Benevenga and Steele, 1979)



Depression or elevation of host plasma amino acid levels and accumulation of some of the end products of amino acid metabolism may contribute to the pathogenesis of trypanosomiasis. Phenylpyruvate is a known inhibitor of adrenaline synthesis, thus this α-keto acid may disrupt host metabolism. In addition, the large quantity of energy rich carbon skeletons lost in the urine should certainly represent a large metabolic loss to the host during periods of metabolic stress from infection.

1.8 AIM OF STUDY

The aims of this study were first to investigate the possible roles of glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) in bloodstream for *T.b. brucei*.

The second aim was to investigate the origin of additional GPT and GOT in plasma of rats infected with *T.b. brucei*.

The third aim was to carry out a survey of aminotransferases in bloodstream form *T.b. brucei* with the hope of finding enzymes unique to these organisms.

CHAPTER 2

MATERIALS AND METHODS

2.1 ORGANISMS

Trypanosoma brucei stock designated (EATRO, 1969) was used in this study. It was originally isolated from a female hyaena (*Crocuta crocuta*) in Serengeti National Park, Tanzania in 1970, (Geigy *et al*, 1971). It was innoculated into rats and mice, collected and frozen as a stabilate. In 1978, it was cloned at the International Centre for Insect Physiology and Ecology (ICIPE), Kenya and proven to be infective to *Glossina morsitans* (tsetsefly) and rodents.

2.2 ANIMALS

Sprague-Dawleyrats 4 - 5 months old weighing 250 - 300gm were used. The animals were housed, given rat pellets and water *ad libitum*.

2.3 REAGENTS

The reagerts used in this study were of analar grade. They were obtained from; British Drug Houses Ltd. Poole England, Sigma Chemical Company, St. Louis

MO. U.S.A., E. Merck A.G. Darmstadt, West Germany and Boehringer Manheim GmbH, West Germany.

2.4 BUFFERS USED FOR MANIPULATION OF TRYPANOSOMES

Two phosphate buffers were used for trypanosome isolation and incubation experiments. The first was phosphate buffered saline containing 57 mM Na_2HPO_4, 3.36 mM NaH_2PO_4, 43.7 mM NaCl and 30 mM D-glucose (Hansen, 1979) adjusted to pH 8 with NaOH and is referred to as PSG. The second buffer was phosphate buffered saline containing 57 mM Na_2HPO_4, 3.36 mM NaH_2PO_4 and 73.7 mM NaCl adjusted to pH 8 with NaOH and is referred to as PS. The buffers were stored frozen at -20°C to avoid bacterial contamination.

2.4.1 Anticoagulant

A stock solution of 5% (w/v) sodium citrate was prepared either in 0.9% (w/v) NaCl, PS or PSG and kept frozen at -20°C.

2.5 MAINTENANCE OF TRYPANOSOMES IN THE LABORATORY

2.5.1 Infection and Bleeding of Laboratory Animals

Rats were infected intraperitoneally with approximately 10^7 trypanosomes in about 0.2 ml blood

or PSG using a 1 ml disposable syringe and a 25 gauge hypodermic needle. The area of injection was wiped with 70% (v/v) ethanol to prevent microbial infection.

The rats were bled on attainment of a parasitemia of over 10^8 trypanosomes/ml of tail blood, usually four days after infection. The animals were anaesthetized with diethyl ether. The chest cavity was opened to expose the heart which was then punctured with an 18 gauge hypodermic needle using a 10 ml plastic syringe containing 0.5 ml 5% (w/v) sodium citrate as anticoagulant in PS or PSG. The blood was sucked gently and placed in 10 ml plastic centrifuge tubes in ice. Ten millilitres of blood could be obtained from a 250 gm rat.

2.5.2 Preparation of Parasite Stabilates

Parasite stabilates were prepared as described by Brohn and Clarkson, (1978). Trypanosomes in PSG or blood were mixed with an equal volume of 10% glycerol (v/v) in PSG. One millilitre volume of the mixture was put into Nunc plastic vials with screw-type caps and placed at 4°C for 30 minutes. The vials were then placed in ice for 30 minutes before being suspended over liquid nitrogen for 2 hours after which they were submerged into the liquid nitrogen and kept frozen at -196°C. When

required, the stabilates were thawed at 30°C and motility of the parasites observed under a microscope.

2.6 ISOLATION OF TRYPANOSOMES

Two methods were used to isolate trypanosomes from infected blood; the diethylaminoethylcellulose (DEAE - cellulose) anion exchanger method (Lanham and Godfrey, 1970) and the differential osmotic lysis method (Njogu and Kiaira, 1982). The latter method was fast and inexpensive.

2.6.1 Preparation of DEAE-Cellulose for Trypanosome Isolation

Well-settled DEAE 52 cellulose (Sigma) powder was suspended in an equal volume of distilled water, stirred thoroughly and left to settle for 30 minutes. The 'fines' were decanted off and the procedure repeated three times using fresh distilled water. The anion exchanger was left overnight in distilled water at 4°C. The water was decanted and replaced with PSG at 4°C. The suspension was stirred thoroughly and left to settle for 30 minutes after which the PSG was decanted. This procedure was repeated three times. The pH of the equilibrated DEAE cellulose suspension was 8. The final suspension ready

for use consisted of PSG : DEAE cellulose 1 : 1 (v/v).

2.6.2 Isolation of Trypanosomes using DEAE 52 Cellulose

Infected rat blood was immediately centrifuged at 500 x g at 4°C for 5 minutes. Three layers were visible. The top layer was of plasma. Below that was a buffy coat containing trypanosomes mainly and a few red blood cells and at the bottom was a pellet of red blood cells and few trypanosomes.

Using a pasteur pipette, the plasma layer was removed. The trypanosome layer (approximately 1 ml) was then carefully removed and placed on ice. This was considered a crude trypanosome preparation. This procedure was necessary to exclude the bulk of red blood cells.

A glass column of 1.3 cm internal diameter and a height of 15 cm was plugged with glass wool at the narrow end and a 'large pore' Whatman filter paper circle (diameter 1.3 cm) placed on the plug. Well-stirred DEAE cellulose anion exchanger slurry in PSG was poured into the column to a height of 10 cm and left to settle for at least 30 minutes at 4°C.

The outlet at the bottom of the column was kept closed to prevent formation of air-spaces in the gel.

After the gel had settled, the outlet at the bottom of the column was opened and the level of PSG brought down slowly to just above the gel surface. The outlet was then closed and the crude trypanosome preparation applied on top of the gel. The outlet was then opened to allow the sample to run into the gel. This was followed by application of cold PSG up to a height of 15 cm. The flow rate was about 1 drop/second. Smears were made on slides at regular intervals to monitor parasite elution from the column. The parasite eluate was collected in a 250 ml erlenmeyer flask placed on ice until no further parasites were evident in the eluate or until red blood cells were detected.

The eluate was centrifuged at 1000 x g for 10 minutes in 10 ml centrifyge tubes at $4^{o}C$. The resultant trypanosome pellet was resuspended either in fresh PSG or PS and kept on ice ready for use.

2.6.3 Isolation of Trypanosomes by Differential Osmotic Lysis

Trypanosomes in the buffy coat (prepared as

described above) were quickly suspended with gentle stirring in hypotonic 1 mM sodium phosphate buffer (pH 7.5) containing 0.3% (w/v) NaCl at 37°C. The final volume of the suspension was 45 mls.

After exactly three minutes, 5 mls of ten times concentrated PSG (10 x PSG) pH 8 was added to the hypotonic buffer suspension. This reconstituted the hypotonic buffer to isotonic concentration. The trypanosome suspension was centrifuged at 1000 x g for 10 minutes at 4°C. The pellet of pure trypanosomes was washed three times by gentle resuspension in fresh PSG or PS and centrifugation at 1000 x g for 10 minutes at 4°C. The final suspension was kept on ice ready for use.

2.6.4 Counting of Trypanosomes

Isolated trypanosomes were suspended in a known volume of either PSG or PS buffer. A sample of this suspension diluted 1:100 or 1:200 was applied to a Neubaeur haemocytometer and trypanosomes counted at 400 times magnification under a microscope. The total number of trypanosomes obtained was calculated according to the following formulae;

$Y \times 10^4 \times$ Dilution factor=Trypanosomes/ml of original suspension.

Trypanosomes/ml of original suspension x Total volume of original suspension = Total number of trypanosomes obtained, where;

Y = Trypanosome count per y number of squares.

10^4 = Constant encompassing the volume and depth of the counting chamber.

For routine parasitemia checks in rats tail blood smears from the animals were observed under a microscope at 400 times magnification.

2.7 PREPARATION OF PLASMA

The rats were anaesthetized as described earlier. A 10 ml plastic syringe attached to an 18 gauge hypodermic needle and containing 0.5 ml of 5% (w/v) sodium citrate in 0.9% (w/v) NaCl was used to suck blood gently from the heart. The blood was put into 10 ml plastic centrifuge tubes on ice and centrifuged at 1000 x g for 10 minutes at 4°C. The plasma which was contaminated by red blood cells was carefully removed and centrifuged at 10,000 x g for 20 minutes at 4°C. The resulting supernatant was plasma free from trypanosomes and most blood cells.

2.8 PREPARATION OF SERUM

Rats were anaesthetized in diethyl ether and the chest cavity opened to expose the heart. The aorta was severed and the blood collected in 10 ml plastic centrifuge tubes placed in ice. The blood was then kept at room temperature for four hours or at 4°C overnight to allow separation of serum. The serum was carefully withdrawn and centrifuged at 1,000 x g for 10 minutes at 4°C. The supernatant was collected and centrifuged at 10,000 x g for 20 minutes at 4°C. The resulting supernatant was serum free from trypanosomes and most blood cells.

2.9 AEROBIC INCUBATIONS

Trypanosomes suspended in 1 ml of either PSG or PS in 50 ml erlenmeyer flasks were placed in a Dubnoff shaking metabolic incubator set at 25°C and a shaking speed of 6.

2.10 TERMINATION OF INCUBATIONS

Two methods were used to stop incubations, before measurements of metabolites. In the first method, an equal volume of ice-cold 14% (v/v) PCA was added to the incubation mixture. The protein precipitate was removed by centrifugation at

1000 x g at 4°C for 5 minutes. The resulting supernatant was neutralized with ice-cold 4N KOH. This neutral protein-free extract was used for metabolite determination.

In the second method described by Bergemeyer (1978), an equal volume of ice-cold 14% (v/v) PCA was added to the incubation mixture. The protein precipitate was removed by centrifugation at 1000 x g at 4°C for 5 minutes. The resulting supernatant was neutralized with an appropriate volume of ice-cold 20% (w/v) K_2HPO_4. The resulting potassium perchlorate precipitate was removed by centrifugation as described above and the resulting supernatant used for metabolite determination. This method was found to be convenient when dealing with large numbers of samples since the amount of K_2HPO_4 required was pre-determined therefore eliminating the danger of exceeding the required pH. Furthermore, the resulting final supernatant was buffered between pH 7.5 and 8.0. Dilution of the protein-free extracts to suit various metabolite assays was made with distilled water.

2.11 PAPER CHROMATOGRAPHY OF AMINO ACIDS

A suspension of 2×10^8 trypanosomes in 3 ml of either PSG or PS containing appropriate substrates

were incubated in 50 ml erlenmeyer flasks at 25°C in a Dubnoff shaking metabolic incubator. Incubations were terminated by the addition of 6 mls of 100% methanol (Williamson and Desowitz, 1961). The lysates were centrifuged at 10,000 x g at 4°C for 10 minutes to remove cellular debris. The clear methanol extract was evaporated to dryness in a rotavapor under vacuum at 60°C. After cooling, the dry extract was dissolved in 0.5 ml 10%(v/v) aqueous isopropanol. Samples of this extract were kept frozen at -20°C until needed. Standard amino acids were also dissolved in 10%(v/v) aqueous isopropanol.

Samples of standard amino acid solution and trypanosomal amino acid extracts were applied as spots 3 cm from the edge of Whatman chromatography papers (size 20 cm x 18 cm) with capillary glass tubes and dried with a hair drier. The chromatography papers were folded into cylinders, stapled and placed in 2 litre air-tight sweet jars containing 50 ml of solvent system. The sample spots were about 2 cm above the level of the solvent. On completion of chromatography, the solvent front was marked and the paper cylinder dried in an oven at 100°C for 10 minutes. The dry paper was then sprayed with 0.2%(w/v) ninhydrin in 100% acetone and heated at 100°C for 5 minutes in an oven. Purple

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spots indicated the presence of amino acids (proline appeared as a yellow spot). These spots were immediately circled with a pencil, and the Rf values calculated.

Various solvent systems were used but those causing tailing were discarded. The best systems resulting in optimum resolution were; n-butanol: acetone: diethylamine: H_2O at ratios of 10:10:2:5 and n-butanol:methylethylketone:ammonia:H_2O at ratios of 50:30:10:10.

The standard amino acids chosen were those known to be found free in the parasite and those which are found to be elevated in the plasma of *Trypanosoma b. brucei* infected animals (Isoun and Isoun, 1978).

2.12 FREEZE-THAWING OF TRYPANOSOMES

Trypanosomes suspensions in tightly capped plastic vials were subjected to freezing in liquid nitrogen for 20 minutes and thawed at 25°C. This procedure was repeated for any number of freeze-thawing cycles required. The lysates were then centrifuged at 10,000 x g at 4°C for 20 minutes and the resulting supernatants used for enzyme assays.

2.13 TREATMENT OF TRYPANOSOMES WITH DETERGENTS

Two detergents were used. Stock solutions of 2%(v/v) Triton X-100 in water and 0.75%(w/v) Digitonin in absolute ethanol were made. Appropriate volumes of these detergent solutions were added to trypanosome suspensions in PSG or PS to achieve the required concentrations. The trypanosome suspensions containing detergent were agitated gently and incubated at 25°C for 10 minutes. Incubations were terminated by centrifugation at 10,000 g for 20 minutes at 4°C. The resulting supernatants were used for enzyme assays.

2.14 SONICATION OF TRYPANOSOMES

Trypanosomes suspensions in PSG or PS were sonicated with a Branson Sonifier (Model W185) with an output of 80 watts for the required time in ice. The lysates were centrifuged at 10,000 x g at 4°C for 10 minutes and the supernatants used for enzyme assays.

2.15 HORIZONTAL STARCH GEL ELECTROPHORESIS OF RAT SERUM AND TRYPANOSOMAL LYSATES

Rat serum was prepared as described earlier.

EDTA and Dithiothreitol dissolved in distilled water was added to a final concentration of 1 mM. Trypanosome pellets were resuspended in a solution of 1 mM EDTA and 2 mM Dithiothreitol for 10 minutes. They were then freeze-thawed once and the lysate centrifuged at 10,000 x g at $4^{o}C$ for 30 minutes. The resulting supernatant and the serum were frozen in liquid nitrogen in convenient amounts and thawed when needed. (Kilgour and Godfrey, 1973; Godfrey and Kilgour, 1976; Gibson et al, 1978; Kilgour et al, 1975).

Two pairs of buffers were used in the starch gel electrophoresis of glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT). The first pair consisted of glycine and NaOH and was used for the electrophoresis of GOT. The tank buffer contained 0.15 M glycine and 0.06 M NaOH (pH 9.5) and the gel buffer contained 0.015 M glycine and 0.006 M NaOH (pH 9.5) (Miles et al, 1977). The second pair of buffers consisted of Tris and citric acid and was used for the electrophoresis of GPT. The tank buffer contained 0.15 M Tris and 0.0075 M citrate (pH 9) and the gel buffer contained 0.015 M Tris and 0.00075 M citrate (pH 9) (Miles et al, 1977). The starch gel was made as described by Smithies (1955, 1959). A suspension of 12%(w/v) hydrolysed starch (Smithies) in gel buffer was heated and the hot gel

de-gassed under vacuum. The hot gel was then poured onto a glass plate containing a trough of dimensions 10 cm x 20 cm to a depth of 2.5 mm, covered and left to cool to room temperature for 15 minutes. The gel was ready for use after being kept at 4°C for 30 minutes.

One centimetre long slots were made in the gel, 4 cm from the cathode end. Samples were applied to the slots by placing boiled cotton threads soaked in the appropriate samples (Kilgour _et al_, 1976; Kilgour _et al_, 1974; Gibson _et al_, 1978; Kilgour and Godfrey, 1973). The gel was then placed in a Shandon electrophoresis tank containing the appropriate tank buffer. The electrophoresis was run at 4°C for 4 hours at a constant voltage of 17 volts/cm and 8 mA. Flannel soaked in tank buffer was used to complete the electric circuit.

Both GOT and GPT activities were developed in 0.1 M sodium phosphate buffer (pH 7.4). For GPT development the coenzyme used was 0.7 mM NADH and substrates were 1 mM α-ketoglutarate and 22.5 mM L-Alanine. The linking enzyme was 15 I.U/ml LDH in 50% glycerol. For GOT development the coenzyme used was 0.7 mM NADH and the substrates were 1 mM α-ketoglutarate and 31.5 mM L-Aspartate. The linking enzyme was 15 I.U/ml MDH in 50% glycerol.

These reagents were mixed in 5 ml of the phosphate buffer and applied on a whatman number 1 filter paper and the wet paper placed on top of the gel. A dry filter paper was applied on top of the wet filter paper to remove excess developer buffer. A heavy glass slab was placed on top of the gel which was incubated at 37°C for 40 minutes.

After incubation, the wet filter paper was observed under ultra violet light. Areas of enzyme activity appeared as dark spots against a bright background. These areas were marked and the filter paper kept for record.

After staining for enzyme activity, the gel was submerged in 0.05%(w/v) coomasie blue in 7%(v/v) acetic acid and incubated at 25°C for 40 minutes. The gel was then rinsed once in 7%(v/v) acetic acid and observed. Protein containing areas were stained dark blue.

2.16 SURVEY OF AMINOTRANSFERASES IN BLOODSTREAM T. BRUCEI

In these experiments 2×10^8 trypanosomes/ml in PS were freeze-thawed once and centrifuged at 10,000 x g for 10 minutes at 4°C. The resulting

supernatant was used in incubations. Neutralized α-ketoglutarate and L-amino acids were added to the supernatant to a final concentration of 10 mM. All solutions were freshly prepared. The lysates were incubated at 25°C for 1 hour in a Dubnoff shaking metabolic incubator in test-tubes. Incubations were terminated as described in Materials and Methods section 2.10. The amount of L-glutamate produced per hour was taken to be a measure of transaminase activity.

2.17 SUBSTRATE AND ENZYME ASSAYS

All assays depended on reactions which could be followed spectrophotometrically by measuring the decreased or increased absorbance of pyridine nucleotides at 340 nm either on a Pye Unicam SP 1800 spectrophotometer coupled to a Pye Unicam SP 22 recorder or a Perkin Elmer recorder at 25°C using cuvettes of 1 cm light path.

During determination of enzyme activities, the assay mixture usually contained the enzyme but not the substrate and was incubated for 2 - 3 minutes in the cell compartment to estimate endogenous activity before initiating the reaction being assayed by addition of substrate. Reactions were followed for 2 - 5 minutes and the enzyme amounts were selected

to give significant reaction rates.

During the determination of metabolites, the assay mixture usually contained the metabolite but not the enzyme which was added later to start the reaction. There was a blank containing all additions except the metabolite.

An extinction coefficient of 6.23×10^{-6} per mole of NADH used or generated was used in all determinations at 340 nm. The concentration of the metabolites in each sample assayed was estimated using the following formula (Bergemeyer, 1974);

$$C = \frac{\Delta E}{6.23} \times \frac{v}{V}$$

where C = Concentration of respective metabolite in µmoles/ml sample.

ΔE = Change in absorbance during the assay due to the presence of volume v of sample in a cuvette containing final volume V of assay mixture.

2.17.1 Substrate Assays

All substrate except L-Alanine were assayed as described by Bergemeyer (1974) in "Methods of

Enzymatic Analysis"

(a) D-Glucose

D-Glucose was assayed in a system containing 255 mM triethanolamine-HCl buffer (pH 7.5), 2.55 mM $MgSO_4$, 10.2 mM ATP, 0.82 mM $NADP^+$, 4 I.U/ml glucose-6-phosphate dehydrogenase (G6PDH) and 1.5 I.U/ml hexokinase (HK). The assays were based on the increase in absorbance at 340 nm as $NADP^+$ was reduced. The reaction was started with ATP. The sequence of reactions in the assay is shown below:

$$\text{D-glucose} + \text{ATP} \xrightarrow[\text{HK}]{Mg^{2+}} \text{ADP} + \text{G6P}$$

$$\text{G6P} + NADP^+ \xrightarrow{\text{G6PDH}} \text{6-phophogluconolactone} + \text{NADPH} + H^+$$

The reaction mixture was incubated for 20 minutes at 25°C.

(b) L-Glutamate

L-Glutamate was assayed in a system containing 290 mM glycine - 232 mM hydrazine buffer (pH 9), 0.97 mM ADP 1.6 mM NAD^+; 4.5 I.U/ml glutamate dehydrogenase (GLDH) free from ammonium ions) was used to start the reaction. The assay was based

on the increase in absorbance at 340 nm as NAD^+ was reduced. The reaction mixture was incubated for 60 minutes at 25°C. The reaction was as follows;

$$\text{L-glutamate} + NAD^+ + H_2O \underset{GLDH}{\overset{ADP}{\rightleftharpoons}} \alpha\text{-ketoglutarate} + NH_4^+ + NADH$$

(c) Pyruvate

Pyruvate was assayed in a system containing 477 mM triethanolamine-HCl buffer (pH 7.6), 4.8 mM EDTA, 0.12 mM NADH and 2.75 I.U/ml lactate dehydrogenase (LDH) was used to start the reaction. The assay was based on the decrease in absorbance at 340 nm as NADH was oxidised. The reaction mixture was incubated for 5 minutes at 25°C. The reaction was as follows;

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

(d) α-KETOGLUTARATE

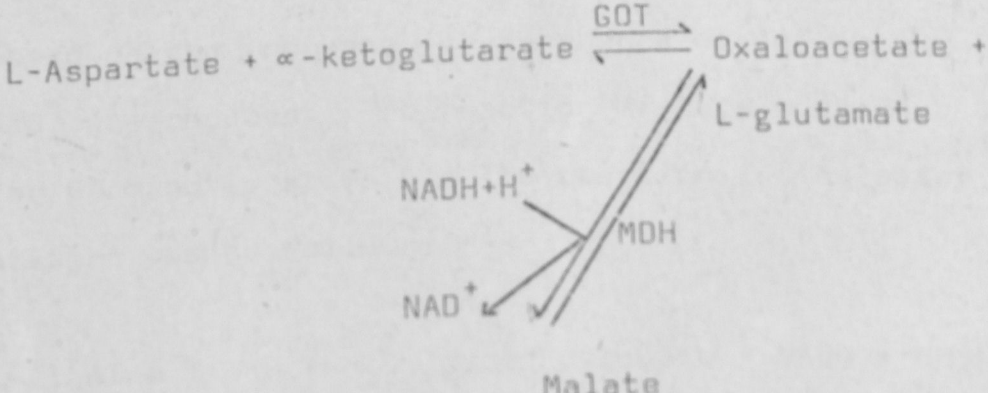
α-Ketoglutarate was assayed in a system containing 15%(w/v) K_2HPO_4, 0.1 mM NADH and 1.8 I.U/ml glutamate dehydrogenase in 2 M $(NH_4)_2SO_4$ was used to start the reaction. The assay was based on the

decrease in absorbance at 340 nm as NADH was oxidised. The reaction mixture was incubated for 20 minutes at 25°C. The reaction was as follows:

$$\alpha\text{-ketoglutarate} + NH_4^+ + NADH \xrightleftharpoons{GLDH} \text{L-glutamate} + NAD^+ + H_2O$$

(e) L-Aspartate

L-Aspartate was assayed in a system containing 61.5 mM sodium phosphate buffer (pH 7.2), 0.18 mM NADH, 3 mm α-ketoglutarate, 17 I.U/ml malate dehydrogenase (MDH) and 2.5 I.U/ml glutamate oxaloacetate transaminase (GOT) was used to start the reaction. The assay was based on the decrease in absorbance at 340 nm as NADH was oxidised. The reaction mixture was incubated for 30 minutes at 25°C. The sequence of reactions in the assay was as follows;



(f) L-Malate

L-Malate was assayed in a system containing 340 mM glycine - 430 mM hydrazine buffer (pH 10) 2.75 mM NAD^+ and 1.3 I U/ml malate dehydrogenase (MDH) was used to start the reaction. The assay was based on the increase in absorbance at 340 nm as NAD^+ was reduced. The reaction mixture was incubated for 60 minutes at 25°C. The reaction was as follows;

$$\text{L-Malate} + NAD^+ \underset{}{\overset{MDH}{\rightleftharpoons}} \text{Oxaloacetate} + NADH + H^+$$

(g) L-Alanine

L-Alanine was assayed as described by Olembo (1980) in a system containing 92 mM glycine-770 mM hydrazine buffer (pH 10) 0.74 mM NAD^+ and 0.38 I.U/ml alanine dehydrogenase (ADH) free from ammonium ions was used to start the reaction. The assay was based on the increase in absorbance at 340 nm as NAD^+ was reduced. The reaction mixture was incubated for 60 minutes at 25°C. The reaction in the assay mixture was as follows;

$$\text{L-Alanine} + NAD^+ + H_2O \overset{ADH}{\rightleftharpoons} \text{pyruvate} + NADH + NH_4^+$$

2.17.2 Enzyme Assays

All enzyme assays were performed at 25°C. The assays were based on the change in absorbance at 340 nm as either NADH was oxidised or NAD^+ ($NADP^+$) was reduced. The assay mixtures were incubated for 2 - 3 minutes to allow completion of endogenous reactions before the reactions were started.

(a) α-Glycerol Phosphate Dehydrogenase (EC 1.1.1.8)

This enzyme was assayed as described by Opperdoes *et al*, (1977a). The assay mixture contained 50 mM sodium phosphate buffer (pH 7.2), 1 mM EDTA, 0.15 mM NADH and 0.5 mM dihydroxyacetone phosphate was used to start the reaction.

(b) Glucose-6-Phosphate Dehydrogenase (EC 1.1.1.49)

This enzyme was assayed as described by Bergemeyer (1974). The assay mixture contained 86 mM triethanolamine - HCl buffer (pH 7.6) 6.9 mM $MgCl_2$, 0.39 mM $NADP^+$ and 1 mM glucose-6-phosphate was used to start the reaction.

(c) Lactate Dehydrogenase (EC 1.1.1.27)

This enzyme was assayed as described by

Bergemeyer (1974). The assay mixture contained 48 mM potassium phosphate buffer (pH 7.5), 0.18 mM NADH and 0.6 mM pyruvate was used to start the reaction.

(d) Pyruvate Kinase (EC 2.7.1.40)

This enzyme was assayed as described by Bergemeyer (1974). The assay mixture contained 85.6 mM triethanolamine-HCl buffer (pH 7.6), 2.5 mM $MgSO_4$ 10 mM KCl, 4.7 mM ADP, 0.2 mM NADH 9.2 I.U/ml lactate dehydrogenase and 0.54 mM phosphoenol pyruvate was used to start the reaction.

(e) Glutamate Pyruvate Transaminase (EC 2.6.1.2)

This enzyme was assayed as described by Bergemeyer (1974). The assay mixture contained 80 mM potassium phosphate buffer (pH 7.4) 800 mM l-alanine, 0.18 mM NADH and 3.7 I.U/ml lactate dehydrogenase. The reaction was started with 18 mM α-ketoglutarate.

(f) Glutamate Oxaloacetate Transaminase (EC 2.6.1.1)

This enzyme was assayed as described by Bergemeyer (1974). The assay mixture contained 80 mM potassium phosphate buffer (pH 7.4), 200 mM

L-aspartate, 0.18 mM NADH, 7.5 I.U/ml malate dehydrogenase and 3.7 I.U/ml lactate dehydrogenase. The reaction was started with 12 mM α-ketoglutarate.

(g) NAD-Linked Malate Dehydrogenase (EC 1.1.1.37)

This enzyme was assayed as described by Bergemeyer (1974). The assay mixture contained 94.6 mM sodium phosphate buffer (pH 7.5) and 0.2 mM NADH. The reaction was started with 0.5 mM oxaloacetate.

(h) NADP -Linked Malic Enzyme (EC 1.1.1.40)

This enzyme was assayed as described by Hoek et al (1976). The assay mixture contained 85 mM Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$ and 50 μM $NADP^+$. The reaction was started with 2 mM L-malate.

2.18 DETERMINATION OF PROTEIN

Protein was determined by the method of Lowry and Roseborough (1951). Protein was precipitated from samples with 5%(w/v) trichloroacetic acid. After centrifugation at 1000 x g for 5 minutes, the clear supernatant was discarded and the pellet resuspended in diethyl ether. The suspension was centrifuged at 1000 x g for 5 minutes and the ether

discarded. The resulting pellet was then dissolved in 1 ml 7%(w/v) NaOH.

Sample protein solutions were diluted so that the final protein concentration was between 30 - 70 μg per 3.0 mls. A standard protein curve was constructed using bovine serum albumin fraction V obtained from Sigma Chemical Company. The optical density of colour developed was read at 750 nm using a Pye Unicam SP 1800 spectrophotometer.

CHAPTER 3

LOCALISATION OF SOME TRYPANOSOMAL ENZYMES BY USE OF DETERGENTS AND FREEZE-THAWING RELEASE METHODS

The pattern of release of glutamate pyruvate transaminase, glutamate oxaloacetate transaminase, NAD-linked malate dehydrogenase, NADP-linked malic enzyme, glucose-6-phosphate dehydrogenase and α-glycerophosphate dehydrogenase from isolated trypanosomes in PSG by increasing detergent concentrations and freeze-thawing cycles was investigated. The detergents used were Triton X-100 and Digitonin. These experiments were performed to ascertain the activities and intracellular localisation of these enzymes in bloodstream form T.b.brucei.

α-Glycerophosphate dehydrogenase was used as a marker for the glycosome and GPT as a marker for the cytosol.

3.1 RELEASE OF GPT, GOT, NAD-LINKED MDH AND α-GPDH BLOODSTREAM FORM T.b. BRUCEI BY TRITON X-100

Results presented in figure 1 show that increasing concentrations of Triton X-100 gradually released GPT, GOT, NAD-linked MDH and α-GPDH from the trypanosomes. The release of α-GPDH activity

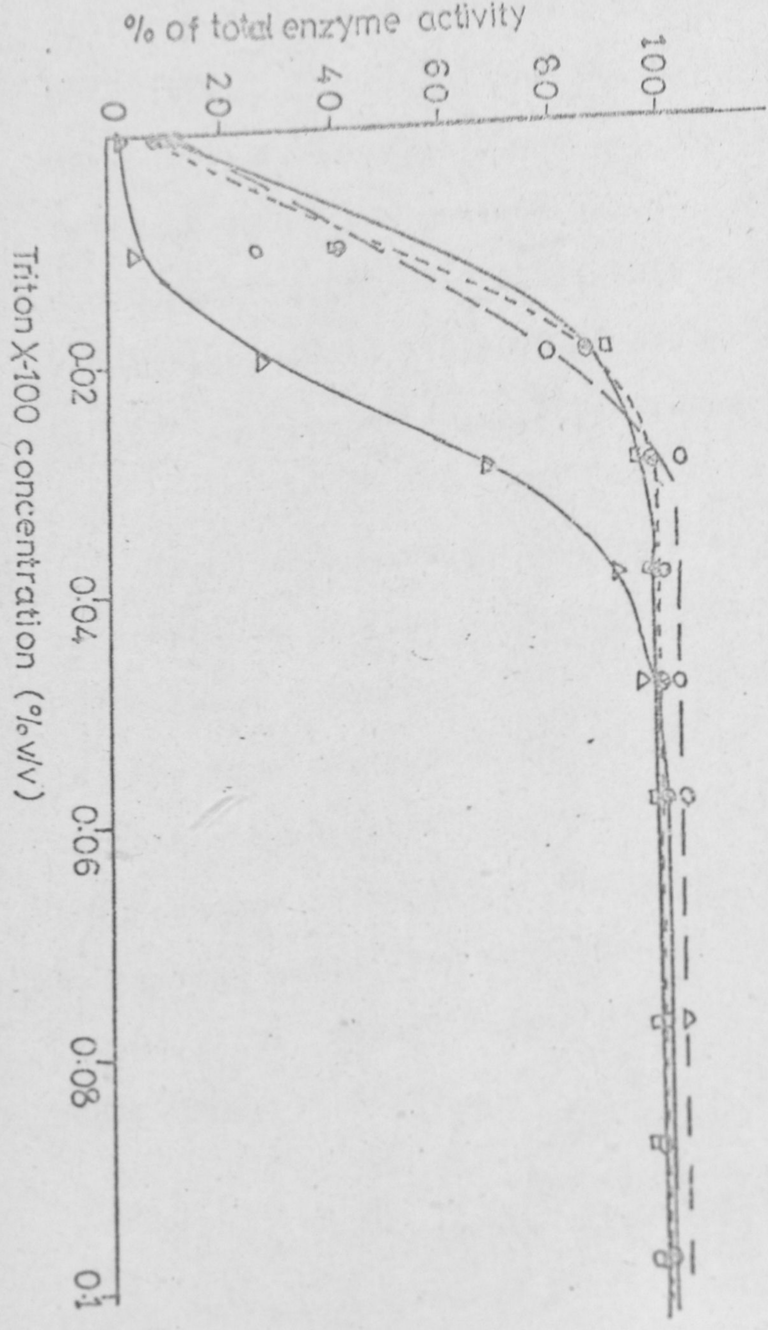
Figure 1

Release of GPT, GOT, NAD-linked MDH and ∝-GPDH from bloodstream form T.b. brucei by Triton X-100. Intact trypanosomes (cell density 10^8/ml) in PSG were incubated at 25°C for 10 minutes with Triton X-100 concentrations ranging from 0 to 0.1% (v/v). Incubations were terminated by centrifugation at 10,000 g for 10 minutes at 4°C and enzymes assayed in the supernatants. The results shown are an average of four individual experiments. Total enzyme activity was taken to be the enzyme activity released by 0.07% Triton X-100.

Total enzyme activities;

GPT	0.526±0.032	µmoles/min/mg protein
GOT	0.075±0.009	"
NAD-Linked MDH	0.140 ± 0.022	"
∝-GPDH	0.490 ± 0.070	"

GPT	-□——□-
GOT	-○- —○-
NAD-Linked MDH	--⊗- - - - ⊗--
∝-GPDH	-△——△-


% of total enzyme activity
0
20
40
60
80
100
0·02
0·04
0·06
0·08
0·1
Triton X-100 concentration (%v/v)

required higher concentrations of Triton X-100 than the other enzymes. The results show that 0.04% Triton X-100 was sufficient to release maximum activities of GPT, GOT and NAD-linked MDH whereas maximum ∝-GPDH activity was released by 0.07% Triton X-100. The maximum enzyme activities released were GPT, 0.526(±0.032); GOT, 0.075(±0.009); NAD-linked MDH, 0.140(±0.022) and ∝-GPDH, 0.490 (±0.070) µmoles/min/mg protein respectively.

There were measurable enzyme activities in supernatants derived from trypanosome suspensions in PSG without detergent. This was taken to be enzyme leakage from damaged trypanosomes in PSG. The percent enzyme activity attributed to leakage from trypanosomes suspended in PSG alone when compared to maximal activities in Triton X-100 were; GPT, 10% (±2.04); GOT, 13%(±2.76); NAD-linked MDH, 9.5%(±2.2) and ∝-GPDH, 3%(±1.00).

The results presented show that GOT and NAD-linked MDH were released in the same pattern as GPT. ∝-Glycerophosphate dehydrogenase was released in a unique pattern.

3.2 RELEASE OF GPT, NADP-LINKED ME, G6PDH AND ∝-GPDH FROM BLOODSTREAM FORM T.b. BRUCEI BY TRITON X-100

Results presented in figure 2 show much semblance to those in figure 1 except that in this experiment NADP-linked ME and G6PDH were studied in addition to GPT and ∝-GPDH. All the enzymes were gradually released from the trypanosomes by increasing Triton X-100 concentrations.

Maximal activities of GPT, NADP-linked ME and G6PDH were released by 0.04% Triton X-100. Maximal activity of ∝-GPDH was released by 0.07% Triton X-100. The maximum enzyme activities released were; GPT, 0.51(±0.055); NADP-linked ME, 0.012(±0.0029); G6PDH, 0.012(±0.003) and ∝-GPDH, 0.485(±0.031) μmoles/min/mg protein respectively.

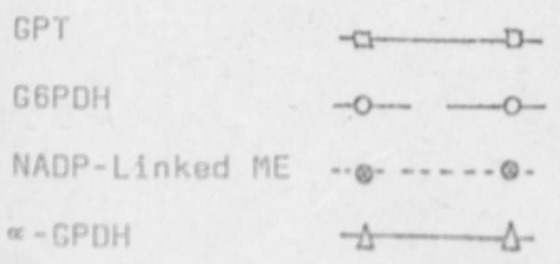
As observed in section 3.1, there were measurable enzyme activities in supernatants derived from trypanosome suspensions in PSG without detergent. By comparison to the maximum activities obtained with Triton X-100, the percentage of enzyme activity attributed to leakage in PSG alone were; GPT, 12% (±3.20), NADP-linked ME, 6%(±0.95), G6PDH, 9%(±2.85) and ∝-GPDH 3.3%(±0.99).

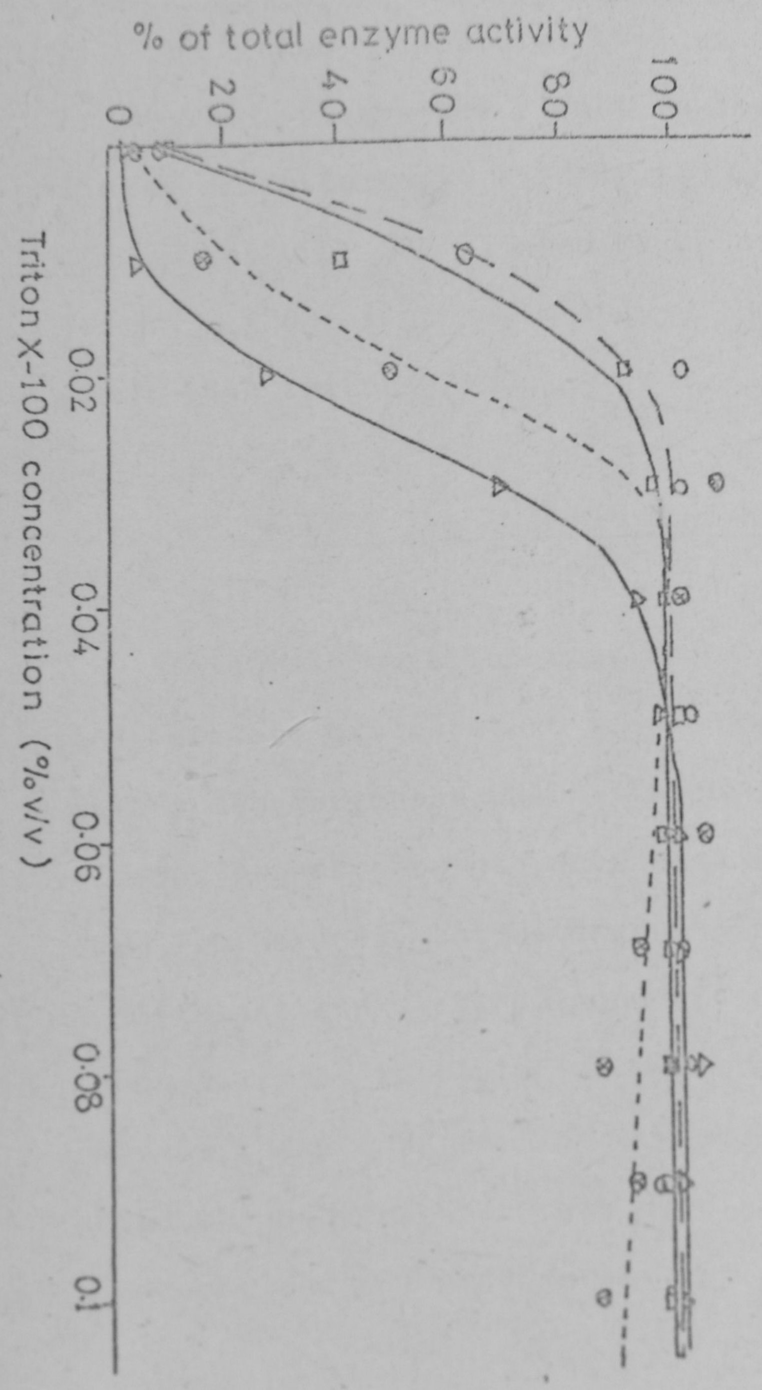
Figure 2

Release of GPT, NADP-linked ME, G6PDH and α-GPDH from bloodstream form T.b. brucei by Triton X-100. Intact trypanosomes (cell density 5×10^8/ml) in PSG were incubated at 25°C for 10 minutes with Triton X-100 concentrations ranging from 0 to 0.1% (v/v). Incubations were terminated by centrifugation at 10,000 g for 10 minutes at 4°C and enzymes assayed in the supernatants. The results shown are an average of four individual experiments. Total enzyme activity was taken to be the enzyme activity released by 0.07% Triton X-100.

Total enzyme activities;

GPT	0.510±0.055	μmoles/min/mg protein
NADP-linked ME	0.012±0.0029	"
G6PDH	0.012 ±0.0029	"
α-GPDH	0.455±0.031	"





% of total enzyme activity
0
20
40
60
80
100
0·02
0·04
0·06
0·08
0·1
Triton X-100 concentration (%v/v)

Glucose-6-phosphate dehydrogenase was released in the same pattern as GPT. Between 0% and 0.03% Triton X-100 concentration, the pattern of release of NADP-linked ME activity fell between that of GPT and α-GPDH. There was slight inhibition of NADP-linked ME activity at high Triton X-100 concentrations. The activity of NADP-linked ME obtained with 0.1% Triton X-100 was approximately 90% of that obtained with 0.04% Triton X-100.

3.3. RELEASE OF TRYPANOSOMAL ENZYMES BY DIGITONIN

To obtain more information on the localisation of glutamate oxaloacetate transaminase, NAD-linked malate dehydrogenase, NADP-linked malic enzyme and glucose-6-phosphate dehydrogenase in bloodstream form T.b. brucei, the detergent-Digitonin, which has a different and distinct mode of action of binding to cholesterol in biological membranes was used to release these enzymes from trypanosomes. α-Glycerophosphate dehydrogenase was used as a marker for the glycosome and GPT as a marker for the cytosol.

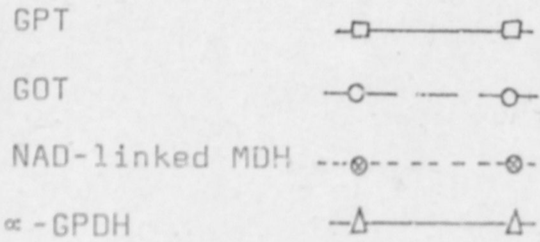
3.3.1 Release of GPT, GOT, NAD-linked MDH and α-GPDH From Bloodstream form T.b. Brucei by Digitonin

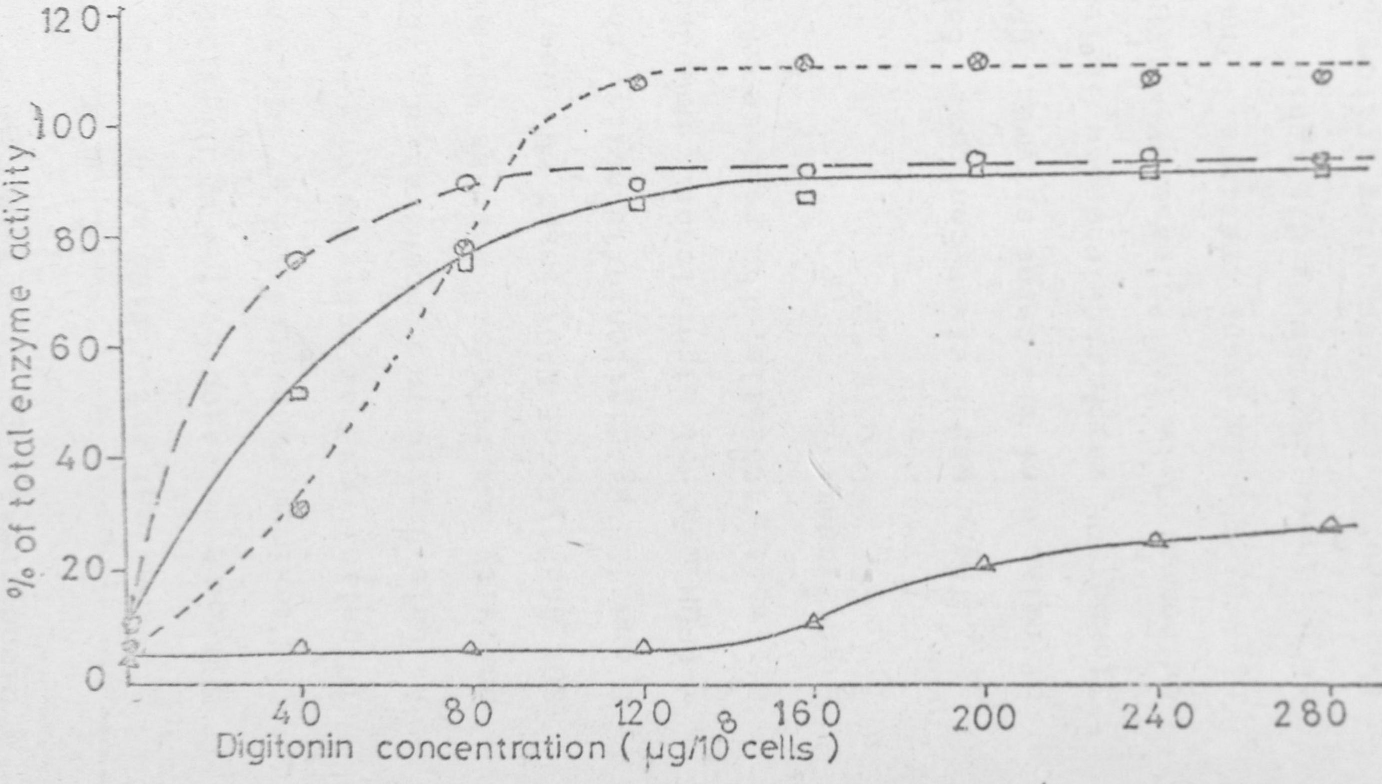
Results presented in figure 3 show that increasing Digitonin concentrations gradually released

Figure 3

Release of GPT, GOT, NAD-linked MDH and α-GPDH from bloodstream T.b. brucei by Digitonin.

Intact trypanosomes (cell density 10^8/ml) in PSG were incubated at 25°C for 10 minutes with Digitonin concentrations ranging from 0 to 280 μg/ml. Incubations were terminated by centrifugation at 10,000 g for 10 minutes at 4°C and enzymes assayed in the supernatants. Total enzyme activity was taken to be the enzyme activity released by 0.07% Triton X-100 (Figure 1). Results shown are average values of four individual experiments.




% of total enzyme activity
0
20
40
60
80
100
120
40
80
120
160
200
240
280
Digitonin concentration (μg/10⁸ cells)

GPT, GOT, NAD-linked MDH and ∝-GPDH from isolated bloodstream T.b. brucei in PSG.

Maximal activities of GPT, GOT and NAD-linked MDH were released by 140 µg Digitonin/10^8 cells. At the maximum concentration of Digitonin used (280 µg/10^8 cells) maximal activity of ∝-GPDH was not released. At this Digitonin concentration, 28% of ∝-GPDH activity was released whereas 90% of GPT, 91% of GOT and 110% of NAD-linked MDH was released. Between 0 and 120 µg Digitonin/10^8 cells, the release of ∝-GPDH was not significant. However above 160 µg Digitonin/10^8 cells, the release of ∝-GPDH was significant.

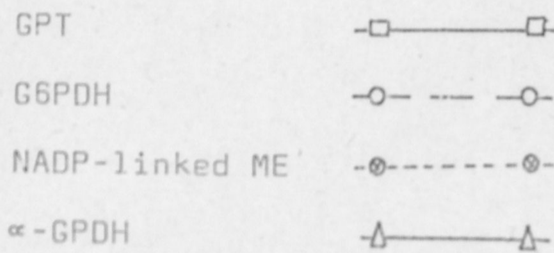
There was a clear contrast between the pattern of release of the three enzymes, GPT, GOT and NAD-linked MDH which required low Digitonin concentrations as compared to the release of ∝-GPDH which required high digitonin concentrations. The pattern of release of these enzymes by Digitonin show similarity to the results obtained using Triton X-100 (Figure 1).

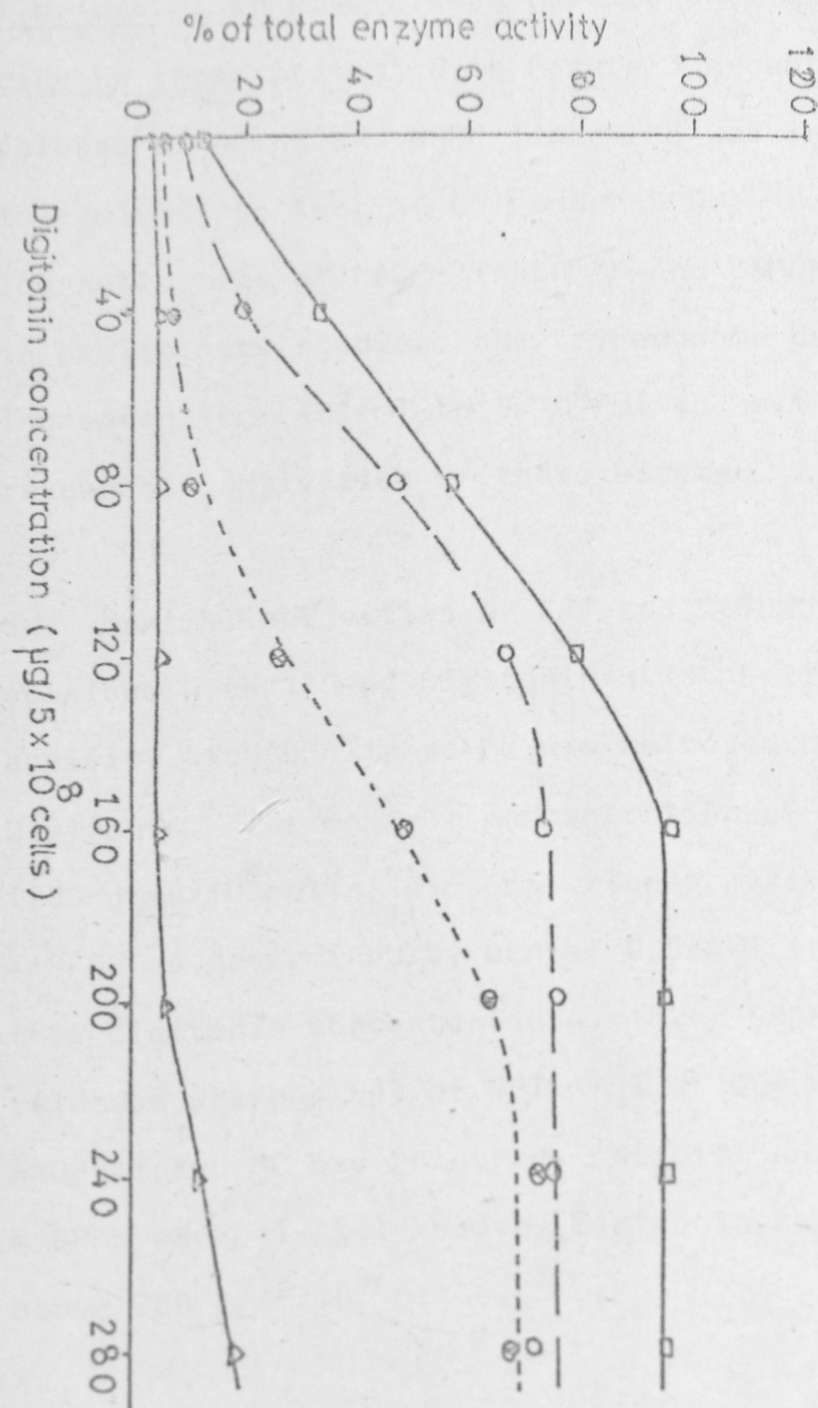
3.3.2. Release of GPT, NADP-linked ME, G6PDH and ∝-GPDH from Bloodstream form T.b. brucei by Digitonin

Results presented in figure 4 show that increasing Digitonin concentrations gradually released GPT,

Figure 4

Release of GPT, NADP-linked ME, G6PDH and α-GPDH from bloodstream form T.b. brucei by Digitonin. Intact trypanosomes (cell density 5×10^8/ml) in PSG were incubated at 25°C for 10 minutes with Digitonin concentrations ranging from 0 to 280 μg/ml. Incubations were terminated by centrifugation at 10,000 g for 10 minutes at 4°C and enzymes assayed in the supernatants. Total enzyme activity was taken to be the enzyme activity released by 0.07% Triton X-100 (Figure 2). Results shown are average values of four individual experiments.





% of total enzyme activity
0
20
40
60
80
100
120
Digitonin concentration (µg/5 x 10^8 cells)
40
80
120
160
200
240
280

NADP-linked ME, G6PDH and α-GPDH from bloodstream T.b. brucei in PSG. These results show much similarity to those presented in Figure 3 except that the release of G6PDH and NADP-linked ME was investigated in addition to that of GPT and α-GPDH. Due to the low activities of NADP-linked ME and G6PDH obtained in preliminary studies, the trypanosome density was increased from 10^8/ml to 5×10^8/ml so as to obtain reasonable activities of these enzymes.

Maximal activities of GPT and G6PDH were obtained with 170 μg Digitonin/5×10^8 cells. Maximal activity of NADP-linked ME was released by 226 μg Digitonin. The maximum concentration of Digitonin used (280 μg/5×10^8cells) did not release maximal activity of α-GPDH as determined by use of 0.07% Triton X-100. At this Digitonin concentrations, 18% α-GPDH activity was released whereas 94% of GPT, 75% of G6PDH and 68% of NADP-linked ME was released. Significant activity of α-GPDH was only released by Digitonin concentrations above 200 μg/5×10^8 cells.

Glucose-6-phosphate dehydrogenase was released in a pattern similar to that of GPT. The pattern of NADP-linked ME release fell between that of GPT and α-GPDH which was released at high Digitonin concentrations. These results are comparable to

those obtained with Triton X-100 (Figure 2) in this study.

Results presented in figures 3 and 4 show that when the trypanosome density was increased, higher Digitonin concentrations were required to release enzymes from the parasites. When the cell density was 10^8/ml, 140 µg Digitonin/ml was required to release maximal activity of GPT whereas when the cell density was 5×10^8/ml, 170 µg Digitonin /ml was required. Similarly when the cell density was 10^8/ml, 28% of α-GPDH activity was released by 280 µg Digitonin/ml. This Digitonin concentration released only 18% of α-GPDH activity when the cell density was increased to 5×10^8/ml.

3.4 Release of Trypanosomal Enzymes by Freeze-Thawing

To complement the results obtained with Triton X-100 and Digitonin on the location of GOT, NADP-linked ME, NAD-linked MDH and G6PDH in bloodstream *T.b. brucei*, a mechanical procedure - freeze-thawing was used to release the enzymes from trypanosomes.

3.4.1 Release of GPT, GOT, NAD-linked MDH and ∝-GPDH from Bloodstream T.b. brucei by Freeze-thawing

Results presented in Figure 5 show that 2 cycles of freeze-thawing rapidly released the maximal activities of GPT, GOT and NAD-linked MDH from the trypanosomes. One freeze-thawing cycle released above 60% of the activities of GPT, GOT and NAD-linked MDH whereas only 16% of ∝-GPDH activity was released. The maximal enzyme activities released by freeze-thawing were; GPT, 73%; GOT, 80%: NAD-linked MDH, 118% and ∝-GPDH, 53%. Seven freeze-thawing cycles were required to release the maximal activity of ∝-GPDH. The release of ∝-GPDH by freeze-thawing was gradual unlike the release of GPT, GOT and NAD linked MDH which was rapid.

The release of GOT and NAD-linked MDH followed a pattern similar to that of GPT. These results are comparable to those obtained with Triton X-100 (Figure 1) and Digitonin (Figure 3).

3.4.2. Release of GPT, NADP-linked ME, G6PDH and ∝-GPDH from Bloodstream T.b. brucei by freeze-thawing

Results presented in Figure 6 show that 2 freeze-thawing cycles released maximal activities of

Figure 5

Release of GPT, GOT, NAD-linked MDH and α-GPDH from bloodstream form T.b. brucei by freeze-thawing. Intact trypanosomes (cell density 10^8/ml) in PSG were subjected to freeze-thawing by freezing in liquid nitorgen for 20 minutes and thawing at 25°C. The suspension were then centrifuged at 10,000 x g for 10 minutes at 4°C and enzymes assayed in the supernatants. Total enzyme activity was taken to be the enzyme activity released by 0.07% Triton X-100 (Figure 1). Results presented are average values of four individual experiments.

GPT	-□———□-
GOT	-○— — —○-
NAD-linked MDH	-⊗------⊗-
α-GPDH	-△———△-

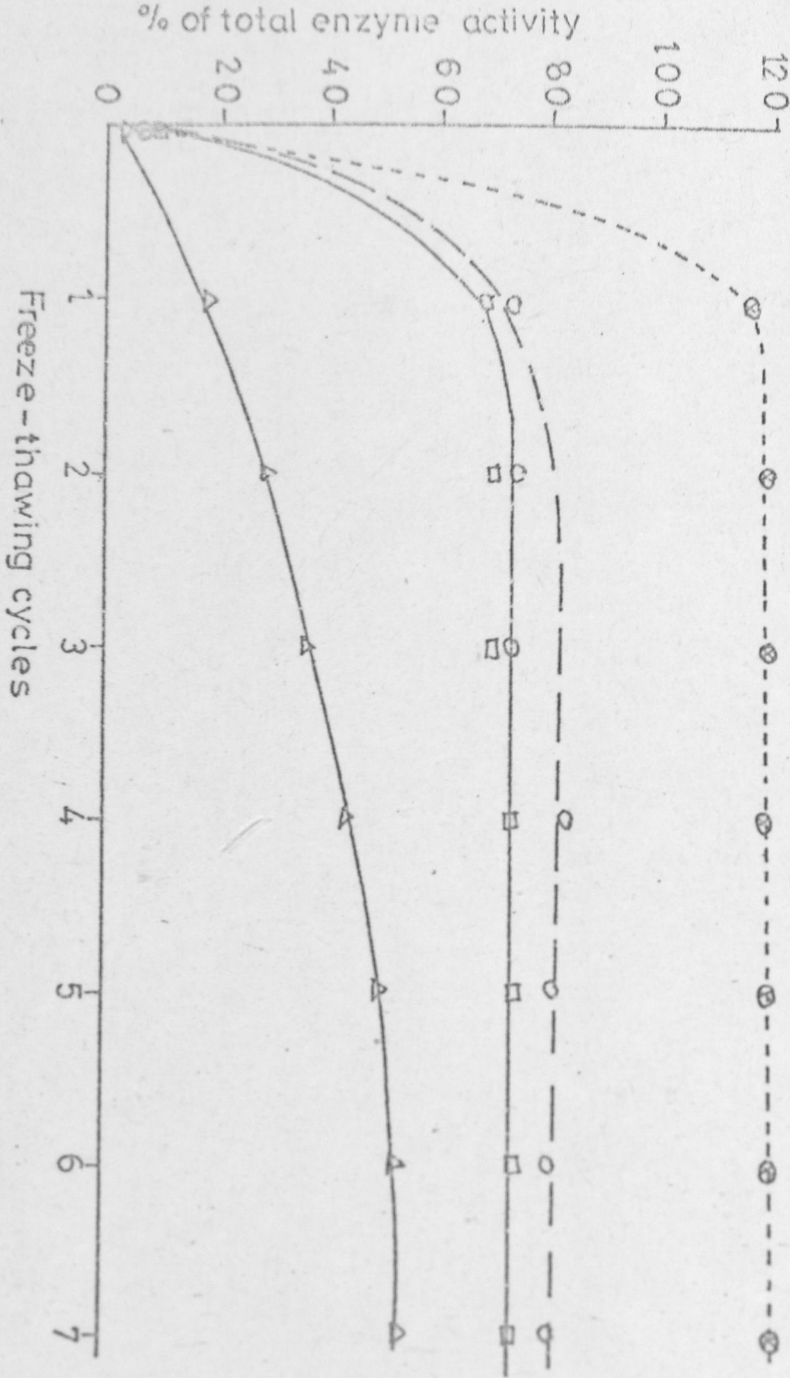
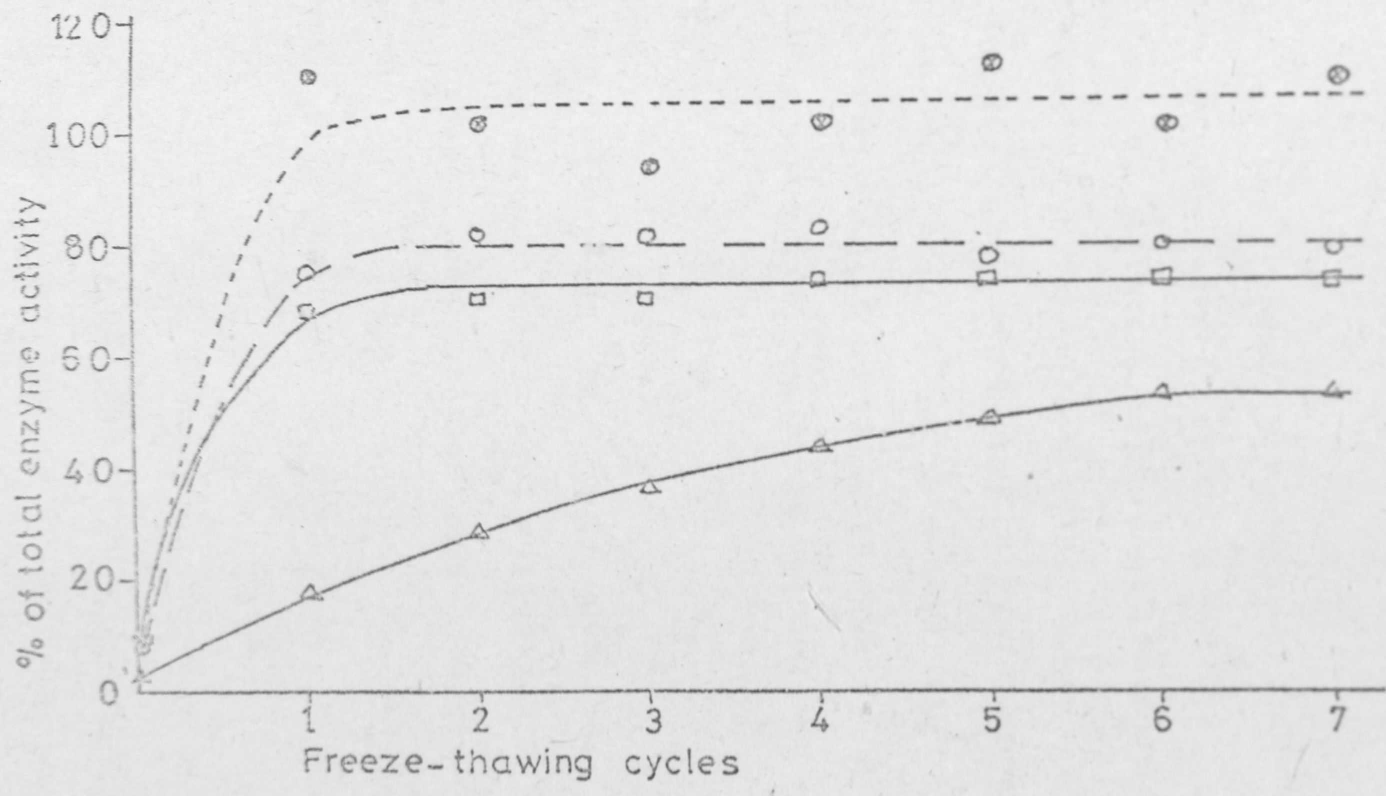

% of total enzynie activity
0
20
40
60
80
100
120
Freeze-thawing cycles
1
2
3
4
5
6
7

Figure 6

Release of GPT, NADP-linked ME, G6PDH and ∝-GPDH from bloodstream form T.b. brucei by freeze-thawing. Intact trypanosomes (cell density 5×10^8/ml) in PSG were subjected to freeze-thawing by freezing in liquid nitrogen for 20 minutes and thawing at 25°C. The suspensions were then centrifuged at 10,000 x g for 10 minutes at 4°C and enzymes assayed in the supernatants. Total enzyme activity was taken to be the enzyme activity released by 0.07% Triton X-100 (Figure 2). Results shown are average values of four individual experiments.

GPT	-□———□-
G6PDH	-○-- --- -○-
NADP-linked ME	-⊗------⊗-
∝-GPDH.	-△———△-



% of total enzyme activity
120
100
80
60
40
20
0
1
2
3
4
5
6
7
Freeze-thawing cycles

GPT, NADP-linked ME and G6PDH. Seven freeze-thawing cycles were required to release maximal activity of ∝-GPDH from trypanosomes. These results are comparable to those obtained for the release of GOT and NAD-linked MDH (Figure 5).

One freeze-thawing cycle released above 65% of the activities of GPT, NADP-linked ME and G6PDH but only 17% of ∝-GPDH activity was released. The maximal enzyme activities released by freeze-thawing were; GPT, 73%; NADP-linked ME, 105%; G6PDH, 80% and ∝-GPDH, 54%.

The results presented here are different from those obtained with Triton X-100 (Figure 2) and Digitonin (Figure 4) where the pattern of NADP-linked ME release fell between that of GPT and ∝-GPDH. Freeze-thawing released NADP-linked ME from trypanosomes in a pattern similar to that obtained for GPT. Glucose-6-phosphate dehydrogenase was released in a pattern similar to that of GPT.

3.4.3 DISCUSSION

Biological membranes are composed of proteins and lipids The lipid component consists of phospholipids and cholesterol. Phospholipid tails contribute a continous non-polar hydrocarbon core to the membrane (Singer and Nicolson, 1972). Biological membranes are physical barriers separating the aqueous compartments with different solute composition. Usually the movement of polar molecules through biological membranes is via specific carrier proteins situated within the membrane. The selective permeability of the biological membranes can be abolished artificially by disrupting the arrangement of the membrane components.

Triton X-100 a non-ionic detergent solubilizes hydrophobic moieties in biological membranes. Digitonin binds to and removes cholesterol residues from biological membranes. Freeze-thawing causes mechanical damage to the membranes. These procedures disrupt the integrity of a biological membrane making it permeable to molecules non-selectively.

An enzyme enclosed by more than one membrane in an organism would require higher detergent concentrations or more cycles of freeze-thawing to release

than one enclosed by only one membrane or within a membrane. An enzyme located inside a small vesicle would be especially difficult to release by the freeze-thawing procedure (Hayashi et al, 1971; Muller, 1973).

In bloodstream form T.b. brucei, α-glycerophosphate dehydrogenase is glycosomal (Opperdoes et al, 1977(a); 1980; 1984) and glutamate pyruvate transaminase is cytosolic (Steiger et al, 1980; Visser and Opperdoes, 1980). The pattern of release of glutamate oxaloacetate transaminase and NAD-linked malate dehydrogenase in this study by freeze-thawing, Digitonin and Triton X-100 was similar to that of GPT indicating that these enzymes are cytosolic in bloodstream form T.b. brucei. This was in agreement with the results of other authors in this field. A cytosolic GOT in bloodstream form T.b. brucei has been reported by Opperdoes and co-workers (1975). Opperdoes et al, (1981) have reported a cytosolic NAD-linked MDH in bloodstream T.b. brucei.

During the present study, no literature was available on the location of NADP-linked malic enzyme and glucose-6-phosphate dehydrogenase in bloodstream form T.b. brucei. Klein and co-workers (1975) have reported a cytosolic NADP-linked ME in T.b. brucei procyclics. They did not investigate the

location of this enzyme in bloodstream forms. In *T. cruzi* epimastigotes, NADP-linked ME is both cytosolic and mitochondrial and is inhibited by high Digitonin concentrations (Cannata, 1984). The bloodstream *T.b. brucei* NADP-linked ME reported here was not inhibited by the Digitonin concentrations used but was inhibited significantly by 0.1% Triton X-100. The pattern of NADP-linked ME release from trypanosomes by Digitonin and Triton X-100 fell between that of cytosolic and glycosomal enzymes. However, the pattern in which the NADP-linked ME was released by freeze-thawing suggested that the enzyme is cytosolic. Since a mitochondrial marker was not used in this study, the localisation of this enzyme in bloodstream form *T.b. brucei* could not be determined conclusively. If the enzyme is located inside a large organelle like the promitochondrion, it would be resistant to release by detergents as observed for glycosomal enzymes but easily released by freeze-thawing as observed for cytosolic enzymes. In bloodstream from *T.b. brucei* NADP-linked ME is most likely located in the promitochondrion and cytosol.

Glucose-6-phosphate dehydrogenase has been assayed in bloodstream trypanosomes. Ryley (1962) found a G6PDH activity of 0.0070-0.0168 µmoles/min/mg/ protein in bloodstream form *T.rhodesiense* homogenates. Reynolds (1975) found a G6PDH activity of 0.0043 µmoles/

min/mg protein in bloodstream T. evansi homogenates. The G6PDH activity obtained in this study with 0.1% Triton X-100 from bloodstream T.b. brucei was 0.012 ±0.003 µmoles/min/mg protein. The pattern of release of this enzyme from the trypanosomes by Triton X-100, Digitonin and freeze-thawing indicated that it is cytosolic.

The localisation of GPT, GOT, NAD-linked MDH and some of the NADP-linked ME in the cytosol of bloodstream T.b. brucei has important implications. It gives credibility to the proposed roles of GPT and GOT in bloodstream trypanosomes. Ryley, (1962) and Reynolds (1975) proposed that GPT, GOT, NAD-linked MDH and NADP-linked ME are involved in a series of coupled reactions leading to a transhydrogenation between NADH and $NADP^+$ in the cytosol of the parasite. The results of the present study are in agreement with the reported observations and proposals. The transhydrogenation pathway would be feasible if the enzymes involved were in the same compartment as reported in this study. Results on the investigation of the roles of GPT and GOT in bloodstream T. b. brucei are discussed in the following chapter.

CHAPTER 4

RAT PLASMA ENZYME LEVELS DURING T.B. BRUCEI INFECTION

As observed with the results on the release of trypanosomal enzymes by detergents and freeze-thawing (Chapter 3) there were significant enzyme activities in the supernatants derived from trypanosome suspensions in PSG not subjected to the enzyme-releasing procedures. It was apparent that enzymes were leaking slightly from the trypanosomes.

Leakage of trypanosomal enzymes into host plasma was investigated in an attempt to explain the origin of additional plasma enzyme activities in animals infected with T.b. brucei. Several authors in this field have made proposals on the origin of additional plasma enzyme activities during trypanosomal infections. Linpi and Sebastian (1958) (quoted by Gray, 1963) proposed that the increase in serum GPT and GOT levels in T.b brucei infected guinea pigs was due to lesions of liver, myocardium, adrenal glands and nervous system. Moon et al (1968) proposed that the increase in serum GPT and GOT in mice infected with T.b. rhodesiense was of trypanosomal origin. Godwin and Guy, (1973) attributed the rise in serum GOT in rabbits infected with T.b. brucei to host cell

necrosis and Stibbs and Seed, (1976) attributed the rise in serum tyrosine aminotransferase in microtus montanus infected with T.b. gambiense to the trypanosomes. These authors did not perform experiments to show presence or absence of trypanosomal enzymes in serum of infected animals.

In this study, the plasma levels of GPT, GOT, P K ,αGPDH and LDH were measured in healthy and T.b. brucei-infected rats. It should be noted that the bloodstream form of T.b. brucei has no lactate dehydrogenase. Male adult Sprague-Dawley rats were infected with 10^4 trypanosomes. Control rats were injected with PSG.

All the rats were tail bled on alternate days and parasitemia determined in infected rats. Results of the enzyme assays are presented in figures 7A to 11.

Figure 7A shows the plasma LDH levels of control non-infected rats. The levels were fairly constant at approximately 23 μmoles/min/100ml plasma. Figure 7B shows the rat plasma LDH activity during T.b. brucei infection. There was no significant alteration of rat plasma LDH activity during infection as it remained at approximately 25 μmoles/min/100ml plasma.

Figure 7A

Normal rat-plasma LDH activity.

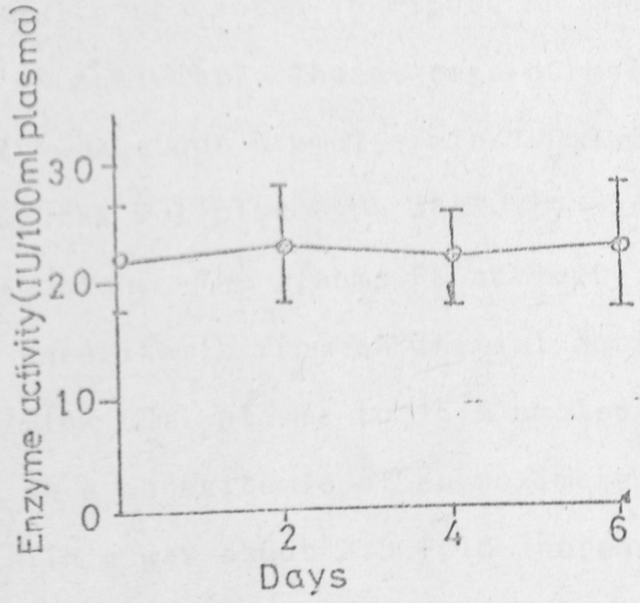
Vertical bars represent standard error.

Figure 7B

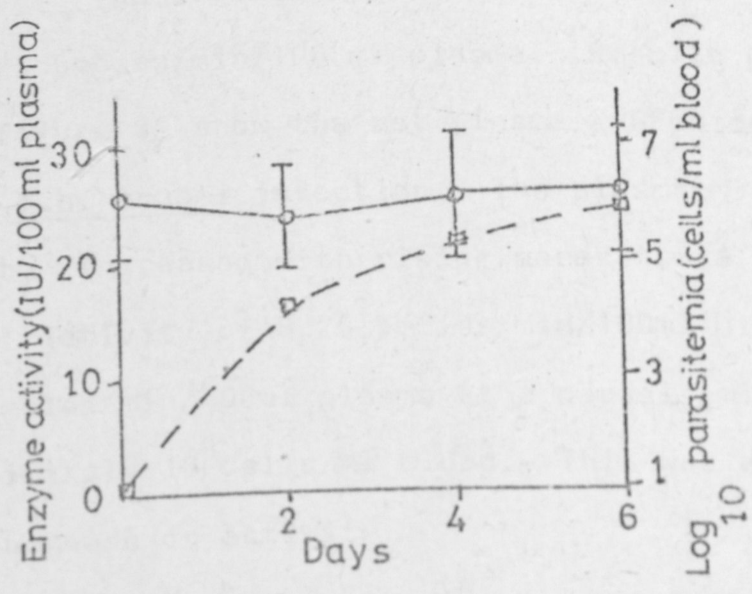
Rat plasma LDH activity during *T.b. brucei* infection. Vertical bars represent standard error.

LDH activity —o———o—

Parasitemia —□— — —□—



Enzyme activity (IU/100ml plasma)
30
20
10
0
2
4
6
Days



Enzyme activity (IU/100ml plasma)
30
20
10
0
2
4
6
Days
Log10 parasitemia (cells/ml blood)
7
5
3
1

Results presented in figure 8A show normal rat plasma PK activity. The average normal plasma PK activity was about 6 µmoles/min/100ml plasma. Figure 8B shows the rat plasma PK activity during <u>T.b. brucei</u> infection. The plasma PK activity increased with rising parasitemia from an initial activity of 6 µmoles/min/100ml plasma to 15.5 µmoles/min/100ml plasma at a parasitemia of approximately 10^8 cells/ml blood. This was about 2.6 fold increase in activity.

Figure 9A shows the normal rat plasma α-GPDH activity. The average normal plasma α-GPDH activity was 0.25 µmoles/min/100 ml plasma. Results presented in figure 9B show the rat plasma α-GPDH activity during <u>T.b. brucei</u> infection. The plasma α-GPDH activity increased with rising parasitemia from an initial activity of 0.25 µmoles/min/100ml plasma to 0.36 µmoles/min/100ml plasma at a parasitemia of approximately 10^8 cells/ml blood. This was a 1.4 fold increase in activity.

Results in figure 10 show the normal and infected rat plasma GPT activity. In the control non-infected rats, the plasma GPT activity remained at approximately 2.6 µmoles/min/100ml plasma. In the infected animals, the plasma GPT activity increased with rising parasitemia from an initial

Figure 8A

Normal rat plasma PK activity.

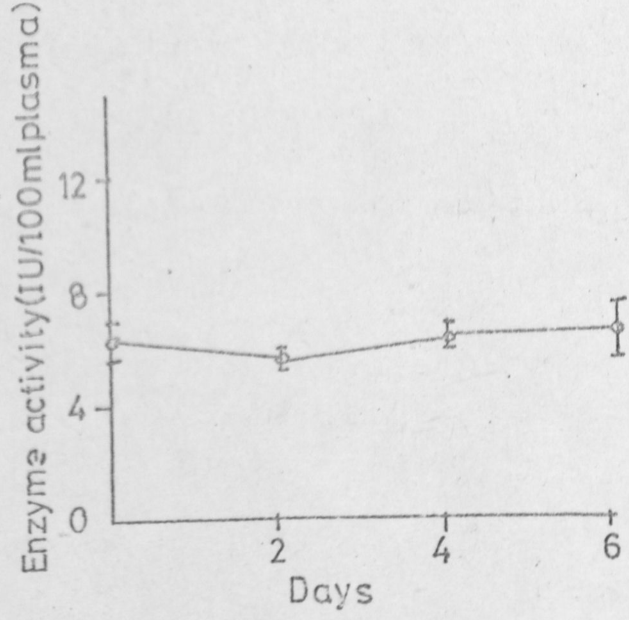
Vertical bars represent standard error.

Figure 8B

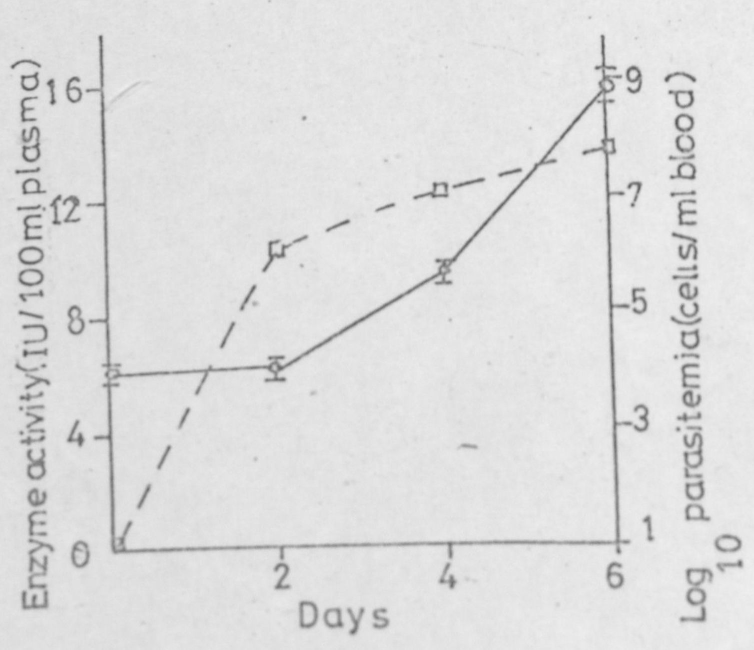
Rat plasma PK activity during *T.b. brucei* infection. Vertical bars represent standard error.

PK activity -o——o-

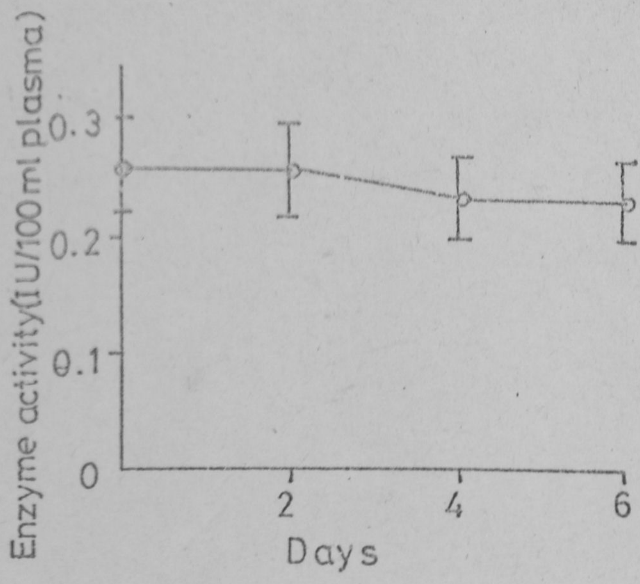
Parasitemia -□- — -□-



Enzyme activity(IU/100ml plasma)
0
4
8
12
2
4
6
Days



Enzyme activity(IU/100ml plasma)
0
4
8
12
16
2
4
6
Days
Log10 parasitemia(cells/ml blood)
1
3
5
7
9


Enzyme activity(IU/100ml plasma)
0
0.1
0.2
0.3
2
4
6
Days

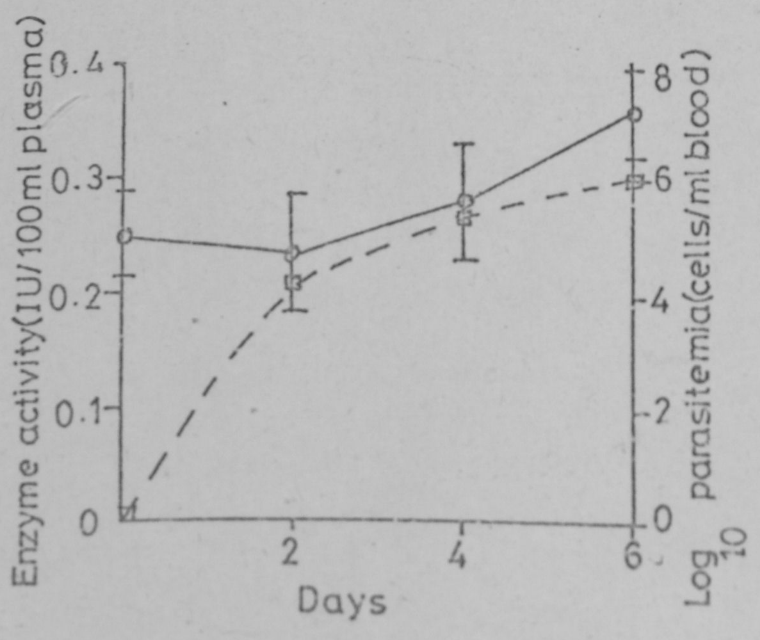

Enzyme activity(IU/100ml plasma)
0
0.1
0.2
0.3
0.4
2
4
6
Days
Log10 parasitemia(cells/ml blood)
0
2
4
6
8

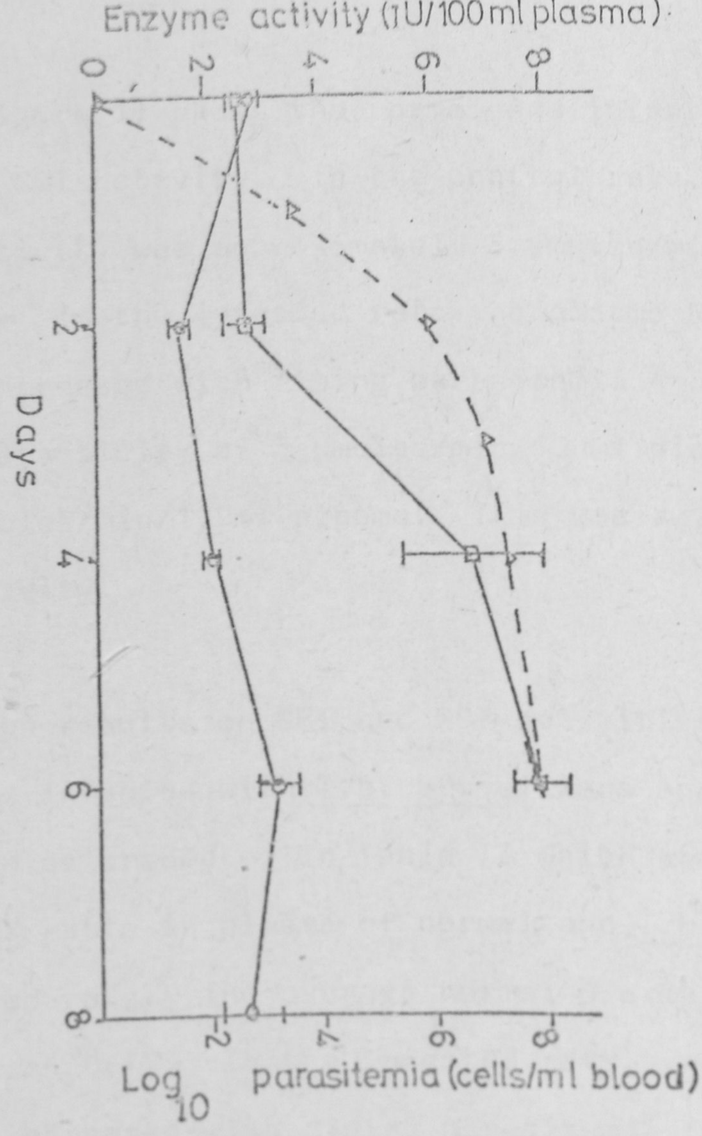
Figure 10

Normal and *T.b. brucei*-infected rat plasma GPT activity. Vertical bars represent standard error.

Normal rat plasma GPT activity -o———o-

Infected rat plasma GPT activity -□———□-

Parasitemia -△- — -△-


Enzyme activity (IU/100 ml plasma)
Days
Log10 parasitemia (cells/ml blood)

activity of 2.7 µmoles/min/100ml plasma to 7.8 µmoles/ min/100ml plasma at a parasitemia of approximately 10^8cells/ml/blood. This was a 4.4 fold increase in activity.

Figure 11 shows the normal and infected rat plasma GOT activity. In the control rats, the plasma GOT activity was approximately 3 µmoles/min/100ml plasma. In the infected rats the plasma GOT activity increased with rising parasitemia from an initial activity of 3 µmoles/min/100ml plasma to 5.8 µmoles/min/100ml plasma. This was a 1.9 fold in activity.

The results on GPT and GOT activities in plasma of rats infected with T.b. brucei were analysed further as presented in Table II which shows the GPT/GOT ratio in plasma of normal and T.b. brucei - infected rats. The average normal plasma GPT/GOT ratio was 0.75. In the infected rats, the GPT/GOT ratio increased with rising parasitemia from an initial value of 0.89 to 1.40 at a parasitemia of approximately 10^8cells/ml blood. This was an indication that the plasma level of GPT during infection was rising faster than that of GOT.

Figure 11

Normal and *T.b. brucei*-infected rat plasma GOT activity.

Vertical bars represent standard error.

Normal rat plasma GOT activity -○———○-

Infected rat plasma GOT activity -□———□-

Parasitemia -△- - - -△-

TABLE II

PLASMA GPT/GOT RATIO IN T.B. BRUCEI INFECTED RATS

DAYS	LOG_{10} PARASITEMIA (Parasites/ml blood)	GPT/GOT RATIO	
		INFECTED	NORMAL
0	0	0.88 ± 0.071	0.76 ± 0.009
2	6.05	1.10 ± 0.090	0.68 ± 0.038
4	7.37	1.45 ± 0.126	0.76 ± 0.038
6	7.78	1.40 ± 0.124	0.76 ± 0.027
8	-	-	0.77 ± 0.027

Parasitemia only applies to infected rats.

GPT and GOT were determined from the same plasma samples.

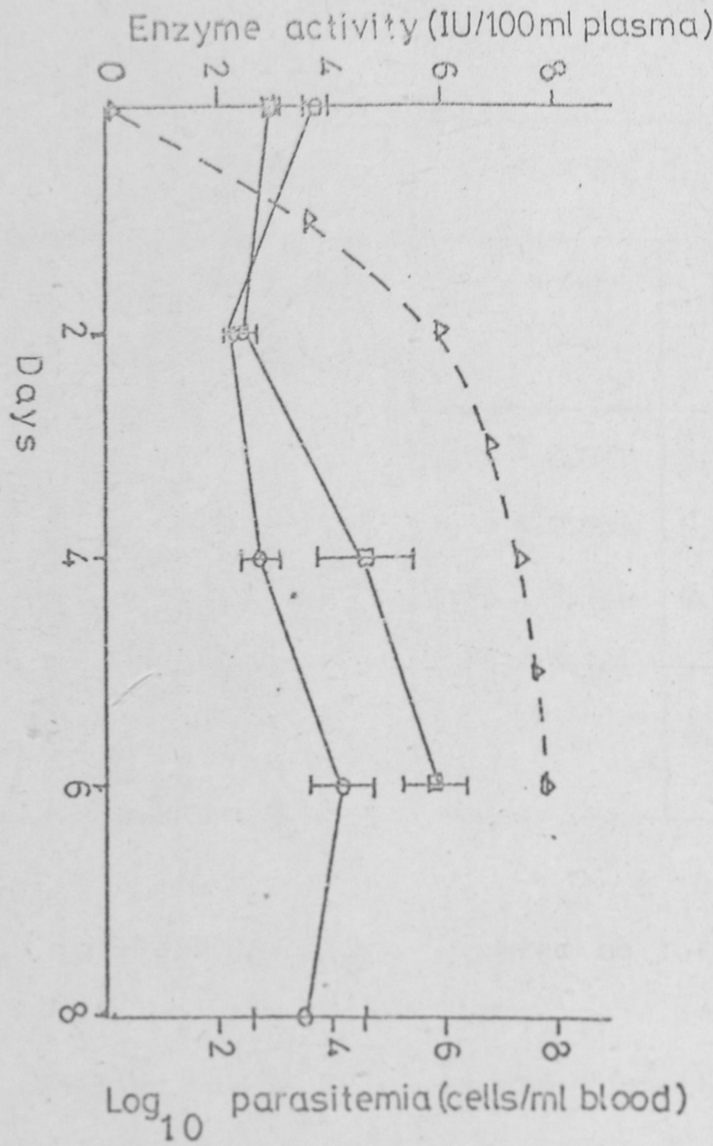

Enzyme activity (IU/100ml plasma)
0
2
4
6
8
Days
2
4
6
8
Log10 parasitemia (cells/ml blood)

TABLE II

PLASMA GPT/GOT RATIO IN T.B. BRUCEI INFECTED RATS

DAYS	LOG_{10} PARASITEMIA (Parasites/ml blood)	GPT/GOT RATIO INFECTED	GPT/GOT RATIO NORMAL
0	0	0.83 ± 0.071	0.76 ± 0.009
2	6.05	1.10 ± 0.090	0.68 ± 0.038
4	7.37	1.45 ± 0.126	0.76 ± 0.038
6	7.78	1.40 ± 0.124	0.76 ± 0.027
8	-	-	0.77 ± 0.027

Parasitemia only applies to infected rats.

GPT and GOT were determined from the same plasma samples.

4.1 STARCH GEL ELECTROPHORESIS OF GPT AND GOT

It was proposed from the results obtained in section 4 that elevated glutamate pyruvate transaminase and glutamate oxaloacetate transaminase in plasma of T.b. brucei - infected rats was due to the trypanosomes. To confirm this proposal, starch gel electrophoresis of normal rat serum, infected rat serum and trypanosomal lysate was performed.

Results presented in figures 12A and 12B show starch gel electrophoresis of normal rat serum, infected rat serum and trypanosomal lysate. The gel sketched in figure 12A was developed for GPT and that shown in figure 12B was developed for GOT. In both cases, the gel showed one bandfor normal serum and two bands for infected serum. Trypanosomal lysate showed one band and it coincide with the second band obtained with infected serum.

Figure 12A

Starch gel electrophoresis of GPT. Sketch of gel developed for GPT and viewed under ultraviolet light. The shaded areas represent regions of enzyme activity. The migration of the bands is drawn to scale.

(+) Anode

(-) Cathode

Figure 12B

Starch gel electrophoresis of GOT. Sketch of gel developed for GOT and viewed under ultraviolet light. The shaded areas represent regions of enzyme activity. The migration of the bands is drawn to scale.

(+) Anode

(-) Cathode

Origin

Normal Serum

(-) Infected serum (+)

Trypanosomal lysate

Origin

Normal serum

(-) Infected serum (+)

Trypanosomal lysate

DISCUSSION

The presence of GPT, GOT, PK and ∝-GPDH in bloodstream form T.b. brucei has been ascertained but the presence of LDH has not been unequivocally demonstrated (Ryley, 1962; Dixon, 1966). Therefore an elevation of plasma LDH during T.b. brucei infections would be attributed to host tissue damage by the trypanosomes since the latter have no LDH.

The enzymes GPT, GOT and PK are cytosolic in bloodstream form T.b. brucei (Oduro et al, 1980; Visser and Opperdoes, 1980; Opperdoes et al, 1977a,b) and ∝-GPDH is glycosomal (Opperdoes et al 1977a; 1980). The enzymes showing highest elevation in plasma of T.b. brucei- infected rats are cytosolic in the parasite thus GPT, GOT and PK were elevated 4.4 fold, 2 fold and 2.6 fold respectively at a parasitemia of approximately 10^8 cells/ml blood. The glycosomal ∝-GPDH was elevated 1.4 fold. In previous experiments (Chapter 3) it was observed that maximal activity of cytosolic enzymes was released from trypanosomes by two freeze-thawing cycles whereas glycosomal enzymes required as many as 7 cycles for release. If trypanosomes in plasma were lysed progressively, cytosolic enzymes

would be released before glycosomal enzymes hence the low elevation of α-GPDH in plasma of T.b. brucei infected rats.

Since both the host and the trypanosomes contain GPT, GOT, PK and α-GPDH, the additional activities of these enzymes in plasma of infected rats may have been of either host, trypanosomal origin or both. Pyruvate kinase and LDH are present in host tissues e.g. heart, skeletal muscle, liver and erythrocytes. As explained earlier, bloodstream trypanosomes contain PK but not LDH. If the elevation observed in the plasma PK activity in the plasma of infected rats was caused by haemolysis, LDH should also have been elevated, but this was not the case. This was an indication that the rise in plasma PK activity during T.b. brucei infection in rats was of trypanosomal origin.

The ratio of GPT/GOT in bloodstream form T.b. brucei measured in this study was 7 (Chapter 3). This value was within the range of ratios obtained by Godfrey and Kilgour, (1973) for bloodstream T.b. brucei. If these two transaminases were to leak from disintegrating trypanosomes into plasma, the final plasma levels of GPT would be higher than that of GOT as shown in Table II. This suggests

that the elevation of these transaminases in the plasma during infection was due to leakage from trypanosomes.

In mammals, tissue damage results in the alteration of the plasma levels of GPT and GOT. For example in cases of liver disease, diseases of the biliary system, muscle and heart diseases, both GPT and GOT are elevated in plasma (Christen and Metzler, 1985). The levels of these enzymes in plasma are thus important indicators of tissue damage. Tissue damage has been reported in trypanosomal infections. Murray et al, (1974) observed splenomegaly, hepatomegaly and myocardial damage in rats infected with T. brucei for a period of eight weeks. Morrison et al, (1978) reported severe lesions in heart, central nervous system, eyes, skeletal muscle, kidney and testicles of dogs infected with T. brucei for 3 - 4 weeks. The extent to which host tissue damage during trypanosomal infections contributes to alteration in plasma enzyme levels has not been established.

Starch gel electrophoresis of normal rat serum infected rat serum and trypanosomal lysate indicated presence of trypanosomal GPT and GOT in infected serum. This finding led to the conclusion that GPT and GOT were leaking from trypanosomus into the plasma.

In conclusion, it is apparent that the origin of additional activities of GPT and GOT in plasma of rats infected with T.b. brucei are the trypanosomes. The origin of the additional activities of PK and α-GPDH in plasma of rats infected with T. brucei can be inferred to be from trypanosomes upon lysis.

CHAPTER 5

THE ROLE OF GPT AND GOT IN BLOODSTREAM T.B. BRUCEI

Following the assays of GPT and GOT in trypanosomes, the study of these two transaminases was extended in an attempt to identify some of their roles in the parasite. The results of this study are presented in Tables III, IV, V, VI, and figure 13.

5.1. UTILIZATION OF GLUTAMATE BY BLOODSTREAM T.B. BRUCEI

In this experiment 10^8 cells/ml PSG were incubated aerobically at 25°C with and without 10 mm L-glutamate. Incubations were terminated every 30 minutes and the concentrations of substrates and products assayed in the post-incubation medium.

The results presented in Table III show that when trypanosomes were incubated in PSG alone under aerobic conditions, pyruvate was the only end product detectable. The quantity of pyruvate produced was twice the amount of glucose consumed. α-Ketoglutarate, L-alanine and L-aspartate were not detectable.

TABLE III

PRODUCTION OF PYRUVATE, L-ALANINE, L-ASPARTATE AND α-KETOGLUTARATE BY T.B.BRUCEI INCUBATED IN PSG AT 25°C UNDER AEROBIC CONDITIONS

Values are µmoles metabolite per 10^8 cells

Time (Mins)	D-glucose Consumption	Pyruvate Production	α-ketoglutarate Production	L-alanine Production	L-aspartate Production
0	0	0	0	0	0
30	1.27	2.75	0	0	0
60	2.21	5.18	0	0	0
90	3.04	7.88	0	0	0
120	4.58	9.34	0	0	0
150	5.52	11.25	0	0	0

When 10 mM L-glutamate was included in the incubation medium (Table IV), pyruvate production was reduced by approximately 10%. L-Alanine and ∝-ketoglutarate were produced in equimolar quantities but L-aspartate was not detectable. Glucose consumption was not affected by addition of 10 mM L-glutamate to the incubation medium.

As shown in figure 13, L-alanine production was a function of L-glutamate concentration in the incubation medium. There was a steady though not linear increase in L-alanine production with increasing L-glutamate concentration. The non-linearity of L-alanine production versus L-glutamate concentration may be explained in terms of limitations caused by the mode of transport of these two amino acids in trypanosomes. Transport of L-alanine and L-glutamate in bloodstream T.b. brucei is carrier-mediated (Voorheis, 1971).

It was not possible to account for 25% of the L-glutamate consumed in terms of either the ∝-ketoglutarate or alanine produced by the trypanosomes (Table IV). This amount was 0.13 µmoles L-glutamate/hr/10^8 cells. This observation prompted investigation of pathways which may consume L-glutamate or convert it to the D-form.

TABLE IV

PRODUCTION OF PYRUVATE, L-ALANINE, L-ASPARTATE AND α-KETOGLUTARATE BY T.B. BRUCEI INCUBATED IN PSG + 10 mM L-GLUTAMATE AT 25°C UNDER AEROBIC CONDTIONS

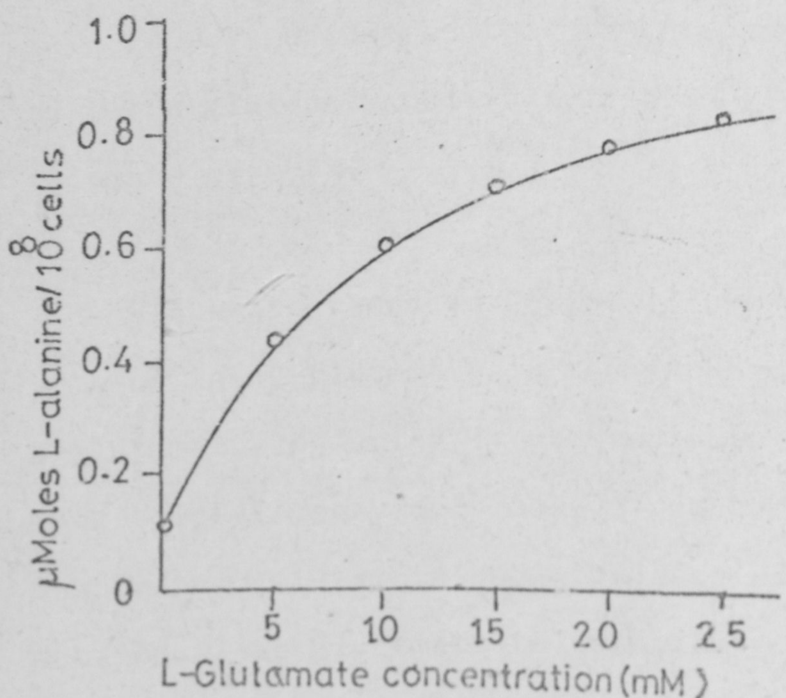
Values are µmoles metabolite produced or consumed by 10^{8} cells

TIME (MINS)	D-glucose Consumption	Pyruvate Production	L-Glutamate Consumption	α-Ketoglutarate Production	L-alanine Production	L-aspartate Production
0	0	0	0	0	0	0
30	1.03	2.23	0.23	0.13	0.12	0
60	2.45	4.68	0.39	0.26	0.34	0
90	3.16	6.89	0.69	0.44	0.54	0
120	4.46	8.44	0.84	0.59	0.71	0
150	5.28	10.13	1.15	0.77	0.84	0

Figure 13

EFFECT OF L-GLUTAMATE ON L-ALANINE PRODUCTION

10^8 cells were incubated aerobically at 25°C in 1 ml PSG containing varying concentrations of L-glutamate for 150 minutes. Incubations were terminated as described in Materials and Methods.


1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
25
μMoles L-alanine/ 10^8 cells
L-Glutamate concentration (mM)

5.2 TEST FOR GLUTAMATE RACEMASE AND GLUTAMATE DECARBOXYLASE IN BLOODSTREAM T.B. BRUCEI

Results presented in Table IV show that 25% of the total glutamate consumed by trypanosomes incubated in PSG containing 10 mM L-glutamate could not be accounted for either by the α-ketoglutarate or L-alanine produced. An attempt was therefore made to identify other reactions which may consume L-glutamate. It was postulated that the trypanosomes contained either a glutamate decarboxylase, a glutamate racemase or both.

Glutamate racemase would, if present, convert some of the L-glutamate in the incubation medium to D-glutamate rendering it undetectable by the L-glutamate-specific enzymatic assay used here to assay this substrate. Glutamate decarboxylase would if present decarboxylate glutamate to gamma amino butyric acid therefore utilising some of the L-glutamate in the incubation medium.

The cells were incubated in PS without glucose so that there would be no transamination between L-glutamate in the incubation medium and pyruvate produced in glycolysis. Results presented in Table V show that when 10^8 cells/ml were incubated in PS

at 25°C in aerobic conditions, L-glutamate was not utilised. The t-Test for paired measurements with a probability of error of 1% showed that there was no significant differences in the concentrations of L-glutamate after incubation with parasites.

TABLE V

TEST FOR GLUTAMATE RACEMASE AND GLUTAMATE DECARBOXYLASE IN BLOODSTREAM T.B. BRUCEI

Values are µmoles L-glutamate/ml

EXPERIMENT NO.	Before incubation (Zero time)	After incubation (2 hrs.)
1	9.80	10.02
2	10.00	10.12
3	9.90	9.85
4	9.90	9.90
5	9.87	9.90
6	9.35	9.70
Average	9.89	9.92

The glutamate assay was tested with a standard solution of L-glutamate and found to be accurate to within 4%. The standard solution was made to approximately the ideal concentration recommended by Bergemeyer (1974) for maximum sensitivity of the assay. Similarly, the test samples were diluted so that the glutamate concentration fell within the ideal concentration.

5.3 PAPER CHROMATOGRAPHY OF AMINO ACIDS

It was proposed in this study that trypanosomes in PSG may convert L-glutamate to other amino acids other than L-alanine. Paper chromatography of amino acid extracts of trypanosomes incubated in PSG and 10 mM L-glutamate confirmed that alanine was the only amino acid product of this incubation.

From these experiments it was concluded that the role of GPT in bloodstream T.b. brucei is to excrete pyruvate generated in glycolysis as alanine. In this study, approximately 10% of the pyruvate generated in glycolysis was converted to alanine.

5.4 UTILISATION OF ASPARTATE BY T.B. BRUCEI LYSATES

It was proposed in this study that GPT and GOT in bloodstream T.b. brucei may be involved in a series of coupled reactions leading to a transhydrogenation between NADH and $NADP^+$.

α-Ketoglutarate produced by transamination of glutamate with pyruvate may transaminate with aspartate in a reaction catalyzed by GOT to form glutamate and oxaloacetate. NAD-linked malate dehydrogenase reduces this oxaloacetate to malate in the presence of NADH. The malate generated by the NAD-linked MDH

reaction is then oxidatively decarboxylated to pyruvate by NADP-linked ME in the presence of $NADP^+$ generating NADPH. Trypanosomal NAD-linked MDH can catalyze the oxidation of malate to oxaloacetate. Oxaloacetate is unstable and may decarboxylate non-enzymatically to pyruvate. For this reason, it was found difficult to demonstrate the oxidative decarboxylation of malate to pyruvate by NADP-linked ME in this study.

Results presented in Table VI show production of malate by trypanosome lysates incubated in PS containing 10 mM L-aspartate. When the lysates were incubated with L-aspartate alone, the amount of malate produced was small. Addition of 10 mM α-ketoglutarate caused minimal increase in malate production. However, when both 5 mM NADH and 10 mM α-ketoglutarate were included in the incubation medium, the amount of malate produced increased significantly from 0.012 µmoles/hr/10^8cells obtained with L-aspartate alone to 0.51 µmoles/hr/10^8cells. It was concluded that the most likely role of GOT in bloodstream form <u>T.b. brucei</u> is to provide substrate for a pathway leading to the formation of NADPH.

TABLE VI

PRODUCTION OF MALATE BY T.B. BRUCEI LYSATES

ADDITIONS	MALATE PRODUCTION µmoles L-Malate/hr/10^8cells
None	N.D
10 mM L-Aspartate	0.012 ± 0.0012
10 mM L-Aspartate 10 mM α-Ketoglutarate	0.018 ± 0.0011
10 mM L-Aspartate 10 mM α-Ketoglutarate 5 mm NADH	0.510 ± 0.0051

In this experiment 10^8cells/ml PS were freeze-thawed once and incubated in the presence of neutralized L-aspartate, α-ketoglutarate and NADH for 1 hr. at 25°C. Incubations were terminated as described in Materials and Methods and malate assayed.

DISCUSSION

Amino acids have been suggested to be physiologically important to bloodstream trypanosomes by being linked to carbohydrate metabolism to remove pyruvate formed during glycolysis and perhaps also contributing to the oxidation-reduction balance in these organisms (Stibbs and Seed, 1973; Hall et al, 1981). Transaminases are important in the metabolism of amino acids. The results of the present study attempt to explain the possible roles of the two transaminases which have been widely reported in bloodstream form T.b. brucei. These are GPT and GOT. The activity of these transaminases reported here in bloodstream T.b. brucei were; GPT, 0.526 ± 0.032 and GOT, 0.075 ± 0.009 µmoles/min/mg protein respectively.

Whole parasites in PSG incubated in aerobic conditions produced approximately 2 moles of pyruvate from 1 mole of glucose consumed. This was consistent with the results obtained by Opperdoes et al (1976) and Brohn and Clarkson, (1980). When L-glutamate was included in the incubation medium, glucose consumption was not altered but pyruvate production was reduced. L-Alanine and α-ketoglutarate were produced in equimolar quantities indicating that GPT catalysed the transamination of L-glutamate with

pyruvate to produce α-ketoglutarate and L-alanine. Production of L-alanine was a function of L-glutamate concentration in the incubation medium. The production of alanine was further confirmed by paper chromatography.

Using paper chromatography, Williamson and Desowitz, (1961) found alanine to be the major free amino acid in bloodstream form _T.b. rhodesiense_. Chappell and Co-workers, (1972) found alanine to constitute more than 47% of the total free amino acid pool in bloodstream form _T.b. gambiense._ They also recovered labelled alanine when bloodstream form _T.b. gambiense_ were incubated with uniformly labelled $[^{14}C]$ glucose. Chappell and Co-workers (1972) speculated that alanine was formed by transamination of glutamate with pyruvate in a reaction catalysed by GPT. The stoichiometry of this reaction was however not investigated. Results presented in the present study led to the conclusion that excretion of pyruvate in the form of alanine is a major role of GPT activity in bloodstream for _T.b. brucei_. The advantage of excreting pyruvate in the form of alanine is however not clear.

About 25% of the L-glutamate consumed by bloodstream _T.b. brucei_ incubated in PSG containing

10 mM-glutamate could not be accounted for in terms of either the α-ketoglutarate or the L-alanine produced. This discrepancy amounted to approximately 0.13 μmoles L-glutamate/hr/2×10^8 parasites. Investigations on possible pathways which could be consuming L-glutamate or converting it to the D-form which would be undetectable by the assay method used did not provide a solution to this discrepancy.

Various amino acid racemases have been reported in micro-organisms. Soda (1971) has reported activity of an unspecific amino acid racemase in Pseudomonas striata and Cardinale (1968) has reported proline racemase activity in that organism. Glutamate racemase activity has been reported in Lactobacillus arabinosus (Wood, 1955; Glaser, 1960). Glutamate racemase activity was not detected in bloodstream form T.b. brucei. These parasite also did not show glutamate decarboxylase activity.

In addition to the role of GPT in the excretion of pyruvate in bloodstream form T.b. brucei, it is proposed in this study that this enzyme may share another role with GOT in a transhydrogenation between NADH and $NADP^+$ (Chapter 1 section 1.4.1). It has been shown in this study that GOT, NAD-linked MDH and possibly part of NADP-linked ME activity

are cytosolic like GPT in bloodstream form T.b. brucei.

Bloodstream form T.b. brucei can utilise acetyl CoA units derived from glucose or threonine catabolism for lipid biosynthesis (Linstead et al, 1977). Reduced NADP is required in fatty acid synthesis. Two main systems for $NADP^+$ reduction have been suggested in trypanosomes; the pentose phosphate pathway and transhydrogenation with NADH. The former pathway requires glucose and the latter aspartate both of which could be obtained from the host plasma (Reynolds, 1975)

In bloodstream form T. evansi, Reynolds, (1975) reported activities of enzymes which may produce NADPH. These were; G6DPH (0.0043 µmoles/min/mg protein), NADP-linked isocitrate dehydrogenase (0.00213 µmoles/min/mg protein) and NADP-linked ME (0.0147 µmoles/min/mg protein). In bloodstream form T.b. rhodesiense, Ryley, (1962) reported activities of enzymes which may produce NADPH. These were somewhat higher than those obtained by Reynolds (1975) in bloodstream T. evansi. These activities were; G6PDH (0.0070 - 0.0168 µmoles/min/mg protein, NADP-linked isocitrate dehydrogenase (0.0040 - 0.0095 µmoles/min/mg protein) and NADP-linked ME (0.0157 - 0.0306 µmoles/min/mg protein). The activity of

NADP-linked ME reported in this study in bloodstream form T.b. brucei was 0.012 ± 0.0029 μmoles/min/mg protein. The high activity of NADP-linked ME as compared to that of G6PDH and NADP-linked isocitrate dehydrogenase suggests that NADP-linked ME is a major source of NADPH in bloodstream trypanosomes.

The enzymes involved in the proposed transhydrogenation of NADH and $NADP^+$ have high activity in bloodstream form T.b. brucei. The activities were; GPT, 0.526 ± 0.032; GOT, 0.075 ± 0.009; NAD-linked MDH, 0.140 ± 0.022 and NADP-linked ME, 0.012 ± 0.0029 μmoles/min/mg protein respectively. Glutamate pyruvate transaminase transaminates pyruvate derived from glycolysis with glutamate derived from host plasma to α-ketoglutarate and alanine. This α-ketoglutamate is transaminated with aspartate derived from host plasma by GOT producing oxaloacetate and glutamate. This oxaloacetate is then reduced to malate by NADH in a reaction catalysed by NAD-linked MDH. Finally NADP-linked ME oxidatively decarboxylates malate in the presence of $NADP^+$ generating NADPH in the trypanosome. The existence of a transhydrogenation pathway is supported by the observation that when trypanosomal lysates were incubated with aspartate and α-ketoglutarate only trace amounts of malate were produced but when NADH was included in the incubation medium, large amounts of malate were

produced. The overall transhydrogenation pathway would be as shown below;

$$\text{Aspartate} + \text{NADH} + \text{NADP} \rightleftharpoons \text{Alanine} + CO_2 + NAD^+ + \text{NADPH}$$

The last step in this transhydrogenation pathway has been reported by several authors working with other tissues. Brdiczka and Pette (1971) have postulated that the function of NADP-linked ME in heart tissue of rabbits, rats, pigs, beef and pigeons would be transhydrogenation yielding NADPH, Lardy et al, (1964) assigned the role of NADP-linked ME in rat liver as production of NADPH for fat synthesis, Hsu, (1970) reported oxidative decarboxylation of malate by pigeon liver NADP-linked ME and Cazzulo et al, (1977) have reported oxidative decarboxylation of malate by NADP-linked ME from *T. cruzi* epimastigotes.

It was concluded in this study that the most likely role of GOT in bloodstream *T.b. brucei* is in a transhydrogenation pathway leading to the transfer of reducing equivalents from NADH to $NADP^+$.

CHAPTER 6

AMINOTRANSFERASES IN BLOODSTREAM T.B. BRUCEI

6.1 A SURVEY OF AMINOTRANSFERASES IN BLOODSTREAM T.B. BRUCEI

The catabolism of most amino acids begin with a transamination. There are no reports in literature of a complete survey of transaminases in bloodstream T.b. brucei, therefore experiments were performed in an attempt to identify aminotransferases in the parasite utilizing α-ketoglutarate as amino group acceptor.

Results presented in Table VII show that bloodstream form T.b. brucei lysates transaminated alanine, aspartate, glutamine, leucine, isoleucine, valine, phenylalanine, tryptophan, tyrosine and methionine significantly with α-ketoglutarate as amino group acceptor. The highest transamination activity was that of alanine. The branched-chain amino acids; valine, leucine and isoleucine were transaminated at similar rates. Among the aromatic amino acids, the highest transamination activity was that of phenylalanine followed by tyrosine and tryptophan. There was novel transamination activity of glutamine, leucine, isoleucine, valine and

TABLE VII

A SURVEY OF AMINOTRANSFERASES IN BLOODSTREAM T. BRUCEI

L-AMINO ACID	µmoles L-Glutamate produced/hr/mg protein
ALANINE	1.444 ± 0.0410 (4)
ARGININE	0.024 ± 0.0010 (4)
ASPARAGINE	0.108 ± 0.0025 (4)
ASPARTATE	0.667 ± 0.0120 (4)
CITRULLINE	0.074 ± 0.0018 (4)
CYSTEINE	0.061 ± 0.0039 (4)
GLUTAMINE	0.330 ± 0.0070 (4)
GLYCINE	0.059 ± 0.0124 (4)
HISTIDINE	0.068 ± 0.0044 (4)
ISOLEUCINE	1.092 ± 0.0152 (4)
LEUCINE	1.022 ± 0.0275 (4)
VALINE	1.132 ± 0.0132 (4)
LYSINE	0.064 ± 0.0054 (4)
METHIONINE	0.645 ± 0.0116 (4)
ORNITHINE	0.029 ± 0.0030 (4)
PHENYLALANINE	1.132 ± 0.0220 (4)
TYROSINE	0.789 ± 0.0137 (4)
TRYPTOPHAN	0.385 ± 0.0141 (4)
PROLINE	0.069 ± 0.0050 (4)
SERINE	0.049 ± 0.0041 (4)
THREONINE	0.039 ± 0.0029 (4)

The values obtained from incubations without amino acid have been substracted from the values shown in Table VII. Values presented are means ± standard error for the number of determinations shown in parenthesis.

methionine by bloodstream form *T.b. brucei* lysates.

The transamination pathway of methionine catabolism in mammals may lead to production of toxic intermediates - methanethiol and hydrogen sulphide (Benevenga and Steele, 1979). It was proposed in the present study that a similar pathway may exist in bloodstream form *T.b. brucei*. The study of methionine transamination by bloodstream form *T.b. brucei* was therefore extended.

6.2 RELEASE OF METHIONINE TRANSAMINATING ENZYME BY FREEZE-THAWING AND SONICATION

In this study, the methionine transaminating activity was referred to as methionine transaminating enzyme. The use of detergents to release the methionine transaminating enzyme from trypanosomes was avoided because of the long incubation period (1 hr.) required to obtain significant transamination. If detergents were used, they may have had unknown effects on the enzyme.

Results presented in Figure 14 show the release of the methionine transaminating enzyme from bloodstream form T.b. brucei by freeze-thawing. A single freeze-thawing cycle was sufficient to release maximal activity of the enzyme (approximately 0.7 μmoles/min/mg protein. Repeated freeze-thawing was found to inhibit the enzyme significantly with five cycles of freeze-thawing reducing the enzyme activity by 26%.

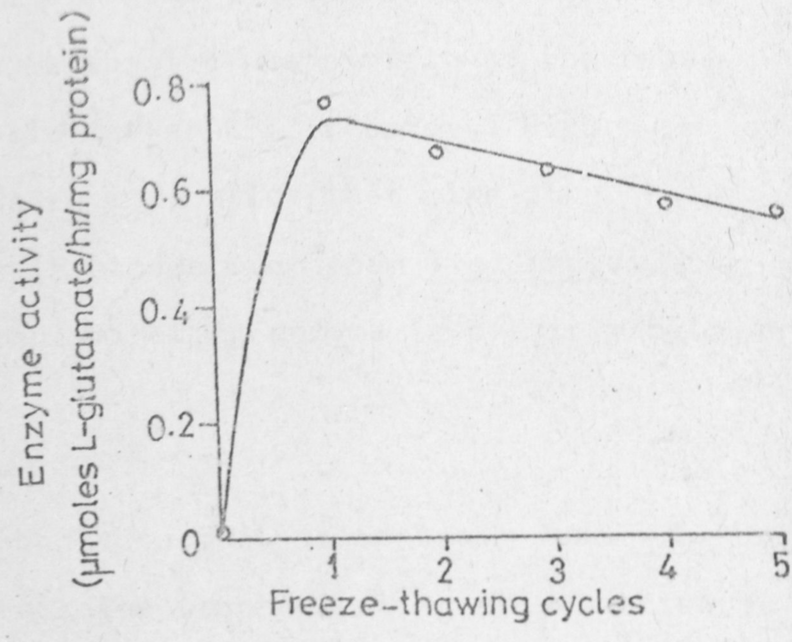
Results presented in Figure 15 show the release of the methionine transaminating enzyme from bloodstream T.b. brucei by sonication at $4^{o}C$. Sixty seconds of sonication released maximal activity of the methionine transaminating enzyme. This value was

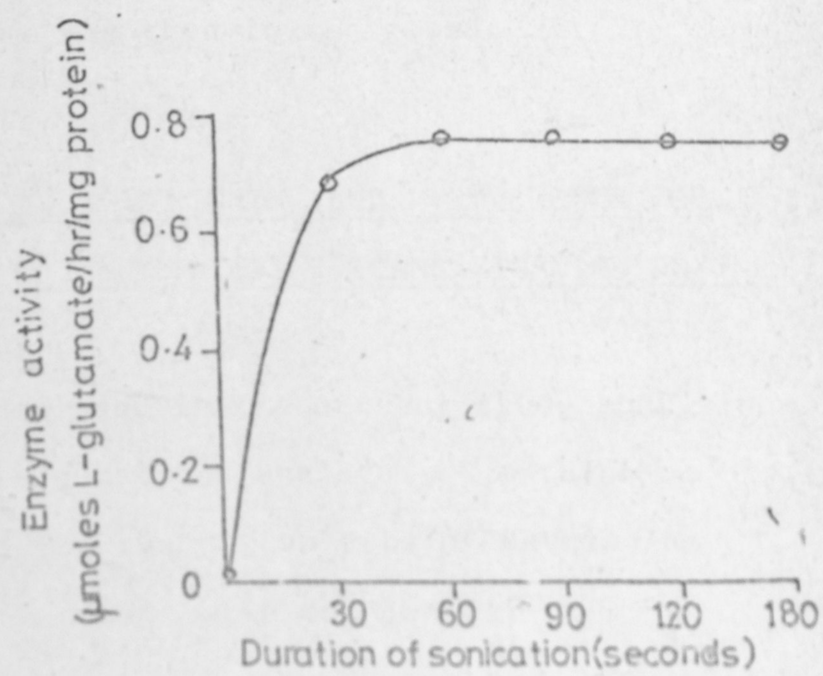
Figure 14

Release of methionine transaminating enzyme by freeze-thawing. Trypanosomes (cell density 2×10^8/ml) in PS were subjected to freeze-thawing. The suspensions were then centrifuged at 10,000 g at 4°C for 10 minutes. The supernatants were incubated at 25°C with 10 mM L-methionine and α-ketoglutarate for 1 hr. The incubations were then terminated and L-glutamate production determined.

Figure 15

Release of methionine transaminating enzyme by sonication. Trypanosomes (cell density 2×10^8/ml) in PS were sonicated at 4°C with a Branson Sonifier Model W185 set at 80 watts for 30 second periods. After each period of sonication, 1 ml of suspension was withdrawn from the sonication tube. These suspensions were centrifuged at 10,000 g for 10 minutes at 4°C. The supernatants were incubated at 25°C with 10 mM L-methionine and α-ketoglutarate for 1 hour. Incubations were terminated and L-glutamate production determined.


Enzyme activity
(µmoles L-glutamate/hr/mg protein)
0.8
0.6
0.4
0.2
0
1
2
3
4
5
Freeze-thawing cycles


Enzyme activity
(µmoles L-glutamate/hr/mg protein)
0.8
0.6
0.4
0.2
0
30
60
90
120
180
Duration of sonication(seconds)

comparable to the activity obtained after one cycle of freeze-thawing. Prolonged sonication upto 180 seconds did not affect the activity of the enzyme.

The release of the methionine transaminating enzyme by freeze-thawing was similar to the release of the cytosolic marker GPT (Chapter 3 Figures 5 and 6). This was an indication that like other known transaminases in bloodstream form T.b. brucei, the methionine transaminating enzyme is possibly cytosolic.

It was concluded that bloodstream form T.b. brucei has a methionine transaminating enzyme which is inhibited significantly by repeated freeze-thawing. The substrate specificity of the methionine transaminating enzyme was then investigated.

6.3. EFFECT OF L-ETHIONINE AND PYRUVATE ON THE ACTIVITY OF METHIONINE TRANSAMINATING ENZYME

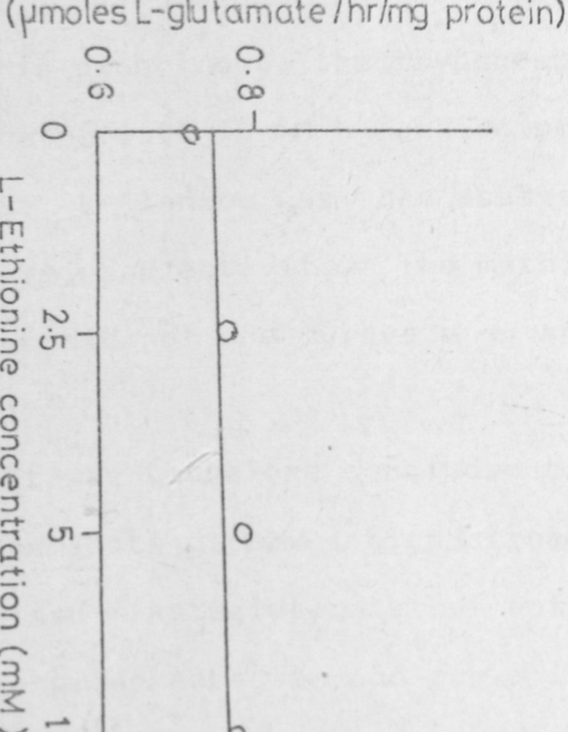
It was proposed in the present study that since ethionine is a substrate analogue of methionine, it would compete for the active site of mathionine transaminating enzyme with methionine.

Results presented in Figure 16 show that ethionine did not have any effect on the activity

Figure 16

Effect of L-ethionine on the activity of methionine transaminating enzyme.

Trypanosome lysates (2×10^{8} cells/ml PS) were incubated at 25°C for 1 hour with a fixed concentration of L-methionine (10 mM) and α-ketoglutarate (10 mM) and varying concentration of L-ethionine ranging from 0 to 20 mM. Incubations were then terminated and L-glutamate production at every L-ethionine concentration determined.


Enzyme activity
(μmoles L-glutamate/hr/mg protein)
0.6
0.8
0
2.5
5
10
20
L-Ethionine concentration (mM)

of the methionine transaminating enzyme even at a concentration of 20 mM and that ethionine was transaminated at a rate similar to that of methionine transamination.

An attempt was also made to use pyruvate as an amino group acceptor in the methionine transamination. In this experiment, the trypanosomal lysates were incubated with 10 mM L-methionine and 10 mM pyruvate in PS. L-Alanine was then assayed as a measure of transaminase activity. The methionine transaminating enzyme did not use pyruvate as an amino group acceptor.

It was therefore concluded that the methionine transaminating enzyme in bloodstream form T.b. brucei utilised α-ketoglutarate but not pyruvate as the amino group acceptor and could use both L-methionine and L-ethionine as amino group donors.

DISCUSSION

The survey of aminotransferases in bloodstream form _T.b. brucei_ showed novel transamination activities of glutamine, leucine, isoleucine, valine and methionine with α-ketoglutarate as amino group acceptor. It was not possible to conclusively show whether this transamination was catalysed by 5 different transaminases specific for each of the 5 amino acids or by non-specific transaminase(s) acting on all the 5 amino acids.

The results obtained in this study on the transamination of glutamine by bloodstream form _T.b. brucei_ lysates were not conclusive because if a glutaminase was present, glutamine would be deamidated to glutamate. Since production of glutamate was taken to be a measure of transaminase activity, production of glutamate by a glutaminase acting on glutamine would yield misleading results. Furthermore, glutamine is easily hydrolysed to glutamate in aqueous solution (Bergemeyer, 1974).

There have been reports of transamination of isoleucine, leucine, valine and methionine with α-ketoglutarate as amino group acceptor in other organisms. Van leuven (1976) found transamination of glutamine,

phenylalanine and methionine by rat brain glutamine aminotransferase. Ikeda and Co-workers (1976) reported rat liver enzyme II or leucine aminotransferase transaminating leucine, ethionine, methionine, nor-valine, nor-leucine and homocysteine. Taylor and Jenkins (1966) reported hog heart enzyme I transaminating valine, leucine, isoleucine and methionine, while Rudman and Meister (1953) reported an aminotransferase B activity in E.coli that transaminated leucine, valine, isoleucine and methionine. Similar transaminase activity was reported in Acetobacter suboxydans (Tachiki and Tochikura, 1975) and Pseudomonas aeruginosa (Norton and Sokatch, 1970).

Cell-free extracts of the hemoflagellate Leishmania donovani, a parasitic protozoa belonging to the family Trypanosomidae, have been reported to catalyse the transamination of aspartate, histidine, methionine, lysine, cysteine, alanine, phenylalanine, tyrosine, tryptophan, valine, ornithine and leucine with α-ketoglutarate as amino group acceptor (Chatterjee and Ghosh, 1957).

The amino acid specificities of the transaminations observed in the present study do not appear to be identical to any of the transaminases in the various

organisms discussed above. The transaminase(s) observed in bloodstream *T.b. brucei* extracts may be similar to the Hog heart enzyme I, *E.coli* aminotransferase B, *Acetobacter suboxydans* aminotransferase or *Pseudomonas aeroginosa* aminotransferase because there was transamination of leucine, valine, isoleucine and methionine. It may be similar to rat liver enzyme II because there was transamination of ethionine or to the transaminases of *Leishmania donovani* because there was transamination of leucine, valine and methionine.

The transamination of methionine by bloodstream *T.b. brucei* lysateswas shown to be specific for α-ketoglutarate and not pyruvate. Further investigations should be carried out on the substrate specificity of other transaminase activities and the extent to which the 5 amino acids are catabolised after the transamination reactions. Such studies may reveal pathways of amino aoid catabolism unique to these trypanosomes which could become targets of new anti-trypanosomal drugs.

CHAPTER 7

CONCLUSIONS

In this study, the role of GPT in conversion of pyruvate to alanine in bloodstream *T.b. brucei* and the stoichiometry of this reaction has been shown. The role of GOT in bloodstream form *T.b. brucei* has been proposed to be in a series of coupled reactions leading to a transhydrogenation between NADH and $NADP^+$. The last enzyme of the proposed transhydrogenation pathway, - NADP-linked ME, was however not conclusively localised though results indicated that it was partly cytosolic. Glucose-6-phosphate dehydrogenase an enzyme generating NADPH was shown to be cytosolic.

The presence of trypanosomal GPT and GOT in serum of rats infected with *T.b. brucei* was demonstrated and bloodstream form *T.b. brucei* lysates were shown to transaminate valine, leucine, isoleucine and methionine with α-ketoglutarate as amino group acceptor.

The results of this study have opened new areas of research. The advantage of bloodstream *T.b. brucei* excreting pyruvate in the form of alanine has not

been established. The extent to which bloodstream form T.b. brucei depend on the transhydrogenation pathway for NADPH production should be further investgated. It is also important to know how essential de novo synthesis of fatty acids is to bloodstream trypanosomes and the effects of inhibition of this system on the parasite. The localisation of NADP-linked ME should be investigated using more sensitive methods.

Further studies should be carried out to establish what other trypanosomal enzymes are present in the plasma of infected animals and the effects these may have on the host. Studies should also be carried out to establish to what extent elevated enzymes in plasma of trypanosome-infected animals arise from host tissue damage.

The novel transaminations by T.b. brucei lysates should be investigated further to obtain information on substrate specificity, inhibition, optimal conditions of activity and also the end products of the reactions. The methionine transaminating enzyme should be studied thoroughly because if the transamination pathway of methionine catabolism occurs in trypanosomes, poisonous intermediates may be released into the host plasma contributing to the

pathogenicity of trypanosomes. Studies should also be carried out to establish the importance of these transaminations to bloodstream trypanosomes. The properties of these transaminase(s) should be compared to those of host mammals with the hope of finding differences which could lead to development of anti-trypanosomal drugs.

From the results of this study it is possible to draw the following conclusions on the amino acid metabolism in bloodstream form *T.b. brucei*:-

(i) Glutamate pyruvate transaminase is involved in the excretion of pyruvate in the form of alanine.

(ii) Glutamate oxaloacetate transaminase is possibly involved in a series of coupled reactions leading to a transhydrogenation between NADH and $NADP^+$ in the parasite.

(iii) Additional activity of glutamate pyruvate transaminase and glutamate oxaloacetate transaminase in serum of rats infected with *T.b.brucei* is of trypanosomal origin.

(iv) Bloodstream *T.b.brucei* lysates can transaminate leucine, isoleucine, valine and methionine with α-ketoglutarate as amino group acceptor.

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