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

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ABSTRACT

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
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.
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CHAPTER ONE

INTRODUCTION

1.1 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-
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), *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*,
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 trypanastigotes to be seen in the blood are long, thin, cylindrical in cross-section and agranular with a free flagellum, known as 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 trypanastigotes 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
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 intra-peritoneal 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 tryponastigotes in
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.
Figure 1. AEROBIC GLYCOLYSIS

Glucose

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-α- glycerophosphate dehydrogenase,
12 L-α- Glycerophosphate oxidase.
Figure 2. ANAEROBIC GLYCOLYSIS

Glucose
1
2
3
F-1, 6-diP
4
Gly-3-P
5
DHAP
6
NAD+
NADH
7
L-α-glycerophosphate
8
9
10
Pyruvate

11

13 - Glycerokinase
1 - 11 are as for Figure 1
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-phosphoglyceric 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.
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; Sargeaunt et al., 1978); Leishmania (Al-Taqi and Evans, 1978); Trypanosoma (Godfrey and Kilgour, 1976; Miles et al.,

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
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) – polycrylamide 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 (Micro-
chiroptera), (Taylor et al., 1982), and subgenus
Trypanozoon (Taylor et al., 1983), as well as to differentiae 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 polycrylamide 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-
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 vesicles, 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.
LABORATORY MATERIALS AND METHODS

2.1 Materials

2.1.1 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% $\text{H}_3\text{PO}_4$. 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°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).
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
Figure 3.  

*T. b. rhodesiense* LSHTM 180F

Man (Dumelo): Mababe, Ngamiland, Botswana

<table>
<thead>
<tr>
<th>Date</th>
<th>Event</th>
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<tbody>
<tr>
<td>6 March 1960</td>
<td>Rat</td>
</tr>
<tr>
<td>26 Oct. 1968</td>
<td>LSHTM 180A</td>
</tr>
<tr>
<td>18 April 1975</td>
<td>LSHTM 180B B1A</td>
</tr>
<tr>
<td>14 May 1976</td>
<td>Mouse</td>
</tr>
<tr>
<td>6 March 1978</td>
<td>LSHTM 180E</td>
</tr>
<tr>
<td>7 March 1978</td>
<td>Mouse</td>
</tr>
<tr>
<td>15 March 1978</td>
<td>LSHTM 180F</td>
</tr>
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- : Syringe Passage
- : Frozen in liquid nitrogen
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.
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-Elvejham 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.
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. 1 KP being the pellet from 1000g centrifugation.
3. TSE is the Tris-Sucrose - EDTA buffer pH 7.8.
Figure 4B.

Subcellular fractionation of trypanosome homogenate

Trypanosome homogenate

\[ \text{1000g, 10 minutes} \times 2 \]

\[ \text{discard 1 KP (pellet) 1 KS (supernatant)} \]

\[ \text{3,400g, 10 minutes} \times 2 \]

\[ 3.4 \text{ KS (supernatant)} \]

\[ 3.4 \text{ KP (pellet)} \]

\[ \text{42,250g, 10 minutes} \times 2 \]

\[ \text{Suspended in TSE and beaded} \]

\[ \text{(pellet) 42 KP} \]

\[ \text{Suspended in TSE and beaded} \]

\[ \text{(supernatant) 105 KS} \]

\[ \text{beaded} \]

\[ \text{105,000g, 1 hour} \]

\[ 105 \text{ KP (pellet)} \]

\[ \text{Suspended in TSE and beaded.} \]

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: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$^3$ were made with 11.2% solution of hydrolyzed potato starch in the appropriate buffer (Table 3, Appendix D). 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
Diagram of an electrophoresis tank

- Figure 5.

- a: bridge buffer
- b: electrode buffer
- c: cooling plate
- d: wick
- e: Melinex sheet
- f: gel plate
- g: cover plate
- h: cover plate
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
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
(0.025% Coomassie blue in 40.5ml methanol: 52 ml H₂O: 0.7 ml glacial acetic acid and destained over a 24-hour period in a solution consisting of 405 ml ethanol, 250 ml H₂O 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 oxidation 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 phosphatase 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-nitrophenyl 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
NH₄OH (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 × 10³ cm²/mole.

Specific activity =

\[ \frac{V}{E \times d \times v \times c \times (protein \times Dt)} \times \Delta E \]

where

- V = total assay volume = 1.00 ml
- E = extinction coefficient of NADH and NADPH at 340 nm = 6.22 × 10³ cm²/mole.
- d = light path = 1 cm
- v = sample volume = 0.01 ml
- 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 (OsO₄) 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.
CHAPTER THREE

RESULTS
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
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 2½ 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 trypanastigotes with less than 10% of the activity in the
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 trypomastigotes 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 phosphoglucomutase (PGM) activity was located in the final supernatant (139KS) in all three trypomastigotes, 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 phosphotase (ACP) activity was determined in sub-
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 Technique

Plate 1

*T. b. rhodesiense* (Liverpool Normal) on agar film. Judging by the eye the sizes of these trypomastigotes 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 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.
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.
Figure 6.

The course of infection of *T. b. rhodesiense* LSHTM 180 in the blood of rats

- Long slender
- Short stumpy

![Graph showing the course of infection of T. b. rhodesiense LSHTM 180 in the blood of rats. The graph plots parasitaemia (No of trypanosomes/field) against hours of infection. There are two peaks: one for long slender forms and one for short stumpy forms.](image-url)
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 Na₂HPO₄ - 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 Na₂HPO₄ - 0.014M NaH₂PO₄, 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.
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 Na₂HPO₄ - 0.038 NaH₂PO₄, pH 7.4; 20 v/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 MgCl₂ pH 7.4; 13.5v/cm; 180 minutes.

Samples 1 - 3 as for Plate 9.
Plate 11

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) - CAE

0.05M Tris - 0.05M maleic acid - 0.005M EDTA - 0.005M MgCl₂ 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

0.65M Tris - 0.063M citric acid pH 8.6; 200v; 25 minutes.

Samples 1 - 6 are as for plate 7.
Polypeptide Profiles from SDS-PAGE

Plate 1A

M* Marker

1. LSHTM 180, LS 1KS
2. LSHTM 180, ST 1KS
3. T. b. rhodesiense (Liv) 1KS
4. LSHTM 180, LS 42KP
5. LSHTM 180, ST 42KP
6. T. b. rhodesiense (Liv) 42KP
7. LSHTM 180, LS 105KP
8. LSHTM 180, ST 105KP
9. T. b. rhodesiense (Liv) 105KP

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

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight in daltons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>68,000</td>
</tr>
<tr>
<td>Carboxy-lyase</td>
<td>58,000</td>
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<tr>
<td>Aldolase</td>
<td>41,000</td>
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<tr>
<td>Myoglobin</td>
<td>17,000</td>
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</table>
Plate 15

M        Marker
1. LSHTM 180, LS                      3.4KP
2. LSHTM 180, ST                      3.4KP
3. **T. b. rhodesiense** (Liv)        3.4KP
4. LSHTM 180, LS                      14.5KP
5. LSHTM 180, ST                      14.5KP
6. **T. b. rhodesiense** (Liv)        14.5KP
7. LSHTM 180, LS                      139KS
8. LSHTM 180, ST                      139KS
9. **T. b. rhodesiense** (Liv)        139KS
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.
<table>
<thead>
<tr>
<th>ENZYME</th>
<th>FRACTION</th>
<th>LSHTM 180 (LS)</th>
<th>LSHTM 180 (ST)</th>
<th>T. b. rhodesiense</th>
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<tr>
<td>Alanine aminotransferase</td>
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<td>E.C.2.6.12; ALAT</td>
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<td>49.0</td>
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<td>Malate dehydrogenase</td>
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<td>Mannose phosphate isomerase</td>
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<td>-</td>
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<td>E.C.2.7.5.1; PGM</td>
<td>139KS</td>
<td>7.0</td>
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</table>

* All enzyme activities are in nmole/min/mg protein, except acid phosphate which is in mole/min/mg protein.
Figure 7. Subcellular distribution of HK

- T. b. rhodesiense, long slender
- T. b. rhodesiense, short stumpy
- T. b. rhodesiense, Liverpool normal

1 = 14.5KP
2 = 139 KP
3 = 139 KS
Figure 8. Subcellular distribution of GPI

- T. b. rhodesiense, long slender
- T. b. rhodesiense, short stumpy
- T. b. rhodesiense, Liverpool normal

1 = 14.5 KP
2 = 139 KP
3 = 139 KS
Figure 9. Subcellular distribution of ICD

- T. b. rhodesiense, long slender
- T. b. rhodesiense, short stumpy
- T. b. rhodesiense, Liverpool normal

1 = 14.5 KP
2 = 139 KP
3 = 139 KS
Figure 10. Subcellular distribution of MDH

- T. b. rhodesiense, long slender
- T. b. rhodesiense, short stumpy
- T. b. rhodesiense, Liverpool normal

1 = 14.5 KP
2 = 139 KP
3 = 139 KS

Specific activity in nmol/min/mg protein
Figure 11. **Subcellular distribution of ALAT**

- **T. b. rhodesiense**, long slender
- **T. b. rhodesiense**, short stumpy
- **T. b. rhodesiense**, Liverpool normal

1 = 14.5 KP
2 = 139 KP
3 = 139 KS

Specific activity in nmole/min/mg protein
Figure 12. Subcellular distribution of G6PD

- T. b. rhodesiense, long slender
- T. b. rhodesiense, short stumpy
- T. b. rhodesiense, Liverpool normal

1 = 14.5 KP  
2 = 139 KP  
3 = 139 KS
Figure 13. Subcellular distribution of PGM

- T. b. rhodesiense, long slender
- T. b. rhodesiense, short stumpy
- T. b. rhodesiense, Liverpool normal

1 = 14.5 KP
2 = 139 KP
3 = 139 KS
Figure 14. Subcellular distribution of MPI

- T. b. rhodesiense, long slender
- T. b. rhodesiense, short stumpy
- T. b. rhodesiense, Liverpool normal

1 = 14.5 KP
2 = 139 KP
3 = 139 KS
Figure 15. Subcellular distribution of Acid Phosphatase

- T. b. rhodesiense, long slender
- T. b. rhodesiense, short stumpy
- T. b. rhodesiense, Liverpool normal

a = 14.5 KP
b = 139 KP
c = 139 KS
3.6 Electron Microscopy

Plate 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 18

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

DISCUSSION AND CONCLUSION
DISCUSSION

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
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-
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
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
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
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
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 interconversion of Krebs cycle intermediates and amino acids.

The distribution of NAD+-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 bloodstream 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 \textit{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 Cycle

Table 2. (Opperdoes et al., 1977)

A comparison of selected enzymes in T. brucei and other organisms

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (n mole/min/mg protein)</th>
<th>T. brucei</th>
<th>Cell-free extract (139KS)</th>
<th>Large granule</th>
<th>Other organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isocitrate lyase</td>
<td></td>
<td>0.2</td>
<td></td>
<td></td>
<td>23^a</td>
</tr>
<tr>
<td>Malate synthase</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>100^b</td>
</tr>
<tr>
<td>NAD+-linked ICD</td>
<td></td>
<td>0.1</td>
<td></td>
<td>2.1^c, 19^d</td>
<td></td>
</tr>
</tbody>
</table>

^a) Tetrahymena pyriformis cell-free extract.
^b) Yeast cell-free extract.
^c) Crithidia luciliae cell-free extract
^d) Rat liver mitochondrion.
these procyclic forms found in the insect vector have a fully functional Krebs cycle.

LSHTM 180 ST has 2½ 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+ as electron accepter and the other NADP+. Both NAD+-linked and NADP+-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
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
other than glucose as a source of energy.

The subcellular fractionation scheme shown in Figure 4B provided the materials 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 typomastigotes, 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
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 tryp-
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
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 CONCLUSION

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
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-
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.
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.
APPENDIX A

LIST OF MATERIALS

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

<table>
<thead>
<tr>
<th>MATERIAL</th>
<th>SUPPLIER</th>
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<tbody>
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<td>Acetic acid (glacial) (AnalaR)</td>
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<tr>
<td>cis-aconitic acid</td>
<td>S</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>BDH</td>
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<td>Alanine aminotransferase</td>
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<tr>
<td>e-Amino-n-caproic acid</td>
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<td>Boric acid (H₃BO₃) (AnalaR)</td>
<td>BDH</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>S</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>S</td>
</tr>
<tr>
<td>Cacodylic acid</td>
<td>S</td>
</tr>
<tr>
<td>Citric acid (AnalaR)</td>
<td>BDH</td>
</tr>
<tr>
<td>Coomasie brilliant blue</td>
<td>BDH</td>
</tr>
<tr>
<td>Copper Sulphate (CuSO₄) (anhydrous)</td>
<td>BDH</td>
</tr>
<tr>
<td>N, N¹ - Diallyltartardiamide (DATD)</td>
<td>Aldrich Chemical Company</td>
</tr>
<tr>
<td>Diethylaminoethyl (DEAE) Cellulose (DE52)</td>
<td>Whatman</td>
</tr>
<tr>
<td>DL-dithiothreitol</td>
<td>S</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>BDH</td>
</tr>
<tr>
<td>Ethylenediamine tetraacetic acid (EDTA)</td>
<td>BDH</td>
</tr>
</tbody>
</table>
Folin Ciocalteu's reagent
Fructose-6-phosphate (diNa salt)
Fructose, -1, 6-diphosphate (triNa salt)
Glucose (AnalaR)
Glucose 1-phosphate with 1% glucose-1, 6-diphosphate
Glucose - 6 - phosphate (mono Na salt)
Glucose - 6 - phosphate dehydrogenase (Baker's yeast, Type XV)
Glucose phosphate isomerase
Glutaraldehyde
Glycine
Heparin
Hydrochloric acid (HCl) (AnalaR)
Inosine
DL - Isocitric acid (tri sodium salt)
Lactate dehydrogenase (hog muscle, 50% glycerol solution)
Magnesium chloride (MgCl₂) (AnalaR)
Malate dehydrogenase (pig heart, 50% glycerol solution)
Maleic acid
Maleic anhydride
DL - Malic acid
Mannose - 6 - Phosphate
Methanol
4-Methylumbelliferyl acetate
MTT
NAD (nicotinamide adenine dinucleotide)
NADH (disodium salt)
NADP (monosodium salt)
p-Nitrophenol
p-nitrophenyl phosphate (dicyclohexyl ammonium salt)
Osmium tetraoxide (OsO₄)
Oxaloacetic acid
2-Oxoglutarate (α-ketoglutarate) (mono K salt)  S
Phenazine methosulphate (PMS)  S
Phosphoric acid (H₃PO₄) (AnalaR)  BDH
Phosphotungstic acid  BDH
Potassium dihydrogen phosphate (KH₂PO₄) (AnalaR)  BDH
Sodium cacodylate  S
Sodium chloride (NaCl) (AnalaR)  BDH
Sodium carbonate (AnalaR)  BDH
Sodium dihydrogen phosphate (NaH₂PO₄)  BDH
diSodium hydrogen phosphate (Na₂HPO₄)  BDH
Sodium hydroxide  BDH
Sodium dodecyl sulphate (SDS)  BDH
Sodium potassium tartrate  BDH
Starch (hydrolysed potato starch)  Connaught Laboratories
Sucrose  BDH
N, N, N¹, N¹-tetramethylethylene-diamine (TEMED)  S
Trichloroacetic acid (TCA)  BDH
Tris (hydroxymethylamine)  BDH
Xanthine oxidase  S
APPENDIX B

Buffers

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

\[
\begin{align*}
\text{Na}_2\text{HPO}_4 & \quad (\text{anhydrous}) \quad 8.094g \\
\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} & \quad 0.468g \\
\text{NaCl} & \quad 2.550g \\
\text{Glucose} & \quad 15.0g
\end{align*}
\]

Made up to 1 litre with distilled water.

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

\[
\begin{align*}
\text{Tris} & \quad 3.031g \\
\text{Sucrose} & \quad 109.53g \\
\text{EDTA} & \quad 0.372g
\end{align*}
\]

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

\[
\begin{align*}
\text{Tris} & \quad 36.42g
\end{align*}
\]

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

\[
\begin{align*}
\text{Tris} & \quad 36.42g \\
1\text{N HCl} & \quad 160ml
\end{align*}
\]

Made up to 1 litre with distilled water.
5. 0.1M Sodium phosphate pH 7.4
   \[ \text{Na}_2\text{HPO}_4 \text{ (anhydrous)} 11.499g \]
   \[ \text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O} 2.964g \]
   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 C

Protein determination (Lowry et al., 1951)

Solution A: 2.0% Na₂CO₃ + 0.02% sodium potassium tartrate
Solution B: 0.5% CuSO₄ · 5H₂O
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)**

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>TANK BUFFER</th>
<th>GEL DILUTION</th>
<th>VOLTAGE IN VOLTS/CM</th>
<th>TIME IN MINS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACP E.C.3.1.2.3</td>
<td>0.15M Trisodium citrate - 0.24M NaH₂PO₄ adjusted to pH 6.3 with NaOH</td>
<td>1 : 39</td>
<td>20</td>
<td>180</td>
</tr>
<tr>
<td>ACON E.C.4.2.1.3</td>
<td>0.1M Tris - 0.028M citrate pH 7.5</td>
<td>1 : 9</td>
<td>20</td>
<td>120</td>
</tr>
<tr>
<td>ES E.C.3.1.1.1</td>
<td>0.1M Tris - 0.1M Maleic anhydride adjusted to pH 7.2 with NaOH</td>
<td>1 : 9</td>
<td>20</td>
<td>180</td>
</tr>
<tr>
<td>GPI E.C.5.3.19</td>
<td>0.162M Na₂HPO₄ - 0.038M NaH₂PO₄ pH 7.4</td>
<td>3 : 37</td>
<td>20</td>
<td>135</td>
</tr>
<tr>
<td>Mdh E.C.1.1.1.37</td>
<td>0.1M Na₂HPO₄</td>
<td>1 : 4</td>
<td>20</td>
<td>90</td>
</tr>
<tr>
<td>MPI E.C.5.3.1.8</td>
<td>0.014M citric acid pH 7.0</td>
<td>1 : 9</td>
<td>20</td>
<td>150</td>
</tr>
<tr>
<td>NH E.C.3.2.2.1</td>
<td>0.04M Tris - 0.44M boric acid pH 6.5</td>
<td>1 : 9</td>
<td>20</td>
<td>180</td>
</tr>
<tr>
<td>PGM E.C.2.7.5.1</td>
<td>0.1M Tris - 0.1M Maleic acid - 0.01M EDTA - 0.01M MgCl₂ Adjusted to pH 7.4 with NaOH</td>
<td>1 : 9</td>
<td>13.5</td>
<td>180</td>
</tr>
<tr>
<td>ALAT E.C.2.6.1.2</td>
<td>0.15M Tris - 0.0075M citric acid, pH 9.0</td>
<td>1 : 9</td>
<td>40</td>
<td>90</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Buffer System</td>
<td>Temp (°C)</td>
<td>Activity (U/mg)</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>----------------------------------------</td>
<td>-----------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>ASAT</td>
<td>0.15M Tris - 0.0075M citric acid pH 9.0</td>
<td>40</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>ME</td>
<td>0.122M Na₂HPO₄ - 0.38M NaH₂PO₄ pH 7.0</td>
<td>37</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>16</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>ICD (NADP)</td>
<td>0.135 Tris - 0.042M citric acid, pH 7.0</td>
<td>20</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>G6PD</td>
<td>0.1M Tris - 0.1M Maleic acid 0.01M MgCl₂ - 0.01M EDTA, adjusted to pH 7.4 with NaOH</td>
<td>20</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>
## APPENDIX D

### ENZYME DEVELOPERS FOR SDS

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>DISTILLED WATER</th>
<th>DEVELOPER BUFFER</th>
<th>ACTIVATORS</th>
<th>CORNENZYMES</th>
<th>LINKING ENZYMES</th>
<th>SUBSTRATES</th>
<th>VISUALIZATION METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACON</td>
<td>4.5ml</td>
<td>B</td>
<td>MgCl (1.0M)</td>
<td>NADP</td>
<td>E.C.1.1.1.42</td>
<td>Cis-aconitc acid (10.0ml)</td>
<td>3PMS 1.0ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.0ml</td>
<td>0.3ml</td>
<td>(ICD) (S-type)</td>
<td>in 0.4M Tris - HCl pH 8.0</td>
<td>MTT 2.0ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV 27uL</td>
<td>ph readjusted to 8.0</td>
<td>Agar 20.0ml</td>
</tr>
<tr>
<td>ACP</td>
<td></td>
<td>F</td>
<td></td>
<td></td>
<td></td>
<td>4 methylumbellifer phosphoryl phosphate 20mg</td>
<td>UV, filter paper</td>
</tr>
<tr>
<td></td>
<td>10.0ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALAT</td>
<td></td>
<td>D</td>
<td></td>
<td>NADH</td>
<td>E.C.1.1.1.27</td>
<td>2-oxoglutarate (100mg/ml)</td>
<td>UV, filter</td>
</tr>
<tr>
<td></td>
<td>5.0ml</td>
<td></td>
<td></td>
<td>(10mg/ml)</td>
<td>(LDH) (B-hog muscle in 50% buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3ml</td>
<td>glycerol - 5,500 u/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1% uL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASAT</td>
<td></td>
<td>D</td>
<td></td>
<td>NADH</td>
<td>E.C.1.1.1.37</td>
<td>2-oxoglutarate (100mg/ml)</td>
<td>UV, filter paper</td>
</tr>
<tr>
<td></td>
<td>10.0ml</td>
<td></td>
<td></td>
<td>(10mg/ml)</td>
<td>(MDH) (B-pig heart in 50% buffer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3ml</td>
<td>glycerol - 6,000 u/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 uL</td>
<td></td>
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</tr>
<tr>
<td>E5</td>
<td></td>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td>4 methylumbelliferyl acetate 20mg</td>
<td>UV, filter paper</td>
</tr>
<tr>
<td></td>
<td>10.0ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPI</td>
<td>2.9ml</td>
<td>B</td>
<td>MgCl (1.0M)</td>
<td>NADH</td>
<td>E.C.1.1.1.49</td>
<td>Fructose - 6-phosphate</td>
<td>3PMS 1.0ml</td>
</tr>
<tr>
<td></td>
<td>13.3ml</td>
<td></td>
<td>(10mg/ml)</td>
<td>(10mg/ml)</td>
<td>G6PD (S-type)</td>
<td>(10mg/ml) 1.20ml</td>
<td>MTT 2.0ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.04ml</td>
<td>0.30ml</td>
<td>IV 100u/ml</td>
<td></td>
<td>Agar 20.0ml</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
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### Table 4. continued

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>DISTILLER</th>
<th>DEVELOPER BUFFER</th>
<th>ACTIVATORS</th>
<th>COENZYMES</th>
<th>LINKING ENZYMES</th>
<th>SUBSTRATES</th>
<th>VISUALIZATION METHOD</th>
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</thead>
<tbody>
<tr>
<td>MDH</td>
<td>H₂O</td>
<td>C</td>
<td>-</td>
<td>NAD</td>
<td>-</td>
<td>DL-Malic acid (1.0 M neutralized with NaOH)</td>
<td>PMS 1.0 ml</td>
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<tr>
<td></td>
<td>2.00 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00 ml</td>
<td>MTT 2.0 ml</td>
</tr>
<tr>
<td></td>
<td>E.C.1.1.1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Agar 20.0 ml</td>
<td></td>
</tr>
<tr>
<td>NADH</td>
<td>H₂O</td>
<td>A</td>
<td>MgCl₂</td>
<td>NADP</td>
<td>-</td>
<td>Mannose-β-phosphate</td>
<td>PMS 1.0 ml</td>
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<tr>
<td></td>
<td>1.50 ml</td>
<td></td>
<td></td>
<td>(1.0 M)</td>
<td>(10 mg/ml)</td>
<td>(10mg/ml)</td>
<td>MTT 2.0 ml</td>
</tr>
<tr>
<td></td>
<td>E.C.1.1.1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Agar 20.0 ml</td>
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</tr>
<tr>
<td></td>
<td>40.4 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00 ml</td>
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<tr>
<td></td>
<td>E.C.1.1.5.4</td>
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<td></td>
<td></td>
<td></td>
<td>0.12 ml</td>
<td></td>
</tr>
<tr>
<td>1CD</td>
<td>H₂O</td>
<td>A</td>
<td>MgCl₂</td>
<td>NADP</td>
<td>-</td>
<td>DL-sodium isocitrate (trisodium salt, 100mg/ml)</td>
<td>PMS 1.0 ml</td>
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<td></td>
<td>1.80 ml</td>
<td></td>
<td></td>
<td>(1.0 M)</td>
<td>(10 mg/ml)</td>
<td>(10mg/ml)</td>
<td>MTT 2.0 ml</td>
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<tr>
<td></td>
<td>E.C.1.1.7.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Agar 20.0 ml</td>
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</tr>
<tr>
<td></td>
<td>13.4 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.40 ml</td>
<td></td>
</tr>
<tr>
<td>PGM</td>
<td>H₂O</td>
<td>A</td>
<td>MgCl₂</td>
<td>NADP</td>
<td>-</td>
<td>Glucose-1-phosphate</td>
<td>PMS 1.0 ml</td>
</tr>
<tr>
<td></td>
<td>2.00 ml</td>
<td></td>
<td></td>
<td>(1.0 M)</td>
<td>(10 mg/ml)</td>
<td>(10mg/ml)</td>
<td>MTT 2.0 ml</td>
</tr>
<tr>
<td></td>
<td>E.C.1.1.1.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Agar 20.0 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.3 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>E.C.2.7.5.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00 ml</td>
<td></td>
</tr>
<tr>
<td>NIL</td>
<td>H₂O</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Xanthine (1.0 ml)</td>
<td>PSM 1.0 ml</td>
</tr>
<tr>
<td></td>
<td>2.60 ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 mg/ml</td>
<td>MTT 2.0 ml</td>
</tr>
<tr>
<td></td>
<td>E.C.3.2.2.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Agar 20.0 ml</td>
<td></td>
</tr>
</tbody>
</table>

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

---

University of Ghana

http://ugspace.ug.edu.gh
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 E

Table 5. Electrophoretic Conditions for Cellulose Acetate

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>BUFFER</th>
<th>VOLTAGE (VOLS)</th>
<th>TIME (MINS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAT</td>
<td>0.66M Tris - 0.08M citric acid, pH 8.6, dil. 1 : 4</td>
<td>200</td>
<td>25</td>
</tr>
<tr>
<td>E.C.2.6.1.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASAT</td>
<td>Tris - barbital - sodium barbital</td>
<td>300</td>
<td>25</td>
</tr>
<tr>
<td>E.C.2.6.1.1</td>
<td>pH 9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G6PD</td>
<td>0.05M Tris - 0.05 M maleic acid - 0.005M EDTA - 0.005M MgCl₂ pH 8.6, dil. 1 : 1</td>
<td>130</td>
<td>55</td>
</tr>
<tr>
<td>E.C.1.1.1.49</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICD</td>
<td>0.006M NaH₂PO₄ - 0.0144M Na₂HPO₄</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>E.C.1.1.1.42</td>
<td>pH 8.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME</td>
<td>0.006M NaH₂PO₄ - 0.0144M Na₂HPO₄</td>
<td>200</td>
<td>50</td>
</tr>
<tr>
<td>E.C.1.1.1.40</td>
<td>pH 8.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. All buffers contained 10% sucrose.
2. Prepared from pre-packaged sachets Cat. No. 5805 supplied by Helena Laboratories (Beaumont, Texas, USA).
### Table 5.

**ENZYME DEVELOPERS FOR CELLULOSE ACETATE ELECTROPHORESIS**

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>DEVELOPER BUFFER</th>
<th>pKa 1.0H Cl-</th>
<th>COENZYMES</th>
<th>LINKING ENZYMES</th>
<th>SUBSTRATES</th>
<th>VISUALIZATION METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAT</td>
<td>0.019% Na₂HPO₄</td>
<td>-</td>
<td>SADH</td>
<td>LDH, 5500 u/ml</td>
<td>Alanine (100mg/ml in developer buffer)</td>
<td>UV, filter paper</td>
</tr>
<tr>
<td></td>
<td>0.081% Na₂HPO₄</td>
<td>-</td>
<td></td>
<td>0.15ml</td>
<td>0.013ml</td>
<td>3-oxoglutarate (100mg/ml) 0.015ml</td>
</tr>
<tr>
<td></td>
<td>pH 7.4 with 50%</td>
<td>-</td>
<td>1.60ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sorbose</td>
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<td></td>
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<tr>
<td>As for ALAT</td>
<td></td>
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</tr>
<tr>
<td>ASAT</td>
<td>As for ALAT</td>
<td>-</td>
<td>SADH</td>
<td>MGDH, 5000 u/ml</td>
<td>Aspartic acid (5mg/ml in developer buffer)</td>
<td>UV, filter paper</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.15ml</td>
<td>0.013ml</td>
<td>2-oxoglutarate (100mg/ml) 0.03ml</td>
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<tr>
<td>DAPD</td>
<td>D.3M Tris - HCl</td>
<td>0.25ml</td>
<td>SADP</td>
<td>-</td>
<td>D-glucose-6-phosphate (10mg/ml)</td>
<td>PMS 0.5ml</td>
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<tr>
<td></td>
<td>pH 7.4</td>
<td></td>
<td>(10mg/ml)</td>
<td></td>
<td>0.50ml</td>
<td>MTT 1.0ml</td>
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<tr>
<td></td>
<td>5.67ml</td>
<td></td>
<td>0.50ml</td>
<td></td>
<td></td>
<td>Agar 10.0ml</td>
</tr>
<tr>
<td>ME</td>
<td>D.3M Tris - HCl</td>
<td>0.20ml</td>
<td>SADP</td>
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<td>DL = Malic Acid neutralized with NaOH</td>
<td>PMS 0.5ml</td>
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<td></td>
<td>pH 7.4</td>
<td></td>
<td>(10mg/ml)</td>
<td></td>
<td>0.60ml</td>
<td>MTT 1.0ml</td>
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<tr>
<td></td>
<td>5.67ml</td>
<td></td>
<td>0.20ml</td>
<td></td>
<td></td>
<td>Agar 10.0ml</td>
</tr>
<tr>
<td>ECD</td>
<td>D.3M Tris - HCl</td>
<td>0.25ml</td>
<td>SADP</td>
<td>-</td>
<td>DL = Isocitric acid</td>
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<td>pH 7.4</td>
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<td>(10mg/ml)</td>
<td></td>
<td>0.40ml</td>
<td>MTT 1.0ml</td>
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<td>0.50ml</td>
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<td></td>
<td>Agar 10.0ml</td>
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</table>
APPENDIX F

SDS-PAGE Solutions and Reagents

Solution A

Tris
3M Tris - HCl buffer pH 8.5 - 8.9
36g

1N HCl
48ml

Made up to 100 ml with distilled water.

Solution B

Tris
1M Tris - HCl pH 7.0
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 H₂O

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

(i) 12% separating gel

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (ml)</th>
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<tbody>
<tr>
<td>A</td>
<td>5.00</td>
</tr>
<tr>
<td>C</td>
<td>17.20</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>10.00</td>
</tr>
<tr>
<td>Distilled water</td>
<td>7.60</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.05</td>
</tr>
<tr>
<td>20% SDS</td>
<td>0.20</td>
</tr>
</tbody>
</table>

(ii) 3% stacking gel

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (ml)</th>
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<tbody>
<tr>
<td>C</td>
<td>2.55</td>
</tr>
<tr>
<td>D</td>
<td>3.00</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>12.00</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.45</td>
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</table>

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

Electrode buffer: Tris - glycine pH 8.3

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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<tbody>
<tr>
<td>Tris</td>
<td>6g</td>
</tr>
<tr>
<td>Glycine</td>
<td>288g</td>
</tr>
<tr>
<td>20% SDS</td>
<td>10.0ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>up to 2 litres.</td>
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</table>

Sample dissolving buffer (SDB)

<table>
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<tr>
<th>Component</th>
<th>Amount</th>
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<tr>
<td>0.05M Tris - HCl pH 7.0</td>
<td>500ul</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>200ul</td>
</tr>
<tr>
<td>20% SDS</td>
<td>200ul</td>
</tr>
<tr>
<td>DTT</td>
<td>0.15g</td>
</tr>
<tr>
<td>Sucrose</td>
<td>a few grains.</td>
</tr>
</tbody>
</table>
## APPENDIX G

### Table 7. STOCK ENZYME ASSAY MIXTURES

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>DISTILLED H₂O</th>
<th>BUFFER</th>
<th>ACTIVATORS</th>
<th>COENZYMES</th>
<th>LINKING ENZYMES</th>
<th>SUBSTRATES</th>
<th>FINAL VOLUME (ml)</th>
<th>NOTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALAT</td>
<td>1.40ml</td>
<td>-</td>
<td>-</td>
<td>0.14ml</td>
<td>0.02ml</td>
<td>8.0ml 8.9% alanine in 0.1M phosphate buffer, pH 7.4</td>
<td>9.36</td>
<td>0.835ml 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.</td>
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<tr>
<td>E.C.2.6.1.2</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>G6PD</td>
<td>5.20ml</td>
<td>3.30ml</td>
<td>0.10ml</td>
<td>1.00ml</td>
<td>-</td>
<td>0.34ml 100mg/ml G6P (0.34mg/ml)</td>
<td>9.94</td>
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</tr>
<tr>
<td>E.C.1.1.1.49</td>
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<td></td>
</tr>
<tr>
<td>GPI</td>
<td>4.40ml</td>
<td>3.30ml</td>
<td>0.15ml</td>
<td>1.00ml</td>
<td>0.10ml</td>
<td>1.00ml 100mg/ml P6P (1.01mg/ml)</td>
<td>9.95</td>
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<td></td>
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<tr>
<td>HK</td>
<td>5.00ml</td>
<td>1.60ml</td>
<td>0.08ml</td>
<td>0.80ml</td>
<td>0.03ml</td>
<td>1.00ml 100mM glucose (10.00mM)</td>
<td>10.01</td>
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</tr>
<tr>
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</table>

*(Note: Enzyme EC numbers and buffer conditions are included for each stock mixture entry.)*
Table 7. continued

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>DISTILLED H₂O</th>
<th>BUFFER</th>
<th>ACTIVATORS</th>
<th>COENZYMES</th>
<th>LINKING ENZYMES</th>
<th>SUBSTRATES</th>
<th>FINAL VOLUME (ml)</th>
<th>NOTES</th>
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</thead>
<tbody>
<tr>
<td>ICD</td>
<td>6.00ml</td>
<td>3.35ml</td>
<td>0.10ml</td>
<td>0.50ml</td>
<td>-</td>
<td>0.05ml 100mg/ml</td>
<td>10.00</td>
<td>Oxaloacetate was made up with 0.5ml 1M HCl and 0.5ml H₂O</td>
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<tr>
<td>E.C.1.1.42</td>
<td>0.3M Tris 1M MgCl₂</td>
<td>HCl pH 8.0</td>
<td>(1.0mM)</td>
<td>(0.50mg/ml)</td>
<td>NADPH</td>
<td>DL-isocitrate</td>
<td>(0.5mg/ml)</td>
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<tr>
<td>MDH</td>
<td>6.40ml</td>
<td>3.30ml</td>
<td>-</td>
<td>0.13ml</td>
<td>-</td>
<td>0.13ml 10mg/ml</td>
<td>9.96</td>
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<td>E.C.1.1.37</td>
<td>0.3M Tris</td>
<td>HCl pH 8.0</td>
<td>10mg/ml</td>
<td>(0.13mg/ml)</td>
<td>NADH</td>
<td>oxaloacetate</td>
<td>(0.13mg/ml)</td>
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<tr>
<td>ME</td>
<td>5.30ml</td>
<td>3.30ml</td>
<td>0.15ml</td>
<td>1.00ml</td>
<td>-</td>
<td>0.25ml</td>
<td>10.00</td>
<td>Malic acid was neutralized with 10N NaOH</td>
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<td>E.C.1.1.40</td>
<td>0.3M Tris 1M MgCl₂</td>
<td>HCl pH 7.4</td>
<td>(1.5mM)</td>
<td>(1mg/ml)</td>
<td>NADPH</td>
<td>malic acid</td>
<td>1M pH 7.0</td>
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<tr>
<td>MPT</td>
<td>5.00ml</td>
<td>3.30ml</td>
<td>0.10ml</td>
<td>0.50ml</td>
<td>0.03ml</td>
<td>1.00ml 10mg/ml</td>
<td>10.03</td>
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<td>E.C.5.3.8</td>
<td>0.3M Tris 1M MgCl₂</td>
<td>HCl pH 7.4</td>
<td>(1.0mM)</td>
<td>(0.50mg/ml)</td>
<td>NADPH</td>
<td>G6PD</td>
<td>(1.00U/ml)</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>100U/ml</td>
<td>10mg/ml M6P</td>
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<td></td>
<td>100U/ml</td>
<td>0.10m</td>
<td>(3.0U/ml)</td>
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<td></td>
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<td></td>
<td></td>
<td>100U/ml</td>
<td>G6PD</td>
<td>(1.00U/ml)</td>
</tr>
<tr>
<td>SUBSTRATES</td>
<td>FINAL VOLUME (ml)</td>
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<td>0.5% D-glucose</td>
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<tr>
<td>0.1M Tris-HCl</td>
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<td>0.2 M/1 M</td>
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<td>0.4 M/1 M</td>
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<td>0.6 M/1 M</td>
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<td>0.8 M/1 M</td>
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<td>1.0 M/1 M</td>
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<td>2.0 M/1 M</td>
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<td>2.3 M/1 M</td>
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<td>2.5 M/1 M</td>
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<td>3.5 M/1 M</td>
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<td>4.0 M/1 M</td>
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<td>5.0 M/1 M</td>
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<td>6.5 M/1 M</td>
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<td>7.0 M/1 M</td>
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<td>7.5 M/1 M</td>
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<td>8.0 M/1 M</td>
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<tr>
<td>8.5 M/1 M</td>
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<td></td>
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<td></td>
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<td>9.5 M/1 M</td>
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</tr>
<tr>
<td>10.0 M/1 M</td>
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</tr>
</tbody>
</table>

Table 7 continued:

- 0.09 mL of all stock assay mixtures were used with 0.01 mL of trypanosome extract.
- The final concentration of the different reagents in the stock assay mixture are given in brackets.

**Reagents**

1. 0.04 mL of 2 M Tris-(hydroxymethyl)aminomethane (HEPES) buffer, pH 9.5
2. 0.01 mL of 10% dithiothreitol (DTT)
3. 0.01 mL of 0.5 M sodium pyrophosphate, pH 5.0
4. 0.01 mL of 10% sodium pyrophosphate standard solution

**Substrate Mixture**

- 5 mg/mL p-nitrophenyl phosphate
- (dicyclohexylaminomethane salt)
- 104.0 mg

**Acid Phosphate Assay**

- Naphthol AS-TR phosphate
- 10.0 mL

- Naphthol AS-TR phosphate
- 10.0 mL
APPENDIX H

Figure 18.  p-Nitrophenol Standard Curve
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