



BOOK NUMBER

QP514.2

A66

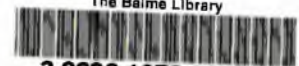
Theses Room

C-1

ACCESSION NO

G348330

The Balme Library

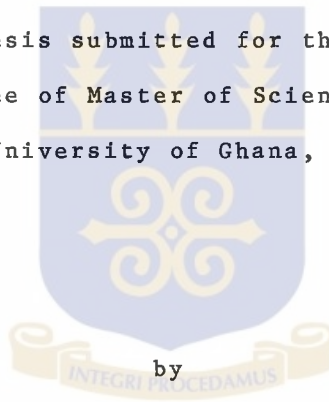


3 0692 1078 5898 5

- 1 -

Monomorphic and Pleomorphic Trypanosoma brucei  
rhodesiense: Biochemical, morphological and  
ultrastructural comparisons.

A thesis submitted for the  
Degree of Master of Science of  
The University of Ghana, Legon



by

Tamara Aboagye-Kwarteng

1983

Department of Biochemistry

A C K N O W L E D G E M E N T S

I will like to thank the University of Ghana and the Department of Biochemistry for arranging for me to do the work contained in this thesis at the London School of Hygiene and Tropical Medicine.

My special thanks go to Dr. K.K. Oduro, my internal supervisor for reading the manuscript and for the helpful suggestions he made.

I am supremely grateful to Dr. W.E. Ormerod, my supervisor at the London School of Hygiene and Tropical Medicine, for all the practical help and advice he gave me with this work. I will also like to thank Professor Wallace Peters, Head of the Department of Medical Protozoology at the London School of Hygiene and Tropical Medicine, for allowing me to work there in the first place.

Finally a big thank you to all my friends in Room 310, Joe, Chris and Shiela for their help and patience.

- 3 -

A B S T R A C T

The long slender and short stumpy forms of pleomorphic T. b. rhodesiense (LSHTM 180) have been compared to each other and to the monomorphic T. b. rhodesiense Liverpool Normal (Liv).

1. The agar film technique showed that morphologically the stumpy LSHTM 180 can be differentiated from the slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv) because it contains lipid granules.

2. Starch gel and cellulose acetate electrophoresis did not show any major differences in the isoenzyme profiles of 8 out of 9 enzymes studied. However, the presence of an aconitase band in the stumpy LSHTM 180 further differentiated it from the slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv).

3. Polypeptide profiles showed the presence of a 58,000 dalton polypeptide in extracts of both the slender and monomorphic trypomastigotes which was absent in the stumpy form.

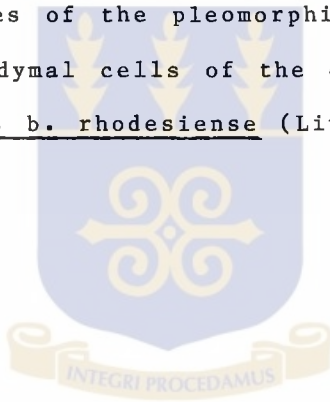
4. Like starch gel and cellulose acetate electrophoresis, enzyme assays gave little information as to

- 4 -

differences between the 3 trypomastigotes. There was, however, a higher NADP-isocitrate dehydrogenase activity in the stumpy form than the other two.

5. Ultrastructural studies showed the stumpy form had more cristae in the mitochondrion than either the slender LSHTM 180 or the monomorphic T. b. rhodesiense (Liv).

6. Trypomastigotes of the pleomorphic strain LSHTM 180 infected the ependymal cells of the choroid plexus, but the monomorphic T. b. rhodesiense (Liv) was unable to do this.



C O N T E N T S

	Page
Acknowledgements - - - - -	2
Abstract - - - - -	3
 <u>Chapter One - Introduction</u>	
1.1 General - - - - -	9
1.2 Biochemistry of Trypanosomes - - -	12
1.2.1 Energy Metabolism - - - - -	12
1.2.2 Hydrolytic Enzymes - - - - -	16
1.3 Characterization of Isoenzymes - -	17
1.4 Polypeptide Profiles - - - - -	19
1.5 The Functional Anatomy of Trypanosomes - - - - -	20
 <u>Chapter Two - Laboratory Materials and Methods</u>	
2. 1 Materials - - - - -	22
2.1.1 List of Materials - - - - -	22
2.1.2 Buffers - - - - -	22
2. 2 Equilibration of DEAE-Cellulose -	22
2. 3 Trypanosomes - - - - -	23
2.3.1 Liverpool Normal (Liv) - - - - -	23
2.3.2 LSHTM 180F - - - - -	23
2. 4 Rodents - - - - -	24
2. 5 Inoculation - - - - -	24
2. 6 Agar Film Technique - - - - -	24
2. 7 Separation of Trypomastogotes from Blood Elements - - - - -	26
2. 8 Preparation of Lysates for Electrophoresis - - - - -	27
2. 9 Subcellular Fractionation of Trypanosome Lysates - - - - -	27
2.10 Protein Determination - - - - -	30
2.11 Starch Gel Electrophoresis (SGE) -	30
2.11.1 Electrophoresis - - - - -	31
2.11.2 Staining for Enzymes - - - - -	31
2.12 Cellulose Acetate Electrophoresis (CAE) - - - - -	33
2.12.1 Enzyme Development - - - - -	33
2.13 SDS-Polyacrylamide Gel Electro- phoresis (SDS-PAGE) - - - - -	34
2.14 Enzyme Assays - - - - -	35
2.14.1 Calculation of Enzyme Activity - -	36
2.15 Electron Microscopy - - - - -	37
2.15.1 Fixation and Embedding - - - - -	37

	Page
<u>Chapter Three - Results</u>	
3.1	38
3.2	38
3.3	39
3.4	39
3.5	46
3.6	64
<u>Chapter Four - Discussion and Conclusion</u>	
4.1	71
4.2	73
4.3	75
4.4	76
4.5	84
4.5.1	84
4.5.2	85
4.6	88
<u>APPENDICES</u>	
A.	92
B.	95
C.	97
D.	99
E.	104
F.	106
G.	108
H.	111
REFERENCES	112

## L I S T   O F   F I G U R E S

1.	14
2.	15
3.	25
4A.	28
4B.	29
5.	32
6.	47

- 7 -

<u>List of Figures (continued)</u>			Page
7.	Subcellular Distribution of	HK - - -	55
8.	"	" GPI - -	56
9.	"	" ICD - -	57
10.	"	" MDH - -	58
11.	"	" ALAT - -	59
12.	"	" G6PD - -	60
13.	"	" PGM - -	61
14.	"	" MPI - -	62
15.	"	" Acid Phosphatase	63
16.	The Glyoxylate Cycle	- - - - -	79
17.	The Protein Standard Curve	- - - - -	98
18.	p-Nitrophenol Standard Curve		112

## L I S T O F T A B L E S

1.	Enzyme Activities	- - - - -	54
2.	A Comparison of Selected Enzymes in <u>T. brucei</u> and other organisms	- - -	79
3.	Electrophoretic Conditions for Starch Gel Electrophoresis (SGE)	- - -	99
4.	Enzyme Developers for SGE	- - - - -	101
5.	Electrophoretic Conditions for Cellulose Acetate	- - - - -	104
6.	Enzyme Developers for Cellulose Acetate Electrophoresis	- - - - -	105
7.	Stock Enzyme Assay Mixtures	- - - - -	108

## L I S T O F P L A T E S

1.	<u>T. b. rhodesiense</u> (Liverpool Normal)	-	43
2.	Slender Forms of <u>T. b. rhodesiense</u> LSHTM 180	- - - - -	44
3.	Giant Form <u>T. b. rhodesiense</u> LSHTM 180		44
4.	Short Stumpy Form, <u>T. b. rhodesiense</u> LSHTM 180	- - - - -	45

## Isoenzyme Patterns

5.	SGE - ACON	- - - - -	48
6.	SGE - MDH	- - - - -	48
7.	CAE - ME	- - - - -	48
8.	CAE - ICD	- - - - -	49
9.	SGE - GPI	- - - - -	49
10.	SGE - PGM	- - - - -	49
11.	CAE - G6PD	- - - - -	50
12.	SGE - NH	- - - - -	50
13.	CAE - ALAT	- - - - -	50

	Page
14. Polypeptide Profiles from SDS-PAGE - -	51
15. Polypeptide Profiles from SDS-PAGE - -	52
 Electron Microscopy	
16. <u>T. b. rhodesiense</u> (Liv) x 20,000 - - -	64
17. <u>T. b. rhodesiense</u> (Liv) x 126,000 with section of tubular mitochondrion - - -	64
18. <u>T. b. rhodesiense</u> (Liv) x 20,000 with double flagellum - - - - -	65
19. LSHTM 180 LS x 20,000, showing nucleus	66
20. LSHTM 180 LS x 126,000 with mitochon- drion, no cristae - - - - -	66
21. LSHTM 180 ST x 20,000 with mitochon- drion and cristae - - - - -	67
22. LSHTM 180 x 80,000, mitochondrion - -	67
23. - 26. Section through choroid plexus of mouse infected with <u>T. b. rhodesiense</u> (Liv) 72 hours after inoculation	
23. x 20,000: trypanosomes and red blood cells - - - - -	68
24. x 80,000: trypanosome in blood vessel	69
25. x 50,000: Golgi body and flagellum - -	69
26. x 126,000: <u>T. b. rhodesiense</u> (Liv) - -	69
27. Sections through choroid plexus of rats infected with LSHTM 180 24 - 27 days after infection. (4 Plates) - -	70

C H A P T E R   O N EINTRODUCTION1.1      General

The trypanosome has been described as a destructive piece of machinery, depriving its host of essential substances while burdening it with unwanted wastes; it inflicts mechanical damage through its incessant physical activity; and it goads the host into responding to its presence and then evades the backlash (Vickerman, 1978).

In two vast areas of the world, tropical Africa and South America, trypanosomes are responsible for serious sickness in man and his domestic animals. They also attack draught animals in the Middle East and tropical Asia. Because the trypanosomiases are mainly associated with rural areas in developing countries, accurate figures are uncertain but the general estimate is that 35 million people are at risk from sleeping sickness in Africa and over 10 million are infected with Chagas disease in Central and South America (WHO, 1976). Throughout Africa other forms of the disease harm domes-

- 10 -



tic stock and the subsequent indirect effects on nutrition and economy are probably more serious than actual human trypanosomiasis.

For reasons related to the diseases they cause, the method of transmission and their morphology and biochemistry, pathogenic trypanosomes are classified into Salivarian (mainly African) and Stecorarian species. The most important Stecorarian species is T. cruzi which causes Chagas disease in man, and is transmitted by triatomid bugs. Salivarian African trypanosomes are tsetse-borne and their distribution coincides with that of their vectors, represented by flies of the purely African genus Glossina. The most important tsetse-borne Salivarian species from the medical and veterinary points of view include T. b. gambiense and T. b. rhodesiense (the causative agents of "gambian" and "rhodesian" sleeping sickness respectively), <sup>and</sup> T. b. brucei, T. vivax and T. congolense, which cause nagana in cattle. Pathogenic salivarian trypanosomes also include two other species, T. evansi and T. equiperdum, which - though not tsetse-borne - are closely related to T. b. brucei. T. evansi is transmitted directly by mechanical inoculators (Tabanid flies), and T. equiperdum, (the causative agent of dourine, a venereal disease of horses) is transmitted by sexual contact between the mammalian hosts. Due to their morphological similarity, T. evansi, T. equiperdum,

- 11 -

T. b. brucei, T. b. gambiense and T. b. rhodesiense have been classified together into the brucei group, T. (Trypanozoon) brucei.

T. b. rhodesiense, like T. b. brucei and T. b. gambiense, is mainly extracellular. The trypanosomes live in the body fluids including the blood and later the cerebrospinal fluid where they grow and divide by binary fission. Recently Abolarin et al., (1982) have shown that intracellular multiple division forms of the parasite occur in the ependymal cells of the choroid plexus.

When a susceptible mammal (e.g. rat or mouse) is cyclically infected with trypanosomes by a tsetse-fly, the first trypomastigotes to be seen in the blood are long, thin, cylindrical in cross-section and agranular with a free flagellum, <sup>known as</sup> the long narrow or LN forms. As the infection develops they become leaf-like, have a flat cross-section and begin to divide in the blood (the long flat or LF form). The LN and LF forms are usually classified together as the long slender or LS form. With the progress of the infection the LF trypomastigotes become short and stumpy, lose the free flagellum and acquire lipid granules. These are called short stumpy or ST forms.

This phenomenon, called pleomorphism, is a variable character among the members of the brucei group. The

- 12 -

most characteristic forms are the LF forms since they are always present in all the species, whereas the proportions of the LN and ST forms, which appear constantly in the tsetse-borne species (T. b. brucei, T. b. gambiense, T. b. rhodesiense) fluctuate in the course of the infection and may disappear completely after prolonged mechanical passages. Thus laboratory strains maintained by syringe passage eventually become monomorphic with only the LF forms present. They are also the only kind of trypanosomes in those species which are normally transmitted by the mechanical method, viz. in T. evansi and T. equiperdum.

Syringe-passaged strains lose their transmissibility by Glossina (tsetse-fly) and their virulence with rodents increases with time (Duke, 1934, 1935). Thus an intraperitoneal inoculation of a syringe-passaged T. b. rhodesiense into a mouse or rat produces a fulminating infection that kills the rodent in 72-90 hours.

## 1.2 Biochemistry of Trypanosomes

### 1.2.1 Energy metabolism

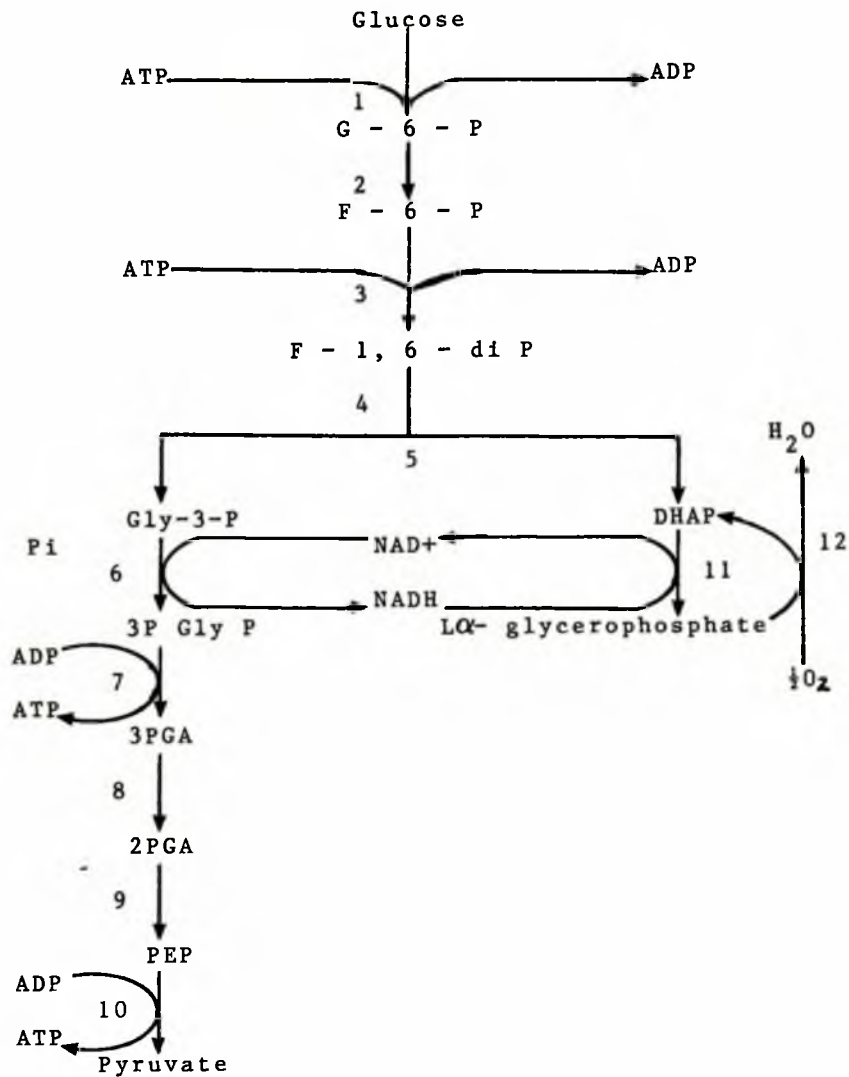
Trypanosomatids contain little or no endogenous energy reserves, and without an exogenous carbohydrate supply they stop respiring and become immobile within a minute (Opperdoes et al, 1976). Bloodstream trypomastigotes in

- 13 -

the brucei group contain some Krebs cycle enzymes, but the cycle is largely inoperative. They are therefore completely dependent on glycolysis for their energy requirements (Bowman and Flynn, 1976), and they metabolize glucose 50 times faster than the mammalian host tissues they infect (von Brand, 1951). Under aerobic conditions, these trypomastigotes catabolize glucose to pyruvate, producing 2 moles ATP per mole glucose. LDH is absent (Dixon, 1966) and NADH generated in glycolysis is reoxidized indirectly by molecular oxygen via DHAP: L- $\alpha$ -glycerophosphate dehydrogenase and L- $\alpha$ -glycerophosphate oxidase enzyme complex are involved (Grant and Sargent, 1960). (Figure 1.) Under anaerobic conditions the continual reoxidation of NADH is achieved by the operation of glycerokinase (Figure 2). Under these conditions equimolar amounts of pyruvate and glycerol are produced (Ryley, 1956; Grant and Fulton, 1957), and ATP production is halved (Brohn and Clarkson, 1978). Anaerobic conditions can be simulated by incubating trypanosomes with salicylhydroxamic acid (SHAM), an inhibitor of L- $\alpha$ -glycerophosphate oxidase. Because of the alternate NADH-reoxidation pathway (Figure 2), SHAM is not lethal to trypanosomes (Opperdoes *et al.*, 1976). A combination of SHAM and glycerol, however, has been shown to represent a potent trypanocidal agent.

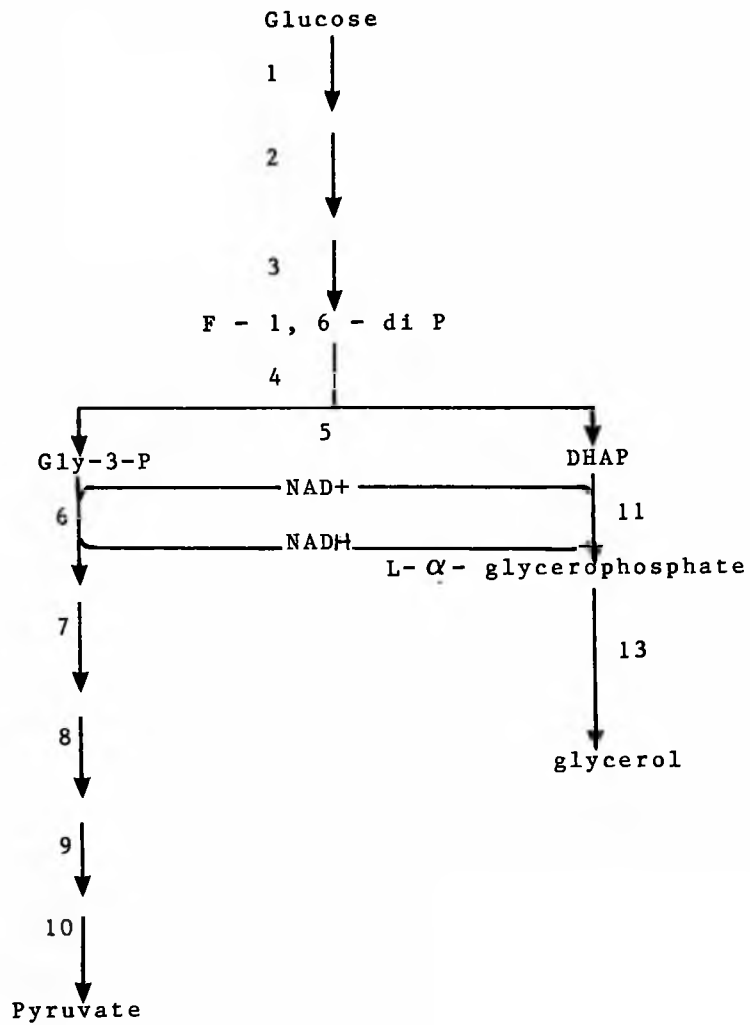
- 14 -

Figure 1.

AEROBIC GLYCOLYSIS

- 1: Hexokinase, 2: Glucose phosphate isomerase,
- 3: Phospho-fructokinase, 4: Aldolase,
- 5: Triosephosphate isomerase,
- 6: Glyceraldehyde-3-phosphate dehydrogenase,
- 7: Phosphoglycerate kinase, 8: Phosphoglycerate mutase,
- 9: Enolase, 10: Pyruvate kinase,
- 11: L- $\alpha$ -glycerophosphate dehydrogenase,
- 12: L- $\alpha$ -Glycerophosphate oxidase.

- 15 -

Figure 2.ANAEROBIC GLYCOLYSIS

1 - 11 are as for Figure 1  
 13 - Glycerokinase

- 16 -

It has been shown in trypanosomes that the glycolytic enzymes involved in the first part of the glycolytic sequence, the conversion of glucose to 3-phospho glyceric acid (3PGA) are located in microbody-like organelles called glycosomes (Oduro, 1977; Oduro et al., 1980; Opperdoes and Borst, 1977). Recently Oduro (personal communication) has confirmed through electron microscope studies that T. b. brucei glycosomes are microbodies. Glycosomes constitute a separate pool of glycolytic intermediates and adenine nucleotides within the cell (Visser et al., 1981). Such compartmentation is probably responsible for the very high rate of glycolysis in the parasite. The glycosome has no parallel in the mammalian host and is therefore a logical target for chemotherapy.

#### 1.2.2 Hydrolytic Enzymes

Trypomastigotes have been shown to contain lysosomes; a bag of enzymes involved in digestion, some of which are acid proteases, leucine aminopeptidase, phosphatases,  $\alpha$ -mannosidases, deoxyribonucleases and lipases (Venkatesan et al., 1977; Steiger, 1975) as well as phospholipases (Tizard et al., 1977). T. brucei trypomastigotes sequester host serum protein by pinocytosis via the flagellar pocket (Langreth and Balber, 1975). The proteins fuse with and are digested by so-called primary lysosomes.

- 17 -

Venkatesan et al., (1977) have shown that the stumpy forms contain higher levels of acid phosphatase and Cathepsin D than the slender forms. This seems to explain the observation made by A. H. Fairlamb (unpublished) that stumpy forms sequester host serum proteins at twice the rate of slender forms. In fact autophagy, the digestion of isolated islands of cytoplasm containing unwanted organelles - also appears to be more active in the stumpy forms (Langreth and Balber, 1975). There is also a marked difference in the localization of acid phosphatase in the two forms. In the slender forms, acid phosphatase is localized in the flagellar pocket and in discrete particles around it, apparently being transported into the pocket for purposes of digestion of particles in the pocket (Jadin and Creemers, 1970; Langreth and Balber, 1975; Venkatesan et al., 1977). In the stumpy forms, however, acid phosphatase is distributed throughout the Golgi zone and the rough endoplasmic reticulum.

### 1.3 Characterization of isoenzymes

Enzyme electrophoresis has been widely used in the characterization of parasitic and non-parasitic protozoa, e.g. Entamoeba (Reeves and Bischoff, 1968; Sargeant et al., 1978); Leishmania (Al-Taqi and Evans, 1978); Trypanosoma (Godfrey and Kilgour, 1976; Miles et al.,

- 18 -

1977). Godfrey and Kilgour (1976) differentiated T. b. gambiense from T. b. brucei and T. b. rhodesiense by its characteristic aminotransferase pattern. Miles et al., (1977) identified two distinct strain groups of T. cruzi circulating in independent sylvatic and domestic cycles in Brazil.

Isoenzymes have been defined as multiple separable forms of enzymes occurring within the same organism and having similar catalytic activities but not necessarily similar molecular structures (Shaw, 1969). Harris and Hopkinson (1976) classified isoenzymes into three major groups.

1. Those arising from multiple genetic loci coding for functionally the same enzyme.
2. Those arising from multiple alleles at a single locus:
3. Those arising from post-translational changes - so-called secondary isoenzymes.

The demonstration of structural differences among isoenzymes by electrophoresis depends largely on the isoenzymes having different electrical charges; therefore a substitution of one amino acid for another with an equal charge will not be detected. Thus two enzymes are not necessarily structurally identical because their electrophoretic mobilities are the same; hence two samples are not necessarily from related organisms because they have the same electrophoretic pattern for one enzyme. However the more enzymes that are examined without any

- 19 -

differences being found between the two samples, the greater the likelihood that the two samples are from related organisms, and if so the closer the relationship.

#### 1.4 Polypeptide Profiles

Like isoenzyme patterns, polypeptide profiles can give an idea of the relationship between two organisms. Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (SDS - PAGE) has been used to study the proteins and RNA's of viruses (Studier, 1973; Laemmli, 1970), and the proteins of bacteria (Swindlehurst et al., 1977).

It has also been used to characterize Trypanosoma, (Schizotrypanum) species from insectivorous bats (Microchiroptera), (Taylor et al., 1982), and subgenus Trypanozoon (Taylor et al., 1983), as well as to differentiate between strains of South American trypanosomes (Taylor and Williams, 1977).

The method involves coating the soluble proteins of an organism with negative charges and electrophoresing them on polyacrylamide gels; this produces characteristic polypeptide profiles for different organisms.

### 1.5 The Functional Anatomy of Trypanosomes

Bloodstream trypanosomes in the brucei group have essentially the same ultrastructure. Pellicular microtubules, longitudinally disposed, lie immediately beneath the plasma membrane of the flagellates body and maintain its elongate shape. The single flagellum arises from a basal body (Kinetosome) submerged in the floor of the flagellar pocket. There is a single mitochondrion which is tubular and runs the length of the trypanosome. It arises from the kinetoplast, a DNA-containing organelle. In the slender forms, internal cristae are almost absent in the mitochondrion, but as the flagellates develop through intermediate to stumpy forms the width of the mitochondrial canal increases and more cristae are formed (Vickerman, 1965). However, in both these forms, the mitochondrion is non-functional with respect to Krebs cycle and electron transport.

In the trypomastigote and epimastigote forms in the tsetse-fly mid gut and salivary glands respectively, the mitochondrion has abundant cristae, with fully functional Krebs cycle enzymes and cytochromes (Vickerman, 1965),

The nucleus has a nucleolus and, as in all animal cells, it is bounded by a double membrane punctured by pores. The outer membrane is continuous with a system of mem-

- 21 -

branous cytoplasmic tubes - the endoplasmic reticulum, many of which are studded with ribosomes.

Golgi apparatus, which are involved in the packaging of cellular secretions into membrane-bound vessicles, lysosomes, are also present in the flagellates.

As mentioned earlier, trypanosomes also contain glycosomes (microbodies) which have the unique biochemical property of containing most of the enzymes of the glycolytic pathway (Oduro, 1977).

In the bloodstream trypomastigotes in the brucei group, the plasma membrane is overlaid by a surface coat of glycoprotein 12-15 nm. thick which contains the variable antigen (VA) of trypanosomes (Vickerman, 1969; Cross, 1975; Fruit et al., 1977), and is responsible for antigenic variation exhibited by the flagellates (Cross, 1978; Vickerman, 1978).

In this study, enzyme activity analysis (total enzyme assays and isoenzyme studies), polypeptide chain profiles and ultrastructural studies have been used to compare the slender and stumpy forms of a pleomorphic strain and a monomorphic strain of T. b. rhodesiense. This is an attempt to discover what changes, if any, take place when a slender trypomastigote becomes stumpy, and when a pleomorphic strain becomes monomorphic.

C H A P T E R   T W O

M A T E R I A L S   A N D   M E T H O D S

LABORATORY MATERIALS AND METHODS2.1 Materials2.1.1 List of Materials

See Appendix A.

2.1.2 Buffers

The quantities of material used to make up buffers are given in Appendix B.

a) For trypanosome separation and maintenance: PSG - phosphate-buffered saline glucose pH 8.0.

b) For cell fractionation: TSE - Tris-sucrose EDTA pH 7.8.

2.2 Equilibration of DEAE-Cellulose (DE-52)

The dry DE-52 was suspended in PSG and the pH adjusted to 8.0 with 5%  $H_3PO_4$ . The material was then washed a minimum of 6 times to remove all fines. The pH and conductivity of the slurry were checked and the material stored at  $-20^{\circ}C$  until required.

## 2.3 Trypanosomes

Two strains of T. b. rhodesiense were used:

2.3.1 Liverpool Normal (Liv). This strain was isolated from man in 1923 (Yorke et al., 1929). It was maintained in mice by syringe passage every 2-3 days until it was frozen in 1960. It produces a very high parasitemia in rats and mice and usually kills the rodent host 72 hours after inoculation. The infection is completely monomorphic - i.e. produces only one type of parasite, the LF. The stabilate was obtained from the LSH & TM cryobank at Winches Farm Field Station.

2.3.2 LSHTM 180F. This strain was isolated from man (Dumelo) in Botswana by Ormerod in 1960 (Apted et al., 1963). Since then it has been passaged through rats and mice and frozen a number of times (Fig. 3). The strain is pleomorphic, and therefore produces a gradation between blood forms of the parasite in the course of infection. The first trypomastigotes seen in the blood about 30 hours after infection are long, slender, cylindrical and agranular (long, narrow or LN forms). As the infection progresses, these gradually become flattened (long flat or LF forms) and finally short, stumpy with increasing number of lipid granules (short stumpy or ST forms).

- 24 -

The course of the infection is shown in Fig. 6. In the present state of passage of the LSHTM 180 strain, the rodent host usually dies 3 - 4 weeks after inoculation.

#### 2.4 Rodents

Adult male and female albino Wistar rats weighing 150-200g were used for the passage and harvesting of trypanosomes.

#### 2.5 Inoculation

A donor rat was inoculated intraperitoneally with trypanosome infected blood. The development of parasitemia was checked by counting the number of motile trypanosomes in a drop of tail blood under a X40 objective of the light microscope 72 - 96 hours after infection. For harvesting trypanosomes 10 - 15 rats were each infected with 0.5ml of heavily infected blood from the donor.



#### 2.6 Agar Film Technique

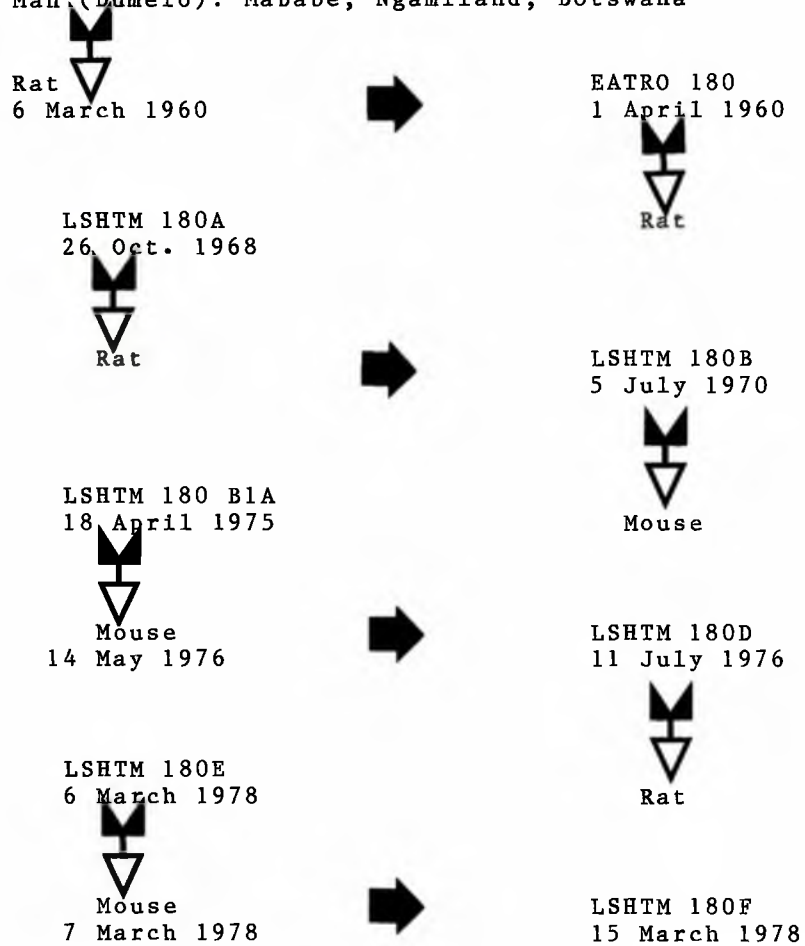

The method is that of Ormerod et al., (1963). It allows the simultaneous counting of trypanosomes and their lipid granules, so enabling one to differentiate between the LF


- 25 -

Figure 3.

T. b. rhodesiense LSHTM 180F

Man (Dumelo): Mababe, Ngamiland, Botswana


 : Syringe Passage

 : Frozen in liquid nitrogen

- 26 -

and ST trypomastigotes. The technique involves making agar slides from 1.2% agar in 0.85% NaCl. A "pinhead" of blood from the infected animal is placed on the slide and sandwiched between a coverslip and the agar film. The slides were left on the bench for about a minute to allow the trypomastigotes to move to the phalanx of the blood film on agar. From a recognizable point on the phalanx, the trypomastigotes were counted per agar field using X100 oil-immersion phase contrast objective on a Zeiss microscope. Counting was continued until the starting point was reached and the number of trypomastigotes with and without granules per field was recorded.

## 2.7 Separation of Trypomastigotes from Blood Elements

When the desired stage of parasitaemia was obtained, the rats were bled under anaesthesia by cardiac puncture using heparin as anticoagulant. The blood was diluted 1:3 with cold PSG and kept on ice.

The trypanosomes were separated from blood elements by the method of Lanham and Godfrey (1970). The infected blood was passed through equilibrated DE-52 in a Buchner funnel with a sintered glass base, and the trypanosomes eluted with cold PSG. The column eluates were collected on ice and centrifuged at 1500g for 20 minutes at 4°C.

- 27 -

The supernatant was discarded and the packed trypanosomes were washed twice by resuspending them in ice-cold PSG before centrifuging again at 1500g for 20 minutes.

#### 2.8 Preparation of Lysates for Electrophoresis

The washed trypanosome pellet was resuspended in an equal volume of "cocktail" containing 2mM each of E-amino-n-caproic acid, dithiothreitol and EDTA in water and mixed thoroughly. The resultant mixture was freeze-thawed in liquid nitrogen 3 times and then centrifuged at 10,000g for 30 minutes at 4°C. The pellet was discarded and 20ul aliquots of the supernatant dropped into liquid nitrogen to form beads which were stored in liquid nitrogen until required.

#### 2.9 Subcellular Fractionation of Trypanosome Lysates

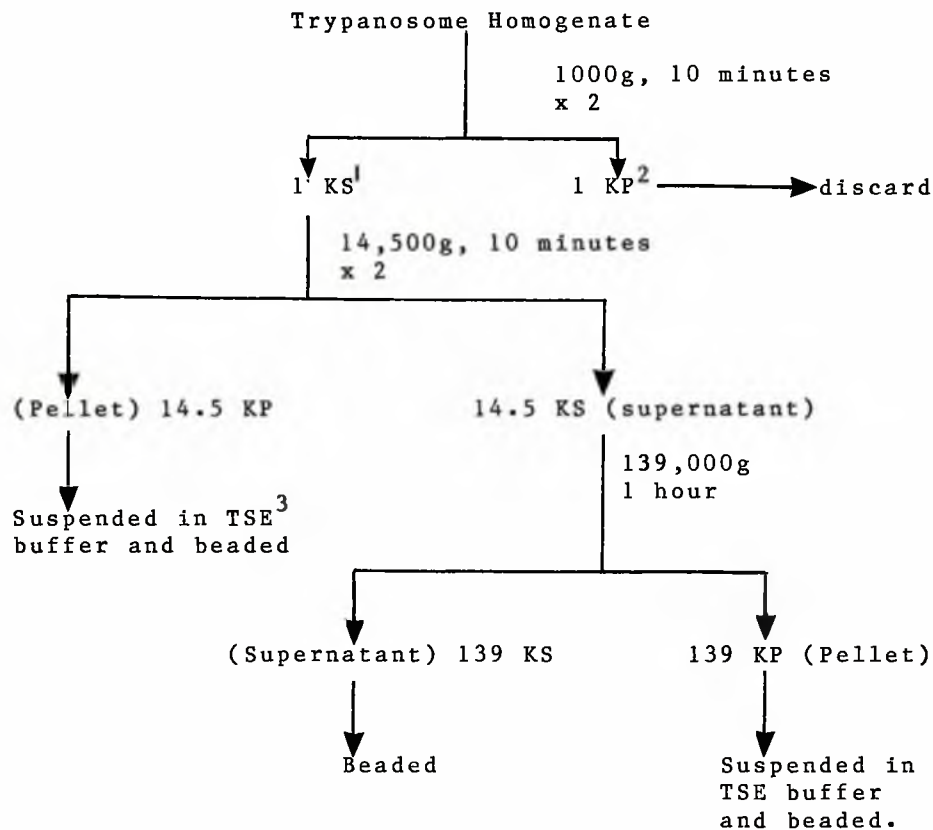
Subcellular fractionation was carried out to provide material to study the distribution of certain enzymes in trypanosomes. Washed trypanosome pellet was suspended in twice the volume of cold TSE buffer pH 7.8 and homogenized on ice in a Potter-Elvehjem homogenizer with a teflon piston for 5 minutes at 300 revolutions/minute. After checking for at least 90% broken cells under a X40 objective, the homogenate obtained was divided into two and treated as shown in Figures 4A. and 4B.

- 28 -

Figure 4A.

Subcellular fractionation of trypanosome homogenate

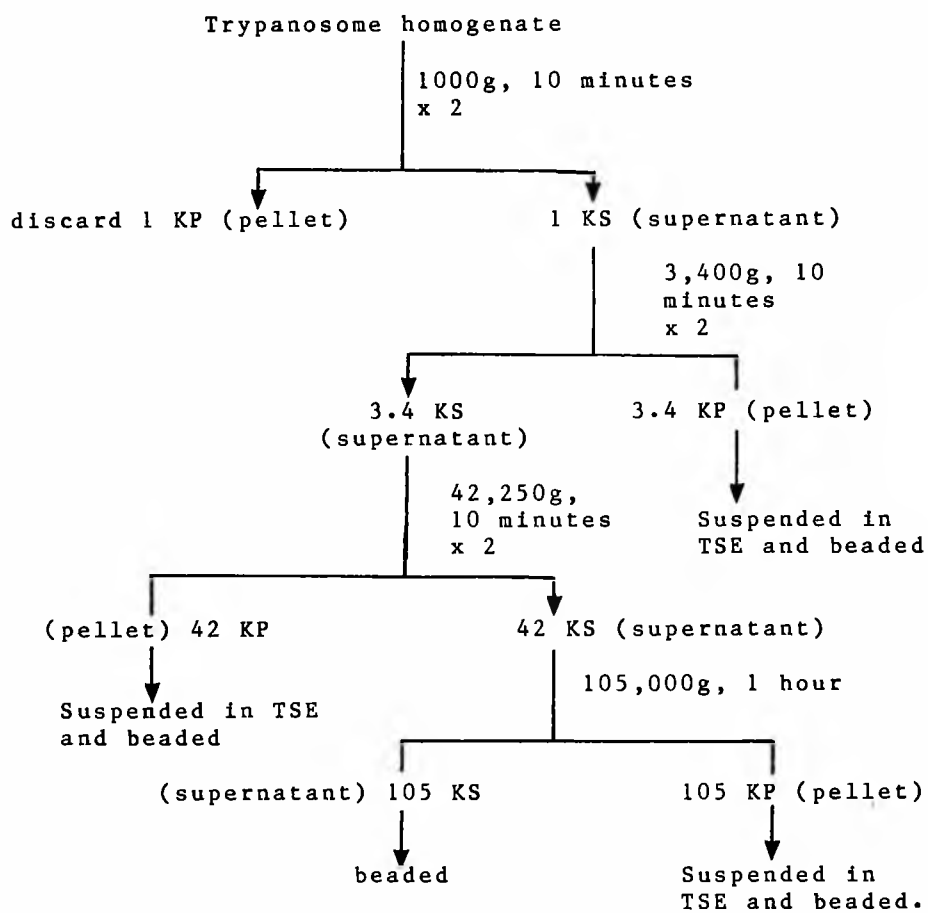
This is the procedure described by Opperdoes et al., (1977).



As well as 14.5 KP, 139 KP and 139 KS, a sample of 1 KS was also beaded. All the beads were stored in liquid nitrogen until required.

1. KS refers to the resultant supernatant after centrifugation at a certain speed, e.g. 1 KS is the supernatant after centrifugation at 1000g.
2. KP is the pellet after centrifugation, e.g. 1KP being the pellet from 1000g centrifugation.
3. TSE is the Tris-Sucrose - EDTA buffer pH 7.8.

- 29 -

Figure 4B.Subcellular fractionation of trypanosome homogenate

All the beads were stored in liquid nitrogen.

## 2.10 Protein Determination

Protein was determined according to the method of Lowry et al., (1951). Detail of reagents and standard curve are shown in Appendix C.

## 2.11 Starch Gel Electrophoresis (SGE)

Electrophoresis was carried out on thin starch gel essentially as described by Smith, (1976) using the modifications and conditions described by Miles et al., (1980). The buffers and electrophoretic conditions are as shown in Table 3, Appendix D

The enzymes analyzed were: acid phosphatase (E.C:3.1.23, ACP), aconitase (E.C.42.1.3, ACON), esterase (E.C.3.1.1.1, ES), glucose phosphate isomerase (E.C.5.3.1.9, GPI), malate dehydrogenase (E.C.1.1.1.37, MDH), mannosephosphate isomerase (E.C.5.3.1.8, MPI), purine nucleoside hydrolase (E.C.3.2.2.1, NH), phosphoglucomutase (E.C.2.7.5.1, PGM), alanine aminotransferase (E.C.2.6.1.2, ALAT), aspartate aminotransferase (E.C.C.2.6.1.1, ASAT), malate dehydrogenase (oxaloacetate decarboxylating) (NADP+) (E.C.1.1.1.40, ME), isocitrate dehydrogenase (NADP+) (E.C.1.1.1.42, ICD), glucose-6-phosphate dehydrogenase (E.C.1.1.1.49, G6PD).

### 2.11.1 Electrophoresis

Thin layer of starch gel plates measuring 0.1 x 14.0 x 21.5 cm<sup>3</sup> were made with 11.2% solution of hydrolyzed potato starch in the appropriate buffer<sup>(Table 3, Appendix D)</sup>. When the gels had solidified and cooled, slots were made in them with a slot template and samples applied to the slots on pieces of boiled cotton thread (Anchor 6-stranded embroidery cotton) cut to size. The tanks for electrophoresis were set up as shown in Figure 5. Electrophoresis was carried out at a constant voltage with variable current, the plates being held at 8°C for the duration of the run by means of a cooling plate. The electrophoretic conditions for each enzyme are shown in Table 3, Appendix D.

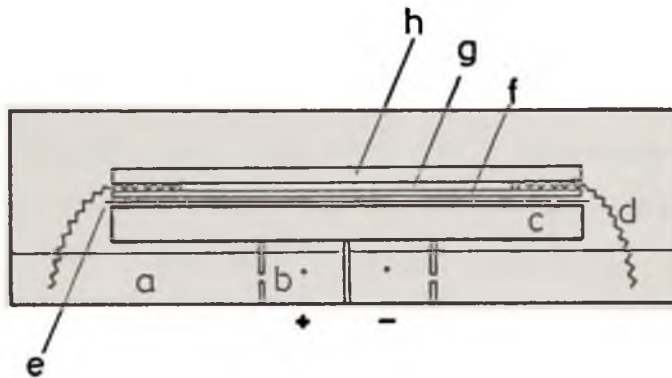
### 2.11.2 Staining for Enzymes

After electrophoresis, zones of enzyme activity were located by applying an appropriate developer solution either as an agar overlay or on filter paper (see Table 4, Appendix D). Agar overlays were used for developers with formazan (MTT) dye-linked reaction. Fluorescent developers were usually applied on filter paper, and the reactions monitored under UV light (360 nm). The developer plates were incubated at 37°C until fully developed as judged by eye, from 10 minutes for ALAT and GPI to 2 hours for ACON. Initially control plates were

- 32 -

Figure 5.

Diagram of an electrophoresis tank



- a: bridge buffer
- b: electrode buffer
- c: cooling plate
- d: wick
- e: Melinex sheet
- f: gel plate
- g: cover plate
- h: cover plate

- 33 -

set up for each enzyme, for which an essential ingredient like a specific enzyme substrate was left out of the developer. This was to ensure that the enzyme activity produced was due to the enzyme in question.

#### 2.12 Cellulose Acetate Electrophoresis (CAE)

CAE was used for the enzymes which gave poor results with thin layer SGE, e.g. ICD, G6PD, ME. Electrophoresis was carried out essentially as described by Lanham et al., (1981). The basic Helena system (Beaumont, Texas) was used, with Titan III cellulose acetate plates (94 x 76mm, Helena Cat. No. 3024). The tank buffers and electrophoretic conditions are shown in Table 5, Appendix E. The cellulose acetate plates were presoaked in the appropriate buffer as per the manufacturer's instructions, and samples loaded with the 8-sample CPK application system (Cat. No. 7092). Electrophoresis was carried out as directed by the manufacturer with a Titan Power supply providing a constant voltage with a variable current.

##### 2.12.1 Enzyme Development

Enzyme developer solutions were applied either as agar underlays or on filter paper. Agar underlays, which were used with MTT dye-linked reactions were made up and

- 34 -

allowed to set before the end of electrophoresis. After the run, the cellulose acetate plate was placed face down on the underlay, excluding all air bubbles, and incubated at 37°C until the zones of enzyme activity were fully developed as judged by the eye. Fluorescent developers were applied on filter paper. The plate was sandwiched together with the developer-soaked filter paper between two rectangular glass plates pressed tightly together with strong bulldog clips, and incubated at 37°C. The development of zones of enzyme activity was monitored under UV light (360 nm). The enzyme developers are shown in Table 6 of Appendix E.

2.13            SDS-Polyacrylamide Gel Electrophoresis  
                  (SDS-PAGE)

SDS-PAGE was carried out in a discontinuous buffer system (Laemmli, 1970), using an LKB2001 Vertical Electrophoresis unit with an LKB 2197 constant wattage power supply. Subcellular trypanosome fractions were separated on a 12% polyacrylamide gel with a 3% stacking gel. (See Appendix F.) Samples (containing about 50ug protein) were dissolved in an equal volume of SDS-sample buffer (Appendix F), boiled for 2 minutes and applied to the gel. Electrophoresis was carried out at 40mA for 5 hours or until the bromophenol blue tracking dye reached the bottom of the gel. Gels were stained overnight in Coomassie blue

- 35 -

(0.025% Coomassie blue in 40.5ml methanol: 52 ml H<sub>2</sub>O:  
0.7 mls glacial acetic acid and destained over a 24-hour  
period in a solution consisting of 405 ml ethanol, 250  
ml H<sub>2</sub>O and 70 ml glacial acetic acid. Gels were dried by  
sandwiching them between a polythene sheet and wet 3MM  
filter paper, clamped tightly to a flat rigid support  
with bulldog clips and left overnight at 37°C.

#### 2.14 Enzyme Assays

The assay procedures used for all enzymes except acid  
phosphatase (E.C.3.1.2.3, ACP) were based on the oxida-  
tion or reduction of pyridine nucleotide co-enzymes.  
Changes in optical density at 340nm were followed at 25°C  
in a Unicam SP1700 UV spectrophotometer coupled to a Unicam  
AR25 Linear Recorder. All assays were carried out in a  
final volume of 1.0ml using a 1cm light path. Acid phos-  
phatase activity was measured by following the release of  
p-nitrophenol from p-nitrophenyl phosphate at 430 nm in  
the same spectrophotometer. Equal volumes (0.5ml) of  
sub-cellular fractionation sample and 0.025M p-nitrophen-  
yl phosphate in 0.1M Acetate buffer pH 5.0 were incubated  
at 38°C for 30 minutes. The reaction was stopped by the  
addition of 2.0 ml 5% trichloroacetic acid. The reaction  
tubes were then centrifuged at 500g for 15 minutes to  
obtain a clear supernatant. Liberated p-nitrophenol was  
estimated at 430 nm after the addition of 1ml of 2M

- 36 -

$\text{NH}_4\text{OH}$  (pH 13.5) to 1 ml of TCA supernatant. A standard curve was prepared with a known range of p-nitrophenol concentration which gave a linear curve (Figure 18, Appendix H). Details of all enzyme assay mixtures are given in Appendix G.

#### 2.14.1 Calculation of Enzyme Activity

##### a) Acid Phosphatase

The amount of p-nitrophenol released, in moles, was determined from a p-nitrophenol standard curve, Figure 18 (Appendix H). The units of activity are mole/min/mg protein.

##### b) Other Enzymes

Enzyme activities were calculated from the rate of production or utilization of reduced pyridine nucleotide using an extinction coefficient at 340 nm of  $6.22 \times 10^3 \text{ cm}^2/\text{mole}$ .

Specific activity =

$$\frac{V}{E \times d \times v \times c (\text{protein})} \times \frac{DE}{Dt} \text{ mole/min/mg protein}$$

where

V = total assay volume = 1.00ml

E = extinction coefficient of NADH and NADPH at 340nm  
=  $6.22 \times 10^3 \text{ cm}^2/\text{mole}$ .

d = light path = 1cm

DE = change in extinction

v = sample volume = 0.01ml

Dt = reaction time

c = protein concentration of sample in mg/ml.

## 2.15 Electron Microscopy

Electron microscopy was used to:

1. compare the ultrastructure of the different trypomastigotes, pure samples of which were obtained by the method of Lanham and Godfrey (1970) described on page 26.
2. study the choroid plexuses of rats infected with the two strains for evidence of intracellular trypomastigotes. To this end, choroid plexuses were dissected from brains of rats which had been infected with either T. b. rhodesiense (Liv) or LSHTM 180 3 days or 3 - 4 weeks respectively after infection.

### 2.15.1 Fixation and Embedding

Material (choroid plexus or trypomastigotes) was immediately placed in 3% glutaraldehyde with cacodylate buffer at pH 7.4 and left for 8 hours, washed overnight in the same buffer, and post-fixed with 1% osmium tetroxide ( $\text{OsO}_4$ ) for 2 hours. The material was then dehydrated in a graded series of 30%, 60%, 90% and 100% methanol, each for 5 minutes duration. The blocks were stained with 1% phosphotungstic acid and embedded in 'Spurr' (Spurr, 1969). Sections were cut with a Cambridge Huxley Ultra-Microtome and observed with a Zeiss Em9 electron microscope.

C H A P T E R   T H R E E

R E S U L T S

### 3.1 Agar Film Technique

The agar film technique shows the morphological differences between the slender and stumpy forms of LSHTM 180 (Plates 2 - 4). Plate 2 shows that the slender trypomastigote forms of LSHTM 180 are agranular and have longer flagella than the stumpy forms (Plate 4) which have lipid granules (arrowed dark spots, Plate 4).

The technique was also used to study the course of the infection of LSHTM 180 in rats. Figure 6 shows that the infection is synchronous, producing alternate peaks of slender and stumpy forms at 72 hours, and 144 hours respectively.

### 3.2 Isoenzyme Patterns on Starch Gel and Cellulose Acetate

The three trypomastigote forms, LSHTM 180 slender and stumpy and T. b. rhodesiense (Liv) had identical isoenzyme patterns for 8 of the 9 enzymes analysed (Plates 6 - 13). The stumpy form of LSHTM 180 showed a single electrophoretic band on starch gel for aconitase (ACON), which was absent in both the slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv) (Plate 5).

### 3.3 Polypeptide Profiles from SDS-PAGE

The polypeptide profiles of subcellular fractions from the three trypomastigotes are shown in Plates 14 and 15. Clear differences exist:

1. between the three trypomastigote forms for each subcellular fraction, e.g. profiles 1 - 3 on each plate;
2. between the subcellular fractions for each trypomastigote; e.g. between profile 1, the 1000g supernatant (1KS) and profile 4, the 42,000g pellet (42KP) of slender LSHTM 180 (Plate 14).

The polypeptide molecular weight range where the greatest differences are found is between 60,000 daltons (60K) and 40,000 daltons (40K). A 58,000 daltons (58K) molecular weight polypeptide is consistently absent from all fractions of the stumpy LSHTM 180, but is present in some fractions of both slender LSHTM 180 and T. b. rhodesiense (Liv), most prominently in the 1KS profiles (Plate 14).

### 3.4 Subcellular Fractionation and Assay of Enzyme Activities

Trypanosome homogenates were fractionated by differential centrifugation to provide large granule (14.5KP), small granule (139KP) and final supernatant (139KS) fractions for all enzyme assays except acid phosphatase. The

- 40 -

results obtained by assaying these fractions are shown in Table 1 and Figures 7 - 15. Figure 7 shows that there is about twice as much hexokinase (HK) in the slender and stumpy LSHTM 180 as there is in T. b. rhodesiense (Liv). LSHTM 180 LS and ST have identical distribution of HK, with about 80% of the activity in the 14.5KP fraction, 18% in the 139KP fraction and 2% in the final supernatant (139KS).

As with HK, the slender and stumpy LSHTM 180 has similar distribution of glucose phosphate isomerase (GPI) (Figure 8). About 60% and 30% of the activity was found in the 14.5KP and 139KS fractions respectively, and the remainder in the 139KP fraction. T. b. rhodesiense (Liv) had a different distribution - 61% of GPI activity was in the 139KS fraction with 24% and 15% in the 139KP and 14.5KP fractions.

All the isocitrate dehydrogenase (ICD) activity of the stumpy LSHTM 180 and the monomorphic T. b. rhodesiense (Liv) was found in the 139KS fraction. In the slender LSHTM 180, about 65% of ICD activity was in the 14.5KP fraction and the rest in the 139KS fraction. The ICD activity of the stumpy LSHTM 180 was  $2\frac{1}{2}$  times and 6 times higher than in the slender LSHTM 180 and monomorphic T. b. rhodesiense (Liv) respectively - Figure 9.

The distribution of malate dehydrogenase (MDH), the other Krebs cycle enzyme studied, was identical in all three trypanomastigotes with less than 10% of the activity in the

- 41 -

14.5KP fraction, about 20% in the 139KP fraction and over 70% in the final supernatant (139KS) - Figure 10. The distribution pattern of alanine amino transferase (ALAT) shown in Figure 11 is similar to that of MDH.

The distribution of glucose-6-phosphate dehydrogenase (G6PD) is shown in Figure 12. The slender and stumpy forms of LSHTM 180 showed about equal G6PD activity which was about 3 times the activity in T. b. rhodesiense (Liv). The three trypanomastigotes showed different distribution patterns. The % distribution in the 14.5KP, 139KP, and 139KS are as follows: LSHTM 180, slender - 34%: 21%: 55%, stumpy LSHTM 180 - 63%: 39%: 8%, T. b. rhodesiense (Liv) 38%: 54%: 8%.

Figure 13 shows that all the phosphoglucosyltransferase (PGM) activity was located in the final supernatant (139KS) in all three trypanomastigotes, with stumpy, LSHTM 180 showing twice as much activity as LSHTM 180 LS and three times as much activity as T. b. rhodesiense (Liv).

Mannose phosphate isomerase (MPI) activity was distributed between the 14.5 KP and 139KS fractions (Figure 14). In T. b. rhodesiense (Liv), MPI was evenly distributed between the two fractions. About 70% of MPI activity in LSHTM 180, LS was located in the 139KS fraction and 30% in the 14.5KP fraction. The reverse was true for the stumpy LSHTM 180.

Acid phosphatase (ACP) activity was determined in sub-

- 42 -

cellular fractions from the differential centrifugation scheme shown in Figure 4B. The distribution of ACP activity is shown in Figure 15. The long slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv) had identical distribution patterns with about 30% of the activity in the 42KP fraction, 60% in the 105 KP fraction and less than 10% in the final supernatant (105KS). The stumpy LSHTM 180 showed 65% activity in 42KP, 35% in 105KP and 5% in 105KS fractions.

Agar Film TechniquePlate 1

T. b. rhodesiense (Liverpool Normal) on agar film. Judging by the eye the sizes of these trypanomastigotes are about the same as the short stumpy form of LSHTM 180 (Plate 4), but unlike them the Liverpool Normal Strain (Liv) is devoid of lipid granules.



Plate 2

The slender forms (LN and LF) of T. b. rhodesiense LSHTM 180.

The LN are the first blood forms which are seen followed by the LF circulating about 30 hours after inoculation. Like the Liv. Strain, these trypomastigotes are agranular

Plate 3

Giant form of T. b. rhodesiense LSHTM 180.

It is a bigger version of the slender form, and is in fact dividing.

PLATE 2

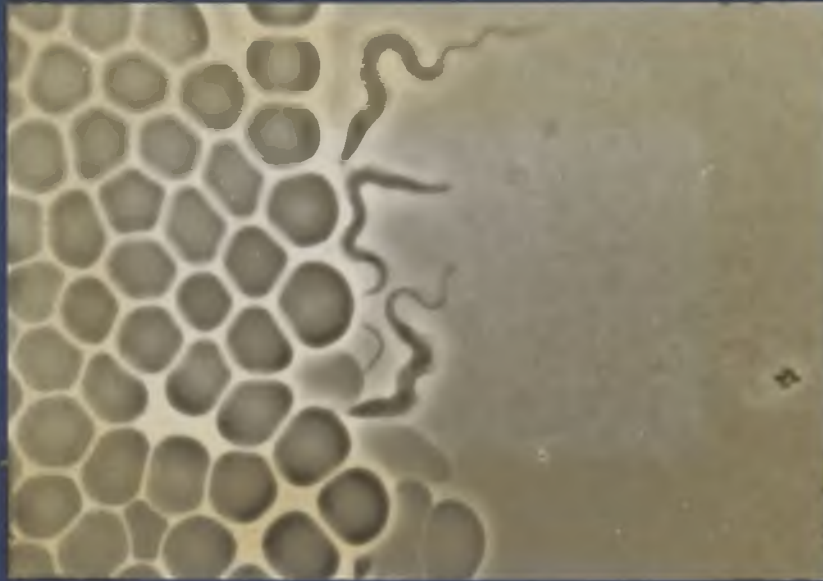


PLATE 3

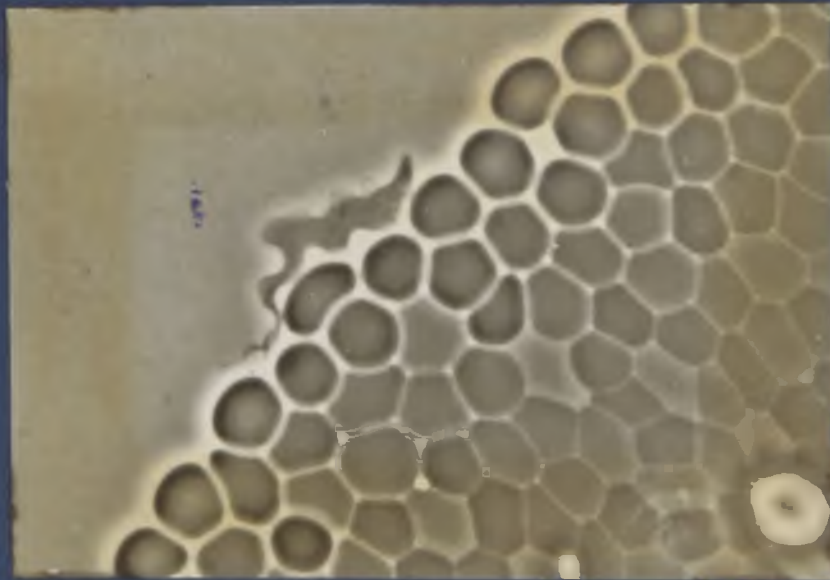


Plate 4

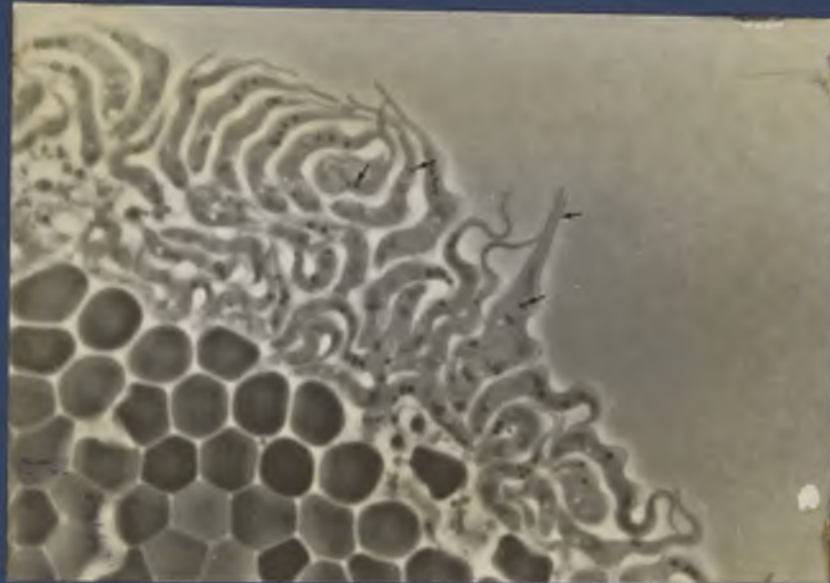
T. b. rhodesiense LSHTM 180, short stumpy form.

These trypomastigotes contain granules which have been shown to be lipoid (Ormerod and Page, 1967).

This form first appears in the blood about 96 hours after inoculation, their numbers increasing until 144 hours after inoculation when they are the only blood forms seen circulating.

The arrowed trypomastigote has at least two flagella and appears either to be dividing by multiple fission or to be arrested at that stage.

PLATE 4

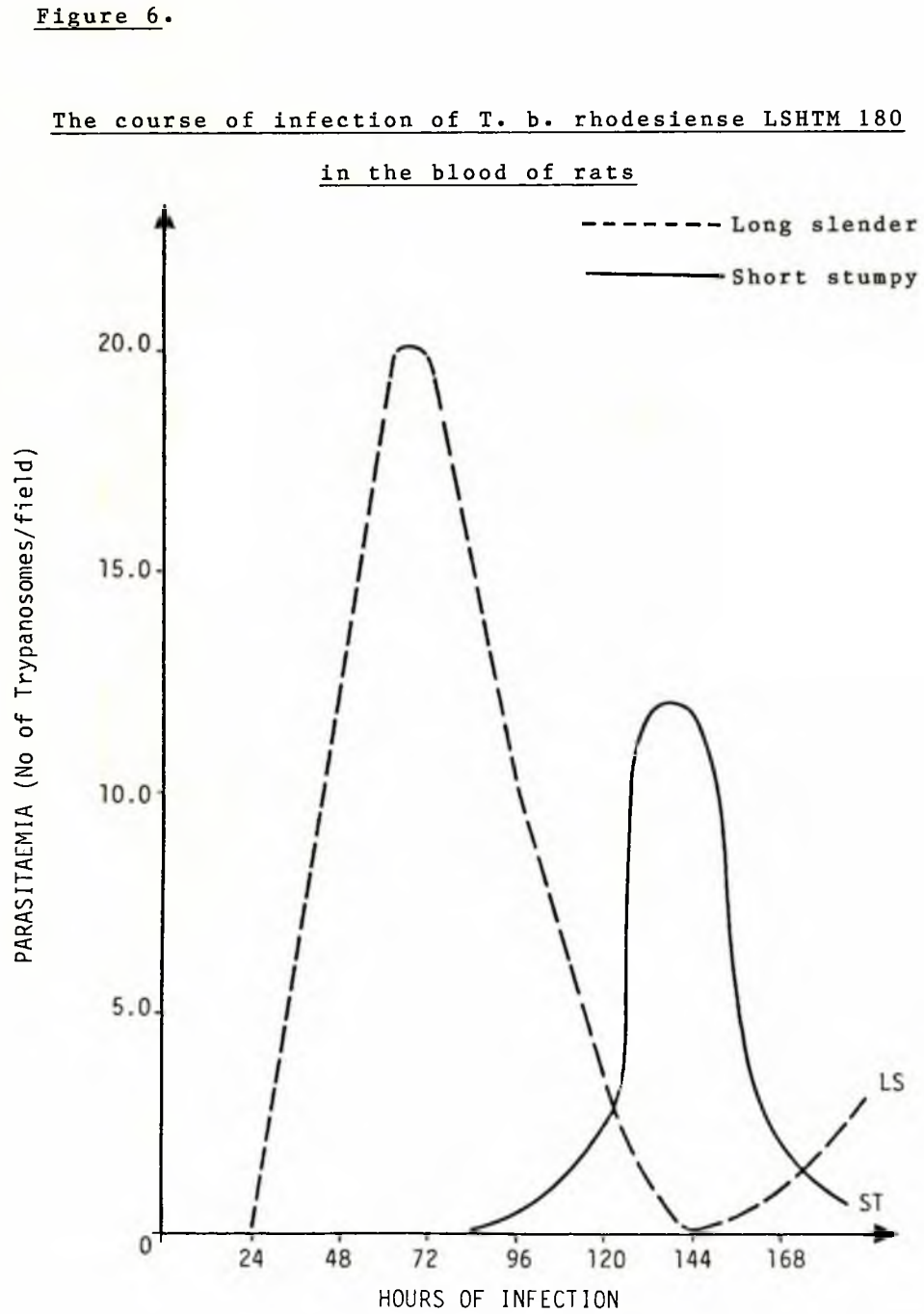


### 3.5 The Course of Infection

The course of infection of LSHTM 180 with particular reference to cytoplasmic inclusions was studied in detail by Ormerod (1963) and by Ormerod and Venkatessan (1971). As shown in Figure 6, the infection produced is sufficiently synchronous to allow pure populations of long slender (agranular) and short stumpy (granular) forms to be isolated at 72 hours and 144 hours respectively. This synchrony, however, occurs only at the beginning of the infection. After the first remission when very few parasites are seen in the blood (about 168 hours), mixed populations co-exist at all times. As the infection progresses, there are repeated rises and falls in blood trypomastigote levels until the rodent host dies 3 - 4 weeks after inoculation.

The Liverpool Normal Strain produces a fulminating infection in the blood without remission, killing the rodent host about 72 hours after inoculation.

- 47 -



- 48 -

Isoenzyme Patterns on Starch Gel and  
Cellulose Acetate

Plate 5

ACONITASE (ACON) - STARCH GEL ELECTROPHORESIS (SGE)

0.1M Tris - 0.028M Citric acid pH 7.5; 20v/cm;

180 minutes.

1. T. b. rhodesiense (Liv)
2. LSHTM 180, ST
3. LSHTM 180, LS

Plate 6

MALATE DEHYDROGENASE (MDH) - SGE

0.1M Na<sub>2</sub>HPO<sub>4</sub> - 0.014M citric acid, pH 7.0; 20v/cm;

90 minutes

Samples 1 - 3 as for Plate 5.

Plate 7

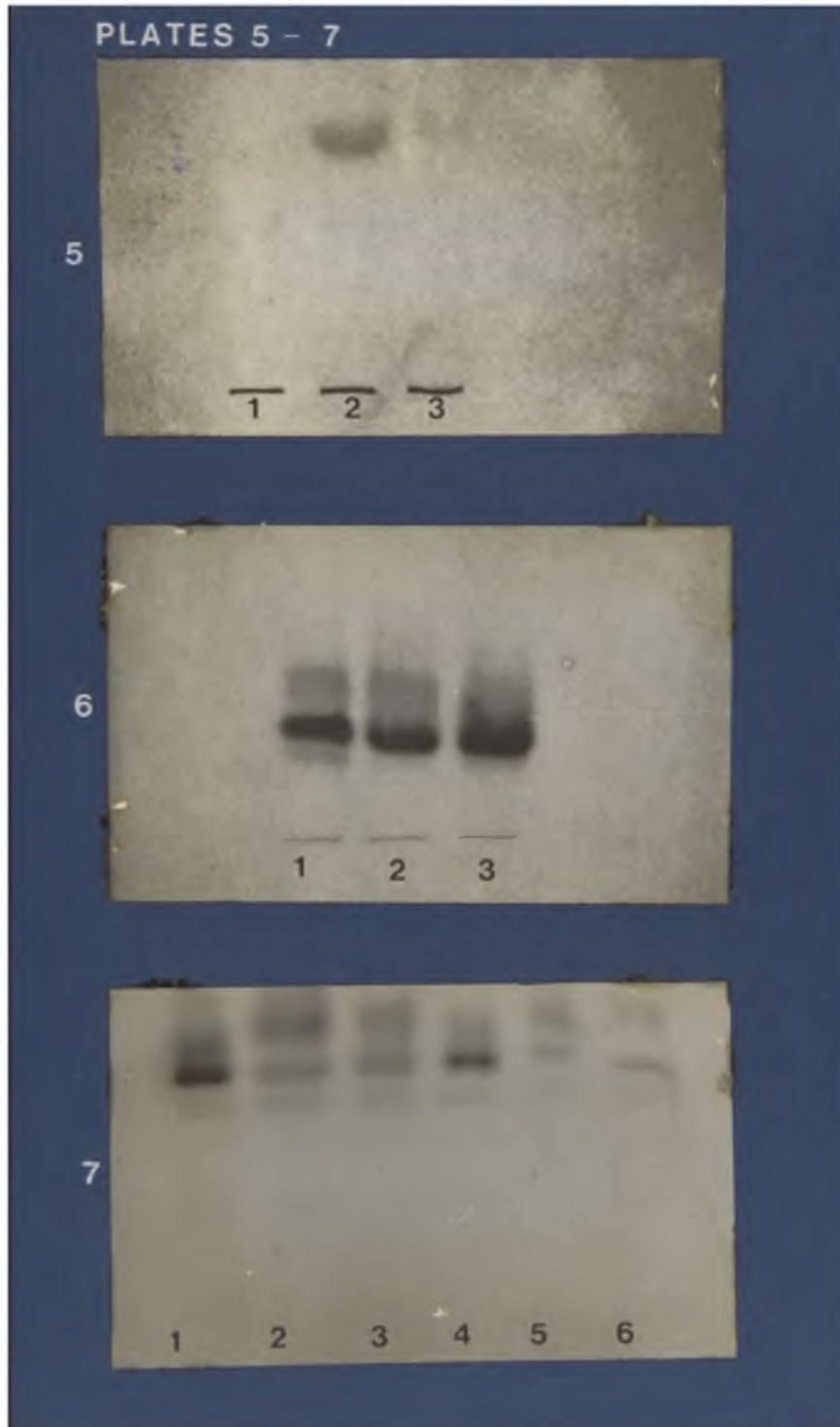
MALIC ENZYME (ME) - CELLULOSE ACETATE ELECTROPHORESIS (CAE)

0.006M Na<sub>2</sub>HPO<sub>4</sub> - 0.014M NaH<sub>2</sub>PO<sub>4</sub>, pH 8.0; 200v;

50 minutes

1. T. b. rhodesiense (Liv) 10 KS\*
2. LSHTM 180, ST 10 KS
3. LSHTM 180, LS 10 KS
4. T. b. rhodesiense (Liv) 139 KP
5. LSHTM 180, ST 139.KP
6. LSHTM 180, LS 139 KP

\* The supernatant from a 10,000g centrifugation of a trypanosome homogenate.



- 49 -

Plate 8

ISOCITRATE DEHYDROGENASE (ICD) - CAE

Conditions the same as ME above.

Samples 1 - 6, as for Plate 7.

Plate 9

GLUCOSE PHOSPHATE ISOMERASE (GPI) - SGE

0.162M  $\text{Na}_2\text{HPO}_4$  - 0.038  $\text{NaH}_2\text{PO}_4$ , pH 7.4; 20v/cm;

135 minutes.

1. T. b. rhodesiense (Liv)
2. LSHTM 180, LS
3. LSHTM 180, ST

Plate 10

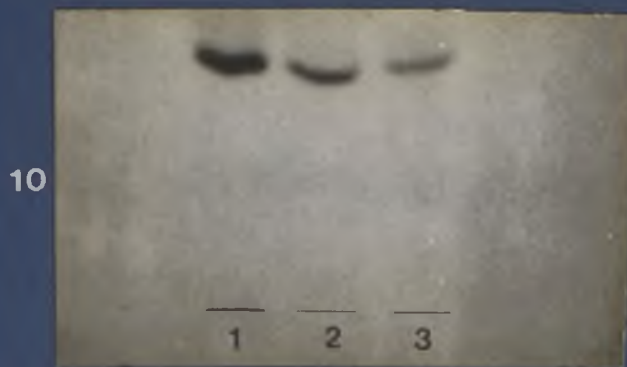
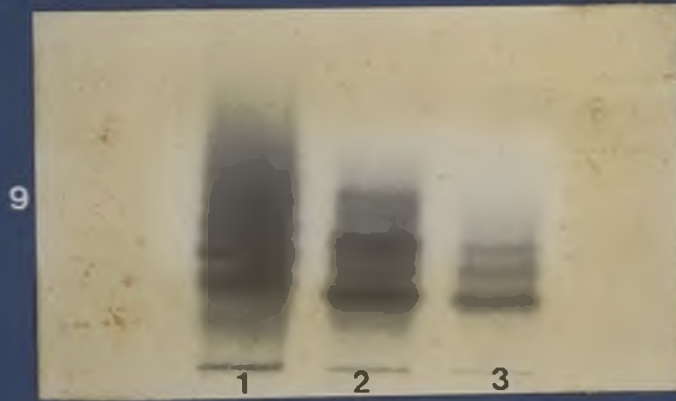
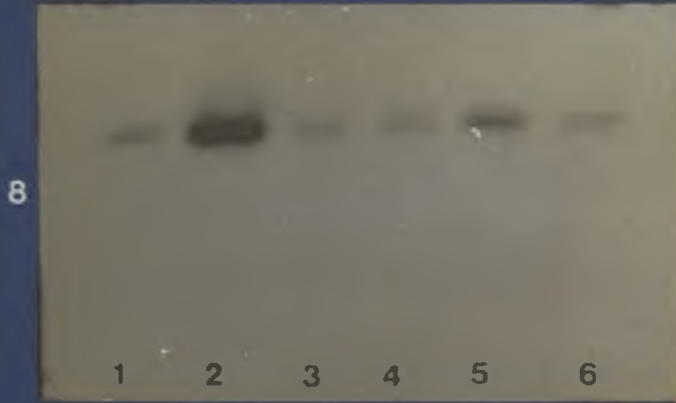
PHOSPHOGLUCOMUTASE (PGM) - SGE

0.1M Tris - 0.1M maleic acid - 0.01M EDTA - 0.01M

$\text{MgCl}_2$  pH 7.4; 13.5v/cm; 180 minutes.

Samples 1 - 3 as for Plate 9.

PLATES 8-10



- 50 -

Plate 11

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) - CAE

0.05M Tris - 0.05M maleic acid - 0.005M EDTA - 0.005M  
MgCl<sub>2</sub> pH 8.6; 130v; 55 minutes.

1. T. b. rhodesiense (Liv) 14.5KP
2. LSHTM 180, ST 14.5KP
3. LSHTM 180, ST 14.5KP
4. T. b. rhodesiense (Liv) 139KP
5. LSHTM 180, LS 139KP
6. LSHTM 180, ST 139KP

Plate 12

NUCLEOSIDE HYDROLASE (NH) - SGE

0.04M Tris - 0.44M boric acid pH 6.5; 20v/cm;  
180 minutes.

Samples 1 - 3 are as for Plates 9 and 10.

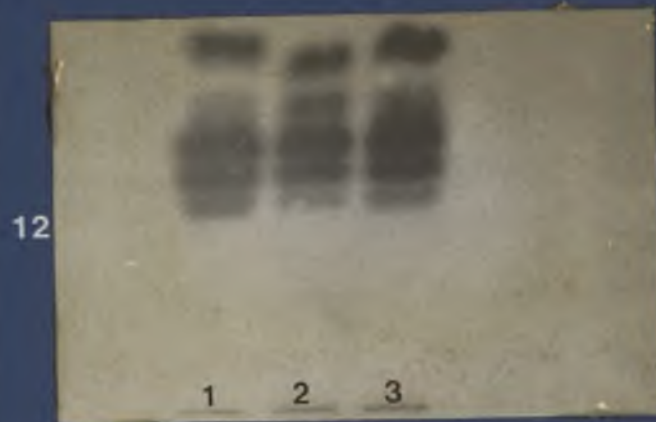
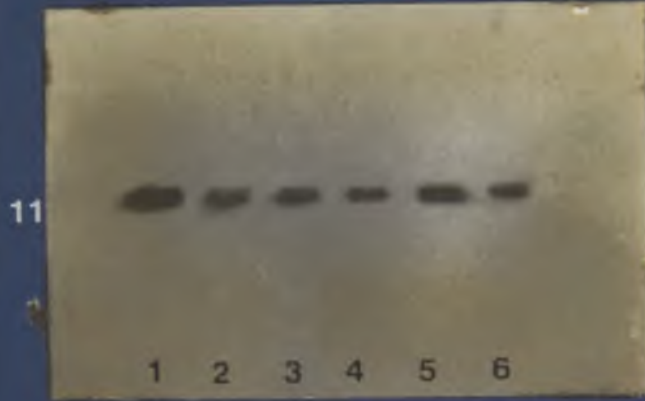
Plate 13

ALANINE AMINOTRANSFERASE (ALAT) - CAE

0.66M Tris - 0.083M citric acid pH 8.6; 200v;  
25 minutes.

Samples 1 - 6 are as for plate 7.

PLATES 11 - 13



- 51 -

Polypeptide Profiles from SDS-PAGEPlate 14

M*	Marker	
1.	LSHTM 180, LS	1KS
2.	LSHTM 180, ST	1KS
3.	<u>T. b. rhodesiense</u> (Liv)	1KS
4.	LSHTM 180, LS	42KP
5.	LSHTM 180, ST	42KP
6.	<u>T. b. rhodesiense</u> (Liv)	42KP
7.	LSHTM 180, LS	105KP
8.	LSHTM 180, ST	105KP
9.	<u>T. b. rhodesiense</u> (Liv)	105KP

\* The marker M was made up of the following proteins:

	Molecular weight in daltons
Bovine Serum Albumin (BSA)	68,000
Caseinase	58,000
Aldolase	41,000
Myoglobin	17,000

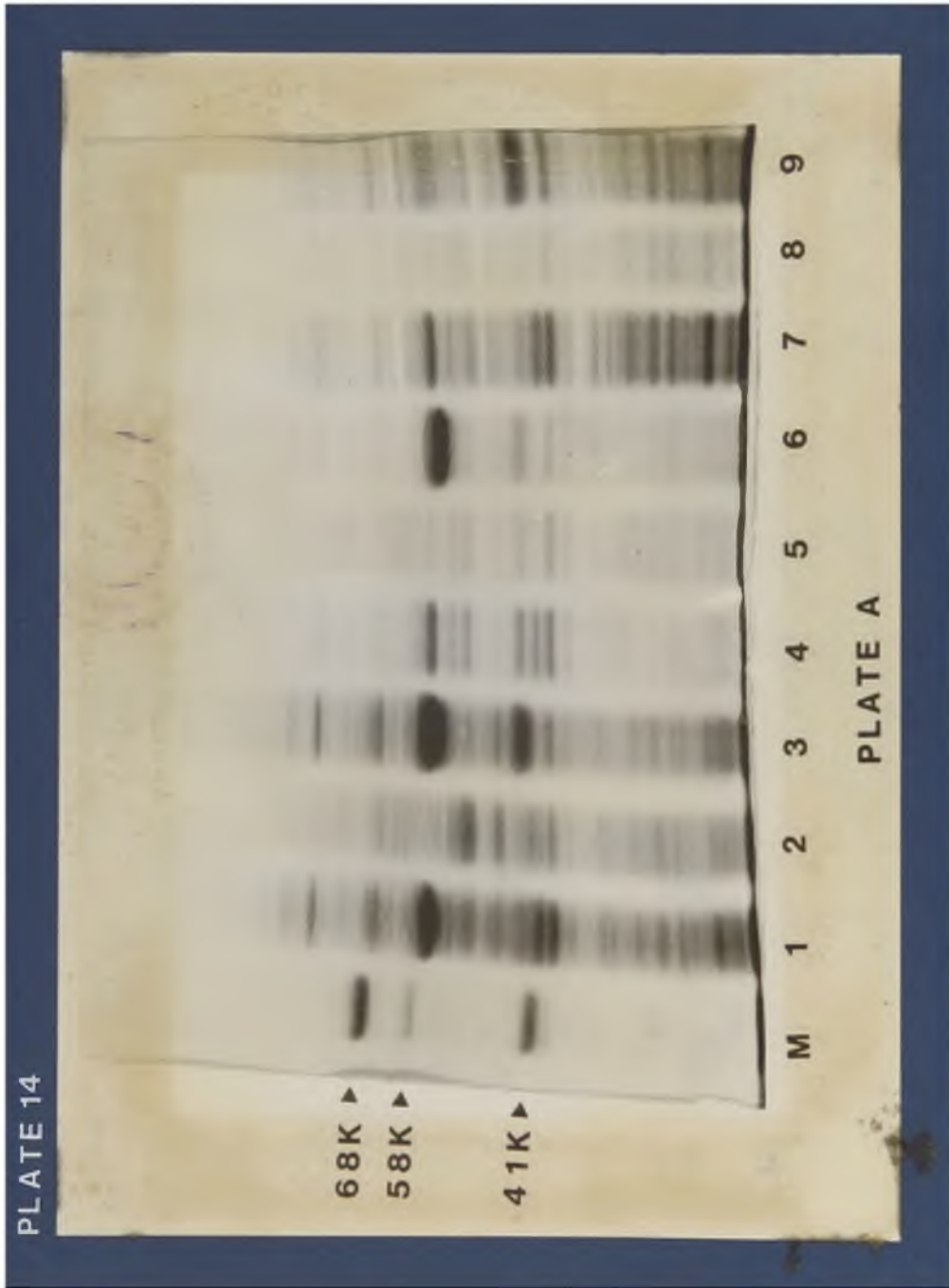
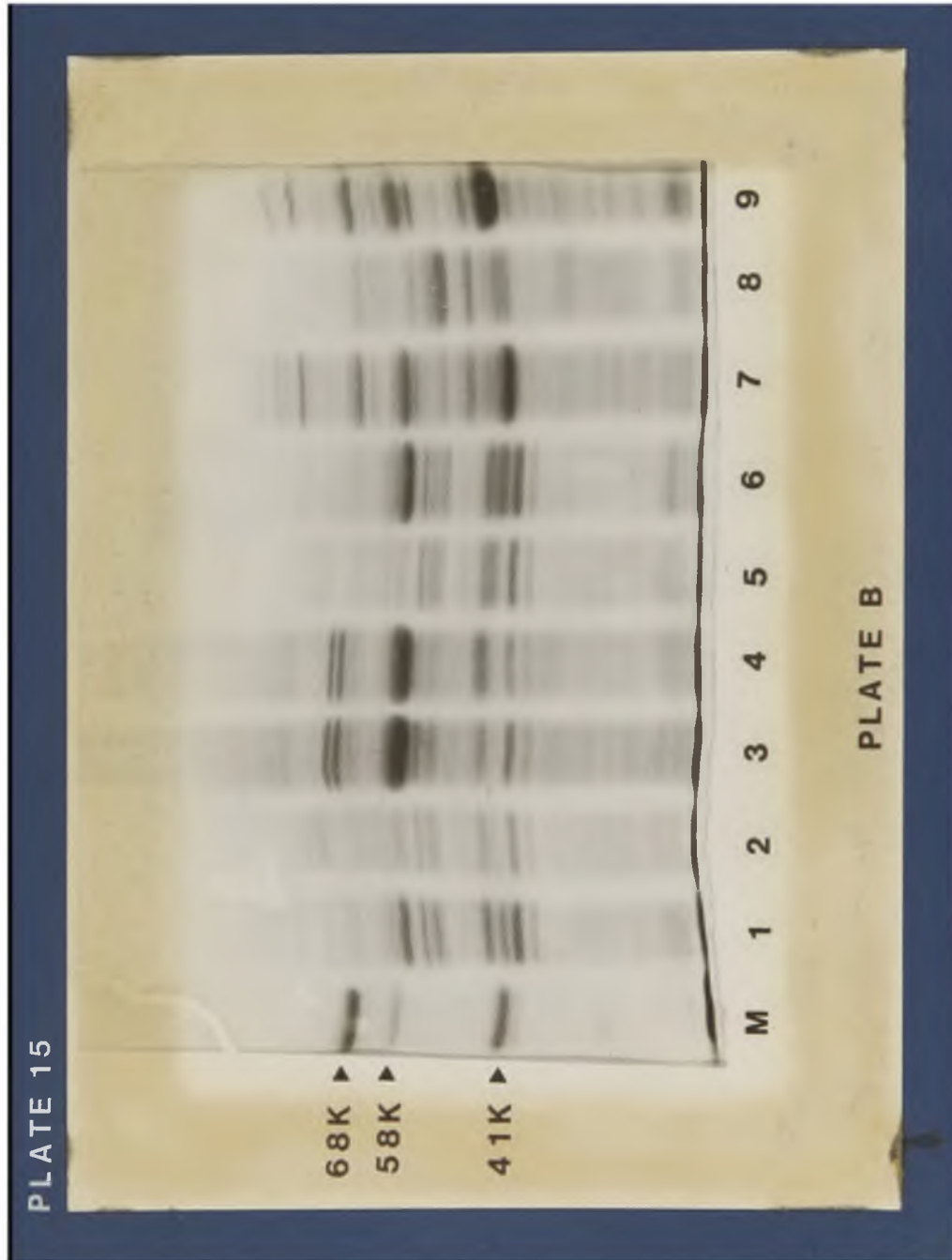


Plate 15

M	Marker	
1.	LSHTM 180, LS	3.4KP
2.	LSHTM 180, ST	3.4KP
3.	<u>T. b. rhodesiense</u> (Liv)	3.4KP
4.	LSHTM 180, LS	14.5KP
5.	LSHTM 180, ST	14.5KP
6.	<u>T. b. rhodesiense</u> (Liv)	14.5KP
7.	LSHTM 180, LS	139KS
8.	LSHTM 180, ST	139KS
9.	<u>T. b. rhodesiense</u> (Liv)	139KS



- 53 -

ENZYME ASSAYS

The enzyme activities measured in the subcellular fractions of the trypanosomes are shown in Table 1, and Figures 7 to 15 show the subcellular distribution of the enzyme.

- 54 -

**Table 1. Enzyme Activities (nmole/min/mg protein)\***

ENZYME	FRACTION	LSHTM 180 (LS)	LSHTM 180 (ST)	T. b. <u>rhodesiense</u>
Alanine aminotransferase E.C.2.6.12; ALAT	14.5KP 139KP 139KS	147.0 335.0 1195.0	126.0 160.0 879.0	54.0 287.0 1320.0
Glucose phos- phate isomerase E.C.5.3.1.9; GPI	14.5KP 139KP 139KS	1847.0 287.0 874.0	1660.0 130.0 697.0	348.0 575.0 1484.0
Glucose-6- phosphate dehydrogenase E.C.1.1.1.49; G6PD	14.5KP 139KP 139KS	21.0 13.0 28.0	49.0 23.0 6.05	9.0 13.0 2.06
Malate dehydrogenase E.C.1.1.1.37; MDH	14.5KP 139KP 139KS	42.0 144.0 539.0	34.0 77.0 273.0	41.0 182.0 619.0
Mannose phosphate isomerase E.C.5.3.1.8; MPI	14.5KP 139KP 139KS	13.0 - 40.0	19.0 - 7.0	5.0 - 5.0
Hexokinase E.C.2.7.1.1; HK	14.5KP 139KP 139KS	1946.0 447.0 53.0	1469.0 309.0 5.4	587.0 435.0 7.4
Isocitrate dehydrogenase E.C.1.1.1.42; ICD	14.5KP 139KP 139KS	3.5 - 1.7	- - 12.0	- - 2.1
Phosphogluco mutase E.C.2.7.5.1; PGM	14.5KP 139KP 139KS	- - 7.0	- - 15.0	- - 4.1
Acid phosphatase E.C.3.1.2.3; ACP	14.5KP 139KP 139KS	10.6 18.3 0.7	24.3 11.0 1.4	15.0 26.0 3.7

\* All enzyme activities are in nmole/min/mg protein, except acid phosphatase which is in mole/min/mg protein.

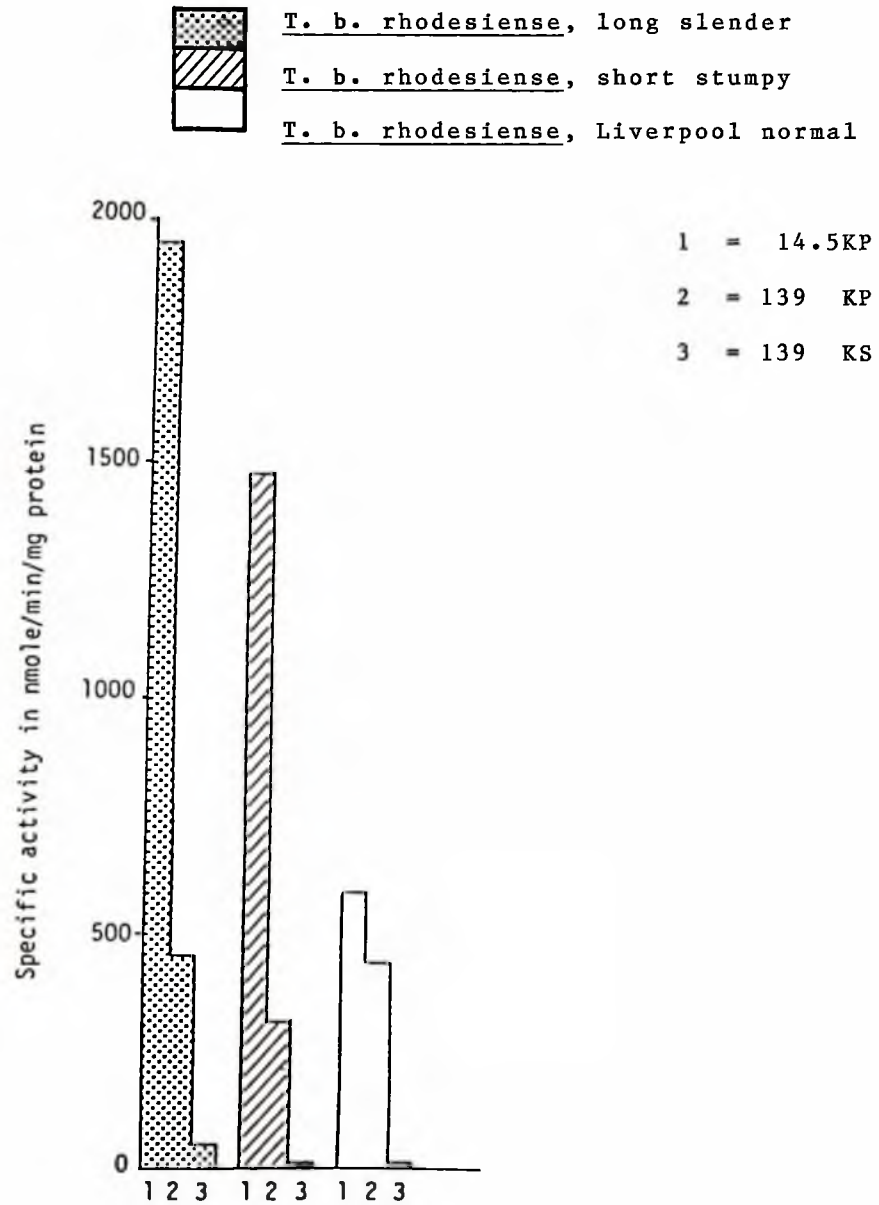
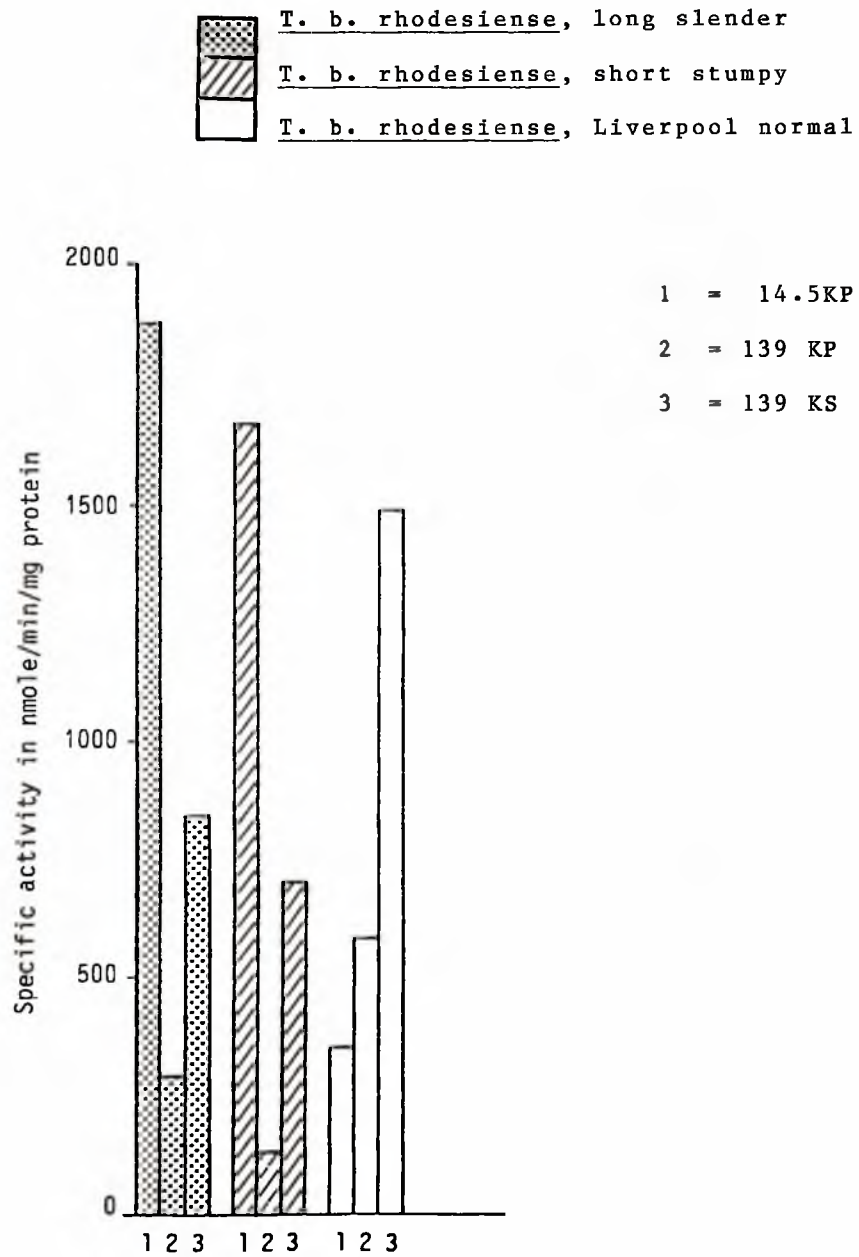
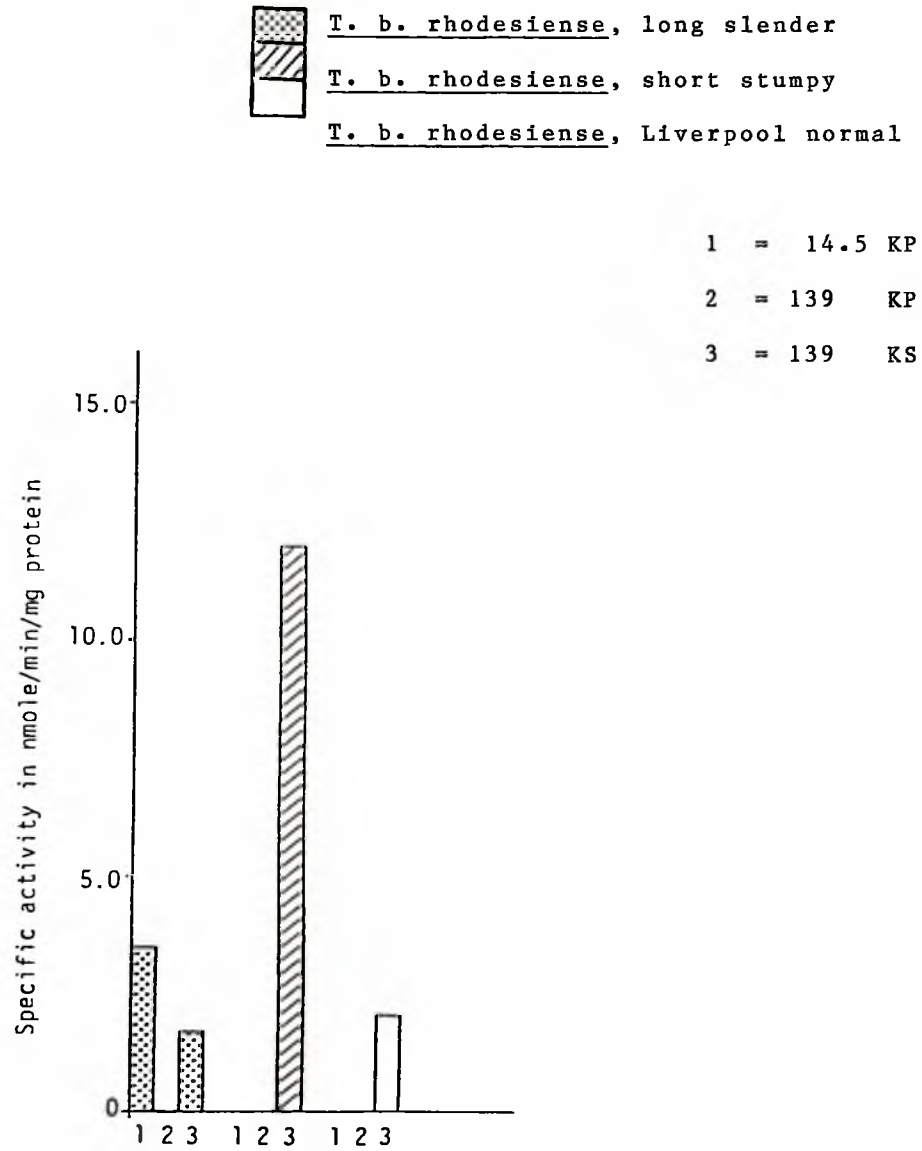
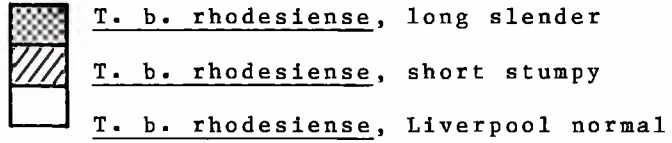
Figure 7. Subcellular distribution of HK

Figure 8. Subcellular distribution of GPI

- 57 -

Figure 9. Subcellular distribution of ICD

- 58 -

Figure 10. Subcellular distribution of MDH

1 = 14.5KP

2 = 139 KP

3 = 139 KS

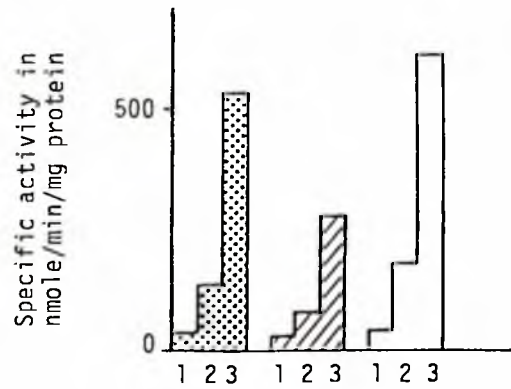


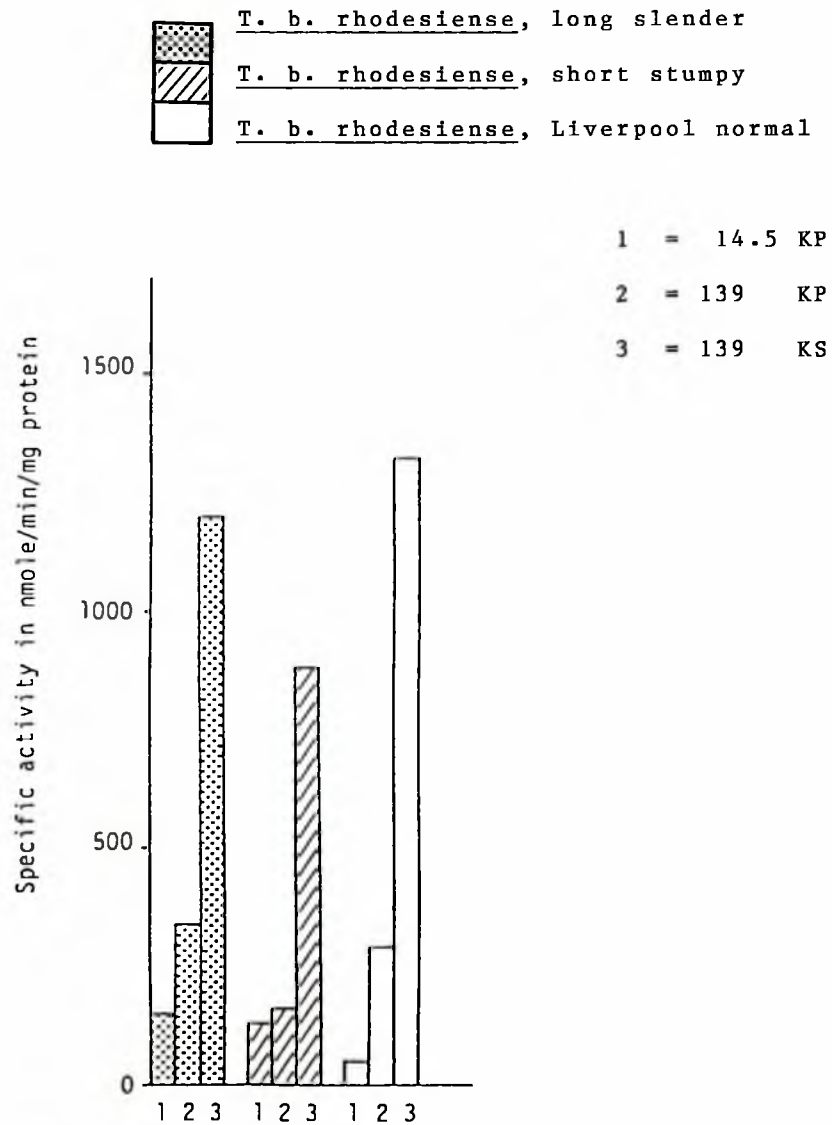
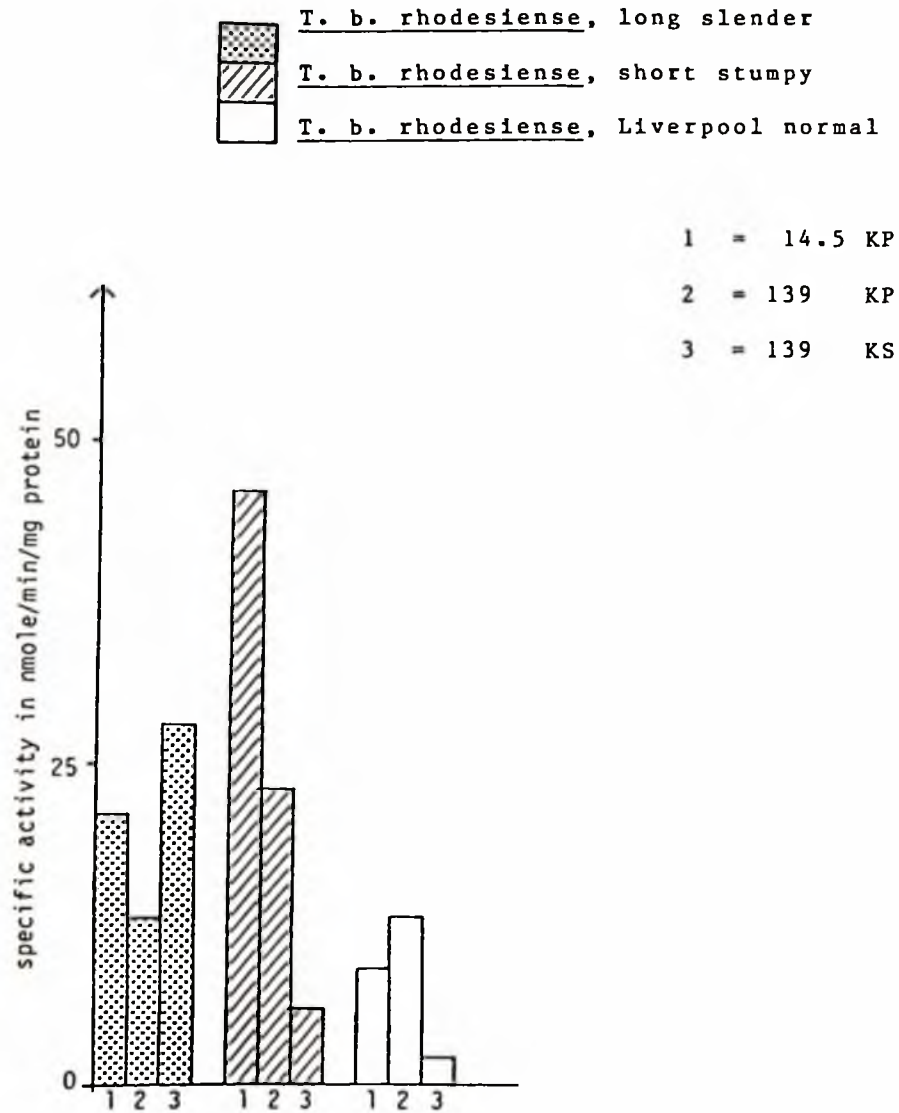
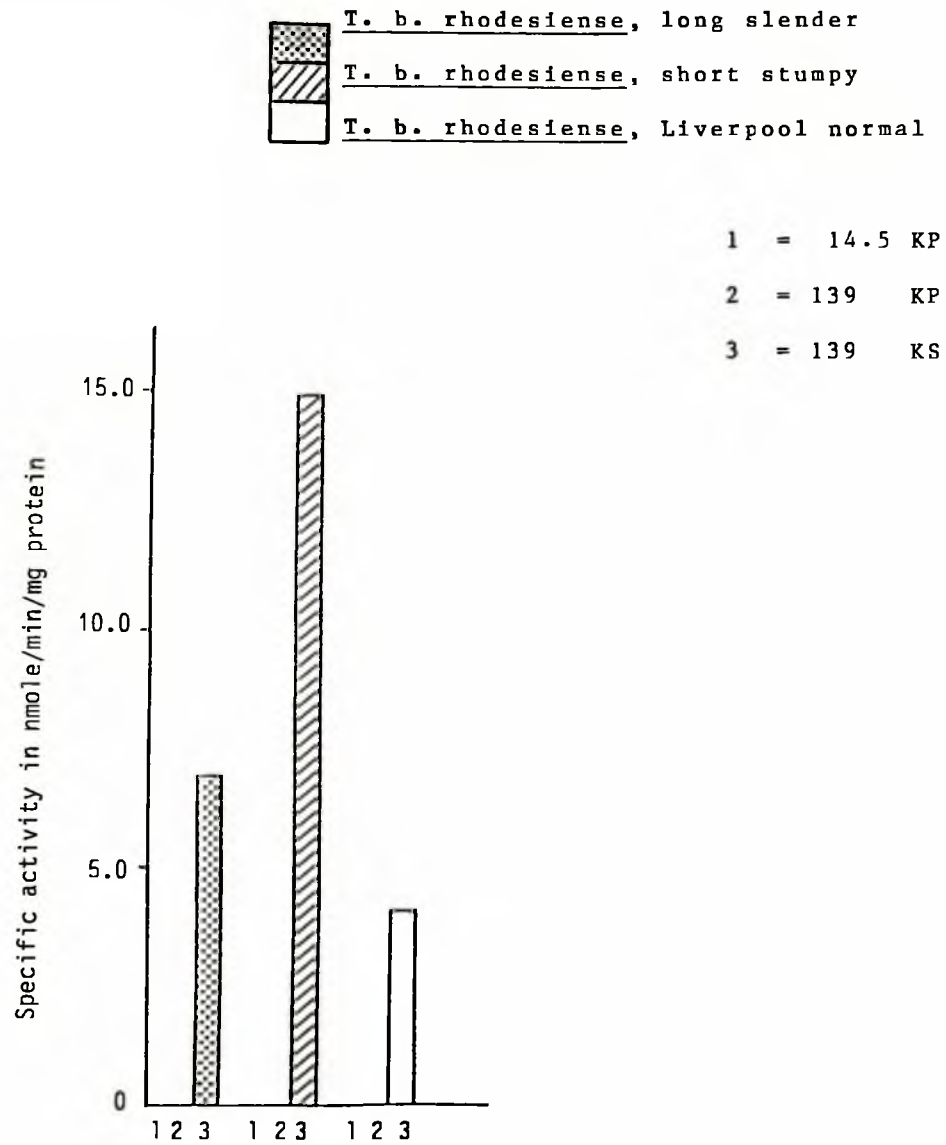
Figure 11. Subcellular distribution of ALAT

Figure 12. Subcellular distribution of G6PD

**Figure 13. Subcellular distribution of PGM**

- 62 -

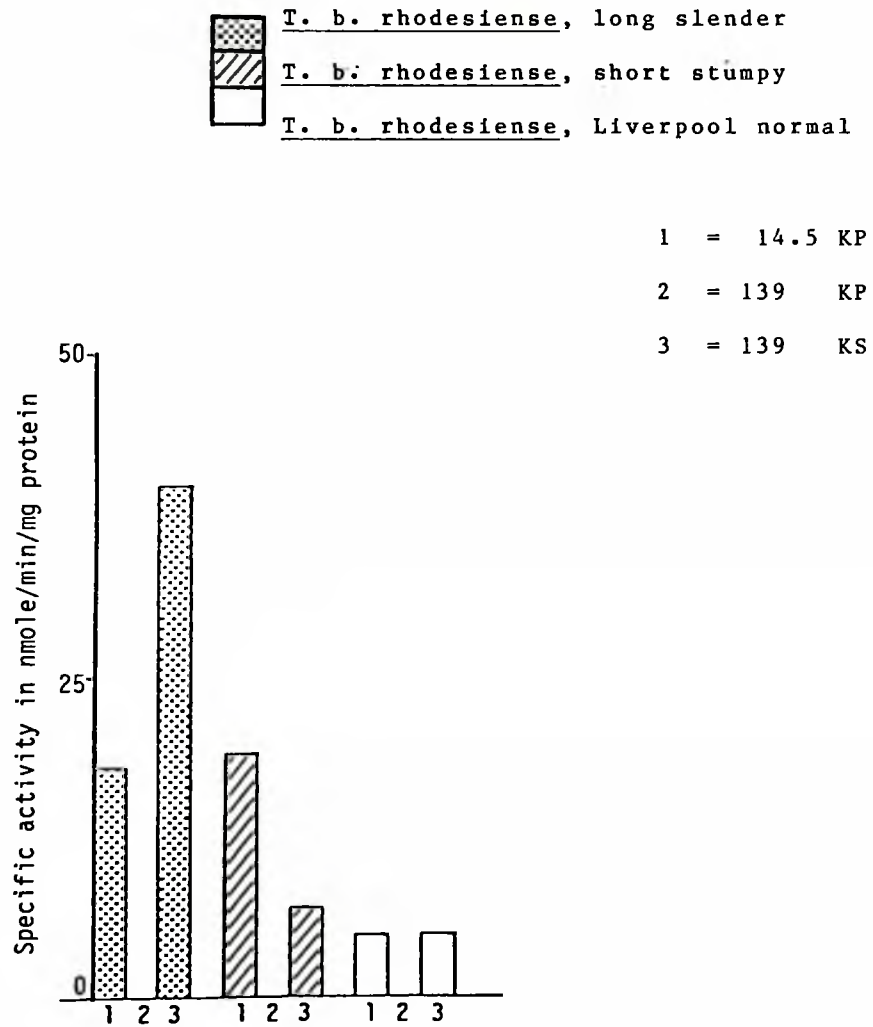
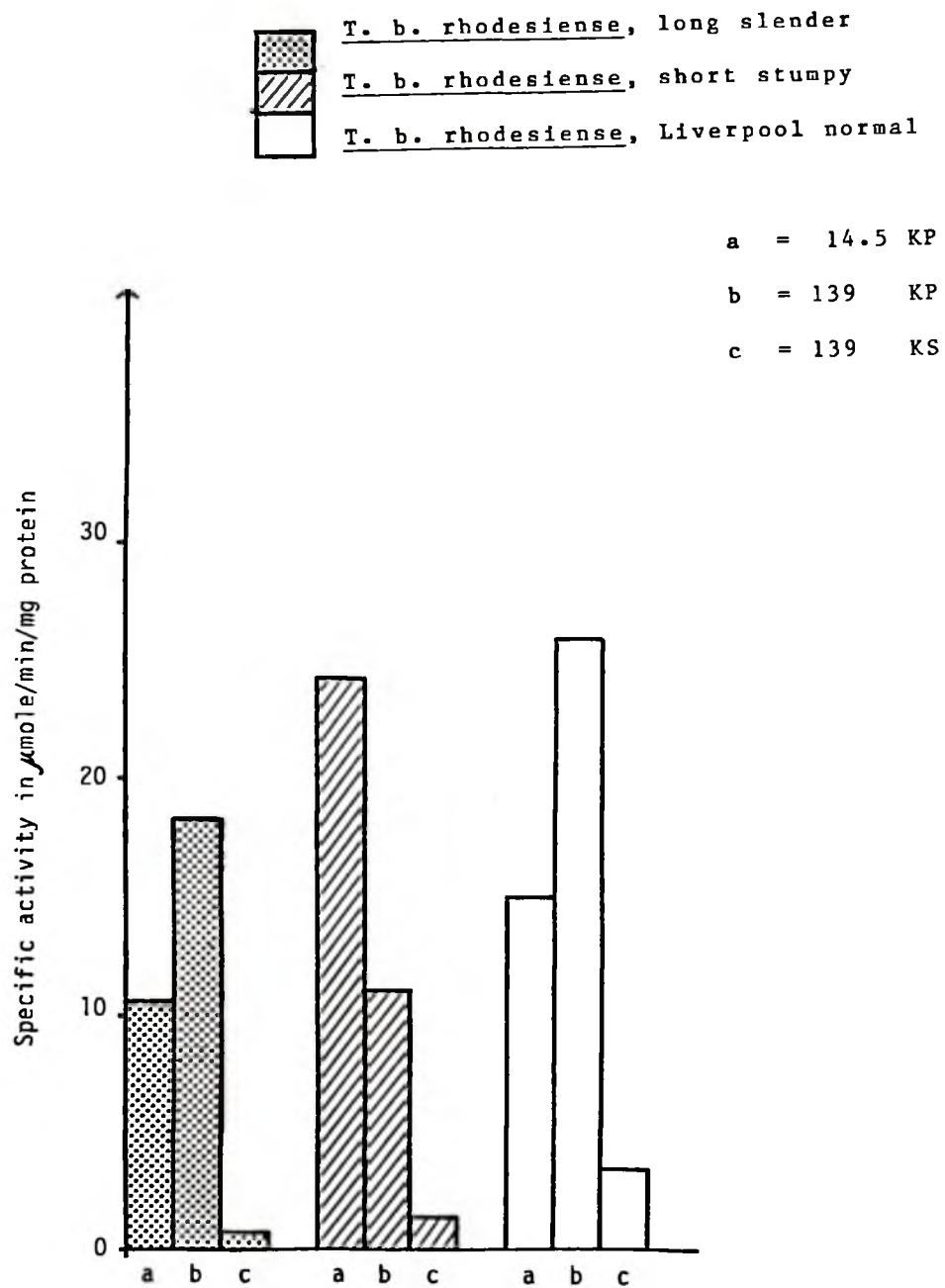
Figure 14. Subcellular distribution of MPI

Figure 15. Subcellular distribution of Acid Phosphatase



## 3.6

Electron MicroscopyPlate 16

Electron micrograph (x 20,000) of T. b. rhodesiense  
(Liv). N = nucleus.

Plate 17

Electron micrograph (x 126,000) of T. b. rhodesiense  
(Liv) showing a section of the tubular mitochondrion  
(M) with a double membrane.

PLATE 16

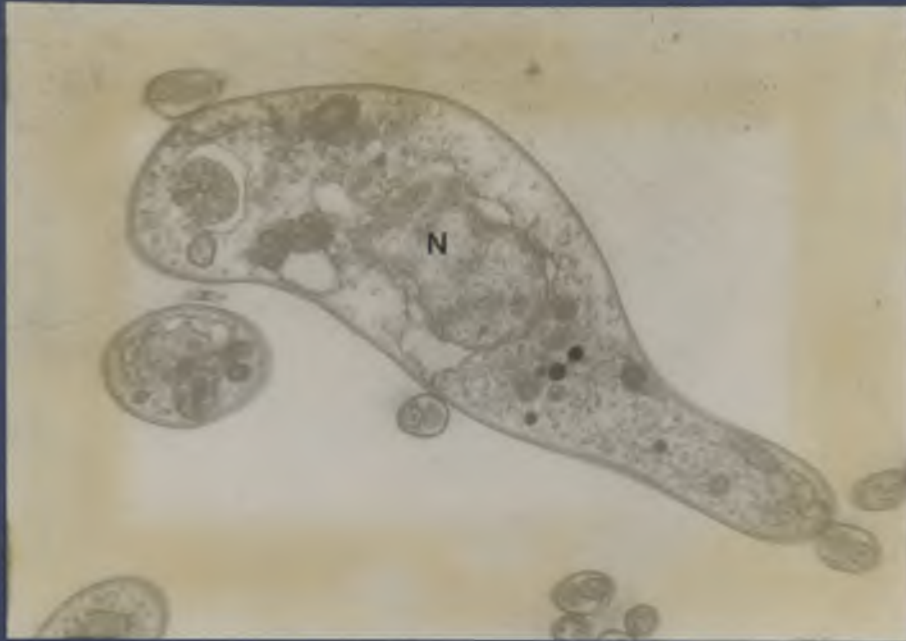


PLATE 17



- 65 -

Plate 18

Electron micrograph (x 20,000) of T. b. rhodesiense  
(Liv) with a double flagellum (narrowed, probably  
dividing.

PLATE 18

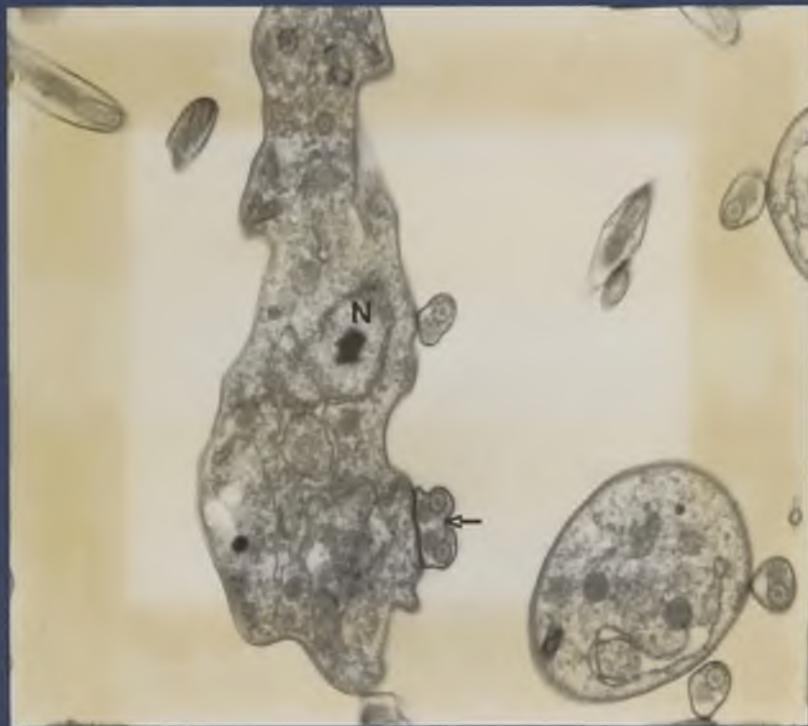


Plate 19

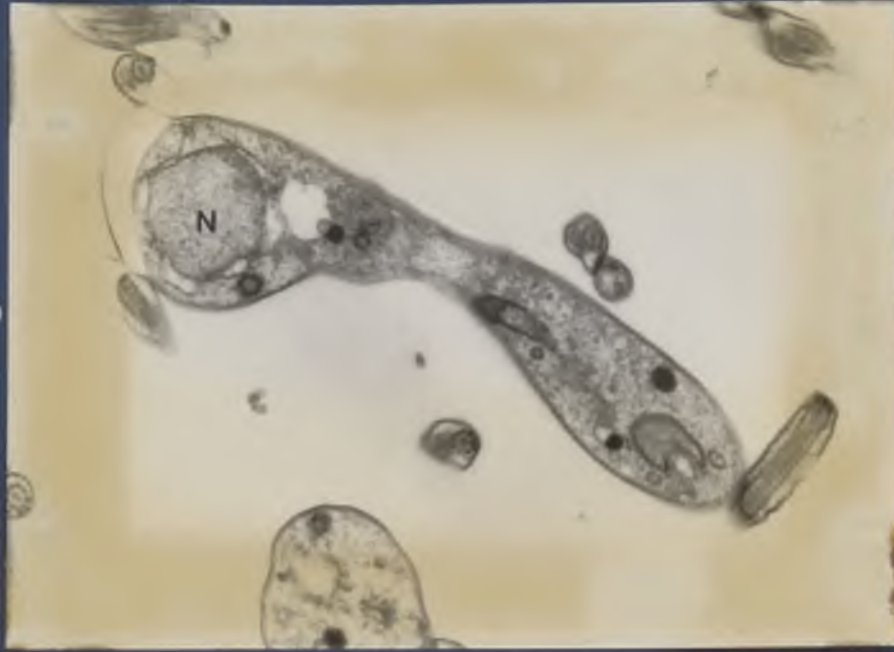
Electron micrograph (x 20,000) of LSHTM 180, LS showing the nucleus (N).

Plate 20

Electron micrograph (x 126,000) of LSHTM 180, LS showing a section of the long tubular mitochondrion (M) bounded by a double membrane but devoid of cristae.

PLATES 19 & 20

19



20

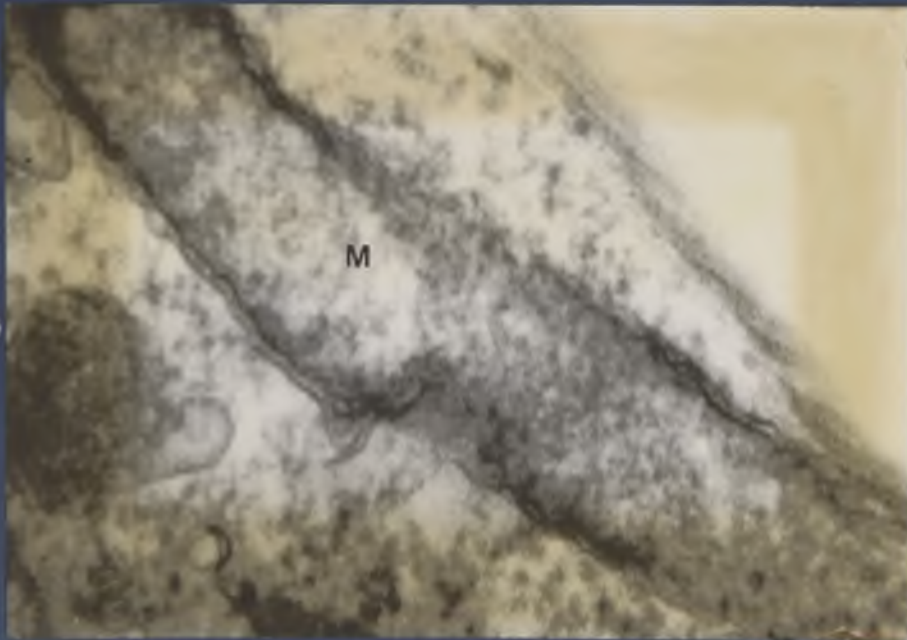


Plate 21

x 20,000 electron micrograph of LSHTM 180, ST, showing the nucleus (N) and nucleolus (Nu) and the mitochondrion (M) with cristae (arrowed).

Plate 22

A close-up (x 80,000) of the mitochondrion of LSHTM 180, ST. (Cristae arrowed.)

PLATES 21 & 22

21



22

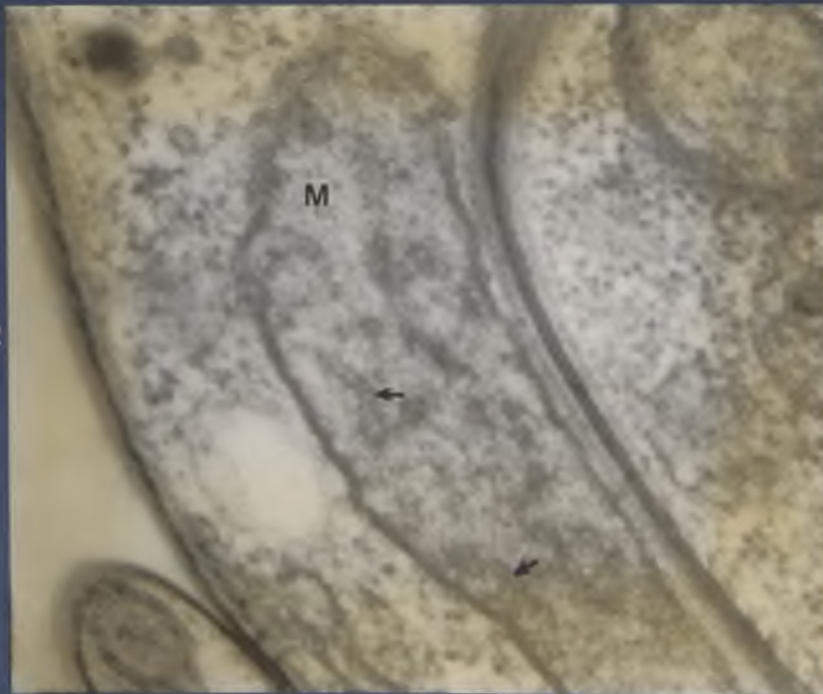


Plate 23

Electron micrograph (x 20,000) of a section through the choroid plexus of a mouse infected with T. b. rhodesiense (Liv) just before death, (i.e. about 72 hours after inoculation), showing trypanosomes (T) and red blood cells (r.b.c.) in a blood vessel.



Plate 24

Electron micrograph (x 80,000) of a section through the choroid plexus of a mouse infected with T. b. rhodesiense (Liv), 72 hours after infection, showing a trypanosome in a blood vessel. N = nucleus.

Plate 25

T. b. rhodesiense (Liv) in a choroid plexus vessel of a mouse showing Golgi body (Gb) and flagellum (f) (x 50,000).

Plate 26

T. b. rhodesiense (Liv) in a choroid plexus blood vessel of a mouse (x 126,000). f = flagellum.

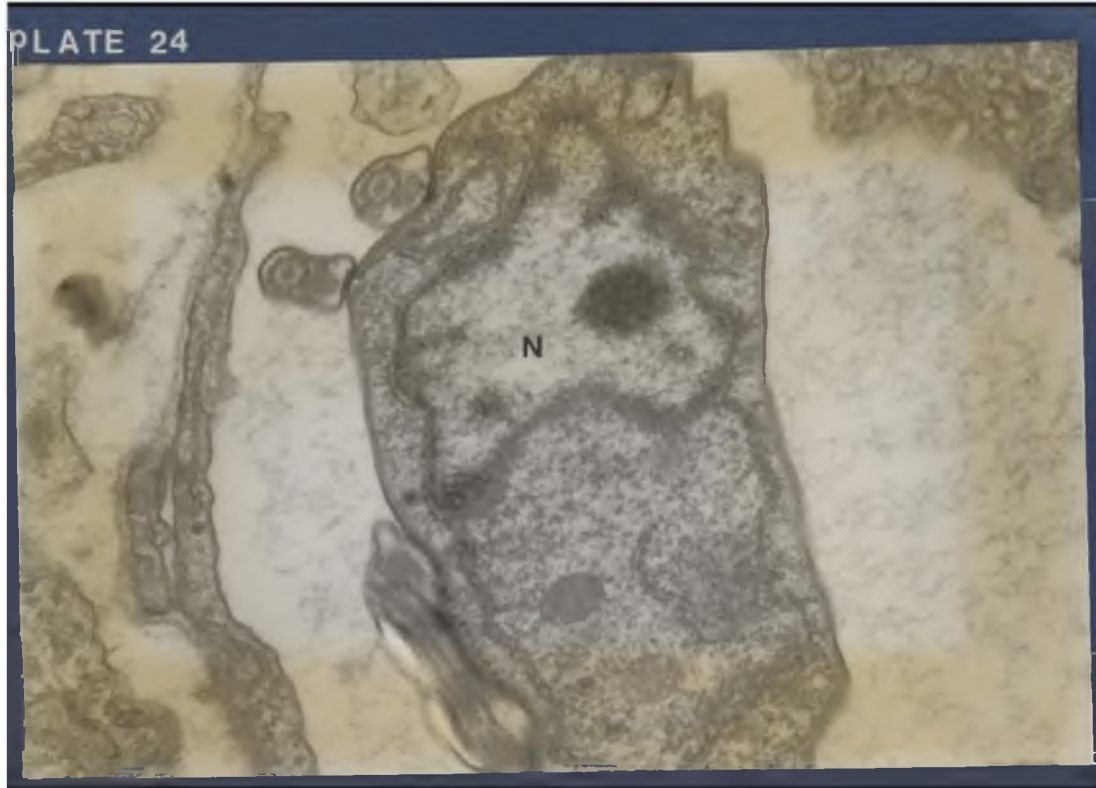


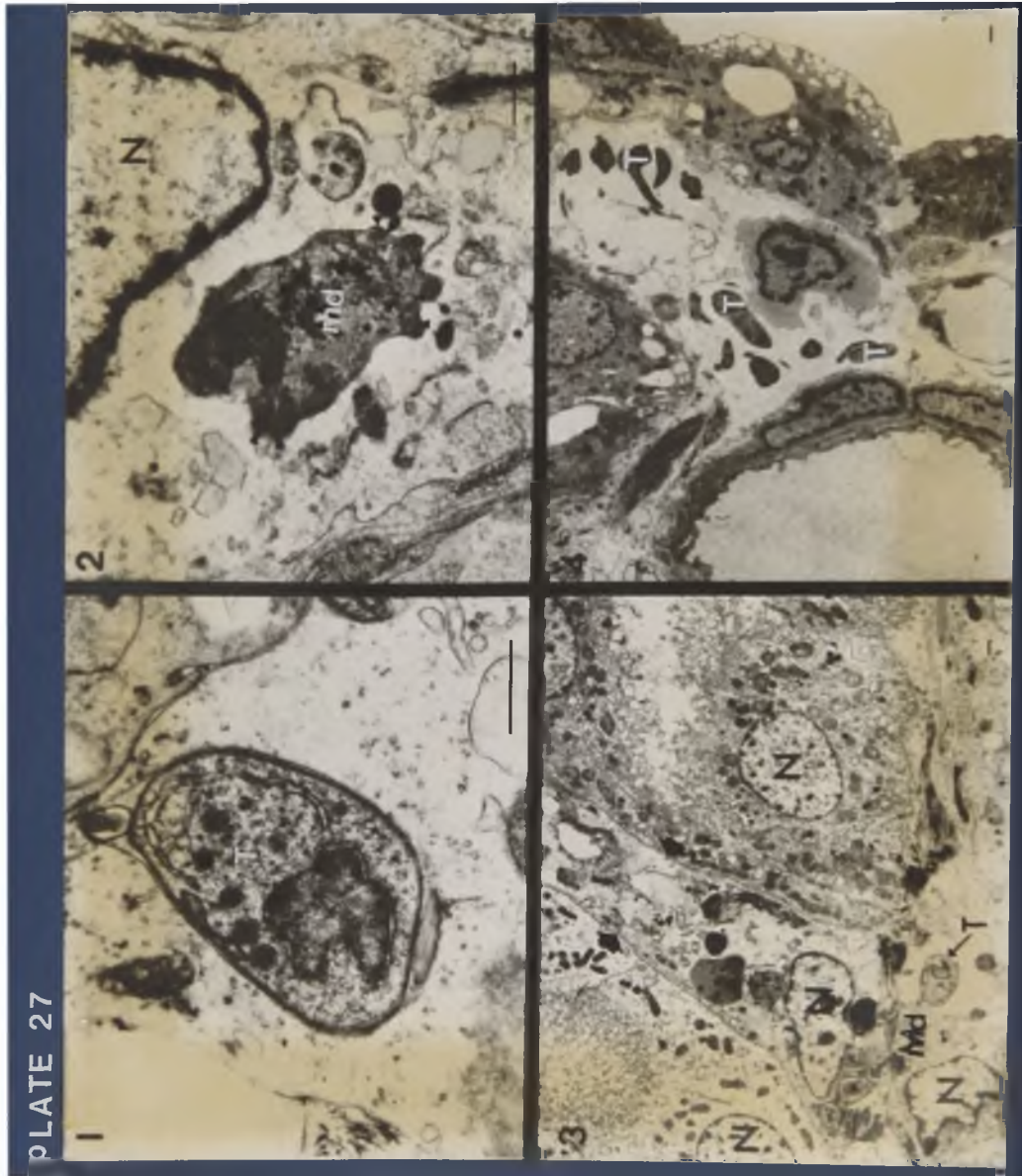
PLATE 26



Plate 27

Electron micrographs of sections through the choroid plexus of rats infected with LSHTM 180 24 - 27 days after infection (Abolarin et al., 1982).

1. Trypomastigote (T) in an intact ependymal cell of choroid plexus.
2. Multiple-division form (md) in intact ependymal cell (N = nucleus of ependymal cell) from rats treated with SHAM and glycerol. Flagellar profiles arrowed.
3. Multiple division form (md) and trypomastigote (T) in degenerating ependymal cell (N = nucleus of ependymal cell). Limiting membranes of adjoining cells arrowed.
4. Liberation of trypomastigotes after destruction of host ependymal cell.



C H A P T E R   F O U R

D I S C U S S I O N   A N D   C O N C L U S I O N

## D I S C U S S I O N

### 4.1 Morphology and Pleomorphism

For many years the pleomorphism of trypanosomes has been studied by drawing individuals by camera lucida and by measuring and recording lengths and other parameters of the drawings (Bruce et al., 1912, Fairbairn and Culwick, 1949). By this method trypomastigotes were divided into "slender", "intermediate" and "stumpy", but the parameters show so much variability that drawing and measuring sufficient numbers becomes a tedious operation. Wijers (1959) further elaborated this method by classifying the trypomastigotes which did not conform to any of the above types either as "long-intermediate" or "short-intermediate", but in the final percentages of categories of "slender", "intermediate" and "stumpy" half the long-intermediates were counted as slender and the other half as intermediate, similarly the short-intermediates were divided among intermediate and stumpy forms. While this method has been useful in relieving much of the tedium of drawing and measuring, it tends to "create" an intermediate form which some workers have considered to be independent and not just a transition from slender to stumpy.

The agar film technique developed by Ormerod (1958), which was used in this study, showed that under phase

- 72 -

contrast microscopy the first trypanosomes to appear in the blood had no cytoplasmic granules, but as the infection developed, granules appeared and increased in number up to the time of the remission when very few trypanosomes are seen circulating in the blood. The use of granules as a parameter for the study of pleomorphism has three clear advantages:

1. it is a less tedious process;
2. it gives an absolute definition of a long thin trypanosome as one without cytoplasmic granules;
- 3, a quantitative assessment of intermediate and stumpy forms can be made according to the number of granules.

Measurement of the overall volume of the trypanosome and of the volumes of cytoplasmic organelles such as the mitochondrion can be obtained from electron microscopic data; this method has also been used to study pleomorphism (Hecker et al., 1972; Hecker, 1980). However, each method produces different types of result and further work is required to study the correlation between the methods.

Monomorphic infection of T. b. rhodesiense (Liv) is so acute that it kills the rodent host at the first peak of parasitemia, and there is no time for the development of more than one form of the parasite, the agranular trypano-

- 73 -

mastigote. The infection of LSHTM 180 in rats is synchronous. This behaviour is not unique either to LSHTM 180 or to the strains obtained in Botswana (Ormerod, 1963); it occurs to some extent in other recently isolated strains of T. brucei, but it is most marked in strains isolated from man in this region. In the absence of proper separation methods of these two forms, LSHTM 180 was particularly useful in obtaining pure populations of both slender and stumpy forms for biochemical and ultrastructural studies.

#### 4.2 Isoenzyme Characterization

LSHTM 180, LS and ST, and T. b. rhodesiense (Liv) all had identical isoenzyme patterns for eight of the nine enzymes examined; only an aconitase (ACON) band was unique to LSHTM 180 ST. The similar isoenzyme patterns for eight enzymes suggests great similarity between the trypomastigotes examined. This means that as far as isoenzymes are concerned, there is little change when a slender trypomastigote becomes stumpy or when a pleomorphic strain becomes monomorphic. It also suggests that both LSHTM 180 and T. b. rhodesiense (Liv) belong to the same zymodeme. Records about where T. b. rhodesiense (Liv) was isolated have become rather confused. It is known that it was isolated in 1923 and was used by Yorke and co-workers for many years at the Liverpool School of

- 74 -

Tropical Medicine (Yorke et al., 1929). It presumably originated from the Luangwa valley, Zambia, where Yorke and others had investigated an outbreak of "Rhodesian" sleeping sickness circa 1912; at any rate, according to Gibson (1979), in the early 1920's T. rhodesiense was known only from Central East Africa. Geographically, the Luangwa valley is close enough to Mababe, Botswana, where LSHTM 180 was isolated, for this close similarity in their isoenzyme patterns to be acceptable.

The absence of aconitase bands in LSHTM 180, LS and T. b. rhodesiense (Liv) was not due to the use of dilute samples because the same result was obtained with more concentrated lysates. Aconitase (ACON), like malate dehydrogenase (MDH) and isocitrate dehydrogenase (ICD), is a Krebs cycle enzyme; while the cycle is not operational in bloodstream trypomastigotes in the brucei group, some of its enzymes have been shown to be present (Bowman and Flynn, 1976), for example MDH and ICD were present in all lysates; but the presence of ACON in only the stumpy LSHTM 180 suggests that:

1. more Krebs cycle enzymes may be formed or activated in the change from slender to stumpy;
2. the activity of some Krebs cycle enzymes may be lost when a pleomorphic strain becomes monomorphic.

It is also significant to note that both the slender form of LSHTM 180 and T. b. rhodesiense (Liv) lack lipid

- 75 -

granules; the absence of ACON in both these forms suggests an even greater similarity between them.

#### 4.3 Polypeptide Profiles

Changes in the genetic code associated with the conversion of a slender trypomastigote to a stumpy or a pleomorphic to a monomorphic strain would be demonstrated by changes in the proteins produced by the organism. These proteins need not necessarily be enzymes. Polypeptide profiles on SDS - polyacrylamide gels allow the study of proteins, both enzymic and non-enzymic. The method involves breaking up the proteins into smaller polypeptides, coating them with negative charges (SDS) and separating them according to their molecular weights. Plates 14 and 15 show the polypeptide profiles of different fractions of the three trypomastigotes. The profiles for different fractions of the same sample of trypomastigotes (e.g. 3.4KP and 14.5KP for T. b. rhodesiense [Liv]) are different; this is to be expected because each fraction contains different organelles and therefore different proteins. The 1000g supernatant fraction (1KS, plate 14) shows the presence of a 58,000 dalton (58K) molecular weight polypeptide in both the slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv), which is absent in the stumpy LSHTM 180. In fact this polypeptide is consistently absent from all the

- 76 -

fractions of the stumpy form. Since the preparation of all the fractions of the three trypomastigotes was done under similar conditions, all the gels run together and the whole repeated four times with consistently the same results, the absence of the 58K polypeptide seems to be an established result and not due to a technical error. Taylor et al., (1982) have suggested that this polypeptide could represent the surface coat glycoproteins, because it stained positively with periodic acid-Schiff. However, this would need to be confirmed for the trypanosomes under study here. If the 58K polypeptide is indeed the surface coat glycoprotein, then it provides another piece of evidence for the greater similarity between the slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv) already suggested on the previous page. Secondly, monoclonal antibodies could be made against it, and it could therefore serve as a more specific tool for differentiating between slender and stumpy trypomastigotes of a pleomorphic strain.

#### 4.4 Enzyme Assay Analysis

Various workers have studied the distribution of enzymes in trypanosomes by subcellular fractionation (Oduro, 1977, Oduro et al., 1980, Opperdoes et al., 1977). Differential centrifugation and subsequent isopycnic sucrose-gradient centrifugation has been used to isolate



and purify the glycosome, a microbody-like organelle, containing some glycolytic pathway enzymes which is unique to bloodstream trypomastigotes (Oduro, 1977, Oduro et al., 1980, Opperdoes et al., 1977). In this study, differential centrifugation has been used to compare the subcellular distribution of selected enzymes. The results show that most of the hexokinase (HK) activity is located in the 14,500g (14.5KP) and 139,000g (139KP) pellets, which is in agreement with those observations made by Oduro (1977, 1980). HK is the first enzyme in the glycolytic pathway and has been shown to be located in the glycosome. Since glycosomes form a pellet at 14,500g (Oduro, 1977, Oduro et al., 1980, Opperdoes et al., 1977), it can be concluded that the 14.5KP fraction in which most of the HK activity is located also contains glycosomes, which have been confirmed by Oduro (personal communication) to be microbodies.

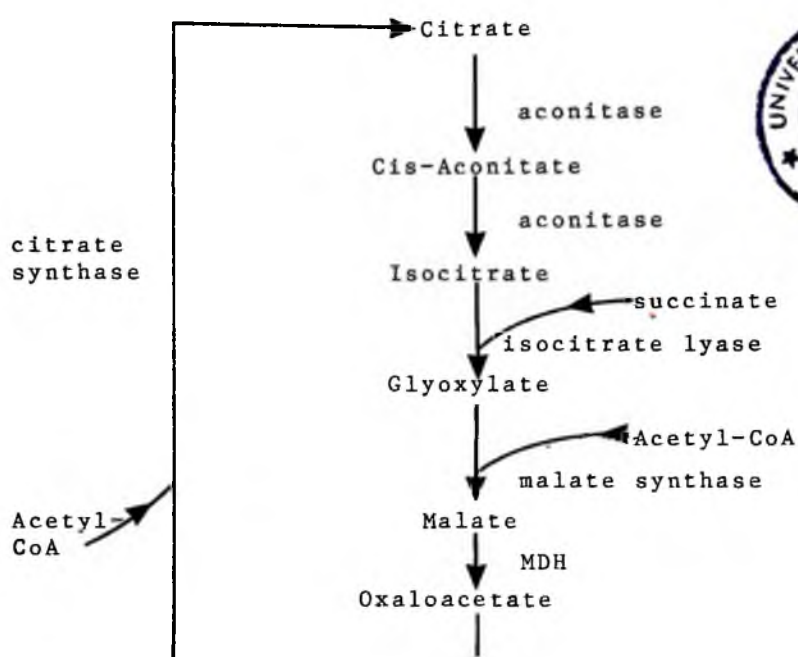
The distribution of glucose-phosphate isomerase (GPI) in T. b. rhodesiense (Liv) differs from those in LSHTM 180 slender and stumpy forms, where most of the GPI activity is located in the 14.5KP (glycosomal) fraction. This difference may be due to leakage of a less tightly bound enzyme during the preparation of the T. b. rhodesiense (Liv) fractions.

The distribution of ALAT in all three trypomastigotes is

- 78 -

identical, most of the activity being located in the 139,000 supernatant (139KS), the soluble fraction. This result confirms the observations made by Visser and Opperdoes (1980) that ALAT is a soluble enzyme. ALAT and other transaminases such as ASAT catalyse the inter-conversion of Krebs cycle intermediates and amino acids.

The distribution of NAD<sup>+</sup>-linked MDH is similar to that of ALAT, most of it being located in the soluble part of the cell (139KS). The presence of high levels of MDH, a Krebs cycle enzyme, which catalyses the reversible oxidation of malate to oxaloacetate remains unexplained in view of the cycle being non-functional in these blood stream trypomastigotes. The operation of a glyoxylate cycle (Figure 16), a modified form of the Krebs cycle which takes place in most plants and microorganisms could explain the presence of MDH in these trypomastigotes. However, as Table 2 overleaf shows, the levels of malate synthase and isocitrate lyase are probably too low to make the cycle operative. MDH seems to be present to generate precursors for anabolic pathways, namely oxaloacetate for the synthesis of the amino acids aspartate and asparagine. The specific activity of MDH in procyclic trypomastigotes of T. brucei is 1600 nmole/min/mg protein (Opperdoes et al., 1981). This is 3 to 5 times as much as the activity observed in the bloodstream trypomastigotes, and is probably due to the fact that

Figure 16. The Glyoxylate CycleTable 2. (Opperdoes et al., 1977)A comparison of selected enzymes in T. brucei and other organisms

Enzyme	Specific activity (nmole/min/mg protein)		
	<u>T. brucei</u>		
	Cell-free extract (139KS)	Large granule fraction	Other organisms
Isocitrate lyase	0.2		23 <sup>a</sup>
malate synthase		1	100 <sup>b</sup>
NAD <sup>+</sup> -linked ICD		0.1	2.1 <sup>c</sup> , 19 <sup>d</sup>

- a) Tetrahymena pyriformis cell-free extract.  
 b) Yeast cell-free extract.  
 c) Crithidia luciliae cell-free extract  
 d) Rat liver mitochondrion.

- 80 -

these procyclic forms found in the insect vector have a fully functional Krebs cycle.

LSHTM 180 ST has  $2\frac{1}{2}$  times to 5 times as much ICD as the slender form of LSHTM 180 and T. b. rhodesiense (Liv). Like MDH, in the absence of a functional Krebs cycle, ICD may also be involved in generating anabolic precursors since it catalyses the oxidation of isocitrate to  $\alpha$ -ketoglutarate which serves as a precursor of amino acids in many transaminations. Most microorganisms and tissues of higher animals contain 2 types of ICD, one type uses NAD<sup>+</sup> as electron acceptor and the other NADP<sup>+</sup>. Both NAD<sup>+</sup>-linked and NADP<sup>+</sup>-linked ICDs occur in the mitochondrion of animal tissues, but the former is found only in the mitochondrion, whereas the latter is found both in the mitochondrion and cytosol. The ICD activity determined in this study located mainly in the cytosol was NADP-linked and was found to be 20 - 100 times the value given for T. brucei bloodstream trypomastigotes by Opperdoes et al., (1977).

G6PD catalyzes the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconate (6PG) in the phosphogluconate pathway. One of the functions of this pathway is to convert hexoses into pentoses, particularly D-ribose 5-phosphate, required in the synthesis of nucleic acids. In animal cells the reactions of the phosphogluconate pathway take place in the cytosol. Most of the G6PD

- 81 -

activity in the stumpy form of LSHTM 180 and T. b. rhodesiense (Liv) appears to be located in the particulate fractions, 14.5KP and 139KP. In the slender form of LSHTM 180, the G6PD activity is fairly evenly divided between the particulate fractions and the cytosol. More work needs to be done to determine if this pathway is fully functional, and its importance to the trypanosomes.

In the cells of higher animals, PGM catalyzes the conversion of glucose-1-phosphate (G-1-P) the end product of glycogen and starch phosphorylase reactions, to G-6-P, which can then enter the glycolytic sequence. Since bloodstream trypomastigotes are not known to contain any endogenous carbohydrate stores (e.g. glycogen), the function of PGM in these parasites is obscure. If it is functional in vivo, then it could be used to convert G-1-P absorbed from the host blood to G-6-P. PGM is located in the cytosol of all the trypomastigotes, its activity in the stumpy form of LSHTM 180 is 2 to 3 times higher than in the other two trypomastigotes. Mannose-6-phosphate, M-6-P, like G-1-P can enter the glycolytic sequence after conversion into one of the glycolytic intermediates. It is converted to fructose-6-phosphate (F-6-P) by mannose phosphate isomerase (MPI). The presence of enzymes like PGM and MPI suggests that these bloodstream trypomastigotes can use monosaccharides

- 82 -

other than glucose as a source of energy.

The subcellular fractionation scheme shown in Figure 4B provided the material for ACP analysis. The scheme is that described by Venkatesan *et al.*, (1977), who located ACP activity in the 42KP and the 105KP pellets which they termed 'heavy lysosomal' and 'light lysosomal' respectively; they also studied the *in vivo* localization of ACP in slender and stumpy trypomastigotes. Cytochemical evidence shows that ACP in the slender forms is localized in the flagellar pocket and in discrete particles around it, conforming to the suggestion of Jadin and Creemers (1970) and Langreth and Balber (1975) that it is transported into the flagellar pocket for purposes of digestion. In the stumpy trypomastigotes Venkatesan *et al.*, (1977) found ACP activity in the Golgi zone and rough endoplasmic reticulum (RER), and suggest that this is due to a different mechanism of intracellular digestion in the stumpy forms. Steiger *et al.*, (1980) have also reported the localization of ACP in both the flagellar pocket and endoplasmic reticulum of a pleomorphic *T. brucei* strain. Without supporting evidence from enzyme cytology, the only conclusions that can be drawn from ACP analysis in this study are:

1. In all three trypomastigotes, most of ACP activity is localized in the 'heavy lysosomal' (42KP) and 'light

lysosomal' (105KP) fractions, as observed by Venkatesan et al., (1977).

2. The slender form of LSHTM 180 and the monomorphic T. b. rhodesiense (Liv) have similar ACP distribution which is different from that of the stumpy LSHTM 180. This is further evidence of the resemblance between the slender and monomorphic trypomastigotes.

Venkatesan et al., (1977) observed that the change in ACP localization from the flagellar pocket in the slender trypomastigotes to the RER and Golgi complex in the stumpy form was accompanied by an increase in the uptake of lipids as well as a 3-4 fold increase in ACP activity in the stumpy forms. From these observations, they suggested that the increased lipid absorption triggered off an increase in ACP activity in the RER and Golgi complex, leading to the autolysis and removal of the stumpy forms from the blood (Figure 6, remission at 144 hours). The results obtained from ACP activity studies (Figure 11) do not support his hypothesis. While the stumpy form of LSHTM 180 shows a higher ACP activity than the slender, the difference is not nearly as large as observed by Venkatesan et al., (1977); moreover the cells of the monomorphic T. b. rhodesiense (Liv) which show the same level of ACP activity as the stumpy are probably too young to have started autolysis at 72 hours. But since the process of cell autolysis would not depend

- 84 -

solely on ACP activity, no firm conclusions can be made until a thorough study of more hydrolytic enzymes is made.

#### 4.5 Electron Microscopy

##### 4.5.1 Choroid Plexus

Trypanosomes in the brucei group are mainly intravascular, but in the latter stages of a pleomorphic infection in rats and mice, they have been seen to be outside the blood vessels and to accumulate in the perivascular spaces in the choroid plexus (Van Marck et al., 1981). The choroid plexus is of special interest both because large numbers of trypomastigotes accumulate in its perivascular spaces and because it forms part of the blood-brain barrier.

Once in the perivascular spaces, the trypomastigotes can enter the ependymal cells lining the choroid plexus. Until recently, these trypanosomes were believed to be entirely extra-cellular, but Abolarin et al., (1982) have shown with plate 27.1 that intracellular forms do occur, albeit infrequently. Blood-stream trypomastigotes divide by binary fission, but plates 27.2 and 27.3 show that the intracellular forms divide by multiple division, and are liberated into the perivascular spaces after the destruction of the host ependymal cell. Once in the host

ependymal cell, these intracellular forms are safe from the trypanocidal action of drugs like suramin which cannot cross the blood-brain barrier, and from the host's immune response. They are therefore thought to be responsible for the tendency to relapse after apparent successful treatment, and spontaneous remissions of African sleeping sickness.

Electron micrographs of the choroid plexus of rats with the monomorphic T. b. rhodesiense (Liv) (Plates 23 - 26) show that unlike the pleomorphic strain these trypomastigotes do not leave the blood vessels. More work needs to be done to establish why these monomorphic trypomastigotes are exclusively intravascular.

1. Is it because the infection does not go on long enough for this to happen?
2. Or is it one of the changes which occurs when a pleomorphic strain becomes monomorphic?

#### 4.5.2 Ultrastructure

The ultrastructural information obtained from electron microscopy shows that some changes occur when a slender trypomastigote becomes stumpy. The mitochondrion of the slender LSHTM 180 has a double membrane, but lacks cristae; but that of the stumpy LSHTM 180 has developed some cristae. The form of the mitochondrion in trypto-

- 86 -

mastigotes of the brucei group varies strikingly from one stage of the life cycle to another. The slender trypomastigotes have few or no cristae, but as they are converted into the stumpy form, more cristae develop. In the trypomastigote forms of the tsetse fly midgut and proventriculus, the mitochondrion has abundant cristae. The epimastigote forms of the salivary glands of the fly maintain this elaborate mitochondrial morphology but in the next stage the trypomastigote metacyclic forms have mitochondria with reduced cristae, rather like the bloodstream forms (Vickerman, 1962, 1966). Bloodstream trypomastigotes metabolize glucose incompletely to pyruvate due, it is generally supposed, to the absence of a functional Krebs cycle in the underdeveloped mitochondrion. This is a rather wasteful use of glucose, so to provide sufficient energy, the trypanosome absorbs massive amounts of glucose from the host's blood, which also supplies the oxygen required to satisfy the demands of the glycerophosphate oxidase system (Fulton and Spooner, 1959; Grant et al., 1961; Danforth, 1967). The limited quantities of glucose and oxygen in the stagnant blood in the fly demand a more efficient use of these resources. To this end the trypomastigotes develop a fully functional mitochondrion, with Krebs cycle enzymes and cytochromes, which is registered by the appearance of cristae.

Vickerman (1965, 1970) has suggested from ultrastructural

- 87 -

and enzyme studies that the stumpy trypomastigote has an advantage in acting as the infective form for the fly because it represents an intermediate stage in the development of the mitochondrion between the 'inactive' mitochondrion of the slender form and the highly active one of the insect form. The results obtained from this study do suggest an increased mitochondrial activity in the stumpy trypomastigote (i.e. increased development of cristae and increased activity of isocitrate dehydrogenase and aconitase). However it has been shown (Mshelbwala [1967] and Elce [unpublished]) that the stumpy trypomastigote is not necessarily the only infective form for the fly. Both workers have demonstrated that the slender trypomastigote can be infective to the tsetse fly. The increased mitochondrial activity of the stumpy LSHTM 180 could also be a pre-adaption to the intracellular form because the same conditions of limited quantities of oxygen and glucose would exist inside the cell as is found in the stagnant blood in the fly.

Plate 17 shows that like the slender form of LSHTM 180, the mitochondrion of T. b. rhodesiense (Liv) have no cristae. T. evansi, a monomorphic trypanosome in the brucei group shows the same characteristic of a non-functional mitochondrion devoid of cristae. The inability of the monomorphic trypomastigote to initiate into culture or infect the tsetse fly could be due to the inactive mitochondrion, however this hypothesis has yet to be adequately investigated.

4.6 C O N C L U S I O N

The two forms of pleomorphic T. b. rhodesiense (LSHTM 180) have been compared to each other and to the monomorphic T. b. rhodesiense Liverpool Normal (Liv).

1. The agar film technique shows that morphologically the stumpy form of LSHTM 180 can be differentiated from the slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv), both of which lack lipid granules; the monomorphic trypomastigote may therefore correspond to the LF form of the pleomorphic strain.

2. Starch gel and cellulose acetate electrophoresis did not show any major differences in the isoenzyme profiles of 8 of the 9 enzymes studied. This suggests that no change has taken place in the genetic information coding for enzymes in the change from slender to stumpy, or from pleomorphic to monomorphic. However, the presence of an aconitase (ACON) band in the stumpy further differentiates it from the slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv).

3. Polypeptide profiles show the presence of a 58,000 dalton polypeptide in extracts of both the slender and monomorphic trypomastigotes which is absent in the

- 89 -

stumpy. The nature of the polypeptide is discussed and it is taken as further evidence of similarity between the two trypomastigotes.

#### 4. Enzyme assay analysis

(i) Like starch gel electrophoresis (SGE) and cellulose acetate electrophoresis, enzyme assays gave little information as to differences between the three trypomastigotes. One interesting result of the enzyme assay analysis was the higher activity of NADP - isocitrate dehydrogenase in the stumpy LSHTM 180 than in the other two trypomastigotes. This suggests an activation of more Krebs cycle enzymes in the stumpy LSHTM 180.

(ii) The distribution of acid phosphatase again suggests a greater similarity between the slender LSHTM 180 and the monomorphic T. b. rhodesiense (Liv).

#### 5. Electron microscopy

##### (i) Ultrastructure

The stumpy LSHTM 180 has more cristae in the mitochondrion than either the slender LSHTM 180 or the monomorphic T. b. rhodesiense (Liv). The presence of aconitase (ACON) and the higher activity of isocitrate dehydrogen-



- 90 -

ase together with the development of more cristae suggest that the stumpy form may be activating Krebs cycle, in preparation for an intracellular stage or for life in the tsetse-fly, as suggested by Vickerman (1965).

(ii) Choroid Plexus

Trypomastigotes of the pleomorphic strain LSHTM 180 leave the blood vessels, enter the perivascular spaces and enter the ependymal cells of the choroid plexus where they divide by multiple-division and can re-enter the blood after destroying the host cell. In contrast, T. b. rhodesiense (Liv) trypomastigotes do not leave the blood vessels during the course of the infection. This may be due to an inability to do so, or lack of time due to the acuteness of the infection.

6. The other results given so far suggest a greater similarity between the monomorphic T. b. rhodesiense (Liv) and the slender form of LSHTM 180. This similarity is probably due to the fact that the monomorphic infection is so acute that there is no time for more than one form (the LF form) to develop, and not to a possible change in the genetic make-up of the monomorphic trypanosome; i.e. monomorphism is probably reversible.

- 91 -

7. The final conclusions of this study are:

(i) There are morphological, ultrastructural and some enzymatic changes when a slender trypomastigote becomes stumpy.

(ii) The monomorphic trypomastigote is equivalent to the long flat (LF) form of the pleomorphic strain studied.

Further work is required to verify the reversibility of monomorphism. This could be done by prolonging the infection by administering small doses of a trypanocidal drug, and determining if stumpy forms develop from a monomorphic strain.

A P P E N D I C E S

- 92 -

APPENDIX ALIST OF MATERIALS

Suppliers: S = Sigma Chemical Company  
 BDH = BDH Chemicals  
 B = Boehringer Mannheim GmbH, Biochemica  
 W = Wessex Biochemicals

<u>MATERIAL</u>	<u>SUPPLIER</u>
Acetic acid (glacial) (AnalaR) - - - - -	BDH
cis-aconitic acid - - - - -	S
Acrylamide - - - - -	BDH
Agar noble - - - - -	Difco (U S A)
L-alanine - - - - -	S
Alanine aminotransferase - - - - -	S
e-Amino-n-caproic acid - - - - -	S
ammonium hydroxide (NH <sub>4</sub> OH) - - - - -	BDH
Ammonium persulphate - - - - -	S
L-aspartic acid - - - - -	S
ATP (Adenosine triphosphate) - - - - -	S
Barbital - Sodium Barbital - - - - -	Helena Laboratories
Boric acid (H <sub>3</sub> BO <sub>3</sub> ) (AnalaR) - - - - -	BDH
Bovine Serum Albumin (BSA) - - - - -	S
Bromophenol blue - - - - -	S
Cacodylic acid - - - - -	S
Citric acid (AnalaR) - - - - -	BDH
Coomasie brilliant blue - - - - -	BDH
Copper Sulphate (CuSO <sub>4</sub> ) (anhydrous) - - - - -	BDH
N, N <sup>1</sup> - Diallyltartardiamide (DATD) - - - - -	Aldrich Chemical Company
Diethylaminoethyl (DEAE) Cellulose (DE52) - - - - -	Whatman
DL-dithiothreitol - - - - -	S
Ethanolamine - - - - -	BDH
Ethylenediamine tetraacetic acid (EDTA) (AnalaR) - - - - -	BDH

Folin Cioculciu's reagent - - - - -	BDH
Fructose-6-phosphate (diNa salt) - - - - -	S
Fructose, -1, 6-diphosphate (triNa salt) - - - - -	S
Glucose (AnalaR) - - - - -	BDH
Glucose 1-phosphate with 1% glucose-1, 6-diphosphate - - - - -	W
Glucose - 6 - phosphate (mono Na salt) - - - - -	S
Glucose - 6 - phosphate dehydrogenase (Baker's yeast, Type XV) - - - - -	S
Glucose phosphate isomerase - - - - -	S
Glutaraldehyde - - - - -	S
Glycine - - - - -	BDH
Heparin - - - - -	Evans
Hydrochloric acid (HCl) (AnalaR) - - - - -	BDH
Inosine - - - - -	B
DL - Isocitric acid (tri sodium salt) - - - - -	S
Lactate dehydrogenase (hog muscle, 50% glycerol solution) - - - - -	B
Magnesium chloride (MgCl <sub>2</sub> ) (AnalaR) - - - - -	BDH
Malate dehydrogenase (pig heart, 50% glycerol solution) - - - - -	B
Maleic acid - - - - -	BDH
Maleic anhydride - - - - -	BDH
DL - Malic acid - - - - -	S
Mannose - 6 - Phosphate - - - - -	S
Methanol - - - - -	BDH
4-Methylumbelliferyl acetate - - - - -	S
MTT - - - - -	S
NAD (nicotinamide adenine dinucleotide) - - - - -	S
NADH (disodium salt) - - - - -	S
NADP (monosodium salt) - - - - -	S
p-Nitrophenol - - - - -	S
p-nitrophenyl phosphate (dicyclohexyl ammonium salt) - - - - -	S
Osmium tetroxide (OsO <sub>4</sub> ) - - - - -	BDH
Oxaloacetic acid - - - - -	S



- 94 -

2-Oxoglutarate ( $\alpha$ - ketoglutarate)	
(mono K salt) - - - - -	S
Phenazine methosulphate (PMS) - - - - -	S
Phosphoric acid ( $H_3PO_4$ ) (AnalaR) - - - - -	BDH
Phosphotungstic acid - - - - -	BDH
Potassium dihydrogen phosphate ( $KH_2PO_4$ )	
(AnalaR) - - - - -	BDH
Sodium cacodylate - - - - -	S
Sodium chloride (NaCl) (AnalaR) - - - - -	BDH
Sodium carbonate (AnalaR) - - - - -	BDH
Sodium dihydrogen phosphate ( $NaH_2PO_4$ ) - - - - -	BDH
disodium hydrogen phosphate ( $Na_2HPO_4$ ) - - - - -	BDH
Sodium hydroxide - - - - -	BDH
Sodium dodecyl sulphate (SDS) - - - - -	BDH
Sodium potassium tartrate - - - - -	BDH
Starch (hydrolysed potato starch) - - - - -	Connaught
	Laboratories
Sucrose - - - - -	BDH
N, N, N <sup>1</sup> , N <sup>1</sup> - tetramethylethylene-	
diamine (TEMED) - - - - -	S
Trichloroacetic acid (TCA) - - - - -	BDH
Tris (hydroxymethylamine) - - - - -	BDH
Xanthine oxidase - - - - -	S

APPENDIX BBuffers

1. Phosphate-buffered-saline glucose (PSG)  
pH 8.0

$\text{Na}_2\text{HPO}_4$ (anhydrous)	8.094g
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.468g
NaCl	2.550g
Glucose	15.0g

Made up to 1 litre with distilled water.

2. Tris-sucrose-EDTA (TSE) pH 7.8

Tris	3.031g
Sucrose	109.53g
EDTA	0.372g

PH adjusted to 7.8 with 5N HCl and the volume made up to 1 litre with distilled water.

3. 0.3M Tris-HCl pH 7.4

Tris	36.42g
------	--------

Tris was dissolved in 700ml distilled water, the pH adjusted to 7.4 with 5N HCl and the volume made up to 1 litre with distilled water.

4. 0.3M Tris-HCl pH 8.0

Tris	36.42g
1N HCl	160ml

Made up to 1 litre with distilled water.

- 96 -

5. 0.1M Sodium phosphate pH 7.4

$\text{Na}_2\text{HPO}_4$	(anhydrous)	11.499g
$\text{NaH}_2\text{PO}_4$	. $2\text{H}_2\text{O}$	2.964g

Made up to 1 litre with distilled water.

6. 0.2M Sodium cacodylate pH 7.4

42.8g	sodium cacodylate
6.9mls	1M HCl

Made up to 1 litre with distilled water.

7. 3% Glutaraldehyde Solution

Distilled water	20 mls
0.2M Sodium cacodylate	15 mls
25% glutaraldehyde	5 mls
1% Calcium Chloride	0.5 mls

APPENDIX CProtein determination (Lowry et al., 1951)

Solution A: 2.0%  $\text{Na}_2\text{CO}_3$  + 0.02% sodium potassium tartrate

Solution B: 0.5%  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

Solution C: 49 vols A + 1 vol B (made up fresh)

Solution D: Folin and Ciocalteu reagent diluted to 1N

Solution E: 1N NaOH

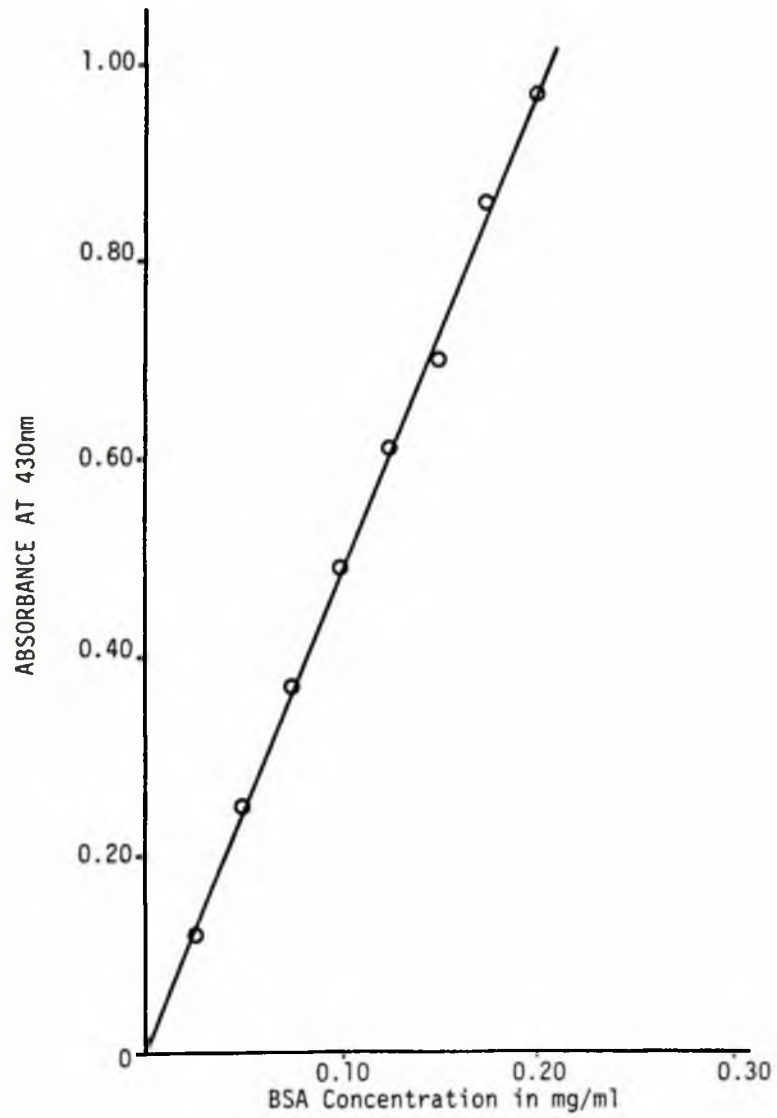
Standard protein solution

5% sterile BSA (Sigma) is diluted 1/100 to give a 0.5mg/ml solution

Procedure

0.1ml of solution E is added to 1ml of protein solution and mixed, followed by 1.0ml of solution C. After mixing, the solution is allowed to stand for 10 minutes. Finally 0.1ml of solution D is added. The absorbance at 660nm is read after 2 hours.

The standard protein curve is shown in Figure 16 overleaf.

Figure 17. Protein Standard Curve

APPENDIX D

Table 3 - Electrophoretic conditions for Starch Gel  
Electrophoresis (SGE)

ENZYME	TANK BUFFER	GEL DILUTION	VOLTAGE IN VOLTS/CM	TIME IN MINS
ACP E.C.3.1.2.3	0.15M Trisodium citrate - 0.24M NaH <sub>2</sub> PO <sub>4</sub> adjusted to pH 6.3 with NaOH	1 : 39	20	180
ACON E.C.4.2.1.3	0.1M Tris - 0.028M citrate pH 7.5	1 : 9	20	120
ES E.C.3.1.1.1	0.1M Tris - 0.1M Maleic anhydride adjusted to pH 7.2 with NaOH	1 : 9	20	180
GPI E.C.5.3.19	0.162M Na <sub>2</sub> HPO <sub>4</sub> - 0.038M NaH <sub>2</sub> PO <sub>4</sub> pH 7.4	3 : 37	20	135
MDH E.C.1.1.1.37	0.1M Na <sub>2</sub> HPO <sub>4</sub> 0.014M citric acid pH 7.0	1 : 4	20	90
MPI E.C.5.3.1.8	0.1M Tris - 0.1M NaH <sub>2</sub> PO <sub>4</sub> pH 7.5	1 : 9	20	150
NH E.C.3.2.2.1	0.04M Tris - 0.44M boric acid pH 6.5	1 : 9	20	180
PGM E.C.2.7.5.1	0.1M Tris - 0.1M Maleic acid - 0.01M EDTA - 0.01M MgCl <sub>2</sub> Adjusted to pH 7.4 with NaOH	1 : 9	13.5	180
ALAT E.C.2.6.1.2	0.15M Tris - 0.0075M citric acid, pH 9.0	1 : 9	40	90

- 100 -

ASAT	0.15M Tris - 0.0075M	1 : 9	40	90
E.C.2.6.1.1	citric acid pH 9.0			
ME	0.122M Na <sub>2</sub> HPO <sub>4</sub> -	3 : 37	16	150
E.C.1.1.1.40	0.38M NaH <sub>2</sub> PO <sub>4</sub> pH 7.0			
ICD (NADP)	0.135 Tris - 0.042M	1 : 9	20	90
E.C.1.1.1.42	citric acid, pH 7.0			
G6PD	0.1M Tris - 0.1M	1 : 9	20	120
E.C.1.1.1.49	Maleic acid 0.01M MgCl <sub>2</sub> - 0.01M EDTA, adjusted to pH 7.4 with NaOH			

## APPENDIX D

ENZYME DEVELOPERS FOR SCE

ENZYME	DISTILLED H <sub>2</sub> O	DEVELOPER BUFFER	ACTIVATORS	COENZYMES	LINKING ENZYMES	SUBSTRATES	VISUALIZATION METHOD
ACON E.C.4.2.1.3	4.5ml	B 4.5ml	MgCl (1.0M) 2.0ml	NADP (10mg/ml) 0.3ml	E.C.1.1.1.42 (ICD) (S-type IV) 27ul	Cis-aconitic acid (0.2M) in 0.4M Tris - HCl pH 8.0, pH readjusted to 8.0	PMS 1.0ml MTT 2.0ml Agar 20.0ml
ACP E.C.3.1.2.1	-	F 10.0ml	-	-	-	4 methylumbelliferyl phosphate 20mg	UV, filter paper
ALAT E.C.2.6.1.2	-	D 5.0ml	-	NADH (10mg/ml) 0.3ml	E.C.1.1.1.27 (LDR) (B - hog muscle in 50% glycerol - 5,500 u/ml 15 ul	2-oxoglutarate (100mg/ml) 0.10ml, L-leucine (120mg/ml buffer)	UV, filter paper
ASAT E.C.2.6.1.1	-	B 5.0ml	-	NADH (10mg/ml) 0.3ml	E.C.1.1.1.37 (RDR) (B - pig heart in 50% glycerol - 6,000 u/ml 10 ul	2-oxoglutarate (100mg/ml) 0.10ml L-aspartic acid 4mg/ml buffer)	UV, filter paper
ES E.C.3.1.1.1	-	K 10.0ml	-	-	-	4 methylumbelliferyl acetate 20mg	UV, filter paper
GPI E.C.5.3.1.9	2.9ml	B 11.3ml	MgCl <sub>2</sub> (1.0M) 0.01ml	NADH (10mg/ml) 0.30ml	E.C.1.1.1.49 G6PD (S-type IV) 100u/ml 0.25ml	Fructose - 6-phosphate (10mg/ml) 1.20ml	PMS 1.0ml MTT 2.0ml Agar 20.0ml

Table 4. continued

ENZYME	DISTILLER H <sub>2</sub> O	DEVELOPER BUFFER	ACTIVATORS	COENZYMES	LINKING ENZYMES	SUBSTRATES	VISUALIZATION METHOD
MDH	2.00ml	C	-	NAD (10mg/ml) 2.0ml	-	DL-Malic acid (1.0N neutral- lead with NaOH) 1.00ml	PHS 1.0ml MTT 2.0ml Agar 20.0ml
E.C. 3.3.1.9							
MP1	-	A	MgCl <sub>2</sub>	NADP	E.C. 5.1.1.9	Mannose - $\alpha$ phosphate (10mg/ml)	PHS 1.0ml
E.C. 5.1.1.8							
	13.3ml		(1.0M) 0.25ml	(10mg/ml) 0.4ml	(GPI) (S-type) IV) 30 $\mu$ l		MTT 2.0ml Agar 20.0ml
					E.C. 1.1.1.49 (G6PD) 100u/ml		
					100ml		
SE	3.20ml	A	-	NADP	-	DL-Malic acid (1.0N neutral- lead with NaOH) 0.12ml	PHS 1.0ml MTT 2.0ml Agar 20.0ml
E.C. 1.1.1.01							
	13.4ml			(10mg/ml) 2.0ml			
LD	1.80ml	A	MgCl <sub>2</sub>	NADP	-	DL-sodium isocitrate (tri- sodium salt, 100mg/ml) 0.40ml	PHS 1.0ml MTT 2.0ml Agar 20.0ml
E.C. 1.1.1.42							
	13.4ml		(1.0M) 0.40ml	(10mg/ml) 2.0ml			
PKR	2.10ml	A	MgCl <sub>2</sub>	NADP	E.C. 1.1.1.49	Glucose-1-phosphate + Glucose-1, $\beta$ -diphosphate (2mg/ml) 2.00ml	PHS 1.0ml MTT 2.0ml Agar 20.0ml
E.C. 2.7.3.1							
	13.3ml		(1.0M) 0.40ml	(10mg/ml) 2.0ml	(G6PD) (S-type) IV) 100u/ml 0.25ml		
NE	3.40ml	A	-	-	E.C. 1.2.3.2 (Naniline ox- idase (5 grade 1) 20ul	lactose (10mg/ml) 1.0ml	PHS 1.0ml MTT 2.0ml Agar 20.0ml
E.C. 3.1.2.1							
	13.2ml						

1. (B) from Boehringer Mannheim Biochemicals, (S) from Sigma Chemical Co Ltd  
2. 1.22 Agar  
3. 2mg/ml PMS, 5mg/ml MTT

- 103 -

APPENDIX D

Developer Buffer

- A. 0.3M Tris - HCl pH 7.4
- B. 0.3M Tris - HCl pH 8.0
- C. 0.3M Tris - HCl pH 8.5
- D. 0.1M Sodium phosphate pH 7.4
- E. 0.1M Sodium phosphate pH 6.5
- F. 0.1M Citric acid - NaOH pH 4.5

APPENDIX ETable 5. Electrophoretic Conditions for Cellulose Acetate

ENZYME	1 BUFFER	VOLTAGE (VOLTS)	TIME (MINS)
ALAT E.C.2.6.1.2	0.66M Tris - 0.08M citric acid, pH 8.6, dil. 1 : 4	200	25
ASAT E.C.2.6.1.1	Tris - barbital - sodium bartibal <sup>2</sup> pH 9.0	300	25
G6PD E.C.1.1.1.49	0.05M Tris - 0.05 M maleic acid - 0.005M EDTA - 0.005M MgCl <sub>2</sub> pH 8.6, dil. 1 : 1	130	55
ICD E.C.1.1.1.42	0.006M NaH <sub>2</sub> PO <sub>4</sub> - 0.0144M Na <sub>2</sub> HPO <sub>4</sub> pH 8.0	200	50
ME E.C.1.1.1.40	0.006M NaH <sub>2</sub> PO <sub>4</sub> - 0.0144M Na <sub>2</sub> HPO <sub>4</sub> pH 8.0	200	50

1. All buffers contained 10% sucrose.
2. Prepared from pre-packaged sachet Cat. No. 5805 supplied by Helena Laboratories (Beaumont, Texas, USA).

## APPENDIX E

Table E. ENZYME DEVELOPERS FOR CELLULOSE ACETATE ELECTROPHORESIS

ENZYME	DEVELOPER BUFFER	1.0N $MgCl_2$	COEXISTERS	LINKING REAGENTS	SUBSTRATES	VISUALIZATION METHOD
ALAT	0.019N $Na_2PO_4$  0.041N $Na_2HPO_4$ pH 7.4 with 50% sucrose 1.605M	-	SDS (10mg/ml) 0.15ml	LM, 5200 w/ml in 50% glycerol 0.013ml	Alanine (100mg/ml in developer buffer) 0.25ml, 2-oxoglutarate (100mg/ml) 0.015ml	UV, filter paper
ASAT	As for ALAT	-	SDS (10mg/ml) 0.15ml	SDS, 8000 w/ml in 50% glycerol 0.015ml	Aspartic acid (5mg/ml in developer buffer) 1.05ml 2-oxoglutarate (100mg/ml) 0.03ml	UV, filter paper
SAPP	0.38 Tris - HCl pH 7.4	0.25ml	SDS (10mg/ml) 0.50ml	-	D-glucose-6-phosphate (10mg/ml) 0.50ml	PHS 0.5ml MTT 1.0ml Agar 10.0ml
ME	0.38 Tris - HCl pH 7.4 8-87ml	0.20ml	SDS (10mg/ml) 0.20ml	-	DL - Malic Acid neutralized with NaOH 0.80ml	PHS 0.5ml MTT 1.0ml Agar 10.0ml
ICD	0.38 Tris - HCl pH 7.4	0.25ml	SDS (10mg/ml) 0.50ml	-	DL - Isosuccinic acid (10mg/ml) 0.40ml	PHS 0.5ml MTT 1.0ml Agar 10.0ml

- 106 -

APPENDIX FSDS-PAGE Solutions and Reagents

Solution A	3M Tris - HCl buffer pH 8.5 - 8.9
Tris	36g
1N HCl	48ml

Made up to 100 ml with distilled water.

Solution B	1M Tris - HCl pH 7.0
Tris	12.14g
Water	up to 100 ml

pH adjusted to 7.0 with conc. HCl.

## Solution C

Acrylamide	28g
DATD	0.735g
distilled water	100ml
SDS solution (20%)	20g SDS/100ml water

## Solution D

1M Tris pH 7.0 (soln. B)	19.2ml
20% SDS	0.8ml
TEMED	0.05ml

Ammonium persulphate solution - 0.14g/100ml H<sub>2</sub>O

Both solution D and ammonium persulphate are freshly prepared prior to mixing gels. Other solutions are stable at 4°C.

- 107 -

Gel composition

## (i) 12% separating gel

Solution	Volume (ml)
A	5.00
C	17.20
Ammonium persulphate	10.00
Distilled water	7.60
TEMED	0.05
20% SDS	0.20

## (ii) 3% stacking gel

Solution	Volume (ml)
C	2.55
D	3.00
Ammonium persulphate	12.00
Distilled water	6.45

Persulphate and TEMED were added immediately prior to degassing to prevent gel setting before or during pouring.

## Electrode buffer : Tris - glycine pH 8.3

Tris	6g
Glycine	288g
20% SDS	10.0ml
Distilled water	up to 2 litres.

## Sample dissolving buffer (SDB)

0.05M Tris - HCl pH 7.0	500ul
Bromophenol blue	200ul
20% SDS	200ul
DTT	0.15g
Sucrose	a few grains.

APPENDIX G  
STOCK ENZYME ASSAY MIXTURES

Table 7.

ENZYME	DISTILLED H <sub>2</sub> O	BUFFER	ACTIVATORS	COENZYMES	LINKING ENZYMES	SUBSTRATES	FINAL VOLUME (ml)	NOTES
ALAT E.C.2.6.1.2	1.40ml	-	-	0.14ml 10mg/ml NADH (0.14mg/ml)	0.02ml 5,200U/ml LDH (11-31U/ml)	8.0ml 8.9% alanine in 0.1M phosphate buffer, pH 7.4 (0.07mg/ml) 0.035ml - KG 100mg/ml - KG (0.37mg/ml)	9.36	0.855ml of assay mixture was incubated with 0.01ml of sample for 15 minutes at 37°C. Then 0.035ml of 100mg/ml - KG was added.
G6PD E.C.1.1.1.49	5.20ml	3.30ml 0.3M Tris HCl pH 7.4	0.10ml 1M MgCl <sub>2</sub> (10mM)	1.00ml 10mg/ml NADP (1.01mg/ml)	-	0.34ml 10mg/ml G6P (0.34mg/ml)	9.94	
GPE E.C.5.3.1.9	4.40ml	3.30ml 0.3M Tris HCl pH 8.0	0.13ml 1M MgCl <sub>2</sub> (15mM)	1.00ml 10mg/ml NADP (1.01mg/ml)	0.10ml 100U/ml G6PD (1.00U/ml)	1.00ml 10mg/ml P6P (1.01mg/ml)	9.93	
HK E.C.2.7.1.1	5.00ml	1.60ml 0.3M Tris HCl pH 7.4	0.08ml 1.0M MgCl <sub>2</sub> (8.0mM)	0.60ml 10mg/ml NADP (0.60mg/ml)	0.03ml 100U/ml G6PD (0.30U/ml)	1.00ml 100mM glucose (10.00mM) 1.5ml 10mM ATP (1.5mM)	10.01	

Table 7. continued

ENZYME	DISTILLED H <sub>2</sub> O	BUFFER	ACTIVATORS	COENZYMES	LINKING ENZYMES	SUBSTRATES	FINAL VOLUME (ml)	NOTES
ICD	6.00ml	3.35ml 0.3M Tris HCl pH 8.0	0.10ml 1M MgCl <sub>2</sub> (1.0mM)	0.50ml 10mg/ml NADP (0.50mg/ml)	-	0.05ml 100mg/ml DL-isocitrate (0.5mg/ml)	10.00	
MDH	6.40ml	3.30ml 0.3M Tris HCl pH 8.0	-	0.13ml 10mg/ml NADH (0.13mg/ml)	-	0.13ml 10mg/ml oxaloacetate (0.13mg/ml)	9.96	Oxaloacetate was made up with 0.5ml 1M HCl and 0.5ml H <sub>2</sub> O
ME	5.30ml	3.30ml 0.3M Tris HCl pH 7.4	0.15ml 1M MgCl <sub>2</sub> (1.5mM)	1.00ml 10mg/ml NADP (1mg/ml)	-	0.25ml malic acid 1M pH 7.0 (2.5mM)	10.00	Malic acid was neutralized with 10N NaOH
MPI	5.00ml	3.30ml 0.3M Tris HCl pH 7.4	0.10ml 1M MgCl <sub>2</sub> (1.0mM)	0.50ml 10mg/ml NADP (0.50mg/ml)	0.03ml 1000U/ml GPI (3.0U/ml) 0.10ml 100U/ml G6PD (1.00U/ml)	1.00ml 10mg/ml 10mg/ml MGP (mg/ml)	10.03	

Table 7. continued

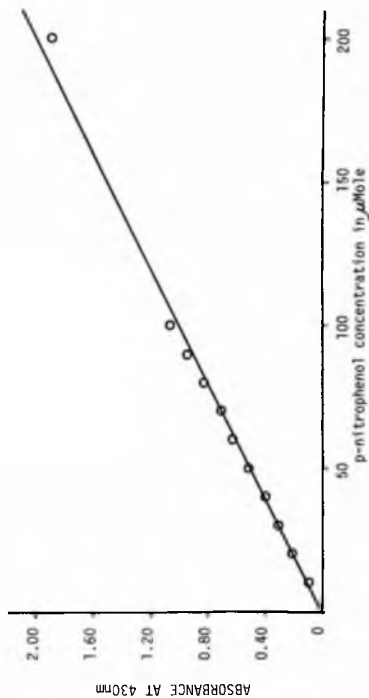
ENTHSE	DISTILLED H <sub>2</sub> O	BUFFER	ACTIVATORS	COENZYMES	LINKING ENZYMES	SUBSTRATES	FINAL VOLUME (ml)	NOTES
PGM K.C.2.7.5.1	1.25ml	7.50ml 0.1M Tri- ethanol- amine-HCl pH 7.4	0.015ml 1M MgCl <sub>2</sub> (15mM) 0.30ml 10mg/ml KOTA (0.3mg/ml)	0.15ml 10mg/ml NADP (0.15mg/ml)	0.03ml 1000ul G6PD (0.30U/ml)	0.75ml 20mg/ml GTP with 1% G-1, 6-ADP (1.5mg/ml)	10.00	

\* 0.99ml of all stock assay mixtures (except ALAT) was used with 0.01ml of trypanosome extract.  
The final concentration of the different reagents in the stock assay mixture are given in brackets.

Acid Phosphate AssayReagentsSubstrate Mixture

- 5% Trichloroacetic acid
  - 2M Mg<sub>3</sub>OH pH 13.5
  - 0.1M acetate buffer pH 5.0
  - p-nitrophenol standard solution
- p-nitrophenyl phosphate  
(di-cyclohexylammonium salt) 104.0mg  
acetate buffer pH 5.0 10.0ml

- 111 -

APPENDIX HFigure 18. p-Nitrophenol Standard Curve

R E F E R E N C E S

- ABOLARIN, M. O., EVANS, D. A., TOVEY, D. G. and ORMEROD, W. E. (1982)  
Cryptic stage of sleeping-sickness trypanosome developing in choroid plexus epithelial cells. British Medical Journal 1982; 285: 1380-1382.
- AL-TAQI, M. and EVANS, D. A. (1978)  
Characterization of Leishmania spp. from Kuwait by isoenzyme electrophoresis. Transactions of the Royal Society of Tropical Medicine and Hygiene, 72: 56-65.
- APTED, F. I. C., ORMEROD, W. E., SMYLY, D. P., STRONACH, B. W. and SZLAMP, E. L. (1963)  
A comparative study of the epidemiology of endemic Rhodesian sleeping sickness in different parts of Africa. Journal of Tropical Medicine and Hygiene, 61, 1.
- BOWMAN, I. B. R. and FLYNN, I. W. (1976)  
Oxidative metabolism of trypanosomes. In: Biology of the Kinetoplastida (Lumsden, W. H. R. and Evans, D. A., eds.), Vol. 1: 435-476, Academic Press, New York.
- von BRAND, T. (1951)  
Metabolism of Trypanosomidae and Bodonidae. In: Biochemistry and Physiology of Protozoa (A. Lwoff, ed.), Vol. 1: 177-234, Academic Press, New York / London.
- BROHN, F. H. and CLARKSON, A. B. J. (1978)  
Quantitative effects of salicylhydroxamic acid and glycerol on Trypanosoma brucei in vitro and in vivo. Acta Tropica, 35: 23-33.
- BRUCE, D., HARVEY, D., HAMERTON, A. E., DAVY, J. B. and BRUCE, M. E. (1912)  
The morphology of the trypanosome causing disease in man in Nyasaland. Proceedings of the Royal Society, Series B, 85: 423-433.
- CROSS, G. A. M. (1975)  
Identification, purification and properties of variant-specific glycoprotein antigens constituting the surface coat of Trypanosoma brucei. Parasitology, (England), 71: 393-417.
- CROSS, G. A. M. (1978)  
Antigenic variation in trypanosomes. Proceedings of the Royal Society of London, B 202: 55-72.
- DANFORTH, W. F. (1967)  
Respiratory metabolism. In: Research in Protozoology, Vol. 1 (Chen, T. T. ed.): 201.
- DIXON, H. (1966)  
Blood platelets as a source of enzyme activity in washed trypanosome suspensions. Nature, London, 210, 428.

- DUKE, H. L. (1934)  
On the transmissibility by Glossina of T. brucei, T. rhodesiense and T. gambiense with special reference to old laboratory strains. Parasitology, 26, 153.
- DUKE, H. L. (1935)  
Old 'laboratory' strains of the polymorphic trypanosomes. Lancet, 229, 665.
- FAIRBAIRN, H. and CULWICK, A. T. (1949)  
The differentiation of the polymorphic trypanosomes. Annals of Tropical Medicine and Parasitology, 43: 90-95.
- FRUIT, J., AFCHAIN, D., PETITPREZ, A., VAN MEIRVENNE, N., LE ROY, D., BOUT, O. and CAPRON, A. (1977)  
Antigenic analysis of a variant-specific component of Trypanosoma brucei brucei: localization on the surface coat with labelled specific anti-bodies. Parasitology (England) 74: 185-90.
- FULTON, J. D. and SPOONER, D. F. (1959)  
Terminal respiration in certain mammalian trypanosomes. Experimental Parasitology, 5, 59.
- GIBSON, W. (1979)  
An Epidemiological Study of Trypanosomes of Subgenus Trypanozoon by Isoenzyme Electrophoresis: PhD. Thesis, London School of Hygiene and Tropical Medicine.
- GODFREY, D. G. and KILGOUR, V. (1976)  
Enzyme electrophoresis in characterizing the causative organism of Gambian trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene, 70: 217-24.
- GRANT, P. T. and FULTON, J. D.. (1957)  
The catabolism of glucose by strains of Trypanosoma rhodesiense. Biochemical Journal, 66, 242.
- GRANT, P. T., SARGENT, J. R. and RYLEY, J. F. (1961)  
Respiratory systems in Trypanosomatidae. Biochemical Journal, 81, 200.
- HARRIS, H. and HOPKINSON, D. A. (1976)  
In: Handbook of Enzyme Electrophoresis in Human Genetics. North-Holland, Amsterdam.
- HECKER, H., BURRI, P. H., STEIGER, R. and GEIGY, R. (1972)  
Morphometric data on the ultrastructure of the pleomorphic blood forms of Trypanosoma brucei. Plimmer and Bradford, 1899. Acta Tropica, 29: 182-98.
- HECKER, H. (1980)  
Application of Morphometry to Pathogenic Trypanosomes (Protozoa, Mastigophora). Path. Res. Pract., 166: 203-217.

- JADIN, J. M. and CREEMERS, J. (1970)  
Etude en microscopie électronique de localisation des hydrolases acides chez les Trypanosomatidae. Archives Internationales de Physiologie et Biochimie, 78: 1001-1002.
- LAEMMLI, U. K. (1970)  
Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature, 227: 680-85.
- LANHAM, S. M. and GODFREY, D. G. (1970)  
Isolation of Salivarian Trypanosomes from Man and Other Mammals using DEAE - Cellulose. Experimental Parasitology 28: 521-34.
- LANHAM, S. M., GRENDON, J. M., MILES, M. A., POVOA, M. M. and ALMEIDA de SOUZA, A. A. (1981)  
A comparison of electrophoretic methods for isoenzyme characterization of trypanosomatids. Standard stocks of Trypanosoma cruzi zymodemes from northeast Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene, 75.5: 742-50.
- LANGRETH, S. G. and BALBER, A. E. (1975)  
Protein uptake and digestion in bloodstream and culture forms of Trypanosoma brucei. Journal of Protozoology (U. S. A.) 22, 40.
- LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951)  
Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-75.
- Van MARCK, F. A. E., LE ROY, D., BECKERS, A., JACOB, W., WERY, M. and GIGASE, P. L. J. (1981)  
Light and electron microscope studies on extravascular Trypanosoma brucei gambiense in the brain of chronically infected rodents. Ann. Soc. belge méd. trop., 61: 57-78.
- MILES, M. A., TOYE, P. J., OSWALD, S. C. and GODFREY, D. G. (1977)  
The identification by isoenzyme patterns of two distinct strain-groups of Trypanosoma cruzi circulating independently in a rural area of Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene, 71: 217-25.
- MILES, M. A., LANHAM, S. M., DE SOUZA, A. A. and POVOA, M. (1980)  
Further enzymic characters of Trypanosoma cruzi and their evaluation for strain identification. Transactions of the Royal Society of Tropical Medicine and Hygiene, 74, 2: 221-36.
- MSHELBWALA, A. S. (1967)  
Infectivity of Trypanosoma rhodesiense to tsetse flies fed through animal membranes. Nature, London, 215, 441.

- ODURO, K. K. (1977)  
Trypanosoma brucei: subcellular distribution and organisation of the enzymes of glycolysis. PhD. Thesis, University of Edinburgh.
- ODURO, K. K., BOWMAN, I. B. R. and FLYNN, I. W. (1980)  
Trypanosoma brucei: Preparation and some Properties of a Multienzyme Complex Catalysing Part of the Glycolytic Pathway. Experimental Parasitology, 50: 240-50.
- OPPERDOES, F. R., AARSEN, P. N., van der MEER, C. and BORST, P. (1976)  
Trypanosoma brucei: an evaluation of salicylhydroxamic acid (SHAM) as a trypanocidal drug. Experimental Parasitology 40: 198-205.
- OPPERDOES, F. R. and BORST, P. (1977)  
Localization of nine glycolytic enzymes in a microbody-like organelle in Trypanosoma brucei: the glycosome. FEBS Letters, 80: 360-64.
- OPPERDOES, F. R., BORST, P. and SPITS, H. (1977)  
Particle-Bound Enzymes in the Bloodstream Form of Trypanosoma brucei. European Journal of Biochemistry, 76: 21-28.
- OPPERDOES, F. R., MARKUS, A. and STEIGER, R. F. (1981)  
Localization of malate dehydrogenase, adenylate kinase and glycolytic enzymes in glycosomes and the threonine pathway in mitochondrion of cultured procyclic trypomastigotes of Trypanosoma brucei. Molecular and Biochemical Parasitology, 4: 291-309.
- ORMEROD, W. E. (1958)  
A comparative study of cytoplasmic inclusions (valutin granules) in different species of trypanosomes. Journal of General Microbiology, 19: 271-288.
- ORMEROD, W. E. (1963)  
A comparative study of growth and morphology of strains of Trypanosoma rhodesiense. Experimental Parasitology, 13: 374-85.
- ORMEROD, W. E., HEALEY, P. and ARMITAGE, P. (1963)  
A method of counting trypanosomes allowing simultaneous study of their morphology. Experimental Parasitology, 13: 386-94.
- ORMEROD, W. E. and PAGE, M. J. (1967)  
Lipid staining of type II granules in trypanosomes. Transactions of the Royal Society of Tropical Medicine and Hygiene, 61, 12.
- ORMEROD, W. E. and VENKATESAN, S. (1971)  
The occult visceral phase of mammalian trypanosomes with special reference to the life cycle of Trypanosoma (Trypanozoon) brucei. Transactions of the Royal Society of Tropical Medicine and Hygiene, 65: 722-35.

- REEVES, R. E. and BISCHOFF, J. M. (1968)  
Classification of Entamoeba species by means of electrophoretic properties of amoebal enzymes. Journal of Parasitology, 54: 594-600.
- RYLEY J. F. (1956)  
Studies on the metabolism of the Protozoa: Comparative carbohydrate metabolism of eleven species of trypanosome. Biochemical Journal, 62: 215-22.
- SARGEAUNT, P. G., WILLIAMS, J. E. and GREENE, J. D. (1978)  
The differentiation of invasive and non-invasive Entamoeba histolytica by isoenzyme electrophoresis. Transactions of the Royal Society of Tropical Medicine and Hygiene, 72: 519-21.
- SHAW, C. R. (1969)  
Isoenzymes: Classification, frequency and significance. International Review of Cytology, 25: 297-332.
- SMITH, I. (Ed.) (1976)  
Chromatographic and electrophoretic techniques Vol. 2: Zone electrophoresis (4th edition). London: Heinemann.
- SPURR, A. J. (1969)  
A low viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure 26: 31-43.
- STEIGER, R. F. (1975)  
Ultrastructure of the surface coat/pellicle complex of Trypanosoma brucei. Acta Tropica (Switzerland), 32: 152-58.
- STUDIER, F. W. (1973)  
Analysis of Bacteriophage T7. Early RNAs and proteins on Slab Gels. Journal of Molecular Biology, 79: 237-48.
- SWINDLEHURST, C. A., SHAH, H. N., PARR, C. W. and WILLIAMS, R. A. D. (1977)  
Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis of Polypeptides from Bacteroides melaninogenicus. Journal of Applied Bacteriology, 43: 319-324.
- TAYLOR, A. E. R. and WILLIAMS, J. E. (1977)  
The Possibility of Characterizing South American Trypanosomes with Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis. Proceedings of the British Society of Parasitology 75, xxiii.
- TAYLOR, A. E. R., EDWARDS, Y. H., SMITH, V. BAKER, J. R., WOO, P. T. K., LANHAM, S. M., and PENNICK, N. C. (1982)  
Trypanosoma (Schizotrypanum) species from insectivorous bats (Microchiroptera): Characterization by polypeptide profiles. Systematic Parasitology 4: 155-68.

- TAYLOR, A. E. R., EDWARDS, Y. H., SMITH, V., MILES, M. A. and GIBSON, W. C. (1983)  
The polypeptide profiles of strains of the Trypanosoma subgenera Schizotrypanum and Trypanozoon: peptideme characterization. Transactions of the Royal Society of Tropical Medicine and Hygiene, Vol. 77, 3: 354-62.
- TIZARD, I. R., NIELSEN, K., MELLORS, A. and ASSOKU, R. K. (1977)  
Free fatty acids and pathogenesis of African trypanosomiasis. Lancet (England) 1, 2 April: 750-51.
- VENKATESAN, S., BIRD, R. G. and ORMEROD, W. E. (1977)  
Intracellular Enzymes and Their Localization in Slender and Stumpy Forms of Trypanosoma brucei rhodesiense. International Journal of Parasitology, 7: 139-47.
- VICKERMAN, K. (1962)  
The mechanism of cyclical development in trypanosomes of the Trypanosoma brucei sub-group: an hypothesis based on ultrastructural observations. Transactions of the Royal Society of Tropical Medicine and Hygiene, 56: 487.
- VICKERMAN, K. (1965)  
Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. Nature, (London) 208: 762.
- VICKERMAN, K. (1966)  
Genetic systems in unicellular animals. Science Progress, Oxford, 54: 13.
- VICKERMAN, K. (1969)  
On the surface coat and flagellar adhesion in trypanosomes. Journal of Cellular Science (England) 5: 163-93.
- VICKERMAN K. (1978)  
Antigenic variation in trypanosomes. Nature (London) 273: 613-617.
- VISSER, N. and OPPERDOES, F. R. (1980)  
Glycolysis in Trypanosoma brucei. European Journal of Biochemistry 103: 623-632.
- VISSER, N., OPPERDOES, F. R. and BORST, P. (1981)  
Subcellular compartmentation of glycolytic intermediates in Trypanosoma brucei. European Journal of Biochemistry.
- WIJERS, D. J. B. (1959)  
Polymorphism in Trypanosoma gambiense and Trypanosoma rhodesiense, and the significance of intermediate forms. Annals of Tropical Medicine and Parasitology, 53: 59-69.
- WORLD HEALTH ORGANIZATION (W. H. O.) - (De Raat, P. and Marinkelle, C. J.) (1976)  
Trypanosomiasis I African - Trypanosomiasis II Chagas disease. Special Programme for Research and Training in Tropical Diseases TDS/WP/76-12.

YORKE, W., ADAMS, A. R. D. and MURGATROYD, F. (1929)  
Studies in chemotherapy I: A method for  
maintaining pathogenic trypanosomes alive  
in vitro at 37° C for 24 hours. Annals of  
Tropical Medicine and Parasitology, 23:  
501-18.