INFLUENCE OF HOST BLOOD AND ITS DIGESTIVE PRODUCTS ON TRYPANOSOME DIFFERENTIATION IN TSETSE FLY, *GLOSSINA MORSITANS MORSITANS* WESTWOOD

BY

EDWARD KINYUA NGUU, BSc. (Hons), MSc. (NAIROBI)

A thesis submitted in fulfilment for the degree of Doctor of Philosophy in the University of Nairobi

1997

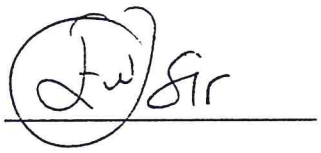
DECLARATION

I, **Edward Kinyua Nguu**, hereby declare that this is my original work and the work described here has not been presented to any other University.

EDWARD KINYUA NGUU

CANDIDATE

This thesis has been submitted for examination with our approval as supervisors.



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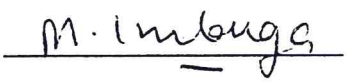

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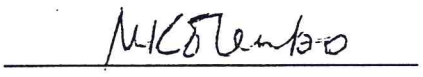

PROF. NORAH K. OLEMBO

PROF. DOMINIC MAKAWITI
CHAIRMAN, DEPARTMENT OF BIOCHEMISTRY

ACKNOWLEDGEMENTS

This work would not have taken off without the scholarship awarded to me by the German Academic Exchanges Services (DAAD) through the African Regional Postgraduate Programme in Insect Science (ARPPIS) of the International Centre of Insect Physiology and Ecology (ICIPE). Therefore I express my sincere appreciation for the scholarship. My sincere gratitude to my first supervisor, Dr. Ellie O. Osir who introduced me into research work. His generous support, guidance, constant encouragement and criticisms throughout my postgraduate studies is invaluable. I am also very grateful to my supervisor, Dr. Mabel Imbuga. Her encouragement, kindness and support was a great inspiration during this study. The useful discussions, support and constant encouragement that I received form my supervisor, Professor Norah Olembo is highly acknowledged. I am also grateful to Professor Dominic Makawiti, Chairman, Biochemistry Department for his understanding and support during this study.

I am most thankful to the Director of ICIPE, Dr. Hans R. Herren, for his support and more so for allowing me to use the ICIPE facilities. I also thank the ICIPE founder Director, Professor Thomas R. Odhiambo for his insight in founding and propping the ARPPIS programme. I also acknowledge the support I got from Drs. V. O. Musewe and Z. T. Dabrowski, the current and former ARPPIS coordinator, respectively.

This work was based on studies involving blood from wild and domestic animals. I wish to express my sincere appreciation to the Kenya Agricultural Research Institute-Wildlife Disease Research Section at Kabete through their leaders Drs. George Orinda, Stanley Mukolwe and D. Malu for supporting my Ph.D study by allowing me to get blood from the wild animals held in captivity without inhibition. I also acknowledge the support from all the technical staff at the KARI-Wildlife Disease Research Section messers Ouma, Agushoma, Kodunga, Githinji and Mutweti. Special thanks to Dr. R. O. Olubayo who personally made alot of effort to assist me in this endeavour and on many other occasions. I also thank Dr. Steve Mihok for his support and encouragement.

I express my sincere thank to my senior colleagues at ICIPE, Drs S. Essuman, N. Massamba, H. Mahamat, M. Vundla, M. Limo for their useful discussions and encouragement. I acknowledge the contributions I got from my fellow ARPPIS scholars H. M. Abakar, D. Ogoyi, E. Omolo, E. Kenya, L. Abubakar, G. Zimba, J. Kongoro, H. Mohammed, F. Massaninga, S. Bengaly, M. Mugunga. The assistance I got from N. Sitayo and J. Kabii is highly appreciated. I also extend my thanks to Mrs R. Kariuki, J. Kagoiya, J. Wanyonje, E. Awuoche, N. Mwikya and H. Banda for the constant supply of the tsetse required for this work.

To my mother Beatrice, brothers and sisters is special thanks for constant prayers during this time. Lastly, but not the least, this work dictated that I spend less time with my family. I therefore express alot of thank to my loving wife, Grace, and our children Amos and Brenda for their understanding and endurance during this period. And the very last, which essentially is first, is to thank the God Almighty, without his blessings nothing is possible.

DEDICATION

TO MY LATE FATHER, AMOS M. NGUU; MY MOTHER, BEATRICE MUTHONI MIGWI; MY WIFE, GRACE AND OUR CHILDREN, AMOS AND BRENDA; MY BROTHERS AND SISTERS.

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ABBREVIATIONS

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
Chromozym-TRY	Carbobenzoxy-val-gly-arg-4-nitrianilide acetate
DEAE-	Diethylaminoethyl-
DFP	Diisopropyl fluorophosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FPLC	Fast protein liquid chromatography
HDL	High density lipoprotein
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
ICIPE	International Centre of Insect Physiology and Ecology
$MgCl_2$	Magnesium chloride
NaCl	Sodium chloride
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
PSG	Phosphate saline glucose

RBCs	Red blood cells
RLOs	Rickettsiae-like organisms
SAS	Saturated ammonium sulphate
SDS	Sodium dodecyl sulphate
STI	Soybean trypsin inhibitor
TBS	Tris-buffered saline
TLF	Trypanosome lytic factor
TPCK	Tosyl phenyl chloromethyl ketone
Tris	Tris-(hydroxy methyl-aminomethane
VATs	Variant antigen types
WBCs	White blood cells

UNIT ABBREVIATIONS

IU	International unit
M	Molar
mCi	Millicurie
mg	illigram
ml	Millilitre
min	Minutes
mmol	Millimoles
mM	Millimolar
Mr	Molecular weight
nm	Nanometre
µCi	Microcurie
µl	Microlitre
µg	Microgram
µM	Micromolar
µmol	Micromole
g	Gravititional constant
pH	-Log hydrogen ion concentration
pI	Isolelectric point
rpm	Revolution per minute
sec	Seconds
v/v	volume by volume

SUMMARY

When tsetse feed on an infected host, the ingested bloodstream trypanosomes undergo a complex developmental process that leads to the formation of the metacyclic forms. Tsetse are obligatory bloodfeeders and display host preferences in their feeding behaviour. Therefore, host blood is an important factor in the transmission of trypanosomiasis. Host blood at the infective meal has been shown to influence trypanosome infection in tsetse. How, the host blood affect trypanosome development, particularly the transformation of bloodstream to procyclic forms, has not been previously established. Therefore, this study investigated how host blood and some of its digestive products influences this process. Morever, since trypsin is one of the bloodmeal induced molecules implicated in the transformation process, this study also investigated the release of midgut trypsin by host blood and blood fractions.

Midgut homogenates from tsetse, *Glossina morsitans morsitans*, that had previously been fed on different host blood samples were tested for their abilities to transform isolated bloodstream *Trypanosoma brucei* into procyclics *in vitro*. Compared to rat and goat blood samples, eland blood had the least capacity to support trypanosome transformation, while buffalo blood showed intermediate capacity. Fractionation of rat blood showed the importance of the cellular portion since both rat and eland red blood cells (RBCs) supported the process. Virtually no transformation was observed in rat and eland plasma or serum fractions. Suspending rat blood cells in eland plasma led to a drastic reduction in transformation rates. Further experiments showed that the RBC membranes were also capable of supporting the process. In addition, the low transformation rates observed in eland blood were due to an inhibitor present in the plasma fraction.

Further studies were carried out to determine the properties of the eland plasma inhibitor. The inhibitor was highly unstable, being labile to heating above 50° C, freezing (-70° C) and

thawing (37° C) and is gradually inactivated during storage. The inhibitor activity was not preserved by lyophilizing fresh plasma. It was sensitive to protease digestion since pronase (1 mg/ ml, 2 h, 30° C) completely abrogated its activity whereas trypsin had only a partial effect at the same concentration. Inclusion of different protease inhibitors in fresh plasma did not stop the inactivation of the inhibitor implying that the loss of activity was not due to endogenous proteolytic activity. The inhibitor was non-dialyzable in a 12-14 kD molecular weight cut-off membrane suggesting that the molecular weight of the factor is greater than 14 kD. Transformation was not inhibited when trypanosomes were pre-incubated in fresh plasma for up to 1.75 h. Solubility studies using saturated ammonium sulphate solution showed that the inhibitor was insoluble above 50 % salt. These results showed that the inhibitor is an unstable molecule.

In an effort to isolate the transformation inhibitor, fresh eland plasma was fractionated using anion-exchange chromatography on DEAE-Sepharose 6B-CL column. The inhibitor was recovered in the bound fraction. Further fractionation using linear salt gradients of between 0-0.5 M NaCl did not resolve the bound proteins. However, a stepwise elution using 0.1, 0.2 and 0.5 M NaCl yielded four peaks none of which had the transformation inhibition property of the whole bound fraction.

As the most abundant products of bloodmeal digestion in tsetse midgut and haemolymph, L-proline and L-glutamine were assayed for their ability to stimulate the transformation of bloodstream trypanosomes. A 0-20 mM concentration of the amino acids in teneral and fed gut homogenates had no influence on this process when tested individually or in combination.

Midgut trypsin activities and isoenzymes stimulated by host blood and blood components were determined as a function of time in order to elucidate the role of trypsin in transformation. Midgut homogenates from *Glossina morsitans* that had been fed on rat and eland blood or blood fractions were used. The trypsin isozymes were labelled using [1,3-^{3}H]

diisopropylfluorophosphate in presence of tosylphenyl chloromethylketone (TPCK). Teneral midguts showed four trypsin isomers of M_r~256, 56, 40 and 30 kD on non-denaturing polyacrylamide gel electrophoresis. The M_r~56, 40 and 30 kD isomers were inhibited during the first 8 h following bloodmeals, in midguts of tsetse that fed on blood or plasma. By 20 h, the inhibition was relieved in midgut of tsetse that fed on whole blood but not plasma. Midguts from tsetse that had fed on plasma showed additional bands of $M_r < 30$ kD at 72 h but did not result in significant change in the activities. Like the isoenzymes, trypsin activities were also inhibited following bloodmeals but stimulated to peak levels around 72 h and 48-72 h in teneral and non-tenerals, respectively. Only slight inhibition of both trypsin isoenzymes and activities occurred in midguts from tsetse that were fed on 50 % (v/v) red blood cell suspensions in saline indicating that plasma was responsible for the inhibitions. Despite initial differences in trypsin isoenzymes and activities following the bloodmeals, peak activities were not significantly different. Significance of the initial inhibition of the M_r~56, 40 and 30 kD isoenzymes during the time when transformation occurs is not known.

Although many factors are involved in the successful development of trypanosomes in tsetse, these results showed the important role played by blood, especially the red blood cells, in the transformation process. Furthermore, the host blood's ability to support this process certainly has a role in modulating infections in tsetse that feeds on such blood. More work is required to answer the questions arising from this work.

CHAPTER 1

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

African trypanosomiasis is a major vector-borne disease that affects both man and livestock in tropical Africa. The causative agent is the protozoan parasite, genus *Trypanosoma* (Hoare, 1972) which is transmitted by tsetse, genus *Glossina* (Diptera: Glossinidae) (Buxton, 1955). Since the trypanosome is digenetic it requires both vertebrate and tsetse hosts to complete its life cycle. Although fossil materials of *Glossina* have been found in Colorado region of America, living species are restricted to sub-Saharan Africa where 30 species and subspecies are known (Jordan, 1986). It is estimated that about 11 million Km^2 of sub-Saharan Africa is under-utilized due to the threat of trypanosomiasis. This has resulted in great economic losses estimated at billions of dollars (ILRAD, 1991). All *Glossina* species are haematophagous, totally dependent on the blood sucking habit (Buxton, 1955). However, different species of *Glossina* have host preferences and this is important in the epidemiology of trypanosomiases (Jordan, 1986). Both male and female tsetse transmit the disease (Hoare, 1972).

Various tsetse control measures have been employed in the management of trypanosomiasis (Jordan, 1986). The traditional methods include bush clearing, destruction of wildlife and the use of insecticides such as Dichlorodiphenyl trichloroethane (DDT) (Buxton, 1955; Jordan, 1986). The major drawback to the use of synthetic

insecticides such as DDT include off-target effects on useful insects and animals. Extermination of game animals which serve as reservior for trypanosomes is also another approach that has been selectively applied (Ford, 1970). However, this approach may be futile since tsetse will always find alternative hosts in livestock and man and this is likely to aggravate the problem (Ford, 1970). Moreover, the indiscriminate application of insecticides, extermination of wildlife and destruction of vegetation are inconsistent with the principals of conservation and could have severe consequences on biodiversity.

The release of sterile males has been used with limited success in some areas (Knippling, 1982; Dame *et al*, 1980). This technique exploits the fact that female tsetse mate only once in their lifetime. The sterile males released into the tsetse infested areas would compete with fertile males thereby suppressing the fecundity to levels below economic injury (Knipling, 1982). However, the application of sterile insect technique (SIT) for tsetse control is labour intensive due to the low reproductive rates and haematophagy (Luger, 1982; Mews, 1971). Secondly, the sterile tsetse are still capable of transmitting disease unless they die earlier than maturation period of trypanosomes. It should be pointed out that SIT has been used successfully to control the screw worm, *Cochliomyia horminivorax*, in North Africa (Knipling, 1982).

Mass trapping of tsetse with the ultimate aim of reducing populations to levels below economic injury has also been used with some success. In recent times, the efficiency of the approach has improved especially with the incorporation of odour baits (Owaga, 1984; 1985; Owaga *et al*., 1988). This involves studying the chemical cues

emanating from animals that helps the tsetse to locate its host (Hassanali, 1986; Hargrove and Vale, 1978; Vale and Hargrove, 1975; Vale, 1982). The behaviour of the fly is also being studied particularly its response to various visual cues aimed at improving the trapping capacity. From this new approach, improved traps, such as the biconical trap, designated NGU trap, has been developed (Brightwell *et al.*, 1987). The main advantages of the trapping method include low cost, ease of application and sustainability. The main drawback is that one trap design may not be suitable for different tsetse species.

The use of trypanocidal drugs for prevention or treatment of trypanosomiasis has also been widely used (Leach and Roberts, 1981). However, in high tsetse challenge areas, the frequency of treatment required to control the disease is economically unacceptable owing to the costs involved (Holmes, 1980). Secondly, the problems of toxicity and resistance development poses major problems. Due to such drawbacks, the possibility of developing vaccine against trypanosomiasis has been an attractive option. However, the phenomena of antigenic variation remains a major impediment to this approach (Vickerman, 1978). Novel strategies for control of vector-borne parasites include blocking transmission at the level of parasite development within the arthropod vector. Such strategies require a thorough understanding of the vector-parasite interaction. In this regard, the understanding of the physiology and biochemistry of tsetse-trypanosome interaction is crucial in designing future control strategies based on disruption of the transmission cycle.

1.2 LITERATURE REVIEW

1.2.1 Tsetse as vectors of trypanosomiasis

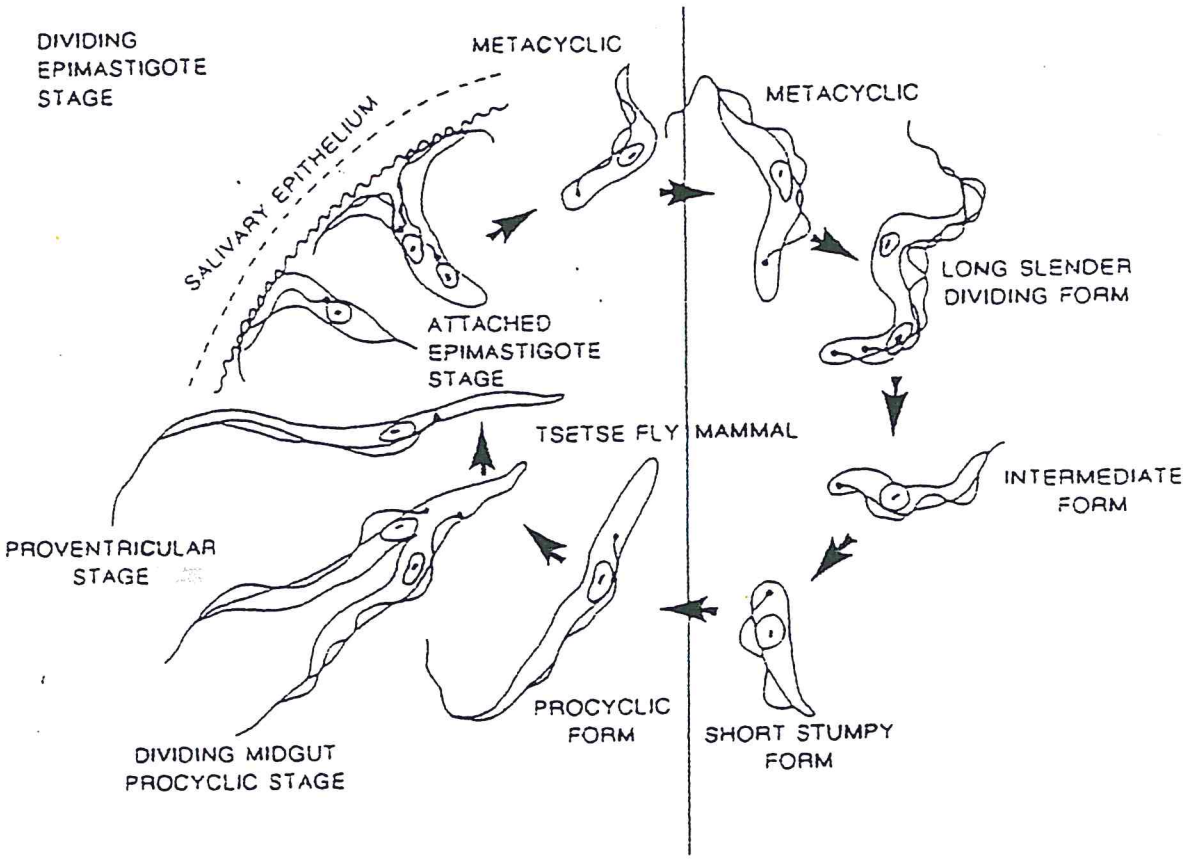
Haematophagous arthropods, notably insects, are major vectors of many pathogens which afflict man and domestic animals. Trypanosomiasis is transmitted by the tsetse bites. The transmission of trypanosomes between wild animals is of no economic value. However, when man and his domestic animals, which are usually susceptible to the pathogenic effects of the trypanosomes, venture into the cycle and becomes hosts of the trypanosome, the disease is of great economic importance (Ascroft 1958; 1959). Unless severely stressed, wild animals are usually able to control parasitaemia and the trypanosome-induced anaemia (Ascroft, 1959; Mulla and Rickman, 1988; Grootenhuis *et al.*, 1990; Mahan *et al.*, 1986). It is for these reasons that wildlife are regarded as reserviors of trypanosomiasis.

All tsetse flies belong to the genus *Glossina* (Jordan, 1986). At the lower taxa, the genus constitutes three well marked species groups namely the *Fusca, Palpalis* and *Morsitans* based on morphological characters and to a lesser extent, on geographical distribution (Jordan, 1986). The *Morsitans* group transmits "nagana" to cattle and horses while both *Morsitans* and *Palpalis* are important vectors of both animal and human sleeping sickness (Jordan, 1986). Being obligatory blood-feeders, infected tsetse with mature infections transmit the trypanosomes during blood meals (Buxton, 1955). Apart from the biological transmission of trypanosomes by *Glossina* (Jordan, 1986), mechanical transmission has also been documented especially among the subgenus *Dutonella* (Molyneux and Ashford, 1983). For example, in South America which is a tsetse free

zone, *Trypanosoma vivax* is transmitted mechanically mainly by the Tabanids and *Stomoxys* species (Molyneux and Ashford, 1983). Wells (1972) argued that although *Glossina* are best known for cyclic transmission of trypanosomiasis, they are still the most efficient mechanical transmitters with *Stomoxys* taking a second position. Other cases of non-cyclical transmission of nagana have been established among the carnivores which become infected with *T. brucei* through carnivory (Sachs *et al.*, 1967) and coital transmission of *T. equiperdum* among the horses (Molyneux and Ashford, 1983).

1.2.2 Trypanosome development in tsetse

The digenetic life cycle of the African trypanosomes requires both the vertebrate and invertebrate hosts (Hoare, 1972) (Scheme I). The whole process involves a series of complex biochemical, physiological and morphological changes that enable the trypanosomes to survive in changing environments (Buxton, 1955; Hoare, 1972). Once bloodstream trypanosomes are ingested with a blood meal, they encounter a hostile environment within the gut (Gooding and Rolseth, 1976), as a result of which most of them are lysed (Maudlin and Welburn, 1987). However, a small proportion of the ingested trypanosomes transform into the procyclic forms thereby escaping lysis (Maudlin and Welburn, 1987; Stiles *et al.*, 1990).


DIVIDING
EPIMASTIGOTE
STAGE
METACYCLIC
SALIVARY EPITHELIUM
ATTACHED
EPIMASTIGOTE
STAGE
METACYCLIC
LONG SLENDER
DIVIDING FORM
TSETSE FLY
MAMMAL
INTERMEDIATE
FORM
PROVENTRICULAR
STAGE
PROCYCLIC
FORM
SHORT STUMPY
FORM
DIVIDING MIDGUT
PROCYCLIC STAGE

The successful development of gut established procyclics culminate in the formation of metacyclic forms which are infective to mammals (Vickerman, 1985; Hoare, 1972). The exact details on the development of procyclics into metacyclic forms is still not clearly understood (Vickerman, 1985). Available information shows that the establishment of procyclics within the midgut requires a lectin which acts as the signal for maturation (Ibrahim *et al.*, 1984; Welburn and Maudlin, 1989). Although there is a recent report on chitinase activity in *Trypanosoma* (Shahabuddin *et al.*, 1993), it is still not clear how the procyclics cross the peritrophic membrane which, essentially, acts as a physical barrier in the midgut (Peters, 1992). Available evidence suggests that the trypanosomes cross after they migrate anteriorly to the proventriculus where the peritrophic membrane is, supposedly, soft (Buxton, 1955). Further migration then proceeds through labial cavity, hypopharynx and finally to the salivary glands (*Trypanozoon*) or proboscis (*Nannomonas*) (Buxton, 1955; Hoare, 1972).

The duration between an infective bloodmeal and maturation of the trypanosome in the vector varies. This process has been shown to be dependent on temperature as well as on the species of trypanosome and the vector involved (Jordan, 1986; Stephen, 1986). The duration is 16-35 days in *Trypanozoon*, 16-25 days in *Nannomonas* and 5-13 days in *Dutonella* (Molyneux and Ashford, 1983; Stephen, 1986). Maudlin and Welburn (1989) have also indicated that maturation of salivary gland infections could also be under genetic control.

The developmental cycle of the sub-genus *Dutonella (T. vivax)* is the simplest among the salivaria trypanosomes. The whole cycle is confined to the tsetse proboscis with no midgut or salivary gland stages. However, the process of *T. vivax* development is poorly understood. It is not clear, for example, why this subgenus does not have a midgut stage despite that the trypanosomes are ingested in the same way as the other subgenus that have midgut stages. Further, it is not clear exactly what happens to trypanosomes in the midgut. This requires further investigation to understand the life cycle. This may be important in designing future control strategies.

1.2.3 Trypanosome-tsetse interaction

An ideal vector-parasite relationship represents a compromise between the need for both the parasite and host to survive. The great economic importance of tsetse is due to biological and not mechanical transmission of trypanosomiasis (Hoare, 1972). The proportion of tsetse that can transmit the disease at any given time is very low (Buxton, 1955; Elce, 1971; Harley, 1971; Jordan, 1986; Stephen, 1986) and this phenomena was recognized quite early (Duke, 1930). It could be that majority of the tsetse in a given population are refractory. The obvious question is why infection prevalence is low and yet the tsetse have the chance of becoming infected. Morever, unlike under natural conditions which may be subject to a lot of variations, controlled laboratory infection studies still results in low rates of infections (Stephen, 1986). Some attention has been directed towards understanding the factors behind this low infection prevalence (Maudlin and

Welburn, 1987; Kaaya, 1989; Shaw and Moloo, 1991; Welburn and Maudlin, 1990; Mihok *et al.*, 1991, 1993). From the current knowledge it is difficult to single out any one factor that would account for the low infection prevalence. These factors include the vertebrate bloodmeal (Moloo, 1981, 1984; Mihok *et al.*, 1991, 1993), the tsetse host (Molyneux and Ashford, 1983; Kaaya, 1989) as well as the type of trypanosomes involved (Maudlin, 1991; Molyneux and Ashford, 1983). For example, some trypanosome stocks are known to thrive better than others within the same hostile environment of tsetse midgut (Maudlin, 1991).

The underlying details on the tsetse-trypanosome interaction are still not well understood. For example, (Kaaya *et al.*, 1986a) showed that bacteria injected into tsetse haemocoele were quickly cleared by phagocytosis. On the contrary, no such response was observed when trypanosomes were injected under similar conditions (Kaaya *et al.*, 1986a). Morever, in *Glossina morsitans morsitans*, nodule formation against invading bacteria but not against injected trypanosomes was also shown (Kaaya *et al.*, 1986b). Cellular immune responses such as melanization and encapsulation are responsible for innate refractoriness of the mosquitoes, *Aedes aegypti*, *Anopheles quadrimaculatus* and *Armigeres subalbatus*, to microfilariae infection (Ham, 1992).

The above observations suggest that the cellular immune responses that are directed against other invading microorganisms in tsetse do not act against trypanosomes. This is probably due to the tsetse inability to recognize trypanosomes as non-self. The salient properties leading to this co-existence merits further investigation in order to

establish how the trypanosomes avoid "immune" recognition by their tsetse host. One strategy adapted by some insect parasites such as microfilariae is molecular mimicry of the host's antigen (Stoffolano, 1986). For example, during penetration into haemocoele, the microfilariae, *Brugia pahangi*, are coated with materials from host midgut to avoid recognition by the hosts' immune system (Sutherlands *et al.*, 1984; Latfond *et al.*, 1985).

1.3 Tsetse midgut factors that influence trypanosome establishment and transmission

1.3.1 Overview

The successful development of trypanosomes is only possible if they can overcome the destructive mechanisms in their hosts. In the vertebrate host, for example, the trypanosomes overcome the host immune system by displaying a wide repertoire of variant antigen types (VATs) on their surface coat (Cross, 1990). The parasitaemic waves in infected mammals is mediated by antibodies directed against exposed variant surface glycoproteins (VSGs) (Vickerman, 1978). Induced VSG specific antibody responses causes clearance of homologous VSG types leaving switched VSG to generate the next and ensuing wave of parasitaemia thereby keeping the vertebrate immune system lagging one generation behind (Vickerman, 1978).

In tsetse midgut, the trypanosomes encounter a hostile environment which includes various digestive enzymes (Cheeseman and Gooding, 1985), lectins (Welburn *et al.*, 1989), agglutinins (Ingram and Molyneux, 1988; Stiles *et al.*, 1990), and trypanolytic proteins (Stiles *et al.*, 1991). These factors kill most of the ingested trypanosomes with

few managing to transform into procyclics which are adapted to the midgut conditions (Maudlin, 1991; Maudlin and Welburn, 1987; Stiles *et al.*, 1990). In addition, the established procyclics require further development into the infective metacyclics and, therefore, have to contend with other barriers, notably the peritrophic membrane (Peters, 1992).

1.3.2 The peritrophic membrane

1.3.2.1 Peritrophic membrane formation

Peritrophic membrane (PM) is a fibrous chitin containing sac-like structure that lines the midgut of insects (Chapman, 1985). Proteins, glycoproteins and mucopolysaccharides are the principal constituents but proteins may account for as much as 50 % dry weight (Peters, 1992). The ubiquitous occurrence of PM among arthropods strongly suggests some essential role in gut physiology (Peters, 1992). Insects PM are divided into two groups according to their mode of formation (Wigglesworth, 1972). Type 1 (or PM1) is secreted throughout the midgut epithelium, while type 2 (PM2) secretion is restricted to specialized group of cells around the cardia. Most adult haematophagous insects secrete PM1 (Lehane, 1976). During PM1 formation, bloodmeal induces its deposition mainly through secretion of preformed products as in *Anopheles stephensi* or through stimulation of *de novo* synthetic activity by epithelial cells as in *Aedes aegypti* (Perrone and Spielman, 1988). Unlike PM1, PM2 is constitutively secreted but deposition is accelerated following a bloodmeal (Lorena and Oo, 1994). It has been

reported that PM1 can also be induced in the absence of blood or proteinaceous matter (Billingsley and Rudin, 1992).

1.3.2.2 Physiological role of peritrophic membrane and its involvement in trypanosome development

The functional significance of PM in the midgut physiology of arthropods is still not well understood although various functions have been assigned to it (Peters, 1992). These include protection of midgut epithelium from abrassive particulate food matter (Wigglesworth, 1972) and acting as a physical barrier to pathogenic infection (Peters, 1992; Richards and Richards, 1977; Yand and Davis, 1977). Its absence especially among the fluid feeding Hemiptera (Chapman, 1985) is in support of the proposed function. Most studies on PM permeability reveal pore size of not more than 10 nm suggesting that it can effectively act as a barrier to many pathogens and particulate matter (Miller and Lehane, 1990; Chapman, 1985). For example, *Leishmania* amastigotes, which transform to promastigotes and divide rapidly within the food bolus, only move to the epithelium after PM breakdown confirming that PM can actually act as a barrier to pathogen invasion (Schlein *et al.*, 1991). Molecules such as lectins that bind to this matrix may reduce permeability to levels that are lethal to the insect (Eisemann and Bennington, 1994).

On the peritrophic matrix, exposed sugars residues are thought to play a crucial role in specificity of vector-parasite interactions (Ponnudurai *et al.*, 1988; Rudin and Hecker, 1989). There is evidence that species differences in the occurrence of these

residues influence specificity of the interaction between the vectors and pathogens they transmit (Rudin and Hecker, 1989; Huber *et al*, 1991). For example, glycosylated receptor molecules on the microvilli and /or the PM in the mosquito midgut are important in enabling ookinete to recognize the midgut wall prior to invasion (Seiber *et al*., 1991). Moreover, some enzymes such as aminopeptidases which have been implicated in refractory mechanisms in some insects, for examples *Anopheles stephensi* to *Plasmodium falciparum* (Feldmann *et al*, 1990) and in vector compatibility in sandflies to various *Leishmania* species are bound to the PM (Ponnodurai *et al*, 1988; Peters and Kalnins; 1985). However, the clear role of PM in midgut physiology and infection has not been fully resolved and requires further studies.

1.3.2.3 Penetration of peritrophic membrane by trypanosomes

In tsetse available information suggests that established procyclics can only penetrate peritrophic membrane in its softer region around the proventriculus (Lehane and Miller, 1991; Buxton, 1955) but not in the other parts which are, supposedly, tougher (Freeman, 1973). This observation qualifies PM as a physical barrier to pathogen development in tsetse. However, it was reported that in *G. m. morsitans*, *T. b. rhodesiense* can penetrate the fully developed membrane in the tougher regions and not just near the proventriculus as earlier suggested (Ellis and Evans, 1977). This suggests that parasites can overcome the PM barrier.

For many parasites, the escape from a bloodmeal is very crucial for successful completion of the life cycle within their respective vector hosts. Ultrastructural studies during *Plasmodium* parasite penetration into haemocoele has revealed that PM is focally disrupted near the apical end of the parasite. This suggests that penetration of the PM by the parasite is an enzymatic process mediated by parasite-produced chitinases, capable of degrading chitin (Huber *et al.*, 1991; Shahabudin and Kashlow, 1994). A recent study reported that *Plasmodium* ookinete chitinase is secreted as a zymogen, prochitinase, which is activated by mosquito secreted trypsins probably by cleavage of one or more lysine residues (Shahabuddin *et al.*, 1993). Inhibition of chitinase by allosamidin, a potent inhibitor of non-fungal chitinases, completely blocked transmission of *P. gallinaceum* in *Aedes aegypti* (Shahabuddin *et al.*, 1993). This suggested that inhibition of midgut proteases would result in lack of chitinase activity thereby blocking penetration of PM by the parasite. Inhibition of trypsin activity could, therefore, have a dual effect as far as parasite development is concerned because trypsin/trypsin-like enzymes are very important digestive proteases in many insects, including haematophagous insects (Applebaum, 1985). This would inhibit bloodmeal digestion and parasite development.

Chitinases have since been demonstrated in many genera of economically important protozoans including *Leishmania, Trypanosoma, Leptomonas, Crithidia* and *Herpestomonas* (Schlein *et al*, 1991; Shahabuddin and Kashlow, 1993). A similar mechanism of escape from bloodmeal exists in the protozoon *Babesia microti* which uses the content of a specialized organelle to penetrate the solid PM1 of the host tick, *Ixodes*

dammini (Rudizinska *et al.*, 1982). These observations suggests that PM may not be as formidable a barrier in some vector-parasite interactions as earlier envisaged.

1.3.3: Midgut trypsins

1.3.3.1 Trypsin stimulation by bloodmeal

Ingestion of a blood meal by haematophagous arthropods stimulates the release of a number of digestive proteases (Gooding, 1973; 1974; 1975; Briegel and Lea, 1975; Borovsky, 1985; 1986). In *Glossina* species, at least six proteolytic enzymes, responsible for bloodmeal digestion, have been reported (Gooding and Rolseth, 1976; Cheeseman and Gooding, 1985). These includes trypsin, trypsin-like, chymotrypsin-like, carboxypeptidases, aminopeptidases among others (Cheeseman and Gooding, 1985). These enzymes are important to the insect because they are involved in bloodmeal digestion (Chapman, 1985). Of these, serine proteases are among the most important in bloodmeal digestion (Applebaum, 1985). Among the serine proteases, trypsin and trypsin-like molecules have received the most attention due to their role in trypanosome differentiation (Imbuga *et al.*, 1992b; Yabu and Takayanagi, 1988).

It has been previously shown that these proteolytic enzymes are released only in the posterior midgut after a bloodmeal (Gooding and Rolseth, 1976; Stiles *et al.*, 1991).

Stiles *et al.* (1991) failed to detect protease activity in the anterior midgut of *Glossina palpalis*. These studies showed that the release of proteases in the posterior midgut follows a periodic pattern peaking around 48-96 hours after a bloodmeal (Abbelle and Decleir, 1991; Stiles *et al.*, 1991; Onyango, 1993).

1.3.3.2 Role of trypsins in trypanosome development

Transformation of bloodstream trypanosomes into procyclic (midgut) forms involves loss of the surface coat (variant surface glycoprotein) as well as other physiological, biochemical and morphological changes (Hoare, 1972; Vickerman, 1985). Loss of surface coat and transformation is attributed to proteolytic activity of midgut trypsins (Yabu and Takayanagi, 1988; Imbuga *et al.*, 1992a; 1992b). Bovine pancrease trypsin has been shown to stimulate bloodstream trypanosome transformation just like midgut trypsins (Imbuga *et al.*, 1992a; Yabu and Takayanagi, 1988). *In vivo* inhibition of midgut trypsin activity by soybean trypsin inhibitor (STI) resulted in reduced transformation rates (Imbuga *et al.*, 1992a). Furthermore, trypsins and trypsin-like enzymes have also been shown to play a crucial role in parasite-vector compatibility in *Phlebotomus papatasi* to *Leishmania* species (Borovsky and Schlein, 1987). Inhibition of certain trypsins by *Leishmania major* and not by *L. tropica* was shown to be the reason behind the successful development of the former in *Phlebotomus papatasi* in which the latter fails to establish (Borovsky and Schlein, 1987).

In *Glossina* species, differences in expression of a chimeric molecules which has both lectin and trypsin activities may be responsible for differences in the abilities of different species to develop infections (Osir *et al.*, 1995; Abubakar *et al.*, 1995). Earlier studies have also shown that high levels of mosquito proteases cause damage to arboviruses (Hardy *et al*, 1983) and *Plasmodium* parasites (Langley, 1975; Yeates and Steiger, 1981). Early escape by parasite from the midgut environment is, therefore, an important strategy that ensures successful development. Studies on mosquito trypsins indicated that they have a range of properties in common with other known trypsins such as molecular weight, substrate and inhibitor specificity as well as amino acid sequence homology (Barillus-Mury *et al.*, 1991). This suggests that the role of trypsins in vector-parasite interactions could be wider than hitherto envisaged.

1.3.4 Lectins

1.3.4.1 Midgut lectins

Lectins or agglutinins are a group of carbohydrate-binding proteins with ubiquitous distribution in nature (Lis and Sharon, 1986; Sharon, 1993) and diverse functions (Barøndes, 1981). The presence of lectins in the midgut, hindgut and haemolymph of tsetse flies has previously been demonstrated (Ibrahim *et al.*, 1986; Ingram and Molyneux, 1990; Welburn and Maudlin, 1990). Presence of lectins in the haemolymph, midgut and the crop of *Rhodnius prolixus*, a vector of *T. cruzi*, has also been shown (Pereira *et al.*, 1981). Midgut lectins are induced in a periodic manner

following a bloodmeal with peak activity around 48-72 h (Abubakar *et al.*, 1995). The presence of lectins in haemolymph has also been demonstrated in other important vectors such as *Simulium* blackflies (Smail and Ham, 1989) and *Anopheles* mosquitoes (Scalzo-Lichtfouse *et al.*, 1990).

The role of midgut lectins in vector-parasite interaction is still not well understood. In *Aedes aegypti*, for example, studies have shown that midgut lectins prevent the migration of *Brugia pahangi* microfilariae into haemocoel (Phiri and Ham, 1990). However, feeding *Aedes aegypti* on the sugar N-acetyl-D-glucosamine, which specifically binds to the lectin, enhances migration of this parasite through the midgut (Huber *et al.*, 1991).

The role of haemolymph lectin in parasite development in vector is not clearly established but they have been implicated in the maturation of midgut established trypanosomes (Welburn and Maudlin, 1990). For example, the differential refractoriness of *Simulium* blackflies to different *Onchocerca* species is thought to be due to haemolymph lectins (Ham, 1988; Ham *et al.*, 1988; Hams and Garms, 1988). It has been demonstrated in tsetse (Croft *et al.*, 1982) and *Rhodnius prolixus* (Pereira *et al.*, 1981) that haemolymph lectins have different sugar specificities to the midgut lectin. In addition, different species of insects have midgut lectins that vary in their sugar specificities.

1.3.4.2 Role of tsetse midgut lectins in trypanosome development

In tsetse, midgut lectins have been implicated in a number of functions. Firstly, they have been shown to mediate lysis of the trypanosomes entering the fly with an infective bloodmeal (Maudlin, 1991). Secondly, they provide a signal for differentiation of the bloodstream trypanosomes that escape lysis (Maudlin and Welburn, 1988a,b). Thirdly, the maturation of established procyclics into the infective metacyclics has been shown to be mediated by lectin (Welburn and Maudlin, 1989).

In tsetse, the involvement of lectin in the susceptibility/ refractory mechanism has been studied mainly by incorporating the specific lectin-binding sugar, D-glucosamine, in the infective bloodmeals (Maudlin and Welburn, 1987). Inhibition of lectin activity using these sugars resulted in higher infection prevalences in both teneral and non-teneral tsetse indicating that lectins play a role in trypanosome development. Furthermore, infection prevalences in tsetse have been shown to be influenced by their midgut lectin titres at the time of an infective feed (Ingram and Molyneaux, 1991). Studies by Maudlin and Welburn (1987) reported very high midgut infection prevalences (> 90 %) in teneral *G. m. morsitans* when D+-glucosamine was included in the infective feed. Similarly, *G. m. morsitans* fed on bloodmeal containing procyclics *T. b. rhodesiense* and 0.06 M galactose developed very high midgut infections (95 %) compared to only 42 % in controls (Welburn *et al.*, 1994). Although higher midgut infections were reported in tsetse that fed on glucosamine, relatively fewer infections (8.3 %) against 45 % in control matured (Welburn and Maudlin, 1989). This clearly shows that lectins do play a role in the

maturation process (Welburn and Maudlin, 1989). In *Glossina palpalis palpalis*, superinfections were only obtained by a combination of D+-glucosamine and D+-galactose (Welburn *et al.*, 1993). This suggested that this tsetse species has a second lectin with galactosyl specificity and its inhibition results in high midgut infection. Welburn and Maudlin (1989) showed that bloodstream *T. congolense* transformed into procyclics but did not mature in *G. morsitans morsitans* maintained on a meal containing D+-glucosamine suggesting that this sugar has no role in the transformation process but is crucial in the maturation process. Although details are missing, the role of lectins in the mechanisms for susceptibility/ refractoriness in vector-parasite interaction appears crucial and requires further investigation.

A recent report on a *Glossina* specific molecule with both lectin and trypsin activities (Osir *et al.*, 1995; Abubakar *et al.*, 1995) is significant in understanding the role of bloodmeal induced molecules in trypanosome development. This molecule has been shown to stimulate trypanosome transformation (Osir and Imbuga, unpublished data). Its presence only in *Glossina* may possibly explain why tsetse flies are the only known vectors of trypanosomes.

1.3.4.3 Lectins attached to peritrophic membrane

Infection prevalences in tsetse have been observed to vary with age, with the tenerals being more susceptible than their non-teneral counterparts (Maudlin, 1991). These differences have been attributed to the levels of protective midgut lectins which are

released after a bloodmeal (Maudlin and Welburn, 1987, 1988a) and are presumably associated with the peritrophic membrane (Lehane and Msangi, 1991). Since tsetse secrete PM2, newly emerged tsetse lack a well formed peritrophic membrane lining their midguts but develops to full line the entire midgut within about 90 hours after eclosion (Lehane and Msangi, 1991). The well developed peritrophic membranes make older tsetse increasingly refractory to infection (Mwangelwa *et al.*, 1987). In non-tenerals, the peritrophic membrane not only act as a physical barrier to trypanosomes (Freeman, 1973) but also harbours the bloodmeal induced anti-trypanosomal lectins, thereby serving a dual role in preventing infection.

Gingrich *et al.* (1982) showed that infection in non-teneral tsetse is possible after prolonged starvation. This observation was attributed to reduced levels of midgut lectins whose stimulation following a bloodmeal is periodic (Welburn *et al.*, 1989) and not lack of peritrophic membrane. However, Lehane and Msangi (1991) disagree with this argument on the basis that high residual levels of midgut lectins (100-200 fold) present in starved non-tenerals compared to tenerals is still sufficient to overcome infection. This implies that the low infection prevalences in non-teneral tsetse could be due to factors other than a physical barrier such as a well formed PM. Furthermore, incorporation of sugars specific to midgut lectins to an infective meal results in higher infections in both teneral and non-tenerals suggesting that lectins and not PM *per se* are responsible for the low infection prevalences in older tsetse (Welburn and Maudlin, 1992; Welburn *et al.*, 1994).

1.4 Endosymbionts

1.4.1 Primary endosymbionts

Many economically important vectors that are dependent on restricted diets such as blood harbour bacterial symbionts which are crucial for their survival by supplying B-group vitamins (Buchner, 1965). Examples of such vectors include ticks, sucking bugs, beetles and lice (Ishikawa, 1990). These bacterial symbionts are either extracellular, for example gram positive *Rhodococcus rhodnii* found in *Rhodnius prolixus,* or intracellular, typically residing within specialized cells called mycetomes. Another category of symbionts includes intracellular reproductive parasites whose common example is the *Wolbalchia* species known to induce cytoplasmic incompatibility in some insects. These bacteria enhances their own transmission through the expression of cytoplasmic incompatibility (Beard *et al.*, 1993). Lack of primary endosymbionts can have devastating effects on the insect vector such as sterility or in, extreme cases, death. The lack of the symbionts would therefore affect the vectors' ability to transmit diseases.

1.4.2 Secondary gut symbionts

Secondary symbionts are not required for the vector host's survival and do not live in well defined mycetomes but live in association within the lower section of the midgut (Ishikawa, 1990). However, their presence may be important in determining vector competence. An example of secondary endosymbionts are the maternally inherited

Rickettsia-like organisms (RLOs) found in the midgut cells of several *Glossina* species (Reinhardt *et al.*, 1972; Pinnock and Hess, 1974).

The presence of RLOs in tsetse is linked to their susceptibility to trypanosomes (Welburn *et al*, 1993). RLOs release the enzyme chitinase that digests chitin resulting in accumulation of glucosamine during the late pupal stage. The glucosamine specifically inhibits midgut lectins. Since midgut lectins have anti-trypanosomal effect, their inhibition by glucosamine results in increased infection prevalences (Maudlin and Welburn, 1988b; Baker *et al.*, 1990). In contrast, refractory tsetse with few RLOs will accumulate relatively less glucosamine making the trypanosomes vulnerable to lectin activity.

Maudlin and Welburn (1988b) established that the proportion of midgut infection of *Trypanosoma brucei* and *T. congolense* maturing was significantly increased when infected tsetse were maintained on a diet that contained D-glucosamine. D-glucosamine was also shown to inhibit *G. m. morsitans* midgut trypsin activity (Osir *et al.*, 1993). This implies that a concerted inhibition of both lectins and trypsins by glucosamine further increases the chances of trypanosomes development. Since midgut lectins increases after bloodmeal, the differences in lectin levels due to RLOs disappears in non-tenerals. Therefore, the involvement of RLOs in the susceptibility/ refractoriness to *Trypanosoma* is restricted only to teneral tsetse. It was also shown that rickettsial infections are not associated with susceptibility of *G. m. centralis* to *T. congolense* infections (Moloo and Shaw, 1989).

1.5 The role of bloodmeal

Tsetse are obligatory bloodfeeders with mammals being the preferred hosts (Jordan, 1986). It is possible to identify the host of tsetse and other haematophagous insects by analysing their bloodmeal content and this has proved useful in the epidemiology of arthropod-borne diseases (Boreham, 1975; Blackwell *et al.*, 1994). Tsetse derives all their metabolic requirements from bloodmeal digestion (Bursell *et al.*, 1974; Kabayo and Langley, 1985). Within the midgut, the blood is held in a sac-like pouch, the peritrophic membrane (Lehane and Msangi, 1991). In the tsetse gut, the crop acts as a temporary store for bloodmeal, while the anterior midgut is the site of rapid diuresis (Gee, 1975). Bloodmeal digestion takes place in the posterior midgut (Dow, 1986) while nutrients absorption occurs in the hindgut where ionic balance is also maintained (Phillips *et al.*, 1986). The release of digestive enzymes to the posterior midgut is under hormonal control and the levels are proportional to meal size at least within 0-24 h of feeding (Gooding, 1974). In *Glossina*, proline is the most abundant amino acid after bloodmeal digestion and is important for energy metabolism (Bursell *et al.*, 1974). High concentrations of this amino acid in haemolymph and flight muscles has been reported (Bursell, 1960, 1963, 1966). It has been shown that during flight, there is a rapid disappearance of proline followed by stoichiometric rise in alanine within the first 2 minutes (Bursell, 1963). The levels of glutamate also increase after the first minute of flight while α-ketoglutarate levels increase 10-fold after 2 minutes. These observations have led to the elucidation of the pathway through which proline is partially oxidized to

alanine to provide energy required for flight activity (Bursell *et al.*, 1974). A similar pathway has now been found in other proline metabolizing insects notably the Colorado potato beetle, *Leptinotarsa decemlineata* (Weeda *et al.*, 1980a,b). It has also been established that following flight, tsetse are capable of re-synthesizing proline from alanine and fat reserve in the fat body (Bursell *et al*, 1974). In this process, alanine is converted to pyruvate by transamination with α-ketoglutarate then carboxylated to oxaloacetate and finally condensed with acetylcoenzyme-A, a two carbon fragment derived from fatty acid oxidation (McCabe and Bursell, 1975; Konji *et al.*, 1984). The α-ketoglutarate so formed undergoes transamination and subsequent reduction to yield proline which is then utilized for energy generation (McCabe and Bursell, 1975; Konji *et al.*, 1984).

Apart from being an important metabolite to tsetse, proline is also a very important source of carbon and metabolic fuel for the midgut form trypanosomes (Evans and Brown, 1972; Srivastava and Bowman, 1971).

1.6 Justification for studying the role of host blood in the differentiation of bloodstream trypanosomes

Previous studies have shown that tsetse infection prevalences upon taking an infective bloodmeal is influenced by the host blood (Mihok *et al.*, 1991, 1993; Moloo, 1981, 1984). Using a diverse range of host blood samples, Mihok *et al* (1993) demonstrated that host blood at the time of infective feed determines the rate at which tsetse developed infection. Generally, wildlife blood samples gave lower infection prevalences than domestic hosts. Further studies have shown that the host blood upon

which tsetse are maintained after an initial infective feed is also important in determining infection prevalences (Moloo, 1984; Mihok *et al.*, 1991). However, the blood factor(s) that influence trypanosome infections in tsetse are not very well understood.

In the blood of some primates and wild Bovidae, trypanolytic factors have been reported (Mulla and Rickman, 1988; Seed *et al.*, 1990; Olubayo, 1991). For example, such factors in plasma of waterbuck (Mulla and Rickman, 1988), buffalo and eland (Reduth *et al.*, 1994), could possibly be directly involved in determining infection prevalences in tsetse feeding on these hosts. Removal of serum from tsetse diet enhanced midgut infection of *Trypanosoma congolense* in *G. m. morsitans* (Maudlin *et al.*, 1984). It has also been shown that some factors found in normal human serum lyse *T. b. brucei* but not *T. b. rhodesiense* and *T. b. gambiense* (Rifkin, 1978) despite the fact that the three trypanosome species are very closely related (Hoare, 1972). The lytic factor has now been identified as a sub-fraction of high density lipoprotein (HDL) (Hajduk *et al.*, 1989). The basis of resistance of the other two members of the brucei sub-group to TLF is still not well understood and is currently under investigation (Hajduk, personal communication). A recent study showed that the binding of TLF to the trypanosome receptors is unaffected such that resistance is as a result of reduced endocytosis (Hager and Hajduk, 1997). Such information may be useful in developing strategies to combat human trypanosomiasis.

Within the tsetse midgut, bloodmeal stimulates the release of various factors namely trypanoagglutinins (lectins), trypanolysins, trypsin/ trypsin-like enzymes which

mediate transformation as well as lysis of ingested trypanosomes (Maudlin, 1991; Imbuga *et al.*, 1992a). Although blood has been shown to be crucial for this process (Imbuga *et al.*, 1992a), it is still not clear how different host blood and blood components influence this process and whether this is related to the observed infection prevalences.

Proline, a product of bloodmeal digestion, is the most abundant amino acid in *Glossina* midgut and haemolymph (Cunningham and Slater, 1974; Vickerman, 1985). The development of procyclics is intimately associated with a switch from gross utilization of glucose to proline. Studies using *T. brucei* have shown that midgut of uninfected tsetse have a much higher concentration of proline than their infected counterparts 24 h after a bloodmeal. This suggests an *in vivo* utilization of proline by the procyclic form trypanosomes (Vickerman, 1985). Even with such information, the role of the bloodmeal digestion products, particularly proline and glutamine, in the transformation of trypanosomes from bloodstream to procyclic forms has so far not been studied and therefore needs to be established.

Significant progress has been made in the elucidation of host blood factors that lyse bloodstream trypanosomes. This is an effort to understand why, for example, wild animals are generally resistant to trypanosomiasis (Ashcroft, 1959). It is crucial to establish how host blood and blood components influence trypanosome transformation in the vector. Although information is available on infection prevalences obtained when tsetse are fed on different host blood, details on how the host blood influences the observed infection prevalences are scarce. Therefore, it is important to establish how host

blood influence the release of the various midgut factors, such as trypsin, that mediate differentiation and lysis of the trypanosomes. To gain insight into this complex problem, the present study was carried out.

1.7 Aims and objectives

The overall aim of this study was to understand the role of host blood in the transformation of bloodstream trypanosome into procyclic forms. The specific objectives were:

(1) Study the influence of host blood (from eland, buffalo, goat and rat) on differentiation of bloodstream trypanosomes.

(2) Investigate the blood component(s) necessary for differentiation.

(3) Study the stimulation of trypsin/trypsin-like enzymes by the host blood and blood components.

(4) Study the role of proline/ glutamine (as products of bloodmeal digestion) on trypanosome differentiation.

CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 Reagents

The reagents used in these studies were of analytical grade. They were obtained from Sigma, UK; Amersham, England; NEN, Du Pont; Serva, Germany; BDH, England; Pharmacia, Uppsala, Sweden; Boehringer-Mannheim GmbH Biochemica, Germany; Biorad, Richmond; Pierce, Rockford, USA; Richter, Budapest, Hungary.

2.2 Animals

Adult Wistar rats 2-4 months old were supplied by the ICIPE Animal Rearing and Quarantine Unit. Eland (*Taurotragus oryx*) and African buffalo (*Syncerus caffer*) were maintained in a tsetse-free environment at the Wildlife Disease Section of the Kenya Agricultural Research Institute (KARI) at Kabete, Kenya. The African goat was maintained at the Large Animal Breeding Unit of the ICIPE.

2.3 Tsetse

Glossina morsitans morsitans were reared in the ICIPE (International Centre of Insect Physiology and Ecology) insectary in Nairobi at a temperature of 25±1° C and a relative humidity of 65-85 %. This colony was established from a Zimbabwean stock maintained by the Tsetse Research Laboratory in Langford, UK. Unless otherwise stated, the tsetse were supplied

as tenerals and maintained at the above conditions for 24 h before the experiments.

2.4 Maintenance of trypanosomes

In this study, pleomorphic *Trypanosoma brucei brucei* of a stock derived from EATRO 1969 was used. The stock was isolated as previously described (Otieno *et al.*, 1983). The trypanosome were originally grown in rats from stabilates that had been cryopreserved in liquid nitrogen. Trypanosomes were maintained in Wistar rats by serial passage and were harvested only at high parasitaemia.

2.5 Collection of blood samples.

Blood samples were regularly collected from eland, rat, buffalo and goat. To collect blood from eland and buffalo by jugular venipuncture, the animals were restrained in a clash while the goat was manually restrained. Heads were held in such a way as to expose their jugular veins and the point of blood collection thoroughly sterilized with 70 % ethanol. Heparin (Richter, Budapest, Hungary) (10 IU/ ml) was used as the anticoagulant. For serum preparation, blood was drawn as before but without anticoagulant. Blood from rat was collected by cardiac puncture into heparinized syringes.

Plasma was obtained from freshly collected blood by centrifugation (1000 x *g*, 15 min, 4° C) in a Heraeus 2 Minifuge (Osterode, Germany). The plasma was carefully separated from the cells and maintained at 4° C before use. For RBC preparation, the cellular portion was washed three times in normal saline (0.85 % (w/v) NaCl). Serum was obtained from the coagulated blood by carefully removing the clot followed by centrifugation (1000 x *g*, 20 min, 4° C) of the

supernatant fraction to remove cells (Bessis, 1973).

2.6 Artificial membrane feeding of tsetse

Tsetse were allowed to feed on the blood samples through a heat sterilised (100° C, overnight) silicon membrane maintained at about 37° C by a warming template (Mews and Ruhn, 1971).

2.7 Isolation of trypanosomes by isopycnic centrifugation

For transformation assays, trypanosomes were isolated from parasitized rat blood as previously described (Grab and Bwayo, 1982). Parasitized blood was mixed with an equal volume of phosphate buffered saline (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4) containing 1 % glucose (PSG). The content was mixed and an equal volume of Percoll (Pharmacia) working solution [8.55 % (w/v) sucrose, 2 % (w/v) glucose in Percoll, pH adjusted to 7.4 with HEPES] added. After thorough mixing, the contents were centrifuged (17,500 x *g*, 4° C, 30 min). The concentrated layer of trypanosomes appearing near the top below the platelet layer was carefully siphoned out using a Pasteur pippette and washed three times in PSG by centrifugation (1,500 x *g*, 4° C, 10 min). Trypanosomes were resuspended in a minimal volume of PSG and the density determined by counting in a haemocytometer with improved Neubauer ruling.

2.8 Transformation assays

Transformation assays were carried out as previously described (Imbuga *et al.*, 1992a). In the *in vitro* assay system, teneral tsetse (24 h after emergence) were allowed to feed on the blood samples through an artificial membrane. After 1 h the tsetse were immobilized by brief chilling and the midguts carefully dissected out. The midguts were gently homogenized and freshly isolated trypanosomes (10^7 trypanosomes/ ml) added. After gently vortexing, the contents were incubated at 25° C. At different times, the incubation mixture was vortexed gently and 5 ml aliquots withdrawn for preparation of thin smears.

In the *in vivo* studies, tsetse were fed on parasitized samples (10^7 trypanosomes/ ml) and the midguts were dissected at various times for preparation of wet smears.

The wet smears were air dried, fixed in absolute methanol for 5 min and stained in Giemsa stain (freshly prepared from a pre-made stock by diluting x 10 in 0.1 M sodium phosphate buffer pH 7.2). Trypanosomes were examined using a Dialux compound microscope (Leitz Wetzler, FRG). Typically, 3-5 groups of 100 trypanosomes each were counted and classified as being typical bloodstream forms, transition forms or midgut forms based on morphological characteristics (Lloyd and Johnson, 1924; Ghiotto *et al.*, 1979). The position of kinetoplast relative to the nucleus, size and mitochondrial staining were the most important morphological characteristics used.

2.9 Preparation of red blood cells

Whole blood samples were centrifuged (1000 x *g*, 4° C, 10 min) to separate cells from plasma. After removing the plasma the "buffy coat" was carefully removed. The remaining RBCs were washed 3 times in normal saline (0.85 % (w/v) NaCl) each time removing the remaining buffy coat.

2.10 Preparation of red blood cell membranes

Red blood cell membranes were prepared by suspending washed cells in 10 volumes of distilled water and leaving them to stand for 30 min at 4° C. The content was centrifuged (10,000 x *g*, 4° C, 30 min) (Bessis, 1973). The pellet was washed once in distilled water then resuspended in normal saline to the original (for eland RBCs) or twice (for rat RBCs) volume of cells. The membrane suspensions were fed to tsetse through an artificial membrane and midguts dissected out for use in transformation assays (section 2.8). Rat RBC membranes were also prepared by one cycle of freezing (-196° C) and thawing (37° C). Membranes were also prepared from rat RBCs by sonicating a 50 % (v/v) cells suspension in normal saline three times for 30 sec at a setting of 10 on a MSE Soniprep 150 sonicator, with 60 second periods of cooling on ice between each burst. The lysates were centrifuged (10,000 x *g*, 4° C, 30 min). The membranes were then washed once in saline then resuspended in an equal volume of the saline before feeding to tsetse through an artificial membrane. Midguts dissected from these tsetse were used in transformation assays.

2.11 Properties of the plasma inhibitor

2.11.1 Thermostability of the inhibitor factor

Seven, 3 ml fresh eland plasma aliquots were separately incubated in a water bath at constant temperatures of 4, 22, 37, 42, 50, 60 and 70° C for 30 minutes. Washed eland RBCs (3 ml) were then added to the plasma aliquots and artificially fed to tsetse. Transformation using dissected midguts was carried out as before.

2.11.2 Effect of freeze-thawing on the stability of the eland plasma inhibitor factor

Fresh eland plasma was divided into six, 3 ml aliquots in 50 ml plastic tubes. The aliquots were separately subjected to 0 (control), 1, 2, 3, 4, and 6 freezing (-70° C) and thawing (37° C) cycles. Washed eland RBCs (3 ml) were added to these samples and fed to tsetse. Midguts dissected from the tsetse were used in transformation assays.

2.11.3 Effect of preincubating isolated trypanosomes in fresh eland plasma on transformation inhibition.

Isolated trypanosomes were suspended in fresh eland plasma for 0, $^1/_2$, 1, and $1^3/_4$ h at room temperature with gentle agitation. After the incubation periods, trypanosome were separated from plasma by centrifugation (2500 rpm, 10 min, 25° C) in a Microfuge. After washing, the trypanosomes were mixed with midgut homogenates dissected from tsetse previously fed on a 50 % (v/v) suspension of eland RBCs in normal saline. Transformation of trypanosomes was then assessed.

2.11.4 Effect of dialysing eland plasma

The following experiment was carried out to assess whether dialysis of eland plasma would result in loss of the inhibitor activity. Fresh eland plasma was dialysed overnight in PBS using a membrane with a molecular weight cut-off (MWCO) of M_r 14,000. The dialysed plasma (3 ml) was mixed with an equal volume of washed eland RBCs and fed to tsetse. Fresh plasma kept at 4° C for the same duration was used as a control. Washed eland RBCs (3 ml) were added to the aliquots and fed to tsetse. Transformation was carried out as before using the dissected midguts.

2.11.5. Effect of lyophilizing plasma on the stability of the inhibitor.

Fresh eland plasma (10 ml) was frozen (-70° C) in a 50 ml plastic tube and lyophilized using a Virtis freeze-drier (Virtis Co., Inc., Gardiner, New York). After this, the weight of the powdered plasma was recorded and stored at -20° C. To assess the effect of freeze-drying fresh plasma on the activity of the transformation inhibitor, a weight equivalent to 3 ml of the original plasma was reconstituted in distilled water, mixed with an equal volume (3 ml) of washed eland RBCs and fed to tsetse. After 1 h, midguts were dissected from these tsetse and used in transformation assays.

2.11.6 Ammonium sulfate fractionation of the eland plasma

Saturated ammonium sulphate (SAS) (pH adjusted to 7.4 by ammonium hydroxide) was added to 50 ml of fresh eland plasma maintained in an ice bath with constant stirring. Protein

fractions were collected from 0-10, 10-20, 20-30, 30-40, 40-50, and 50-60 % (wt/v) SAS by centrifugation (10,000 x *g*, 4° C, 15 min). At each SAS precipitation step, the insoluble proteins were obtained by centrifugation (10,000 x *g*, 4° C, 30 min). The pellets were redissolved in a minimum volume of PBS and dialysed extensively in the same buffer to remove excess salt. A 2 ml protein solution of each fraction was mixed with an equal volume of washed RBCs and fed to tsetse. Transformation assays using the dissected midguts were then carried out.

2.11.7 Effect of proteases on the plasma inhibitor

2.11.7.1 The effect of pronase

Fresh eland plasma was divided into four, 3 ml fractions. The fractions were incubated in a water bath (30° C, 30 min). Aliquots of a freshly prepared stock pronase (Sigma, St. Louis, MO, USA) (5 mg/ml in PBS) was added to the eland plasma aliquots to attain a final concentration of 0 (control), 0.05, 0.1 and 1.0 mg/ ml, respectively. The content was mixed and incubated (2 h at 30° C). After this period, the pronase treated plasma was dialysed in PBS (4° C, 2 h).

For transformation assays, washed eland RBCs were mixed with the pronase treated plasma in a 1:1 ratio and fed to tsetse. Transformation using the dissected midguts was carried out.

2.11.7.2 Effect of trypsin

The effect of treating eland plasma with bovine pancrease trypsin (Serva, Feinbiochemica, Heidelberg) was assessed as described under section 2.11.7.1. except that the

trypsin inhibitor, tosyl lysine chloromethyl ketone (TLCK), was added and incubated (30° C, 20 min), before dialysis. Washed eland RBCs were mixed with the trypsin treated plasma on a 1:1 ratio and fed to tsetse. Transformation using midguts dissected from the tsetse was then carried out.

2.11.8 Effect of protease inhibitors on the stability of the inhibitor factor

The effect of protease inhibitors on the stability of eland plasma inhibitor factor was assessed as previously described (Reduth *et al.*, 1994) except that diethylpyrocarbonate was used instead of leupeptin. To 6 ml of freshly obtained eland plasma, the protease inhibitors were added to the following final concentrations; 40 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) (predissolved in a minimum volume of isopropanol), 2 mM DL-dithiothreitol (DTT) (Sigma, St. Louis, MO, USA), 10 mg/ ml aprotinin (Boehringer Mannheim GmbH, FRG), 4 mM EDTA and 150 mM diethylpyrocarbonate (Serva, Feinbiochemica, Heidelberg).

The 6 ml plasma with protease inhibitors was divided into two, 3 ml portions. Another two, 3 ml portions of fresh plasma without inhibitors were included as controls. The fractions were stored for 6 days at either -20° C or 4° C.

After this period the protease inhibitors were dialysed for 24 h with several changes of PBS followed by dialysis in 0.15 M NaCl for 2 h. Washed eland RBCs (3 ml) were mixed with each of the dialysed plasma aliquots and fed to tsetse. Midguts were dissected and transformation assays carried out.

2.12 Fractionation of eland plasma.

A DEAE-Sepharose CL-6B (Pharmacia, Uppsalla, Sweden) column (1.5 x 18 cm) was equilibrated overnight in 20 mM Tris-HCl buffer, pH 7.4. The flow rate was adjusted to 13.2 ml/h. Fresh eland plasma (9 ml) dialysed against the equilibrating buffer was loaded onto the column at the same flow rate. Elution of the unbound proteins was carried out by washing the column in equilibrating buffer until the protein absorbances at 280 nm were equal to that of the blank.

A stepwise gradient 0.1 M, 0.2 M, and finally 0.5 M NaCl in equilibrating buffer was used to elute the bound proteins. The resulting fractions were pooled, concentrated by dialysis in polyethylene glycol (PEG) powder then dialysed in Tris-buffered saline (0.15 M NaCl in 20 mM Tris-HCl, pH 7.4) to remove the excess salts. The ability of the protein fractions to inhibit transformation were then assayed.

2.13 Protein estimation

Protein estimation was carried out by the Bicinchoninic acid (BCA) protein assay method (Pierce, Co.) according to the manufacturers' protocol. Bovine serum albumin (BSA) fraction V (Serva, Heidelberg, Germany) was used as the protein standard. Absorbance was measured at 562 nm using a model DU-50 Beckman spectrophotometer (Beckman, Palo Alto, CA., USA).

2.14 Polyacrylamide gel electrophoresis (PAGE)

Native polyacrylamide gel electrophoresis (Native-PAGE) was performed as described by Laemmli (1970). The gradient gels (4-20 % acrylamide) were cast using gradient maker (BRL).

Where necessary, samples were extensively dialysed against PBS prior to electrophoresis. Samples were dissolved in an equal volume of sample buffer (0.13 M Tris-HCl, 20 % glycerol, 0.002 % bromophenol blue, pH 6.8) and mixed thoroughly prior to loading onto the gel. Electrophoresis was carried out at a constant voltage (70 volts) at room temperature in 25 mM Tris-HCl, 192 mM glycine pH 8.3.

After electrophoresis, the gels were stained overnight for proteins with 0.6 % Coomassie Brilliant Blue in methanol, acetic acid and distilled water in ratios of 50:9.2:40.8, respectively. The gels were then treated with several changes of destaining solution (methanol, acetic acid, distilled water; 50:9.2:40.8) at room temperature. Native molecular weight of the proteins were estimated from the relative migration of the high molecular standards (Pharmacia).

2.15 Effect of proline/ glutamine on transformation

The influence of proline and glutamine on bloodstream trypanosome transformation was investigated, *in vitro*, using two assay systems. One utilised teneral midgut homogenates while the other one used fed homogenates. Groups of ten, 24 h old teneral *G. morsitans* were dissected and midguts homogenised. L-proline or L-glutamine (Sigma, St. Loius, USA) (from a stock freshly prepared in PBS) was added to homogenates to the following final concentrations; 0 (control), 2, 4, 6, 8, 10, 12, 14, 16, 20, and 50 mM. Freshly isolated trypanosomes were added (10^7 trypanosomes/ ml) and mixed. Transformation was assessed as already described. This experiment, was repeated using the same proline and glutamine concentrations but in midgut homogenates from tsetse that were fed on rat blood. Transformation was assessed as above.

2.16 Qualitative and quantitative studies on trypsins stimulated by host blood and blood fractions

2.16.1 Trypsin assays

Trypsin activity was assayed using a chromogenic substrate, carbobenzoxy-val-gly-arg-4-nitrianilide acetate (Chromozym-TRY; Boehringer Mannheim; FRG). The reaction mixture contained midgut homogenate appropriately diluted to 0.95 ml in 0.1 M Tris-HCl, pH 8.0 and maintained at 30° C. The reactions were initiated by the addition of 40 mmol of substrate (predissolved in 0.1 M Tris-HCl pH 8.0) and the increase in absorbance at 405 nm monitored using a Beckman model DU 50 spectrophotometer (Palo Alto, Calif, USA) fitted with a thermostat control. The change in Molar extinction coefficient at 405 nm (e_{405} = 8,800; Erlanger *et al.*, 1961) was used to determine the amount of substrate hydrolysed; 1 unit of trypsin is the amount of enzyme required to hydrolyse 1 mmol Chromozym-TRY min^{-1} (30° C).

2.16.2 Qualitative analysis of trypsin/ trypsin-like enzymes in tsetse midgut after feeding on different blood samples

Trypsin isoenzyme analysis using radioactive inhibitor [1, 3 ^{3}H]-Diisopropyl fluorophosphate (DFP) was carried out as previously described (Borovsky and Schlein, 1988). Teneral *Glossina m. morsitans* (24 h after emergence) were fed on the following blood samples; rat blood, eland blood, 50 % (v/v) eland RBC suspension in normal saline, 50 % (v/v) eland RBC suspension in saline, rat plasma and eland plasma. At 0, 2, 5, 8, 20, 24, 48, and 72 h after feeding, six tsetse flies were dissected from each group and their midguts separately

homogenized in 60 ml of 0.1 M Tris-HCl, pH 7.8 containing 0.1 M $CaCl_2$. The homogenate was centrifuged (10,000 rpm, 10 min, 4° C) in a Microfuge Model 5415 C (Eppendorf, Netheler, Hinz, GmbH, Hamburg). The equivalent of one midgut (10 ml) of the supernatant was removed for trypsin analysis. Before separation by native-PAGE, the 10 ml supernatants were first incubated with 10 mM of cold tosylamide-2-phenyl chloromethylketone (TPCK) (Sigma, St. Louis, MO, USA), a chymotrypsin inhibitor for 6 h at 4° C. Three mCi of [1, 3 ^{3}H] DFP (specific activity 35 mCi/mmol, NEN, Du Pont) was added, vortexed and then incubated (20 h, 4° C). An equal volume of native-PAGE sample buffer was added, mixed and centrifuged (10,000 rpm, 2 min, 4° C) to remove any debris. The labelled trypsins were separated on native-PAGE (4-20 % acrylamide). The gels were stained using Coomassie Blue. The radioactivity signal in the gel was enhanced by incubating in Amplify™ (Amersham, UK) for 30 min followed by drying in a Hoefer slab gel drier model SE 1160 (Hoefer Scientific Instruments, San Francisco, USA). The gels were exposed to X-ray films (Fuji RX) for 7 days at -70° C. To reveal trypsin bands the films were developed after seven days.

2.17 Statistical analysis

Statistical analysis were performed using the Statistical Analysis System (SAS, 1985). Means were tested for significance using Analysis of Variance (ANOVA). Rejection of the null hypothesis was based on 0.05 % confidence interval.

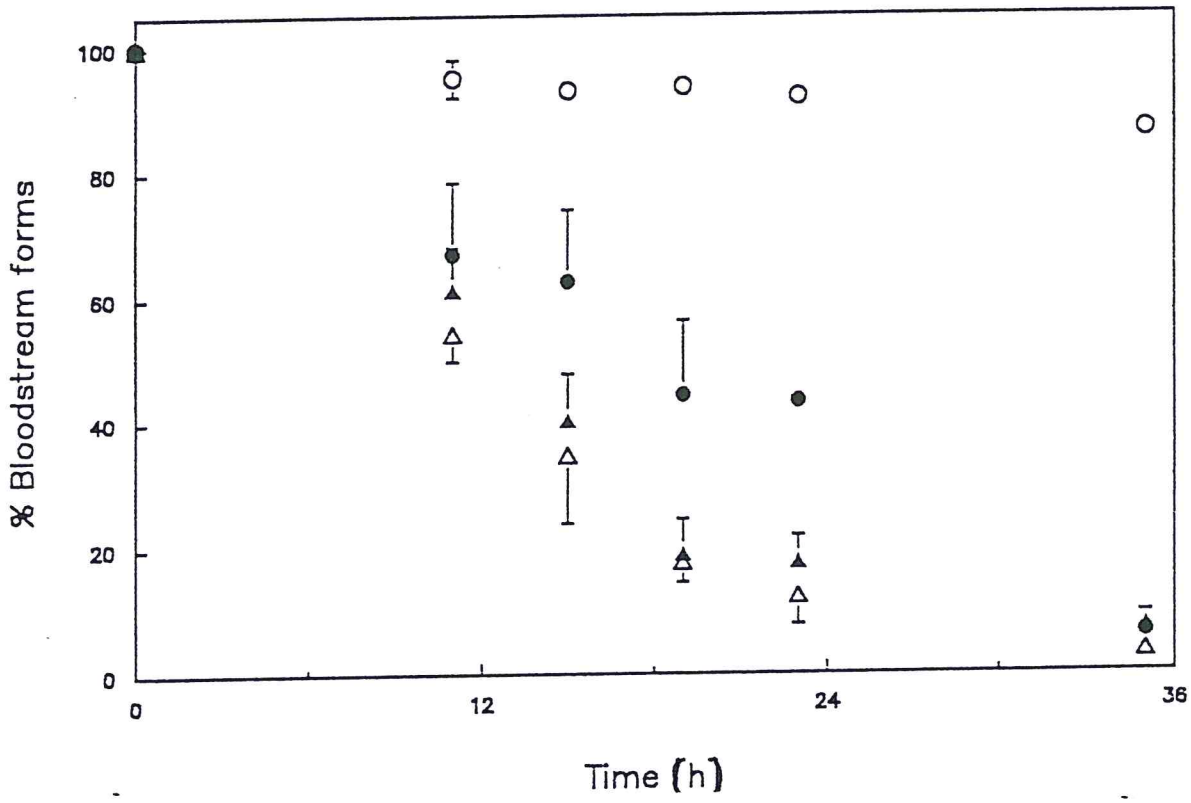
CHAPTER 3

3.0 RESULTS AND DISCUSSION

3.1 ROLE OF HOST BLOOD, BLOOD COMPONENTS AND AMINO ACIDS (PROLINE AND GLUTAMINE) IN TRYPANOSOME TRANSFORMATION

3.1.1 Trypanosome transformation in various host blood samples

Transformation of bloodstream trypanosomes by midgut samples prepared from tsetse previously fed on eland, buffalo, goat and rat blood samples was carried out *in vitro*. Transformation occurred readily in assays involving rat and goat blood samples (Fig. 1). Approximately 50 % of the bloodstream trypanosomes had transformed into either transition or procyclic (midgut) forms after 10 h of incubation at 27° C. In contrast, buffalo blood supported a slower transformation rate, with 50 % of the trypanosomes having transformed after 18 h. Trypanosomes incubated with midgut homogenates from tsetse that had fed on eland blood had very low transformation rates. In this case, less than 10 % of the trypanosomes had transformed compared to 60 %, 80 % and 85 % in buffalo, rat and goat blood, respectively. Apart from the low transformation rates, lysis of trypanosomes occurred in all experiments where whole eland and buffalo blood was used.


% Bloodstream forms
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36
Time (h)

3.1.2 Influence of blood samples from different elands on trypanosome transformation

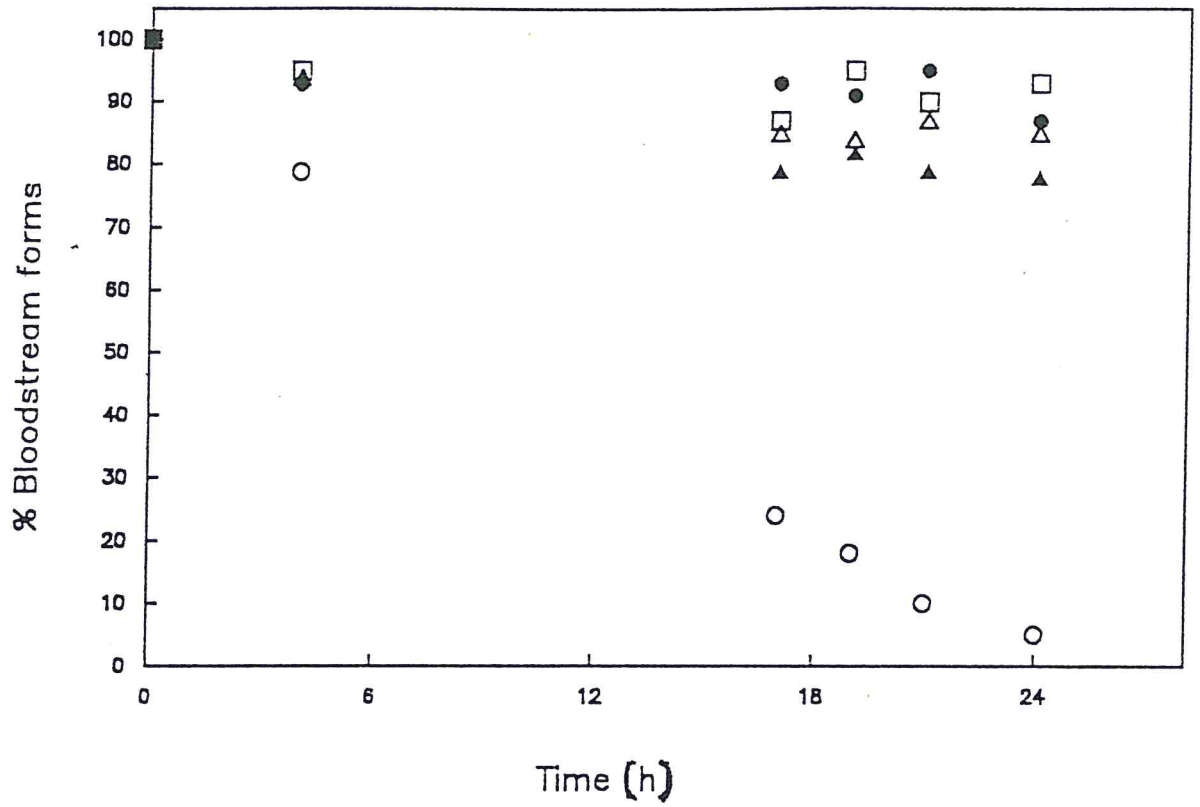
Since blood samples from eland number 7695 did not support transformation, it was necessary to establish whether blood samples obtained from other elands, held together in captivity, had similar transformation inhibition properties. Blood was obtained from four elands aged between 1-6 years, born and bred in captivity in a tsetse free environment. The identification numbers and institutions to which the elands were assigned were given as 7788 (International Livestock Research Institute), 7695 (International Centre of Insect Physiology and Ecology), 7827 and 7843 (Kenya Agricultural Research Institute). Eland number 7843 was the youngest. However, blood from its mother was not tested. Figure 2a clearly showed that fresh blood from all the elands did not support trypanosome transformations. Based on transformations after 24 h (Fig. 2b), there were no significant differences among the blood samples from the four elands ($P < 0.05$). However, transformations between the elands samples and the rat blood (control) were significant ($P < 0.05 = 0.001$)

In order to elucidate the blood factor(s) involved in inhibiting trypanosome transformation, all subsequent experiments were carried out using rat and eland blood samples as positive and negative controls, respectively. The results given in this study therefore, are based on blood samples collected from one eland (number 7695).

3.1.3 The effect of mixing eland and rat blood on trypanosome transformation

The transformation of trypanosomes by midgut homogenates of tsetse fed on mixtures of eland and rat blood samples was studied. The results showed that increasing the proportion of eland blood in rat blood led to a progressive decrease in the transformation rates (Fig. 3). Virtually no transformation occurred in 100 % eland blood.

(a)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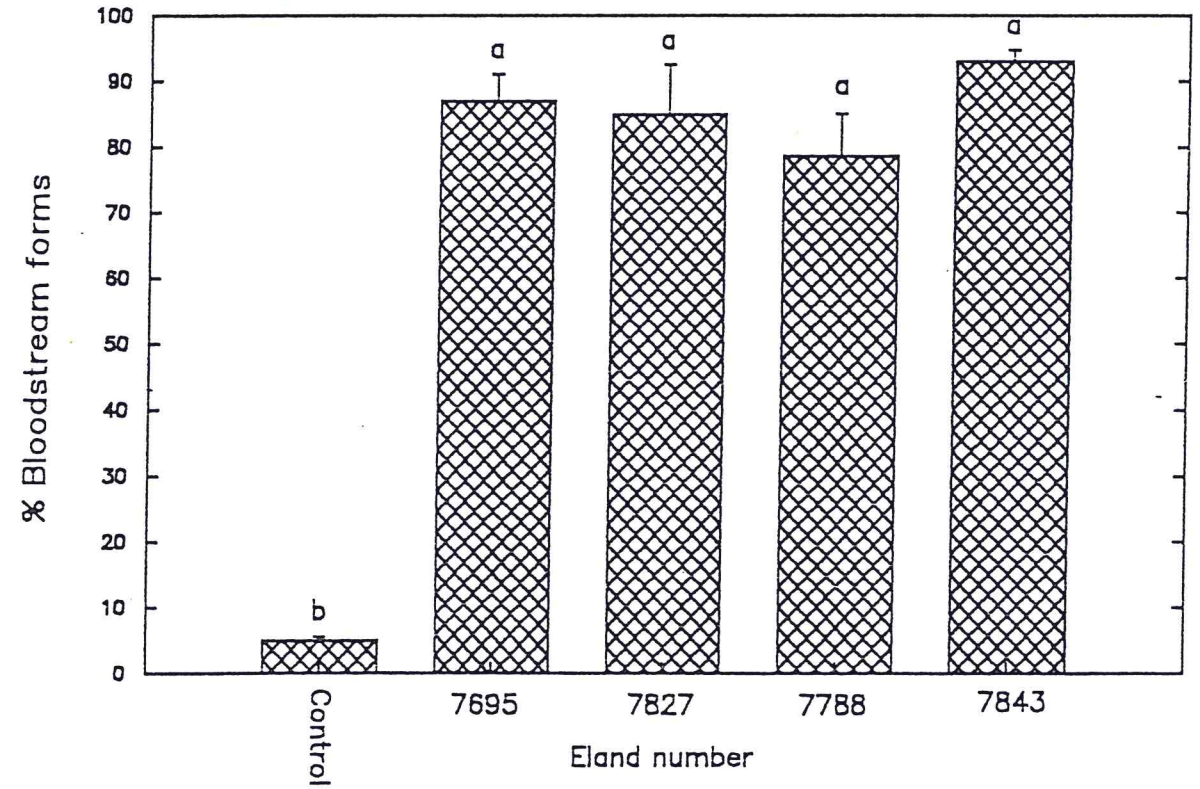
Time (h)

(b)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Control
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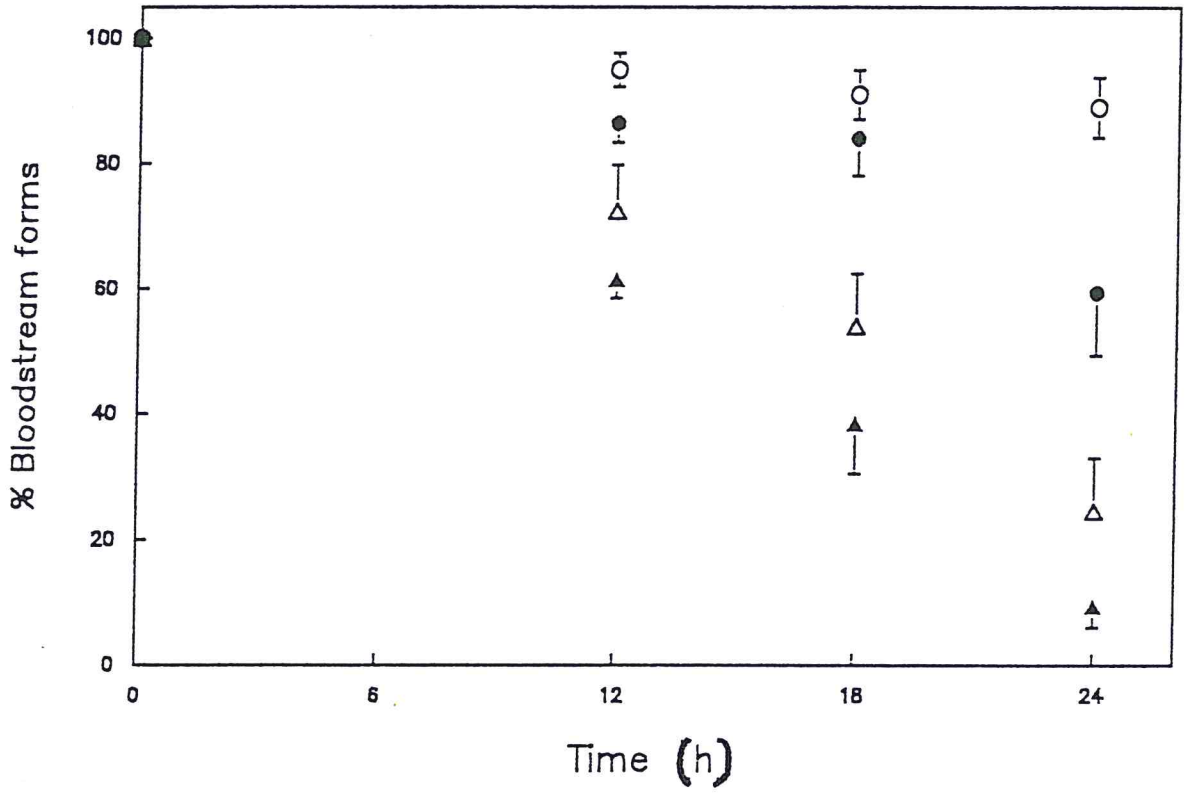
7788
7843
Eland number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Time (h)

3.1.4 Trypanosome transformation in rat blood fractions

Transformation of the trypanosomes occurred only in rat blood fractions that contained cells, for example, total cells suspended in either plasma or serum (Fig. 4). Plasma or serum alone or a mixture of the two did not support the process. A 50 % (v/v) total cell suspension in normal saline also supported transformation, except that, compared to whole blood, a delay in the start of the process was observed (Fig. 5). By 18 h, about 62 % of the trypanosomes were still untransformed in homogenates from tsetse fed on a 50 % (v/v) cell suspension compared to 42 % in homogenates from tsetse that had fed on whole blood (control). On the other hand, a reduction in the concentration of cells resulted in a decrease in transformation rates such that by 18 h, 83 % were not transformed (Fig. 5). A similar observation was made using a 50 % (v/v) RBC suspension in saline (data not shown). However, removal of RBCs from rat whole blood resulted in very low rates of trypanosome transformation (data not shown).

3.1.5 Trypanosome transformation in eland blood fractions

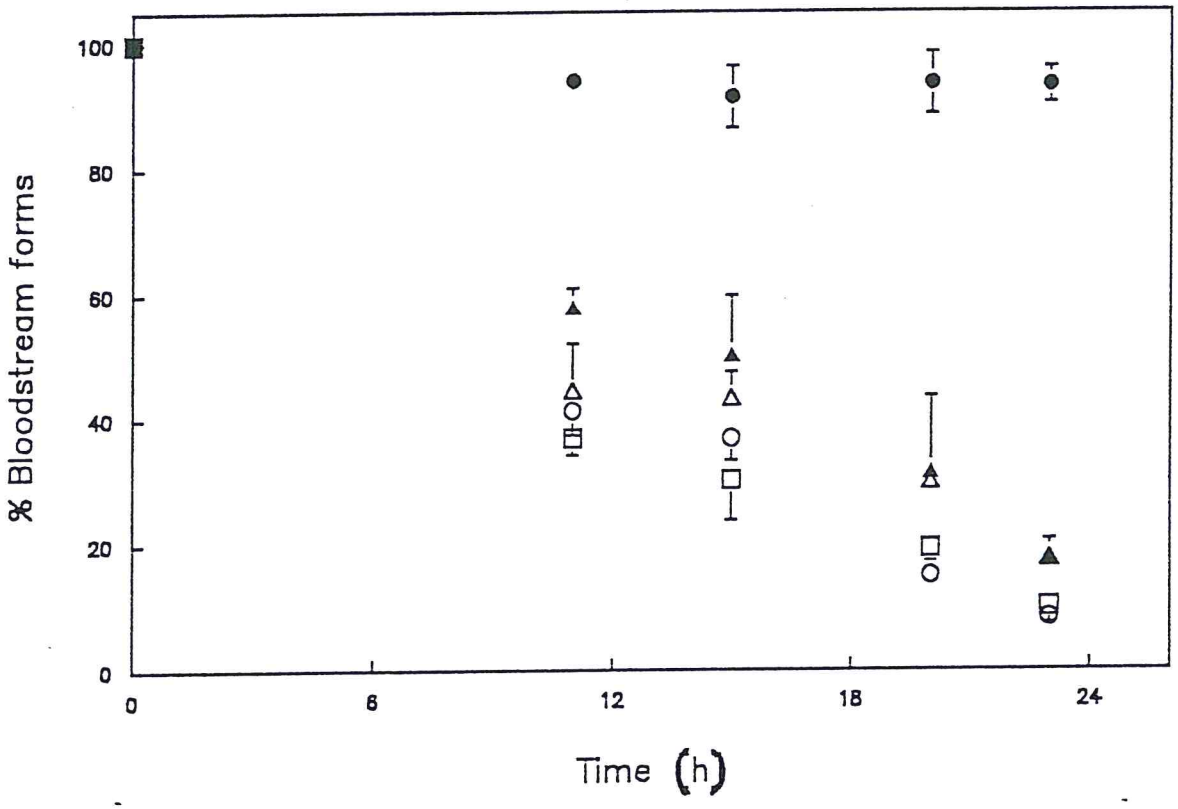
The fractions prepared from whole eland blood showed very low capacities to support trypanosome transformation. Plasma, serum or total cells suspended in either plasma or serum gave very low rates of trypanosome transformation (Fig. 6). In all these cases, approximately 85 % of the trypanosomes were still untransformed after 24 h of incubation. In contrast, the process was almost complete by the same time in rat whole blood (Fig. 6). However, a 50 % (v/v) suspension of eland total cells in saline gave rates of trypanosomes transformation as control (rat blood) (Fig. 7a). These result indicated that the low capacity of whole eland blood to support trypanosome transformation was due to the plasma fraction. The ability of a suspension of eland cells in rat plasma to support transformation was also studied. In this case, a transformation rate comparable to

that of whole rat blood was observed (Fig. 7a). Thus, eland cells suspended in rat plasma gave a better transformation rate than when suspended in saline. As was the case with rat blood fraction where RBCs were removed, a similar fraction from eland blood did not support the process (Fig. 7a).

The ability of midgut homogenates prepared from tsetse fed on washed rat blood cells suspended in eland plasma to transform trypanosomes was also examined. The low rate of transformation observed in this case suggested the inhibitory nature of eland plasma (Fig. 7b).

3.1.6 Trypanosome transformation in red blood cell membranes

Since RBCs prepared from rat or eland blood samples supported trypanosome transformation, it was considered of interest to assess whether the RBC membranes might be sufficient for the process. In this experiment, various methods were used to achieve cell lysis. Brief sonication using a probe led to complete cell lysis in both cases. However, the eland RBCs appeared to disintegrate completely under these conditions resulting in poor recovery of the membranes. In contrast, rat RBCs gave a good yield of the membranes. One cycle of freezing and thawing also resulted in a poor yield of eland RBC membranes while rat RBCs gave a good yield. It was only hypoosmotic lysis in water which gave a good yield of the eland RBCs membranes. The RBC membranes from rat and eland supported transformation of the trypanosomes (Fig. 8). Transformations in homogenates from tsetse fed on rat and eland RBC membranes were higher compared to rat blood control (Fig. 8). By 18 h, more than 90 % of the trypanosomes in RBC membranes were transformed compared to 70 % in rat blood control.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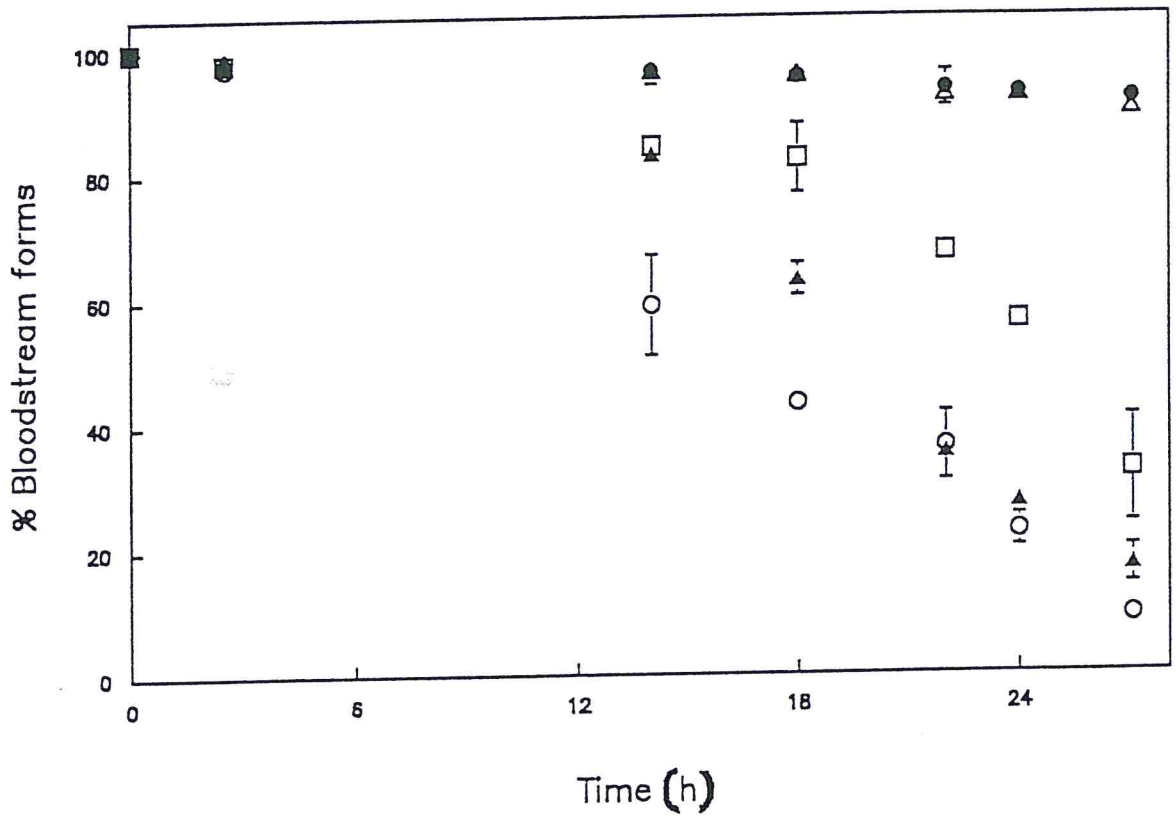
Time (h)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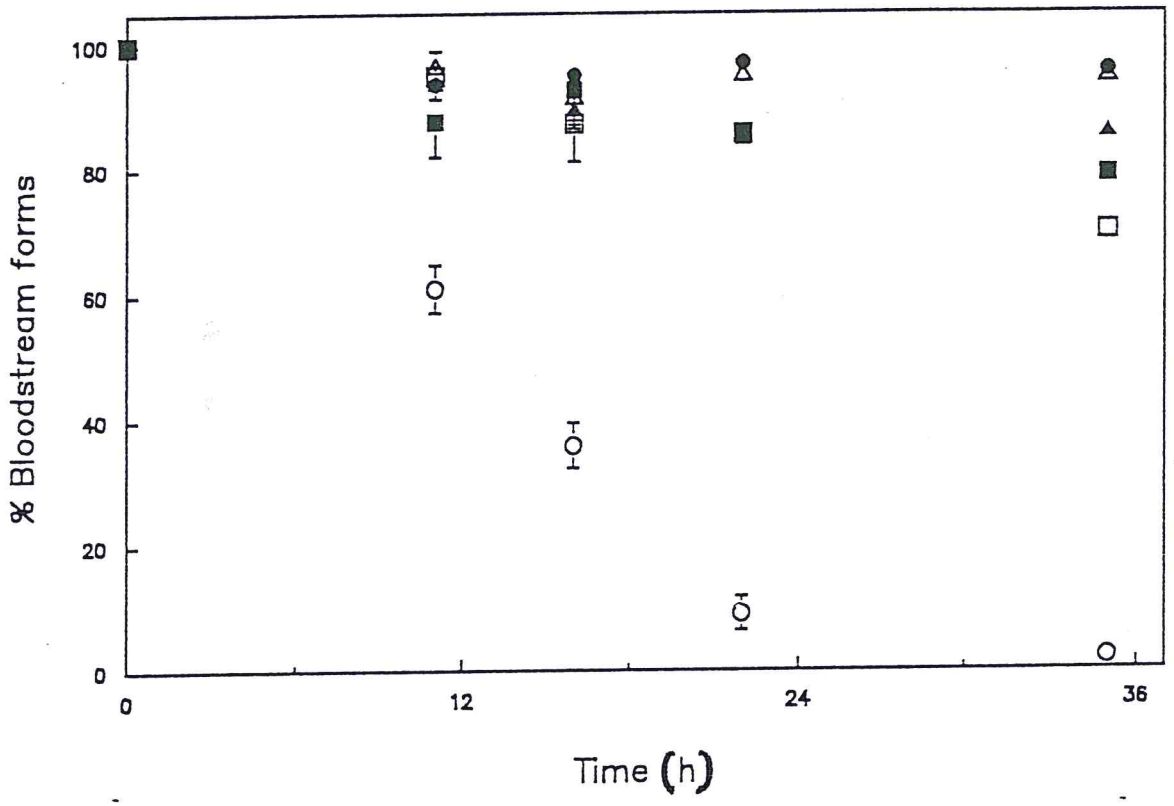
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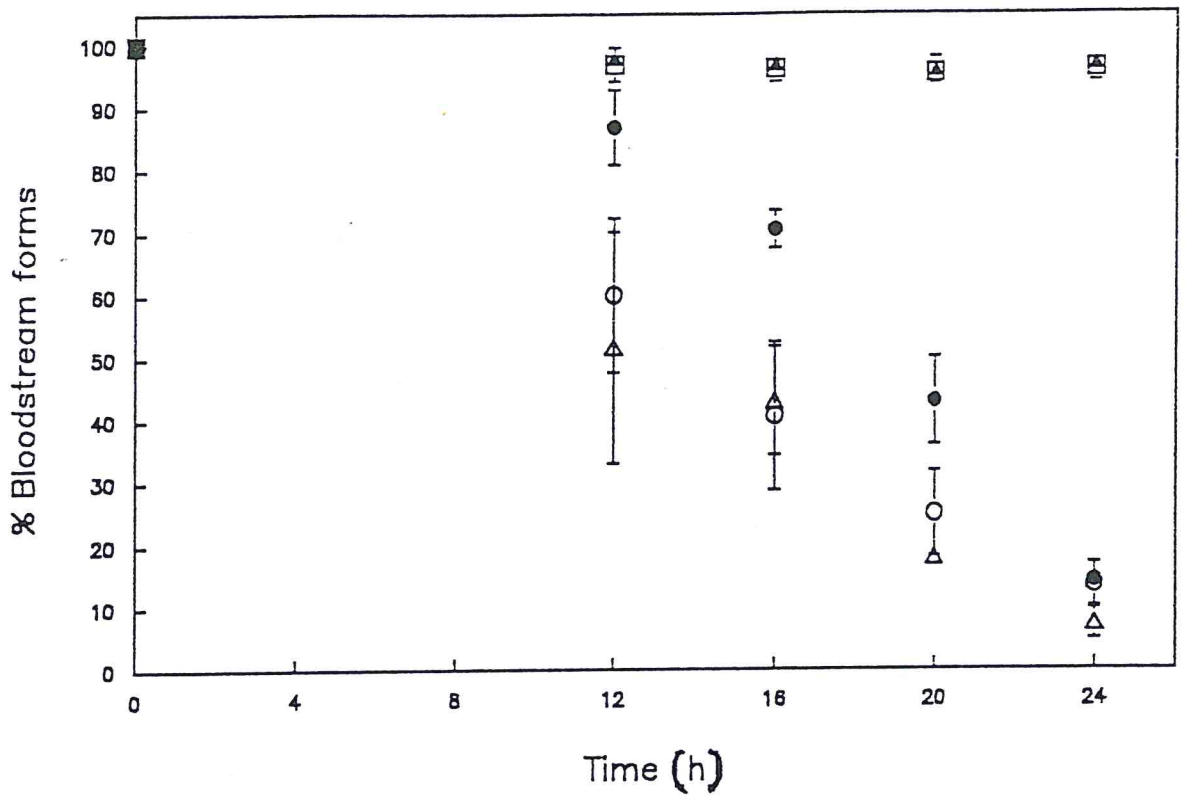
Time (h)

(a)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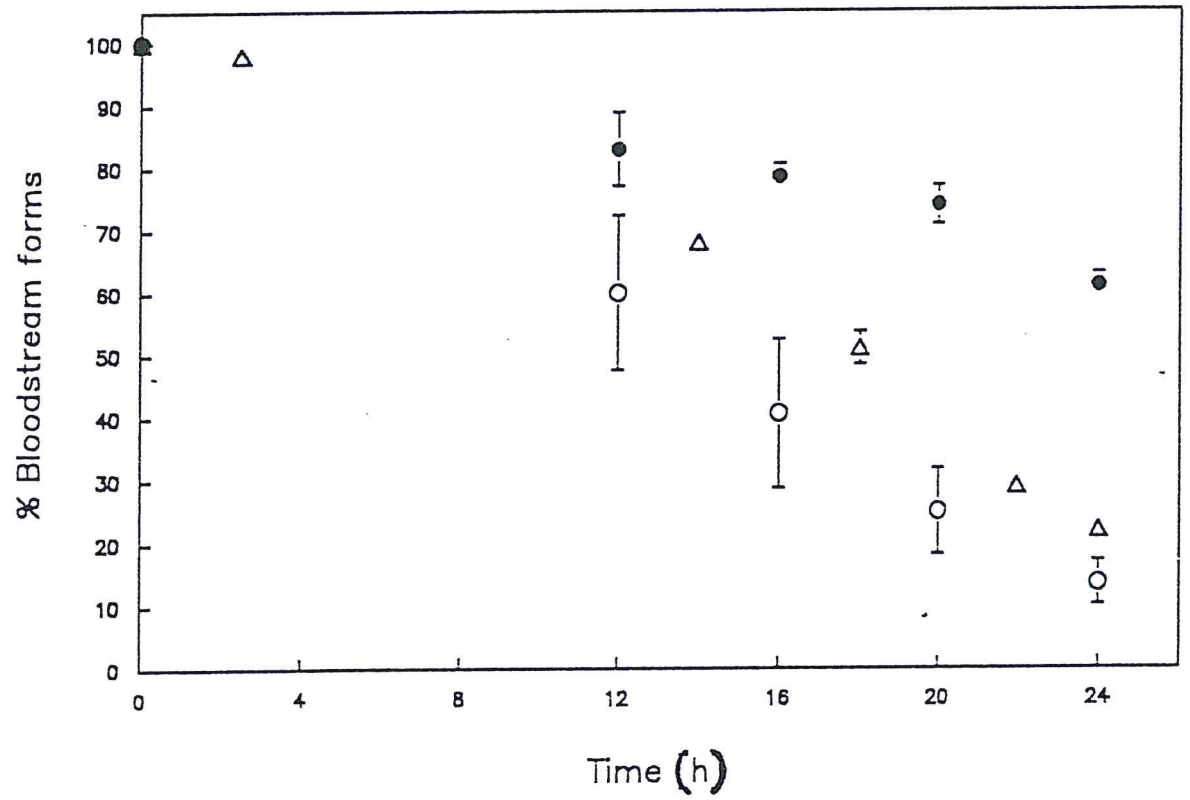
(b)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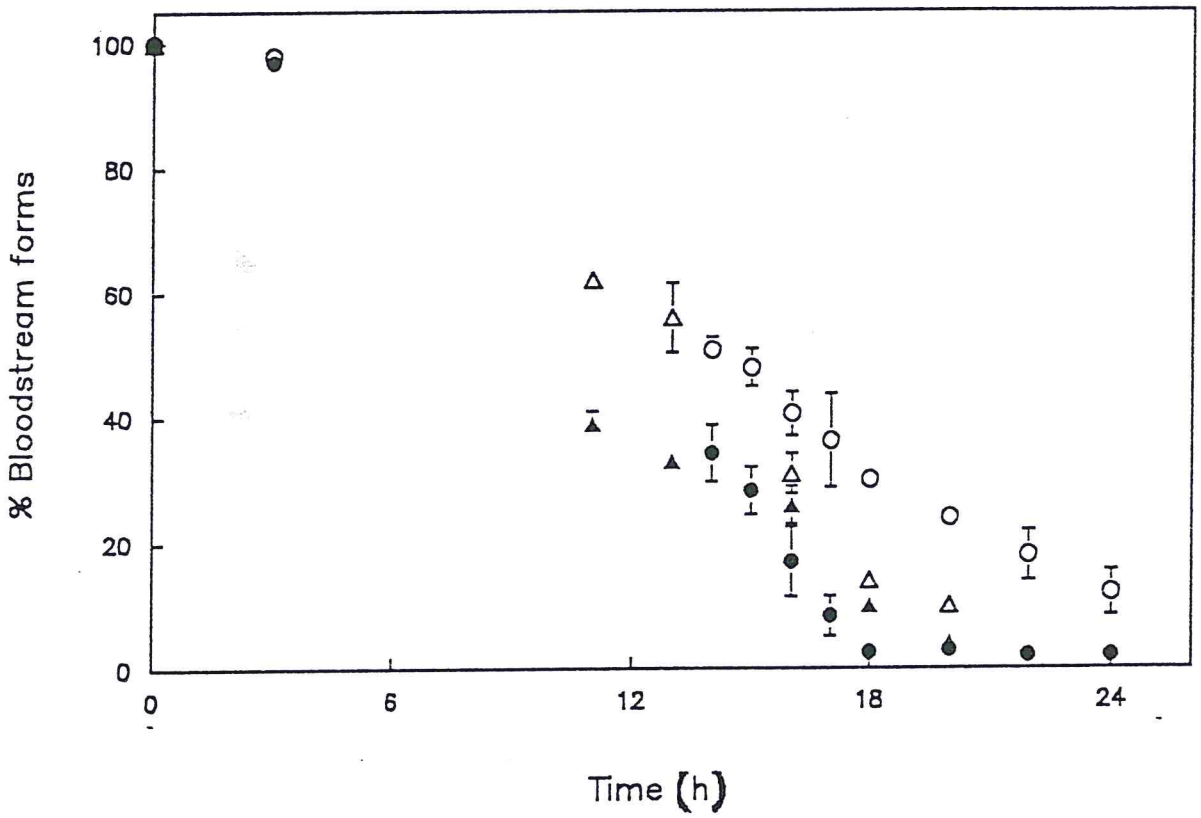
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Time (h)

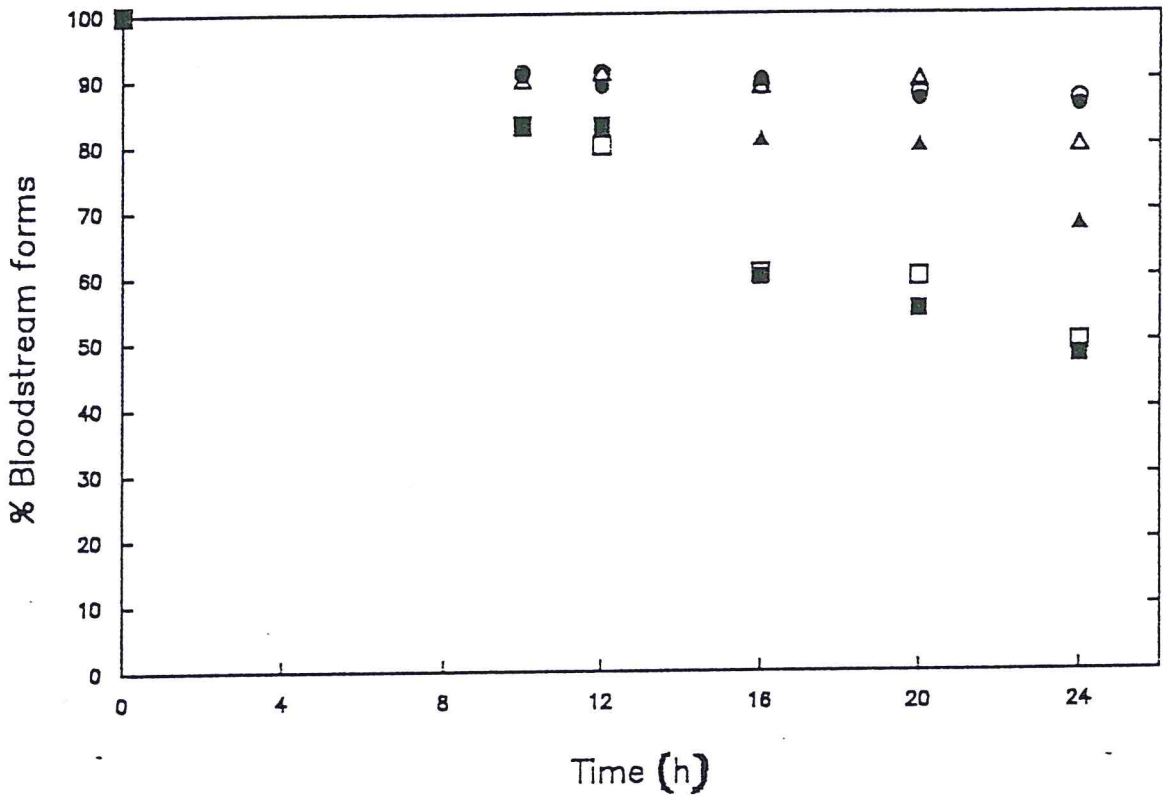
3.1.7 Properties of the inhibitor for trypanosome transformation in eland plasma

3.1.7.1 Effect of temperature on the plasma inhibitor

Thermostability of the eland plasma inhibitor for transformation was studied by incubating aliquots of eland plasma for 30 minutes at temperatures of 4° C, 22° C, 37° C, 42° C, 50° C, 60° C and 70° C prior to the transformation assays. Heating plasma above 50° C resulted in loss of the inhibitor activity whereas negligible loss occurred below 42° C (Fig. 9). Incubating the plasma for 30 min at 4° C, 22° C, and 37° C had no effect on the inhibitor activity. Heating the plasma for 30 min at 70° C led to formation of an insoluble gel that could not be utilized in the assays.

3.1.7.2 Effect of freezing and thawing

The effect of freezing (-70° C) and thawing (37° C) on the stability of the eland plasma inhibitor was studied by subjecting fresh plasma aliquots to several freeze-thawing cycles. Freeze-thawing resulted in loss of the inhibitor activity (Fig. 10). Based on transformation rates after 24 h, one freeze-thawing cycle resulted in about 30 %, while two or more cycles resulted in more than 50 % loss of activity, respectively. Whereas the activity decreased tremendously following two or more freeze-thawing cycles, it was not abolished even by six cycles (Fig. 10).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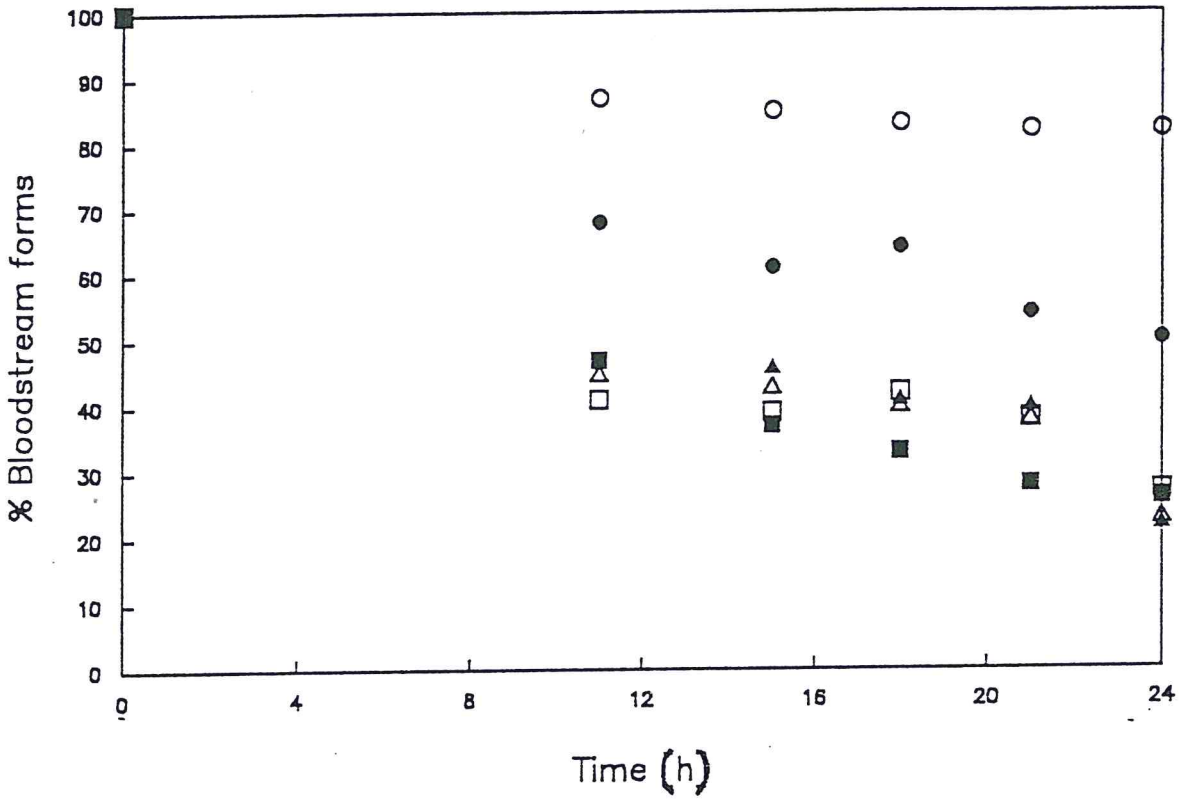
Time (h)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Time (h)

3.1.7.3 Effect of fresh eland plasma on trypanosome transformation

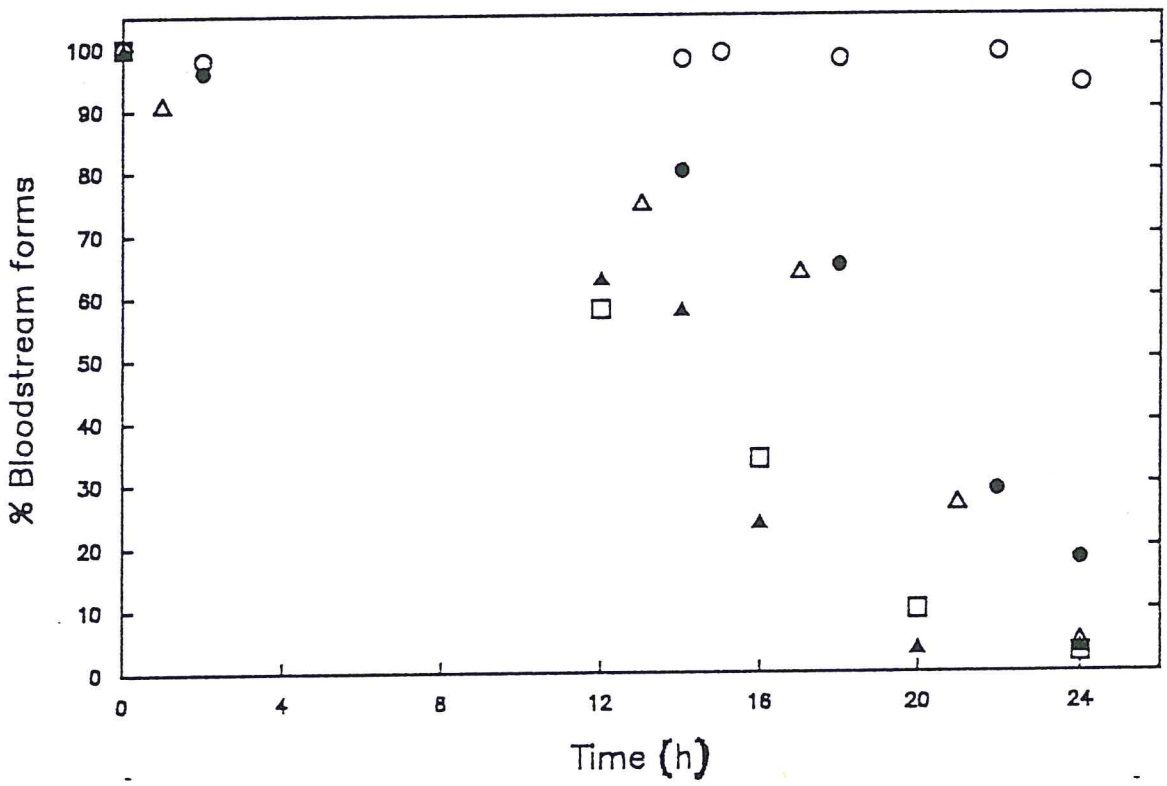
Preincubation of isolated trypanosomes for various length of time in fresh eland plasma before they were transferred to an *in vitro* transformation system did not affect their ability to transform normally (Fig. 11). No inhibition on transformation was evident in these trypanosomes even after 1.75 h of preincubation (Fig. 11). Longer incubation of the trypanosomes was avoided due to trypanolytic effect of eland plasma (Reduth *et al.*, 1994).

3.1.7.4 Effect of dialysing plasma

Effect of dialysing fresh plasma on the inhibitor activity was studied. This was necessary to have an idea about the molecular size of the inhibitor so that activity is not lost through dialysis. Plasma was dialysed for 24 h at 4° C in a 12-14 KD molecular weight cut off (MWCO) membrane and assayed for its ability to inhibit transformation. Both the dialysed plasma and one kept at 4° C for a similar duration showed similar capacities to inhibit transformation (Fig. 12). The slight loss of activity in both aliquots is consistent with storage effect (section 3.7.6) during the 24 h period. This results indicated that the inhibitor factor has a molecular weight greater than M_r~14 KD.

3.1.7.5 Stability of the eland plasma inhibitor to lyophilization

The effect of storing eland plasma in lyophilized form on the inhibitor activity was assessed. Storage of lyophilized plasma for 1 week did not preserve the inhibitor activity. Both the lyophilized plasma and one kept at -70° C for a similar duration showed similar transformation inhibition capacity (Fig. 13). Fresh plasma (used as a control) showed high inhibition on transformation (Fig. 13).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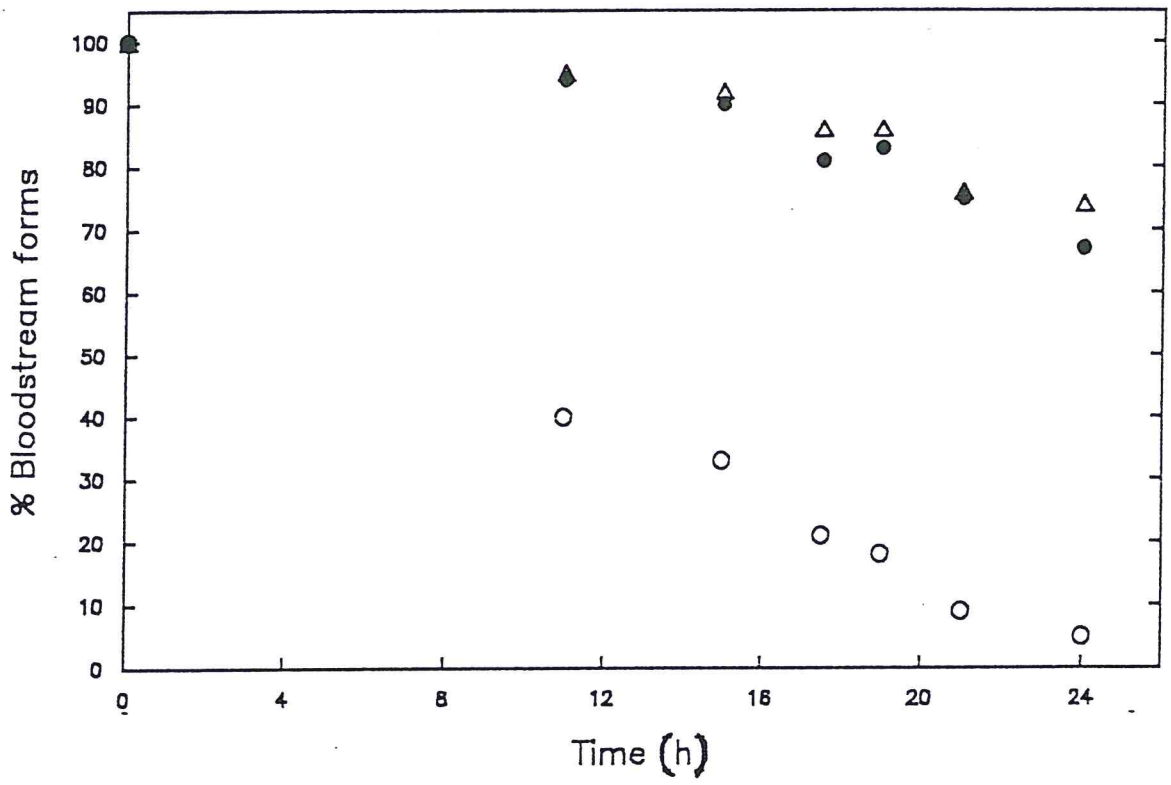
Time (h)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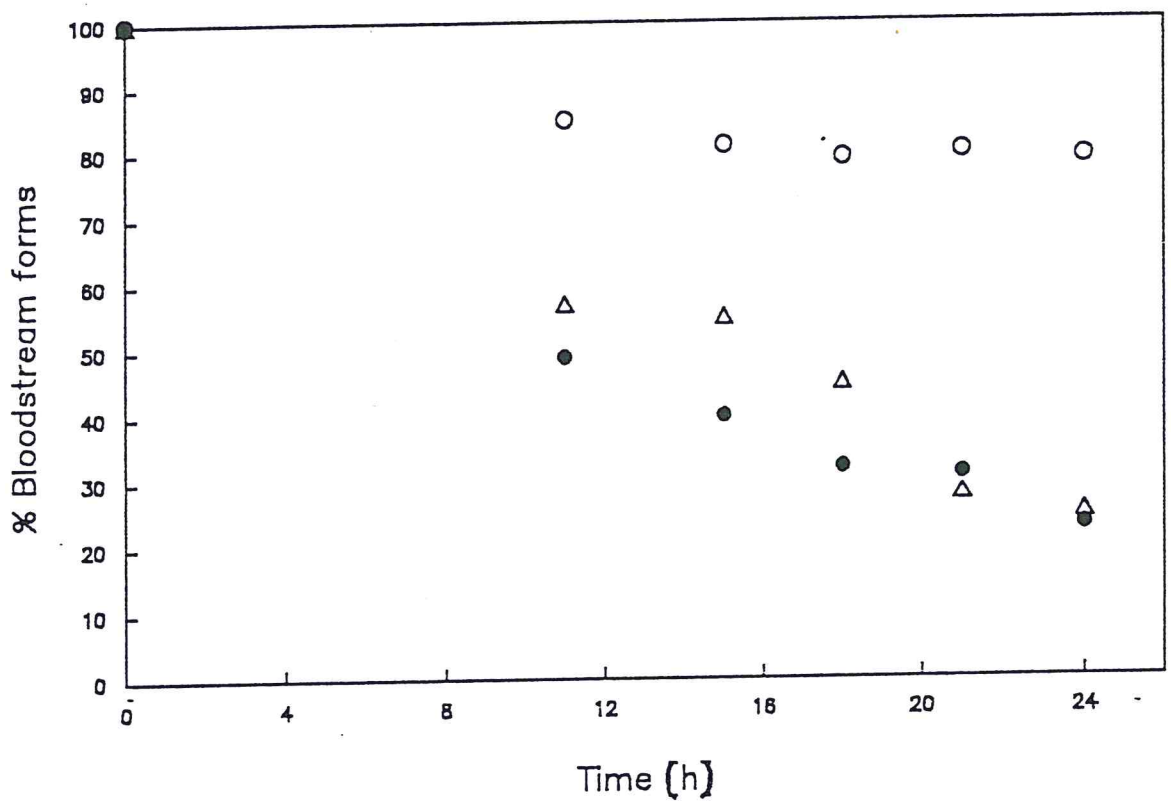
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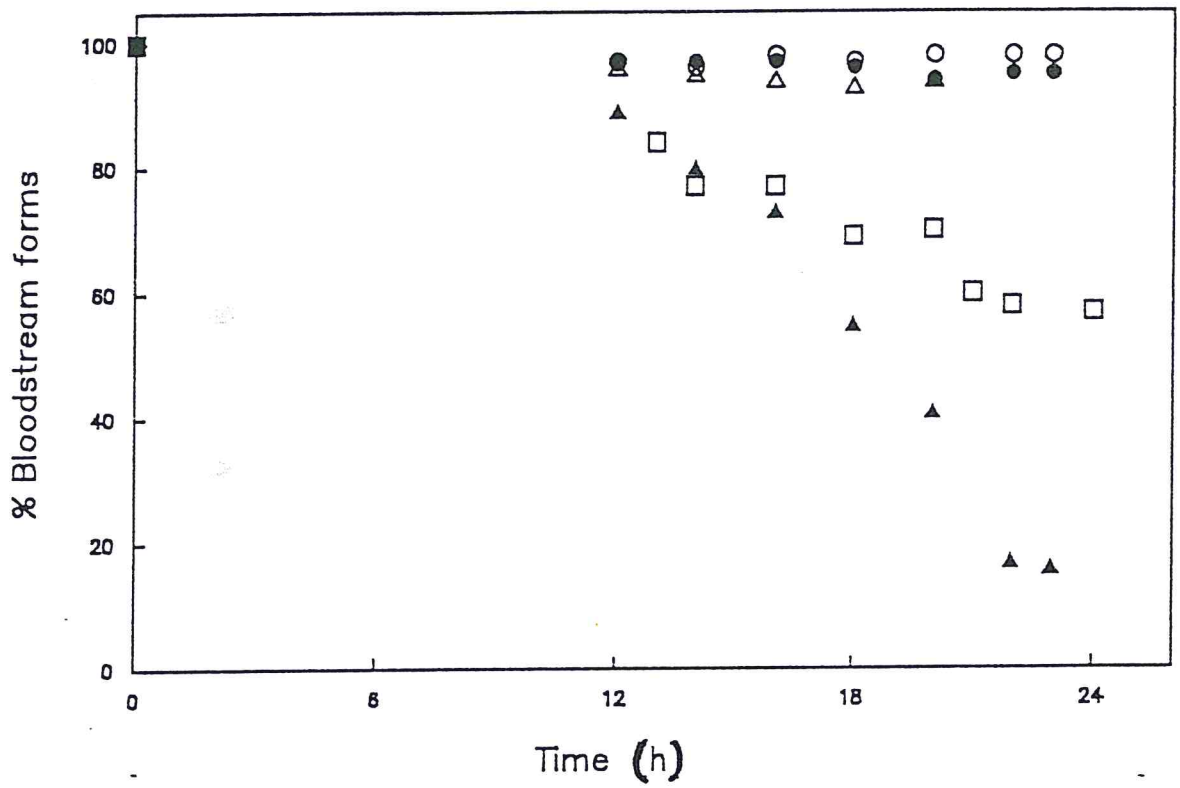
Time (h)

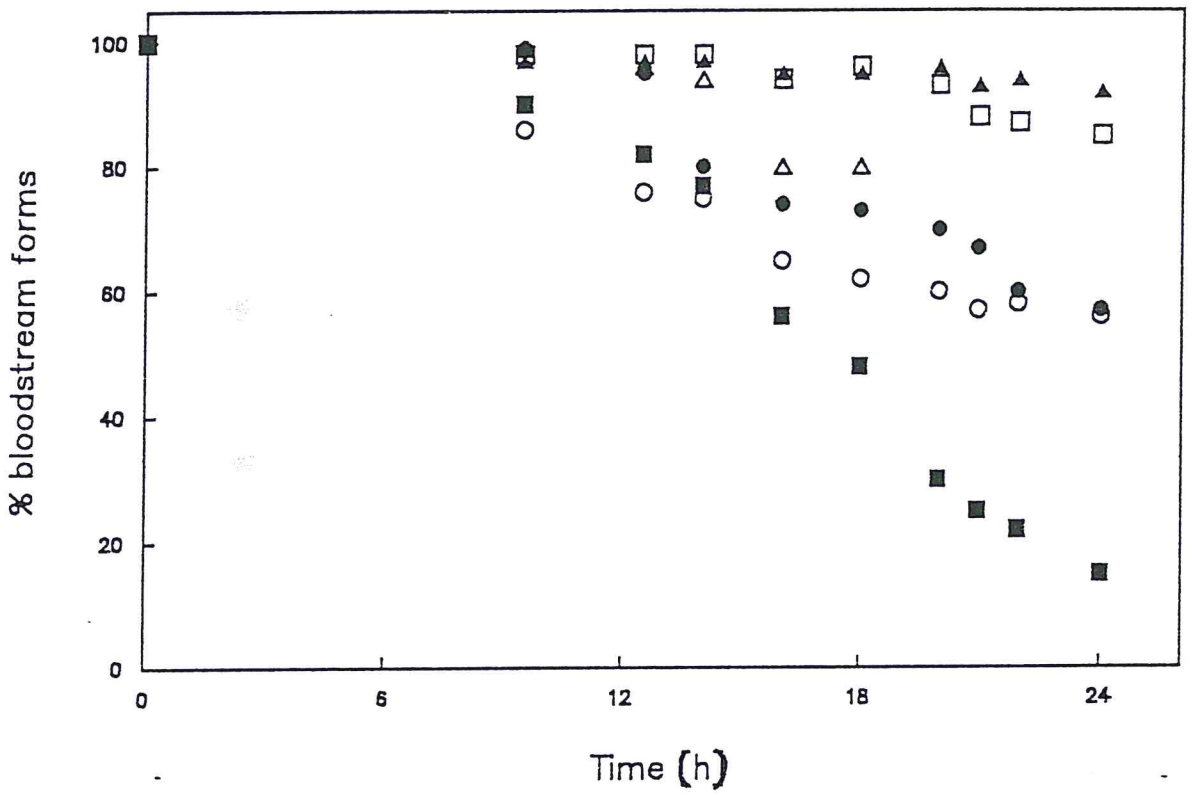
3.1.7.6 Loss of inhibitor activity during storage

Figure 14 clearly shows that the inhibitor activity was highest in fresh plasma and was gradually lost during storage. Based on transformations after 24 h, about 50 % and 80 % of the inhibition activity was lost after 3 and 7 days at 4° C, respectively (Fig. 14). The result also shows that RBCs (whether fresh or one week) did not have any effect on the inhibitor activity confirming that the inhibitor is only present in the plasma.

3.1.7.7 Ammonium sulphate fractionation of eland plasma

Various saturated ammonium sulphate (SAS) fractions prepared from fresh eland plasma were assayed for their abilities to inhibit trypanosome transformation. The results showed that most of the inhibitor activity was in the 30-40 % and 40-50 % SAS fractions (Fig. 15). The 20-30 % SAS fraction had a high lytic effect on trypanosomes hence it was not possible to include it in the transformation assays. The control, which was eland plasma stored at 4° C for the same duration, lost some of its inhibitory activity within the same duration. The high salt concentration in the fractions had somehow preserved the inhibitor activity during isolation. It was not possible to study the effect of a 0-10 % fraction on transformation since it always formed an insoluble gel. Therefore, 0-20 % SAS fraction, which did not gel, was used instead.


% Bloodstream forms
100
80
60
40
20
0
0
6
12
18
24
Time (h)


% bloodstream forms
100
80
60
40
20
0
0
6
12
18
24
Time (h)

3.1.7.8 Effect of Proteolytic enzymes on plasma inhibitor activity

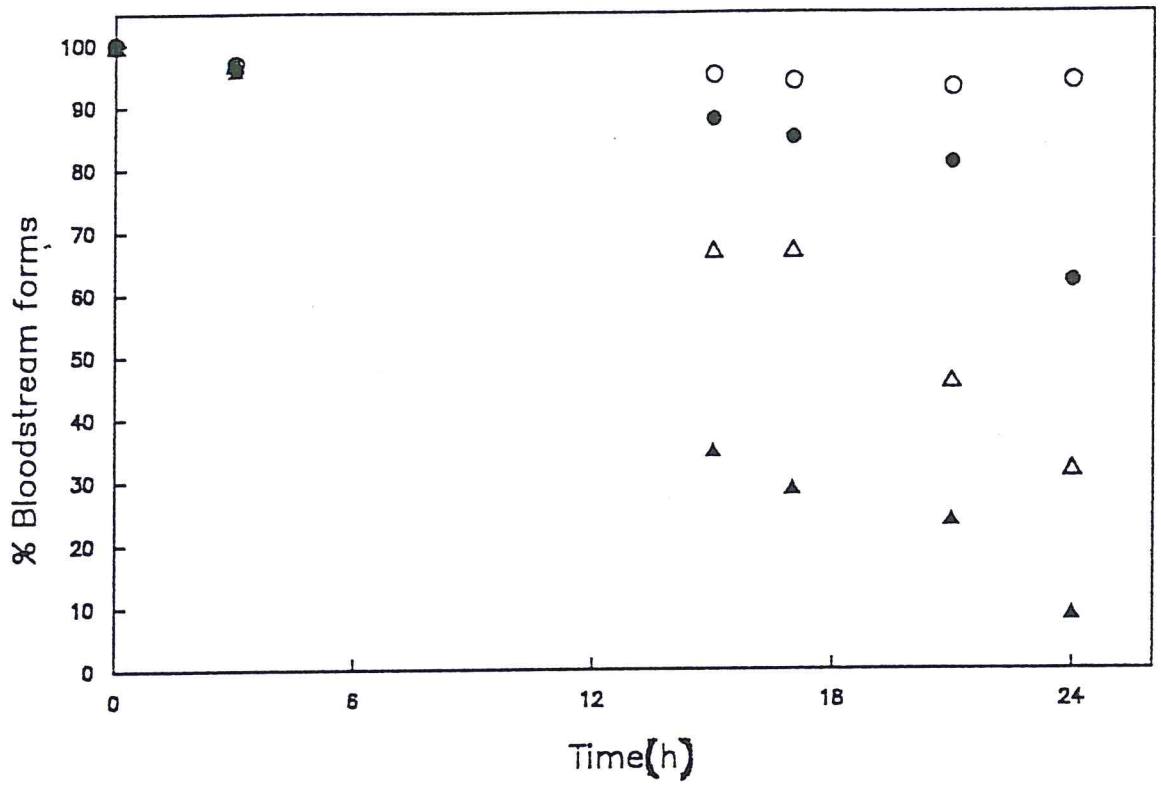
Treatment of fresh eland plasma with pronase (2 h, 30° C) led to loss of the inhibitor activity (Fig. 16a). The loss of the activity increased with enzyme concentration used. A complete loss of activity resulted when plasma was treated with 1 mg/ml pronase for 2 h (Fig. 16a). Similarly, the use of bovine pancreas trypsin at the same concentration resulted in reduction of the inhibitor activity although it was not completely abolished (Fig. 16b). A 0.05 mg/ ml trypsin had negligible effect. In contrast, a similar concentration of pronase resulted in a 30 % loss of the inhibitor activity based on transformations after 24 h (Fig. 16a).

3.1.7.9 Effect of protease inhibitors on the stability of the plasma inhibitor

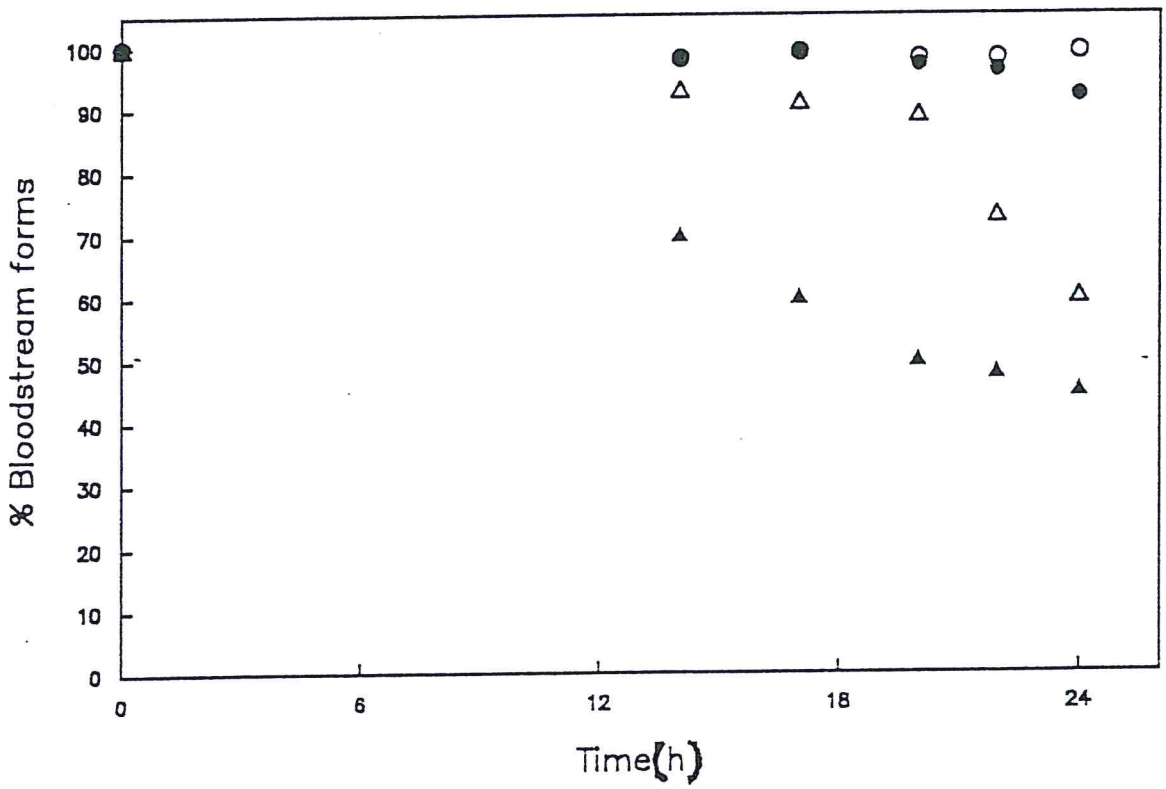
Different protease inhibitors were added to fresh eland plasma aliquots before storage for 6 days at either 4° C or -20° C. Although there was substantial loss of activity in all aliquots, those that were stored at -20° C retained slightly more activity than those stored at 4° C (Fig. 17).

Table 1 summarises the ability of various rat and eland blood fractions to support transformation.

(a)


% Bloodstream forms
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
Time(h)

(b)


% Bloodstream forms
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
Time(h)

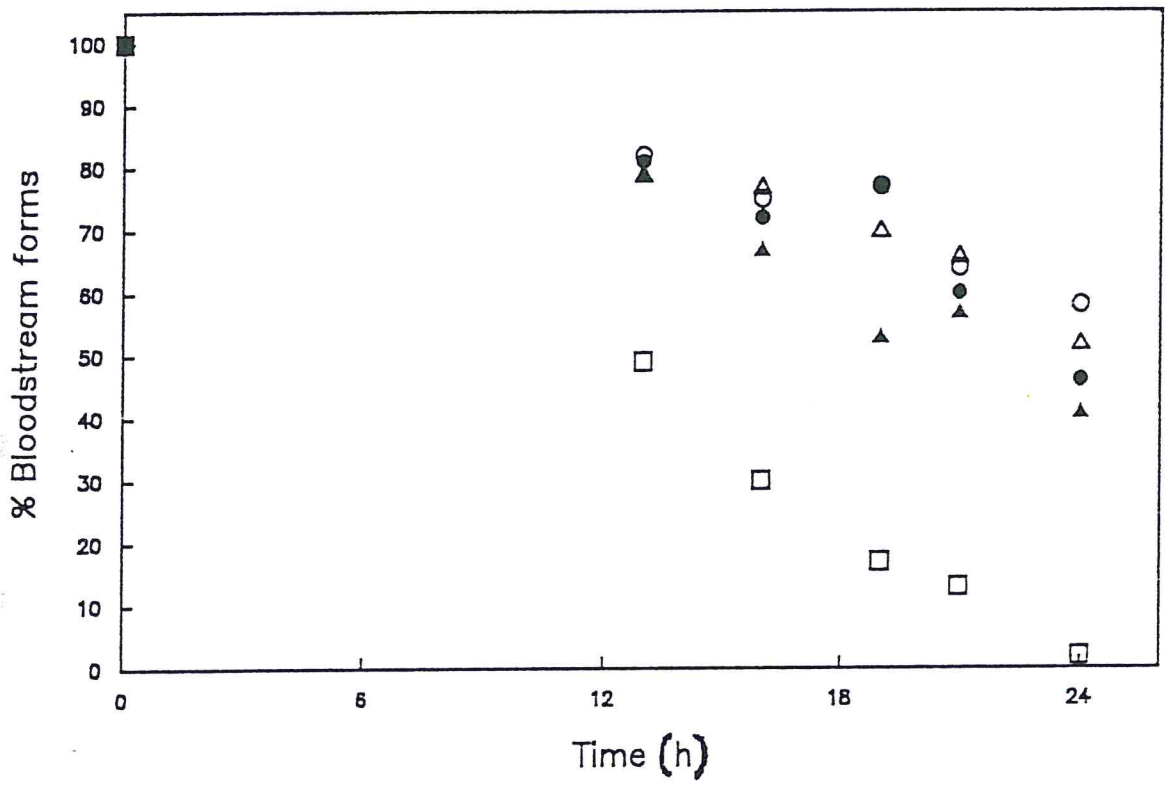

% Bloodstream forms
100
90
80
70
60
50
40
30
20
10
0
0
6
12
18
24
Time (h)

Table 1

Ability of various rat and eland blood fractions to support/inhibit trypanosome transformation

Fraction	Supports transformation
Fresh rat blood	+
Fresh eland blood	-
Rat RBCs	+
Eland RBCs	+
Rat blood fraction without RBCs	-
Eland blood fraction without RBCs	-
Rat plasma	-
Eland plasma	-

3.1.8 Anion-exchange chromatography of plasma

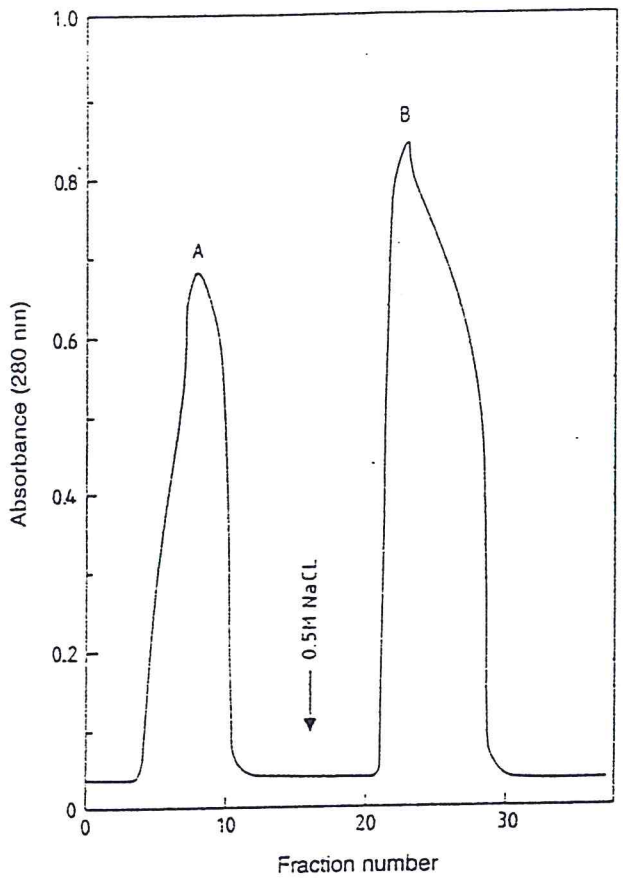
Since the eland plasma inhibitor was precipitated in the 30-50 % (wt/v) saturated ammonium sulphate fraction (Fig. 15), the initial efforts to isolate this factor utilized this fraction followed by gel filtration on Sepharose 6B-CL column. However, assays of the fractions after concentration and dialyses in PBS, showed very little inhibitor activity (Data not shown).

Due to the rapid loss of activity, it was important to try isolation procedures that would take the shortest time possible. Thus, fresh plasma was fractionated by gel filtration on FPLC using Superose 6 column but several runs were necessary to obtain enough protein samples to carry out transformation assays. After concentration and dialyses in PBS, the fractions showed very little inhibitor activity (Data not shown). Further attempt to isolate the plasma inhibitor was made by using ion-exchange chromatography on DEAE-Sepharose-6B-CL. Fresh plasma was initially fractionated into unbound and bound fractions (Fig. 18a). Assays of these fractions showed activity only in the bound fraction (Fig. 18b). Since the inhibitor was in the bound fraction, further attempts to isolate the molecule was carried out using linear salt gradient (0-0.5 M NaCl). None of the four salt gradients separated the bound fraction into distinct peaks (Fig. 19). Consequently, a strategy involving stepwise gradient elution with various salt concentrations (0.1, 0.2, and 0.5 M NaCl) in equilibration buffer was adopted. With this approach, the bound fraction was resolved into 4 distinct peaks (Fig. 20a). Two peaks were eluted in 0.1 M NaCl while both 0.2 M and 0.5 M gave single peaks (Fig. 20a). Assays of the fractions from these peaks showed very little inhibitor activity (Fig. 20b). There was slightly more activity in peaks C and D when compared to the other peaks. However, since the procedure before assays involved chromatography, concentration and dialysis and took more than 30 hours, it is possible that the activity was lost during this time (Fig. 20b).

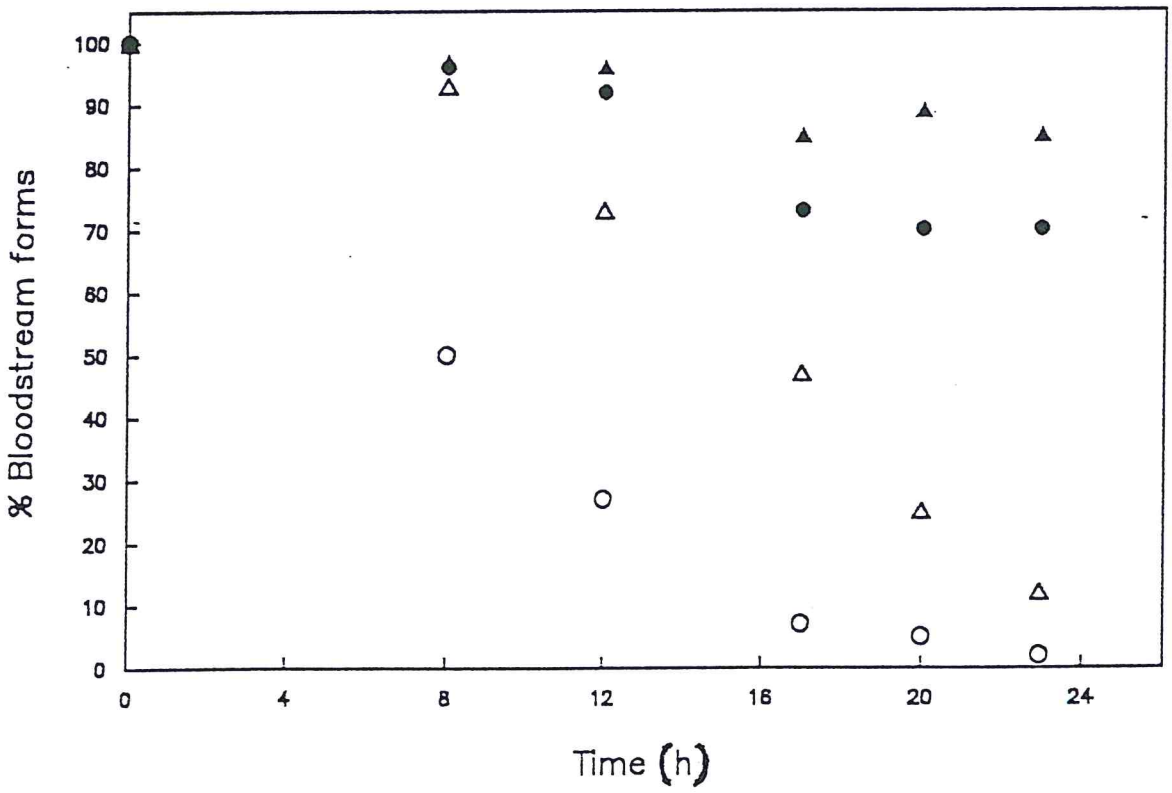
3.1.9 Effect of proline and glutamine on transformation

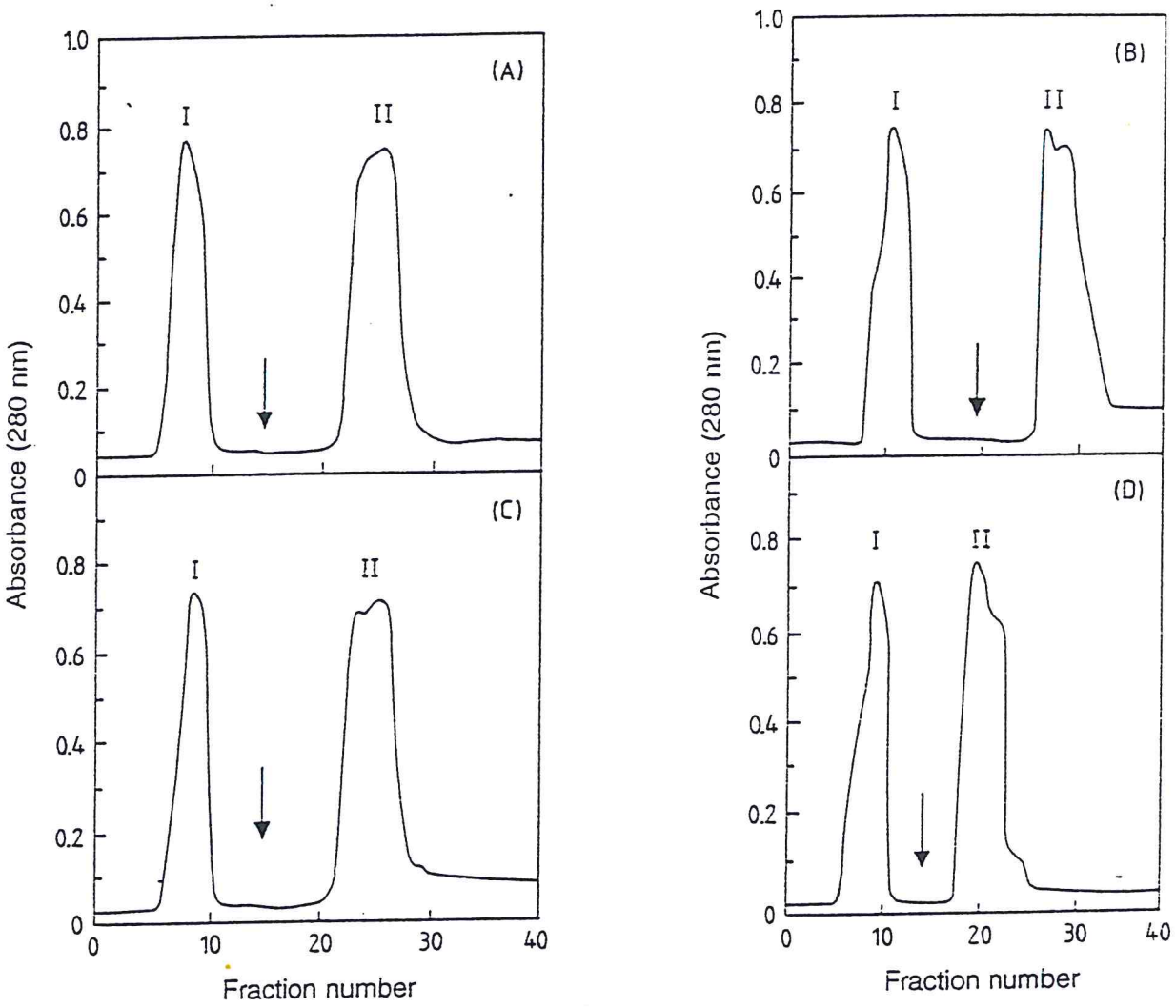
Compared to the other amino acids, proline and glutamine are present in higher concentration in tsetse after bloodmeal digestion. Their role in the transformation of trypanosomes from bloodstream to procyclic forms was investigated. Since unfed gut homogenates had earlier been shown not to stimulate transformation (Imbuga *et al.*, 1992a), the amino acids were added to these homogenates at concentrations of 0-20 mM and tested for their ability to stimulate this process. The results showed that L-proline did not stimulate transformation (Fig. 21). Midgut homogenates from tsetse that had fed on rat blood (as a control) supported normal transformation. Similarly, trypanosome transformation using midgut homogenates from tsetse that had fed on rat blood in which L-proline was included did not stimulate significantly higher transformations from the control (Fig. 22). When the assays were repeated using either L-glutamine or a combination of L-proline and L-glutamine at the above concentrations, there was no significant stimulation on transformation due to the amino acids (data not shown). Since proline concentration of upto 50 mM has been reported in tsetse (Cunningham and Slater, 1974), a similar concentration of proline and glutamine did not stimulate transformation *in vitro* (data not shown).

(a)

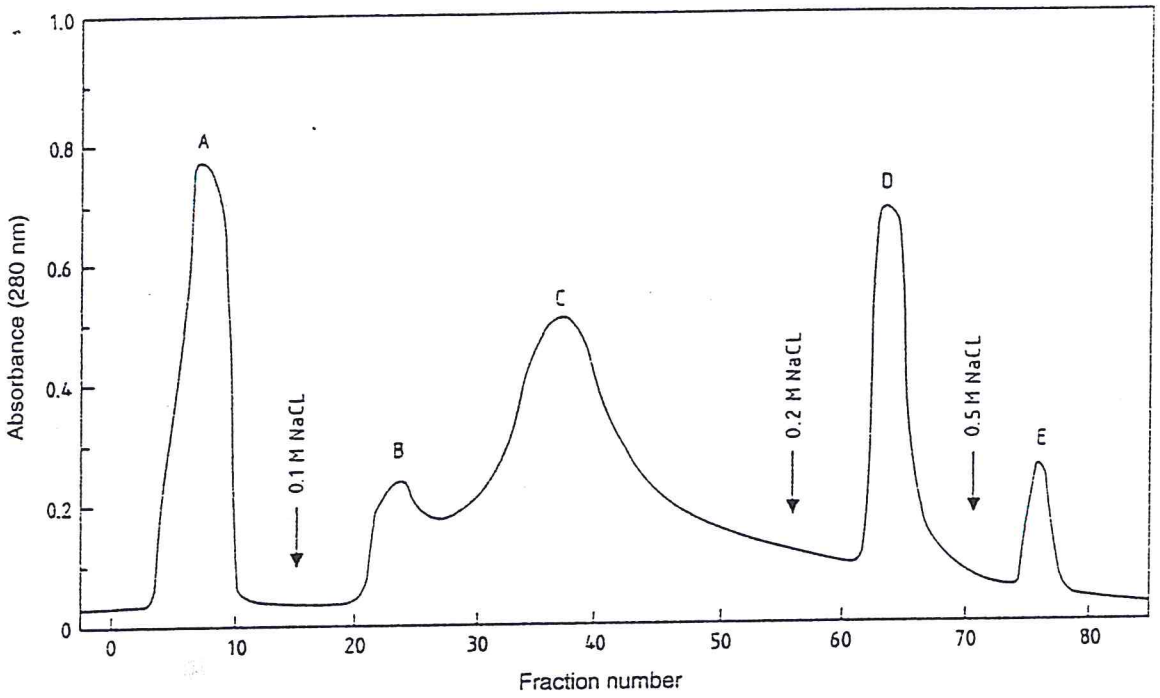

1.0
0.8
0.6
0.4
0.2
0
Absorbance (280 nm)
A
B
0.5M NaCL
0
10
20
30
Fraction number

(b)

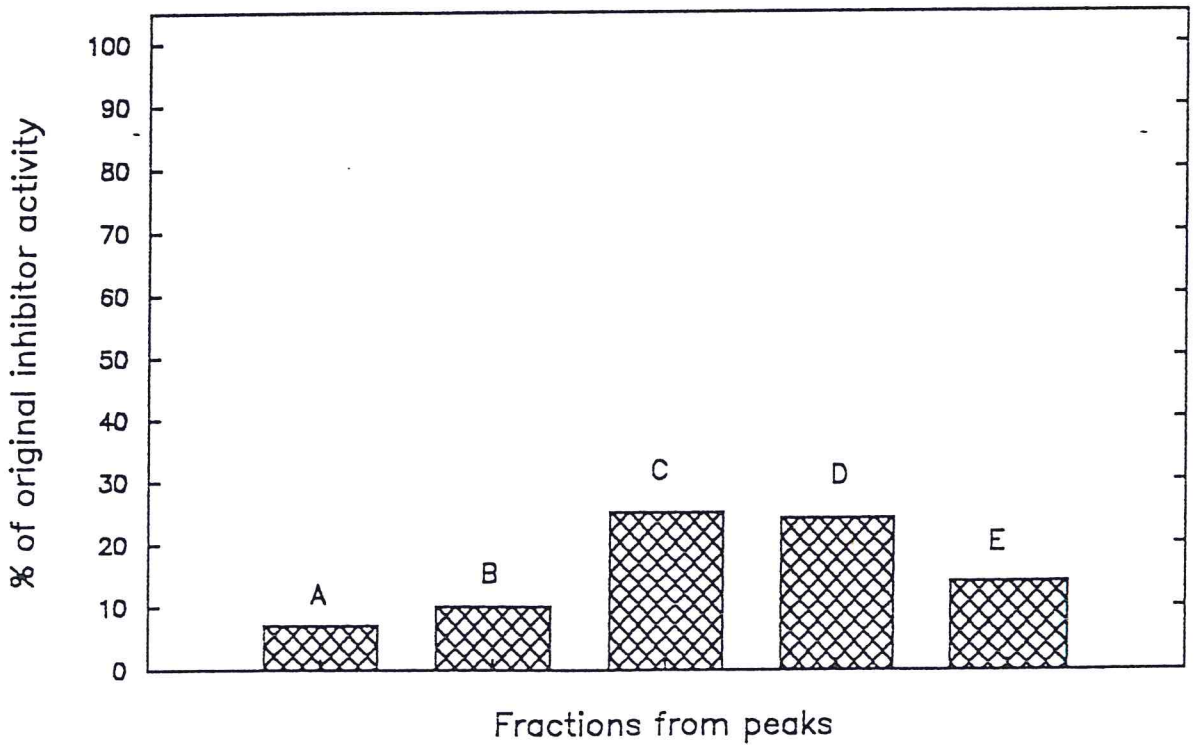

100
90
80
70
60
50
40
30
20
10
0
% Bloodstream forms
0
4
8
12
16
20
24
Time (h)

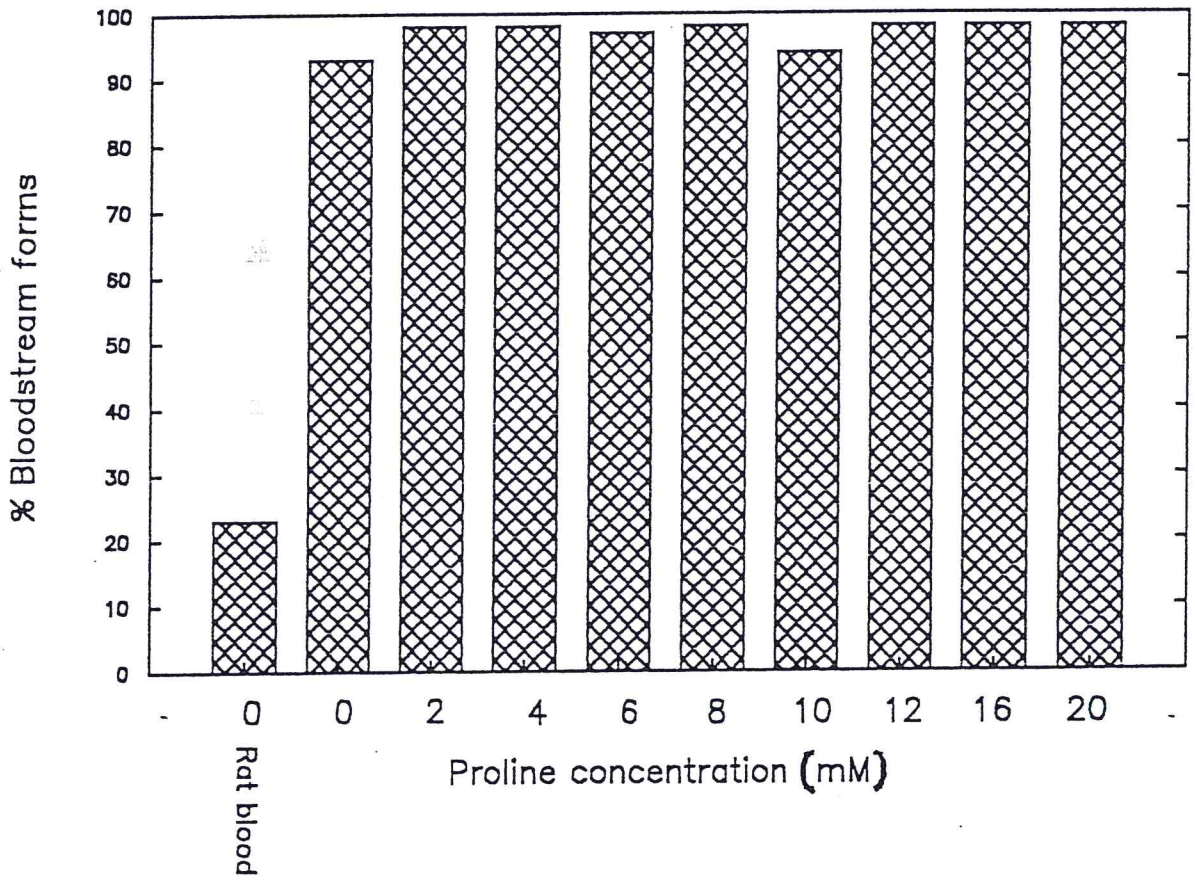

(A)
(B)
(C)
(D)
I
II
Absorbance (280 nm)
Fraction number
0
0.2
0.4
0.6
0.8
1.0
10
20
30
40

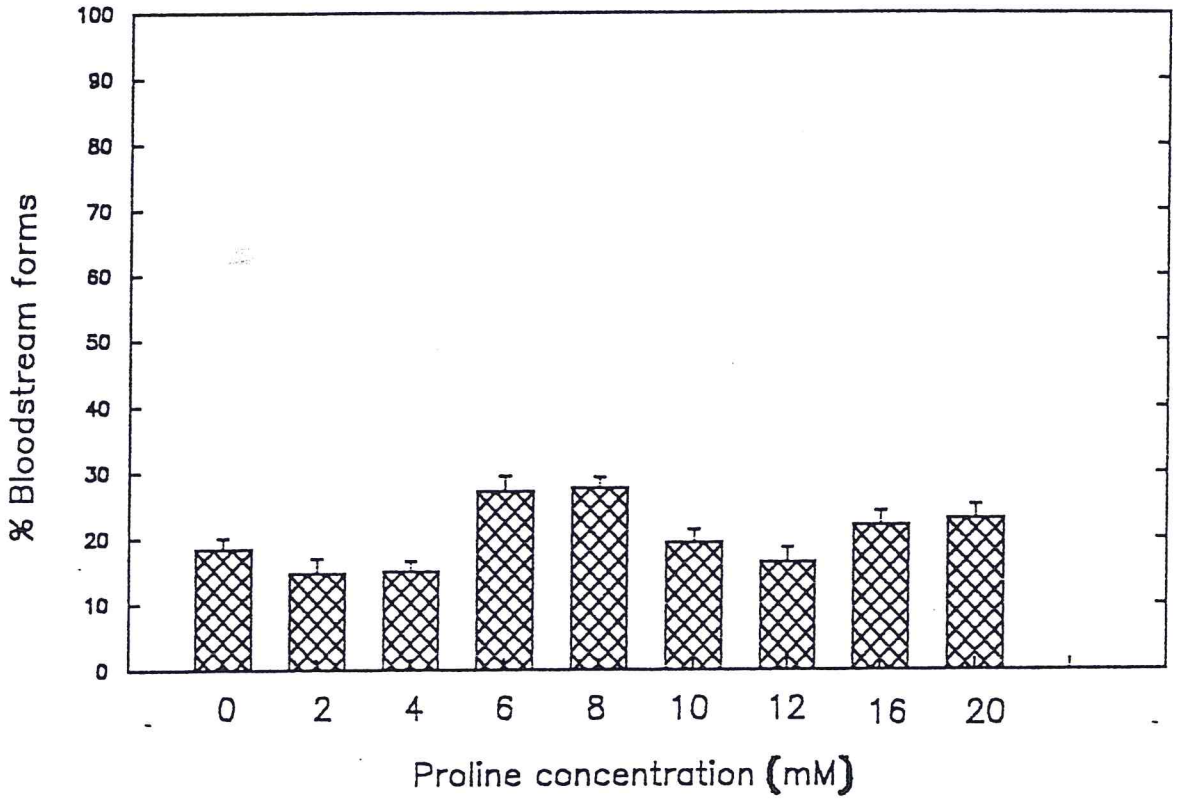
(a)


Absorbance (280 nm)
1.0
0.8
0.6
0.4
0.2
0
A
B
C
D
E
0.1 M NaCL
0.2 M NaCL
0.5 M NaCL
0
10
20
30
40
50
60
70
80
Fraction number

(b)


% of original inhibitor activity
100
90
80
70
60
50
40
30
20
10
0
A
B
C
D
E
Fractions from peaks


% Bloodstream forms
100
90
80
70
60
50
40
30
20
10
0
0
0
2
4
6
8
10
12
16
20
Rat blood
Proline concentration (mM)


100
90
80
70
60
50
40
30
20
10
0
% Bloodstream forms
0
2
4
6
8
10
12
16
20
Proline concentration (mM)

3.2 Discussion

3.2.1 Role of host blood in the transformation of bloodstream trypanosomes

An important step in the establishment of the gut-adapted trypanosomes (*Trypanozoon* and *Nannomonas*) within the tsetse vector involves transformation of the trypanosomes from bloodstream into the procyclic (midgut) forms (Vickerman, 1985). This process is accompanied by high trypanosome mortality mediated by several midgut factors that include lectins, trypsin or trypsin-like molecules and lysins (Maudlin, 1991; Osir *et al.*, 1993). In both susceptible and refractory tsetse, the extent of trypanosome mortality appears to be dependent on the type and quantity of these factors as well as on the trypanosome species ingested in the blood meal. Since the midgut factors are bloodmeal-induced, the infection prevalence would be expected to be influenced by the type of host blood at the time of an infective meal. This point has already been demonstrated by Moloo (1981) and Mihok *et al.* (1993), although it is presently unclear exactly how host blood influences the types and quantities of midgut factors released. Using a relatively simple *in vitro* system for studying transformation of bloodstream trypanosomes into procyclic (midgut) forms, the results of the present study provide some insight into this complex problem.

Among the four host blood types tested, those from rat and goat supported transformation of *T. brucei brucei*, while buffalo and eland blood samples gave intermediate and low transformation rates, respectively. A recent study reported low infection prevalence by *T. congolense, T. brucei* and *T. simiae* in *Glossina morsitans* when the infective blood originated from cow and eland (Mihok *et al.*, 1993). The same study also showed that wildlife hosts found that goat blood produced a high infection prevalence. In these studies the wildlife blood supported lower infection prevalence than blood of domestic hosts (Mihok *et al.*, 1991, 1993; Moloo, 1981, 1984). Since the blood samples that gave low rates of trypanosome

transformation are the same ones that gave lower infection prevalence, it may be assumed that the two processes are closely linked. Those trypanosomes that transform efficiently in the fly midgut are much more likely to reach maturation than those that do not.

The results of this study have shown that RBCs are an important blood component in trypanosome transformation. In contrast, the WBCs did not support the process. Imbuga *et al.* (1992a) showed that transformation of bloodstream *Trypanosoma brucei* was supported only by midgut homogenates obtained from tsetse that had been fed on blood. Furthermore, stimulation of the process by bovine pancreas trypsin occurred only in presence of blood (Imbuga *et al.*, 1992a), suggesting that blood played a crucial role in this process. Yabu and Takayanagi (1988) also reported that transformation of bloodstream trypanosome could be stimulated by bovine pancreas trypsin only in presence of mammalian cell feeder layer. The role played by the RBCs or the cell feeder layer in facilitating transformation of the trypanosomes remains a matter of conjecture.

Previous studies by Gingrich *et al.* (1982) reported high midgut infection prevalence of *T. b. rhodesiense* in *G. morsitans* that had been fed on serum-free meals. Maudlin *et al.* (1984) also showed that removal of serum from blood greatly increased midgut infection rates of *T. congolense* in *Glossina morsitans*. The results of Gingrich *et al.* (1982) are supported by the results of this study which showed that midgut homogenates prepared from tsetse that had been fed on serum or plasma from rat or eland gave very low rates of trypanosome transformation. Furthermore, the process was readily supported by washed cells. Moreover, the lytic properties of plasma from humans and baboons (Gillet and Owens, 1992), buffalo (Reduth *et al.*, 1994) and waterbuck (Mulla and Rickman, 1988) may have a direct contribution to the low infection prevalence observed in tsetse maintained on these hosts. In addition to the trypanolytic factor in eland plasma (Reduth *et al.*, 1994), the present study has clearly demonstrated the presence of an

inhibitory factor(s) for trypanosome transformation. The ability of bloodmeal taken up with trypanosomes to facilitate the transformation process appears crucial for successful establishment of an infection in tsetse. Following a bloodmeal, several trypanolytic factors are released into the fly midgut. These factors reach peak levels after 48-72 h (Abubakar *et al.,* 1995; Stiles *et al.,* 1991; Osir, Imbuga and Abubakar, unpublished data). Some previous studies carried out in our laboratory showed that, compared to bloodstream trypanosomes, the procyclic parasites are not lysed by high levels of trypsin *in vitro* (Osir and Imbuga, unpublished data). Indeed, under *in vivo* conditions, the procyclic trypanosomes thrive in an environment of high trypanolytic activity. The ability of procyclic trypanosomes to withstand such conditions has been exploited in characterizing wild infections through *in vivo* procyclic expansion from gut homogenates of wild tsetse (Mihok *et al.*, 1994). From the observed differences in the abilities of host blood to support transformation, a relationship between infection prevalence and transformation rates may be deduced. Blood samples with poor capacities to support transformation lead to low infection prevalence since the trypanosomes are lysed before they get a chance to transform into procyclics. In contrast, those host blood that support high transformation rates enable the ingested trypanosomes to escape the harsh midgut environment thus resulting in high infection prevalence. However, it should be pointed out that transformation of the trypanosomes into procyclics is only the first step in a series of complex changes that eventually result in the establishment of infection in tsetse (Vickerman, 1985). These changes are under the control of other factors within the fly.

3.2.2 Properties of eland plasma inhibitor for trypanosome transformation

Domestic animals succumb very easily to trypanosomiasis when they venture in endemic areas and this has led to low livestock productivity in tsetse infested areas (Jordan, 1986). In contrast, wild bovids, unless severely stressed, do not readily succumb to such infections (Ascroft,

1959; Murry and Dexter, 1988). They are usually capable of controlling parasitaemia below detection levels and show little or no pathological signs of the disease (Ascroft, 1959). In addition, the development of parasite-induced anaemia, a major pathogenic feature of bovine trypanosomiasis, is effectively controlled by wildlife (Murry and Dexter, 1988).

Wild bovids use both immunological and non-immunological responses to overcome the pathological effect of trypanosome infections. The production of antibodies to specific trypanosomal antigens is responsible, in part, for the control of parasitaemia (Rurangirwa *et al.*, 1986; Olubayo, 1991). Studies have shown that both buffalo and eland, born of trypanosome free parents and bred in captivity in a tsetse-free environment, are capable of efficiently controlling trypanosome infection. This is achieved by antibody mediated responses while showing few or no pathological signs (Dwinger *et al.*, 1986; Grootenhuis *et al.*, 1990; Olubayo *et al.*, 1990). This observation suggests that these hosts have innate mechanisms for controlling trypanosome infections.

Antibody-independent responses, notably serum trypanocidal factors, have also been identified as the other mechanism by which the parasitaemia is controlled (Reduth *et al.*, 1994, Mulla and Rickman, 1988, Olubayo, 1991). Trypanocidal factors have been demonstrated in sera of buffalo, eland (Reduth *et al.*, 1994), and waterbuck (Mulla and Rickman, 1988) that have not been previously exposed to trypanosome infection. Apart from wild bovids, other animals such as the ground dwelling primates have also evolved mechanisms for overcoming trypanosome infections. The plasma of these animals contain a trypanosome lytic factor (TLF), a high density lipoprotein (HDL) (Seed *et al.*, 1990). A similar factor, found in human plasma, confers protection against *T. b. brucei* infection (Lorenz *et al.*, 1994; Seed *et al.*, 1993). This protection is absent in individuals with hepatic disease owing to their low levels of circulating HDL (Abebe *et al.*, 1988; Lux *et al.*, 1973). Although different subclones of trypanosomes show variations in

their sensitivities to the TLFs in ground dwelling primates (Seed *et al.*, 1990) and humans (Lorenz *et al.*, 1994), this factor certainly is important in determining whether tsetse taking infected blood meals from these animals develop infections.

The finding in this study of an inhibitor for transformation of bloodstream trypanosomes in fresh eland plasma is interesting. Such a factor has not been previously reported. The factor was shown to be highly labile. Interestingly, the African buffalo, which shares with eland the same mechanism for controlling parasitaemia did not have this factor (Nguu, Osir and Imbuga, unpublished). It has also been previously shown that buffalo serum does not affect differentiation of *T. congolense* metacyclics into bloodstream forms *in vitro* (Olubayo and Brun, 1992). This finding is in support of our observation.

It is still unclear how host blood affects infection prevalence in tsetse. The serum trypanocidal factors are likely to be partly responsible for low trypanosome infection prevalence in tsetse that feed on such blood. Reduth *et al.* (1994) screened a large number of domestic and wild hosts for trypanocidal activity and found it mainly in buffalo and eland and to a lesser extent, waterbuck. In buffalo serum, trypanocidal activity was found to be associated with a molecule (M_r~150 kD) that was insoluble in 50 % ammonium sulphate (Olubayo, 1991). The findings by Gingrich *et al.* (1982) that *T. b. rhodesiense* infection prevalence increased significantly in *G. morsitans* fed on serum free blood fraction indicated that serum factors are involved in modulating infection in tsetse. This observation was confirmed by Maudlin *et al.* (1984) who showed that serum free infective meals resulted in significantly higher *T. congolense* midgut infection prevalence in *Glossina morsitans*. Other than serum factors, nucleated red blood cells have also been reported to support very low parasite infection prevalence in haematophagous vectors. For example, the sandfly, *Plebotomus papatasi*, a competent vector of *Leishmania tropica* when maintained on rodents, failed to develop infections when maintained on turkey

blood (Schlein *et al.*, 1983).

The sensitivity of the eland plasma inhibitor to treatment with proteolytic enzymes suggested that it is a protein. Rifkin (1978) showed that the human plasma trypanocidal activity was abolished by trypsin treatment of gel filtration fractions but not in whole plasma. This observation may be attributed to presence of trypsin inhibitors in whole plasma (Huang, 1971) but not in gel filtration fractions. Similarly, the result of this study shows that the highest trypsin concentration used (1 mg/ ml), which was 10 times what Rifkin (1978) used, only showed partial loss in the eland inhibitor activity. In contrast, a similar concentration of pronase completely abolished the eland plasma inhibitor activity. Seed and Sechelski (1989) demonstrated that plasma from *T. brucei* infected mice contain a factor that inhibits the transformation of long slender trypanosomes into short stumpy forms. Unlike the mice inhibitor factor, the one in eland plasma is present in animals that had not been exposed to trypanosomes. Secondly, it blocks transformation of bloodstream-form trypanosomes into procyclics. Therefore, it remains to be elucidated whether the two inhibitors are similar. In addition, preincubation of isolated trypanosomes in fresh plasma for 1.75 h did not affect their subsequent ability to transform. This suggested that the plasma inhibitor does not have a direct effect on the trypanosomes' ability to transform. Possibly, it interacts with the midgut factors that mediate transformation. Based on the stability studies, it is possible that the eland plasma inhibitor is completely different from the trypanocidal factor found in eland and buffalo plasma (Reduth *et al.*, 1994). The trypanocidal factors are relatively stable making their purification feasible (Rifkin, 1978; Reduth *et al.*, 1994; Seed *et al.*, 1990). For example, the activity of the human plasma TLF could be preserved by storage at -70° C (Rifkin, 1978; Gillet *et al.*, 1992). The instability of the eland plasma inhibitor was not due to proteolytic degradation since addition of protease inhibitors to plasma did not stop loss of activity.

Although we were unable to purify the plasma inhibitor due to its instability, it constitutes an additional mechanism by which trypanosome infection in tsetse is modulated by host blood. Since activity of the inhibitor is lost during storage, this implies that a tsetse taking a blood meal directly from host will have even lower infection prevalences than those reported by Mihok *et al.* (1993). The factor(s) in eland plasma that inhibits transformation certainly requires further investigations to understand the mechanism(s) involved in this interaction. This would increase the current knowledge on tsetse-trypanosome interaction.

3.2.3 Influence of proline and glutamine on trypanosome transformation.

The development of African trypanosomes from bloodstream to procyclics forms is accompanied by major changes in their nutrient uptake, energy metabolism and ultrastructure (Opperdoes, 1985; Vickerman, 1985). The most significant metabolic change is a switch from the gross utilization of glucose to proline as the major energy source (Bowman and Flynn, 1979). Lower proline levels has been reported in tsetse midguts infested by rapidly dividing *T. brucei* procyclics compared to uninfected ones 24 h after bloodmeal (Vickerman, 1985). This is not surprising considering that procyclic forms actively utilise proline not only for energy provision but also as a carbon source for growth and development (Evans and Brown, 1972; Srivastava and Bowman, 1971). This metabolic strategy is essential since proline is the most abundant amino acid in tsetse midgut and haemolymph (Cunningham and Slater, 1974; Vickerman, 1985). Active proline catabolism by midgut stage parasites appears widespread among trypanosomatids (Evans and Brown, 1972; Krassner and Flory, 1977; Silvester and Krassner, 1976). For example, *Leishmania donovani* and *Trypanosoma cruzi* have been shown to utilize proline in the midgut of sandflies and triatomine bugs, respectively (ter Kuile and Opperdoes, 1992; Silvester and Krassner, 1976). As in tsetse, proline is also abundant in the midgut of sandfly and triatomine

bug (ter Kuile and Opperdoes, 1992; Madrell and Gardiner, 1980).

For a long period, proline and glutamine have been identified as essential ingredients in the cultivation of trypomastigote procyclic culture forms in semi-defined media (Vaucel and Fromentin, 1967; Brun and Jenni, 1987; Evans, 1978). However, it has not been previously clear how the two amino acids may influence trypanosome transformation from bloodstream to procyclic forms. Unlike the case of procyclic cultures, these amino acids are not as crucial in the maintenance of bloodstream cultures *in vitro* (Brun and Jenni, 1987; Hirumi and Hirumi, 1994).

This study showed that the amino acids, proline and glutamine do not stimulate trypanosome transformation. This result suggested that although blood is important for the transformation process, the digestive products, proline and glutamine do not stimulate this process. Earlier studies have shown that tricarboxylic cycle (TCA) intermediates, citrate/ cis-aconitate, stimulates the transformation of bloodstream form trypanosomes to procyclics, *in vitro* (Brun and Schonenberger, 1981; Czichos *et al.*, 1986). Proline and glutamine are 5 carbon metabolites which are catabolized via the TCA cycle for energy generation. It is still not clear how these 6 carbon TCA cycle intermediates, namely, citrate and cis-aconitate stimulate transformation and not proline or glutamine. During degradation, the 5 carbon skeleton of both proline and glutamine enter the TCA cycle via a-ketoglutarate which has not been shown to stimulate transformation. This may possibly explains why the two amino acids had no effect on transformation.

In a previous study, Ross (1987) demonstrated that the transformation of *T. congolense* to metacyclic trypomastigote stage was dependent upon the concentration of proline or glutamine. Similarly, proline and to some extent, glutamine was also shown to stimulate transformation of *T. cruzi* epimastigotes to the metacyclic stage (Homsy *et al.*, 1989; Contreras *et al.*, 1985). Although several of these studies were based on the effect of amino acids on trypanosome transformation,

no information was hitherto available on the effect of proline and glutamine on the transformation of trypanosomes from bloodstream to procyclic forms before their subsequent transformations to the infective metacyclic forms.

CHAPTER 4

4.0 RESULTS AND DISCUSSIONS

4.1 TRYPSIN STIMULATION BY HOST BLOOD AND BLOOD COMPONENTS IN *GLOSSINA MORSITANS* AND THEIR ROLE IN TRYPANOSOME TRANSFORMATION

4.1.1 Trypsin isoenzyme in tsetse midguts fed on rat and eland blood and blood fractions

This study was initiated to collaborate trypsin isoenzymes with the trypanosome transformations in midgut homogenates from tsetse that were fed on different host blood and blood fractions. A radiolabelled trypsin inhibitor, [1,3-^{3}H]diisopropyl fluorophosphate (DFP), in presence of a chymotrypsin inhibitor, tosylphenylalanine chloromethyl ketone (TPCK), was used to specifically label the midgut trypsins. Trypsin isoenzyme profiles were progressively determined in midguts at 0, 2, 5, 8, 20, 24, 48 and 72 h after the bloodmeals. Teneral midgut homogenates showed four trypsin bands estimated as M_r~256, 56, 40 and 30 kD (Fig. 23). Immediately after the bloodmeals, homogenates from tsetse that were fed on blood and plasma had the M_r~56, 40 and 30 kD bands completely inhibited while the 256 kD was partially inhibited (Fig. 23, 24 and 25). The inhibition of these bands persisted up to 8 h post-bloodmeal (Fig. 26). By 20 h, the inhibition of the M_r~56, 40 and 30 kD isomers was relieved in blood fed tsetse midguts but persisted in midguts of tsetse that fed on plasma (Fig. 27). Signals obtained in the 0-72 h after the bloodmeals progressively increased with the strongest at 72 h. There was a

30 kD band at 0 h (Fig. 23) in midgut dissected from tsetse that had fed on rat blood that was absent at 2 h, 5 h and 8 h (Fig. 24, 25 and 26). Although blood and plasma samples showed an initial inhibition of the M_r~56, 40 and 30 kD trypsins soon after the bloodmeals, the isomer and radioactivity signals obtained at 48 and 72 h (Fig. 29 and 30) were the same as those from midguts of tsetse that fed on the other blood fractions. By 48 h, additional trypsin bands of M_r lower than 30 kD appeared only in midgut homogenates of tsetse that fed on plasma fraction and were more prominent at 72 h (Fig. 29 and 30).

4.1.2 Trypsin activities in tsetse midguts fed on rat and eland blood and blood fractions

This experiment was meant to study trypsin activities stimulated by different host blood and blood fractions and whether they bear any relationship to the trypanosome transformation supported by midgut homogenates from tsetse that fed on these fractions. Trypsin assays were carried out using a chromogenic substrate, Chromozym-TRY. Changes in absorbances as a function of time were measured at 405 nm in a spectrophotometer. Figure 31a shows that the trypsin stimulated by the different blood fractions in tenerals followed a periodic pattern peaking around 72 h. It was interesting to establish that immediately after the bloodmeal, enzyme activities in teneral tsetse decreased sharply before a stimulation phase ensued. This drop in activity was much more pronounced in midguts of tsetse that were fed on either blood or plasma compared those fed on 50 % (v/v) cell suspensions in saline. Trypsin activities stimulated by the blood fractions were not significantly different but decreased to nearly teneral levels by 144 h in all the midguts.

Figure 31b shows similarly obtained results when 72 h non-teneral tsetse were re-fed on similar host blood/ blood fractions as those in figure 31a. As in the case of tenerals, an initial drop in the (peak) trypsin activities was observed in all the midguts but the drop was highest in midguts from tsetse that fed on either whole blood or plasma. Trypsin activities were higher in the 72 h non-tenerals than in tenerals at the peak activities. However, the peak activities were around 48-72 h in both teneral and non-tenerals.

Fig. 23. **Native-PAGE of labelled midgut trypsins 0 h after bloodmeals.** Groups of *G. m. morsitans* were fed on rat and eland blood and blood fractions. Midguts were removed, homogenized then labelled in [1, 3 ^{3}H]-DFP. The homogenates (equivalent of one midgut) were separated on PAGE. **Lane 1**, unfed midgut; homogenates from tsetse fed on: **lane 2**, rat whole blood; **lane 3**, eland whole blood; **lane 4**, 50 % (v/v) rat RBCs in saline; **lane 5**, 50 % (v/v) eland RBCs in saline; **lane 6**, rat plasma; **lane 7**, eland plasma.

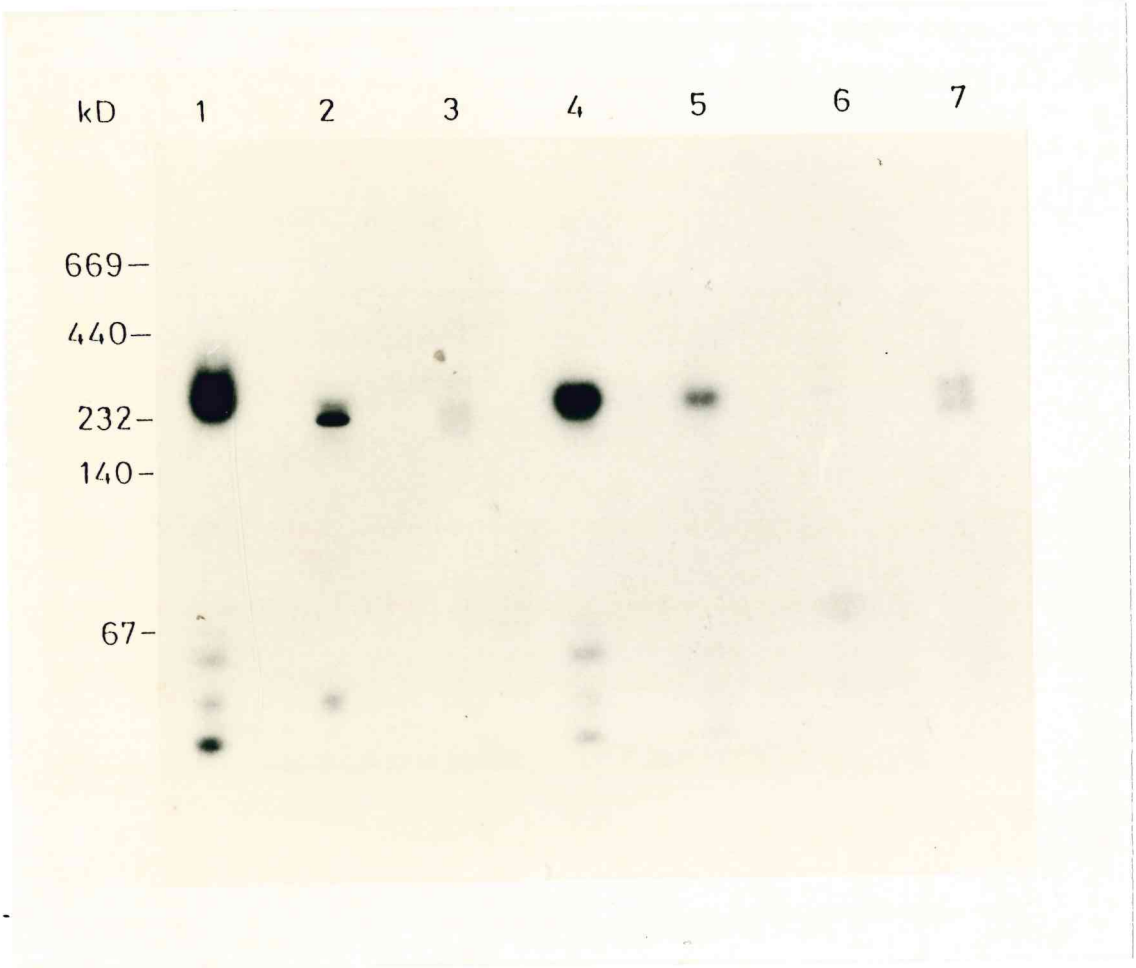

kD
1
2
3
4
5
6
7
669–
440–
232–
140–
67–

Fig. 24. **Native-PAGE of labelled midgut trypsins 2 h after bloodmeals.** The details are as in figure 23 except that midguts were obtained after 2 h.

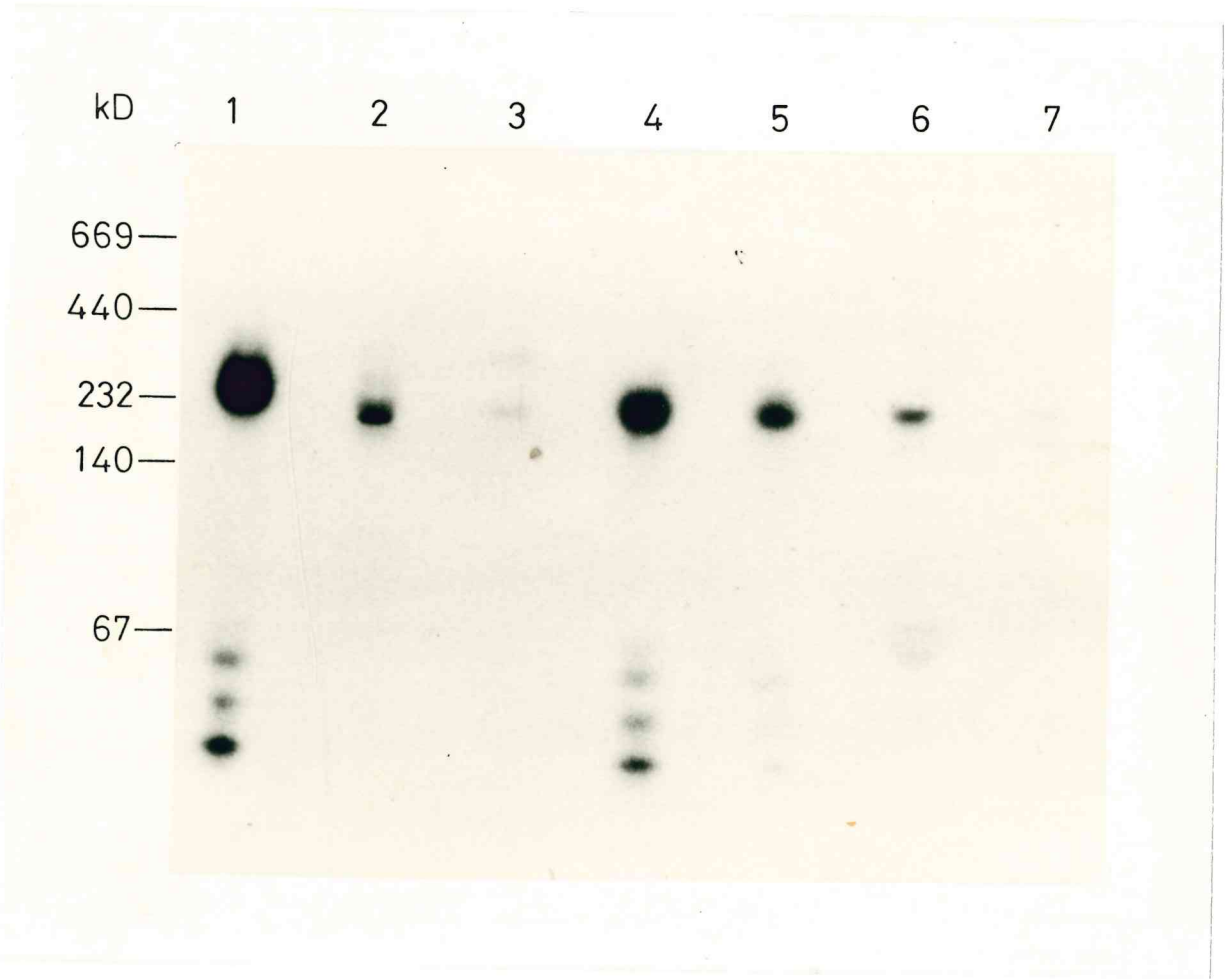

kD
1
2
3
4
5
6
7
669
440
232
140
67

Fig. 25. **Native-PAGE of labelled midgut trypsins 5 h after bloodmeals.** The details are as given in figure 23 except that midguts were obtained after 5 h.

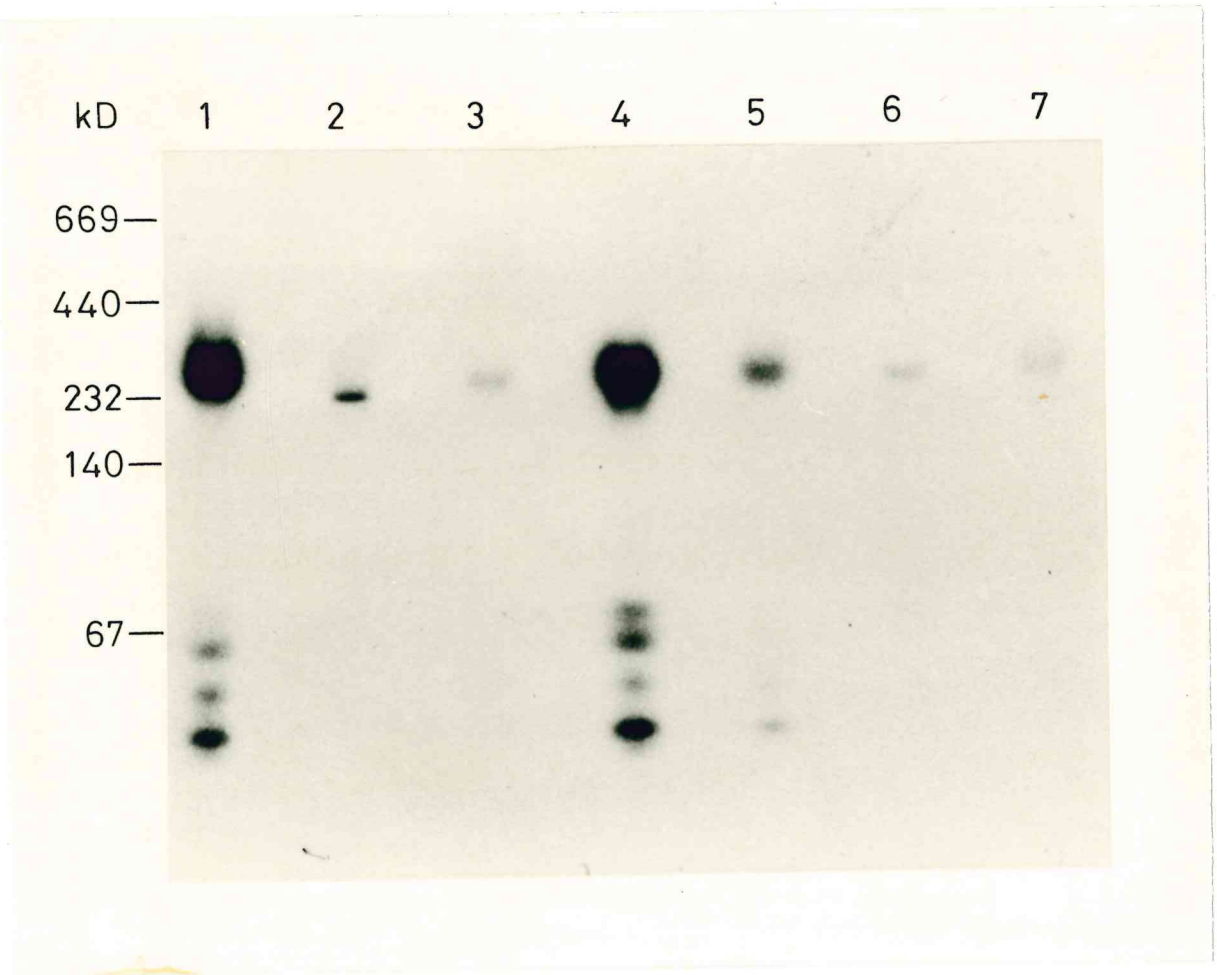

kD
1
2
3
4
5
6
7
669
440
232
140
67

Fig. 26. **Native-PAGE of labelled midgut trypsins 8 h after bloodmeals.** Details are as in figure 23 except that midguts were obtained after 8 h.

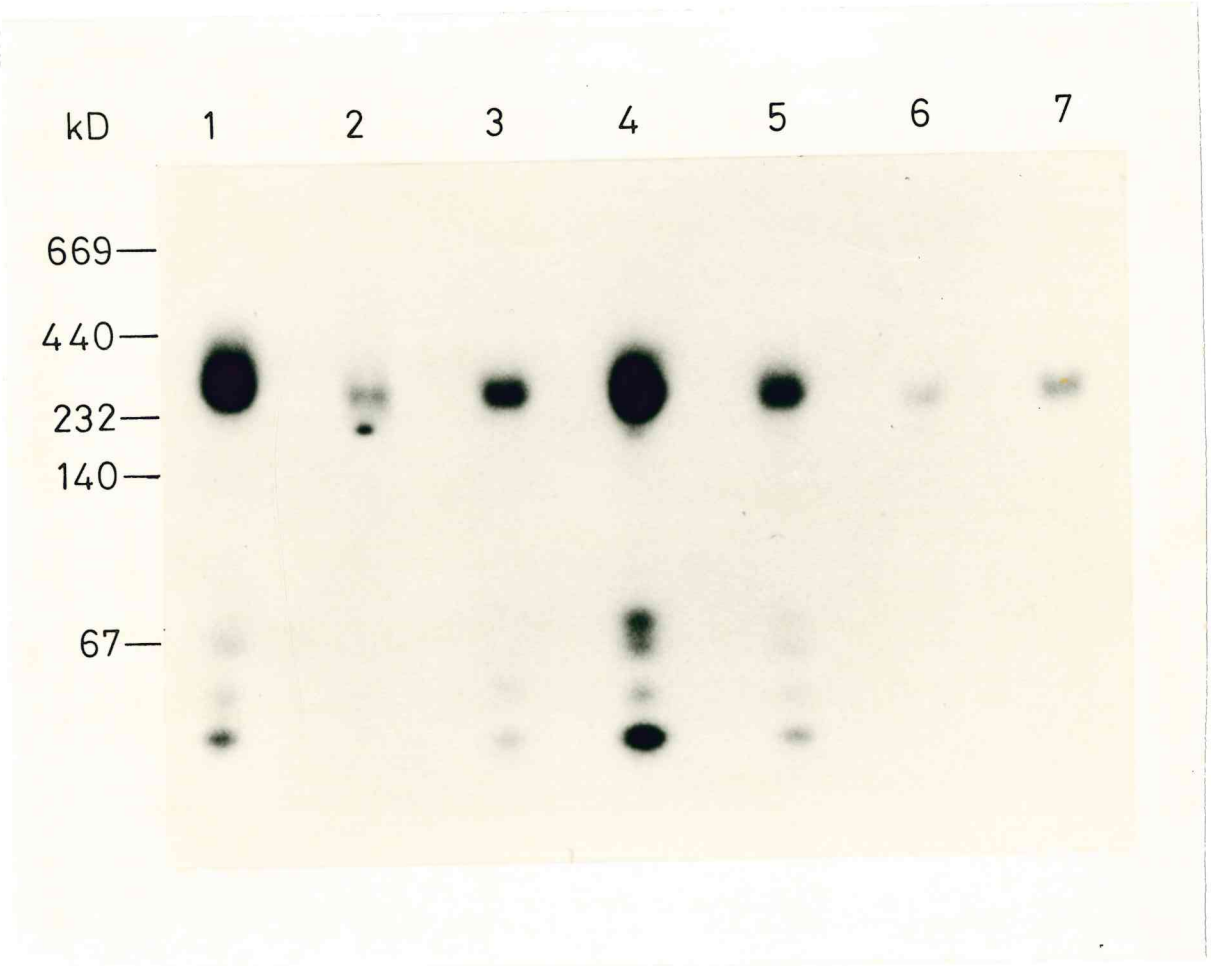

kD
1
2
3
4
5
6
7
669
440
232
140
67

Fig. 28. **Native-PAGE of labelled midgut trypsins 24 h after bloodmeals.** Details are as in figure 23 except that tsetse were dissected after 24 h.

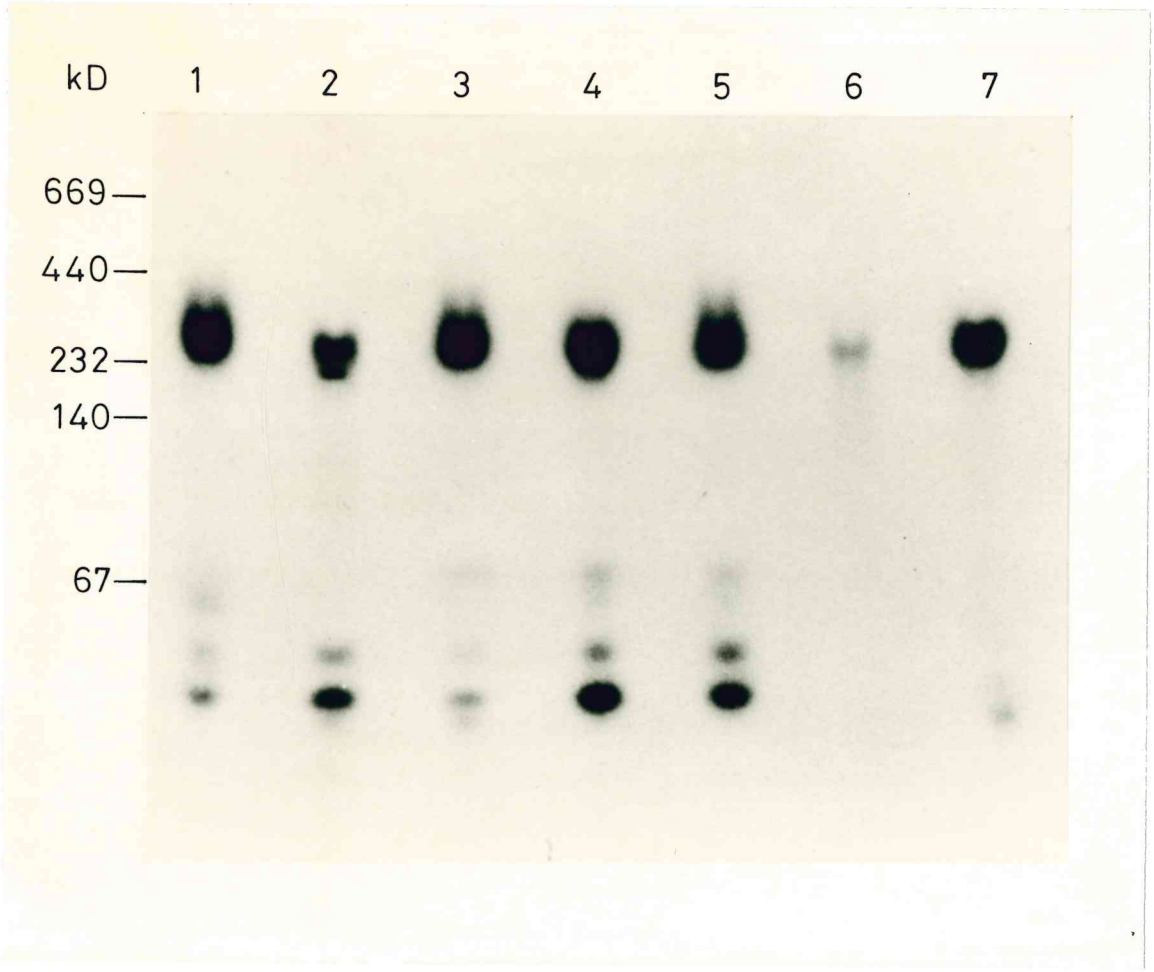

kD
1
2
3
4
5
6
7
669
440
232
140
67

Fig. 27. **Native-PAGE of labelled midgut trypsins 20 h after bloodmeals.** Details are as in figure 23 except that midguts were obtained after 20 h.

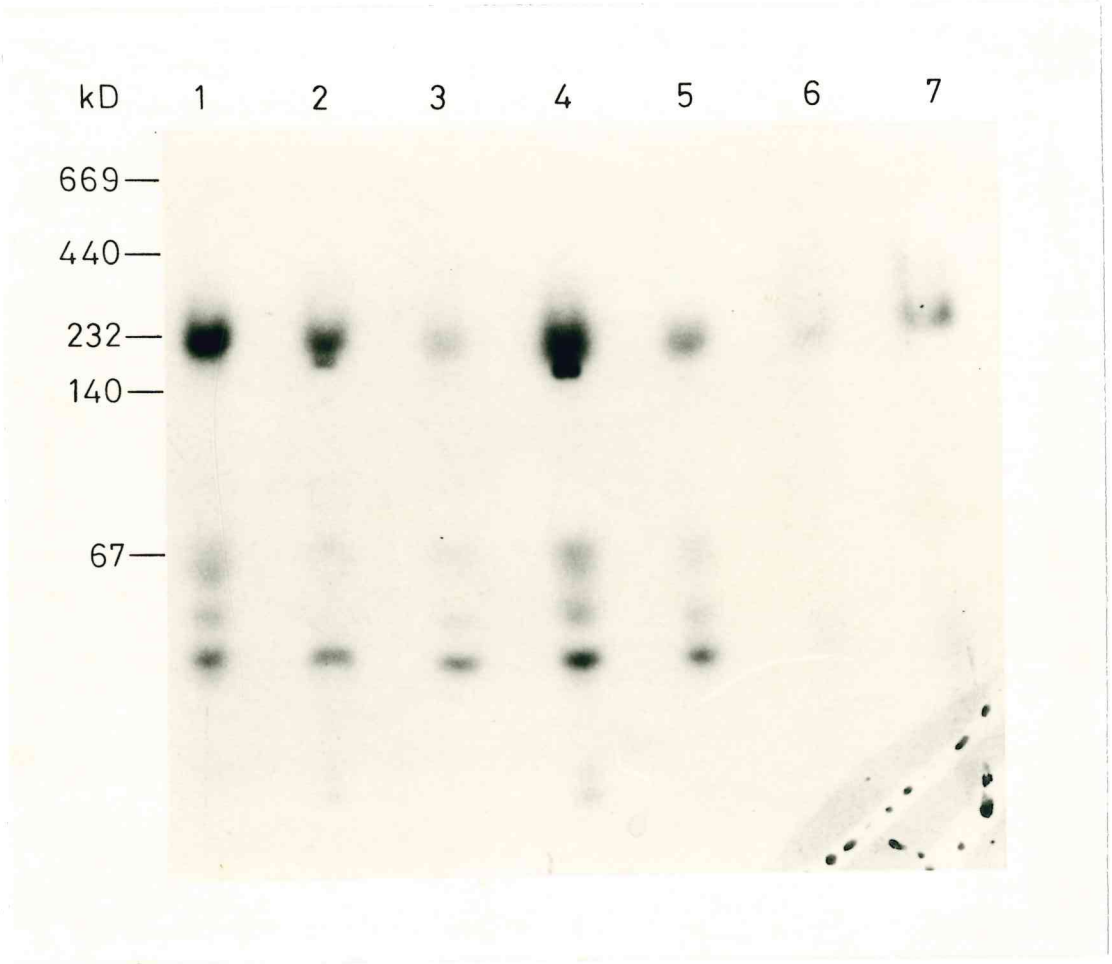

kD
1
2
3
4
5
6
7
669
440
232
140
67

Fig. 29. **Native-PAGE of labelled midgut trypsins 48 h after bloodmeals.** The rest is as in figure 23. **Lane 1**, unfed midgut; homogenates from tsetse fed on: **lane 2**, rat whole blood; **lane 3**, eland whole blood; **lane 4**, rat plasma; **lane 5**, eland plasma; **lane 6**, 50 % (v/v) eland RBCs in saline;

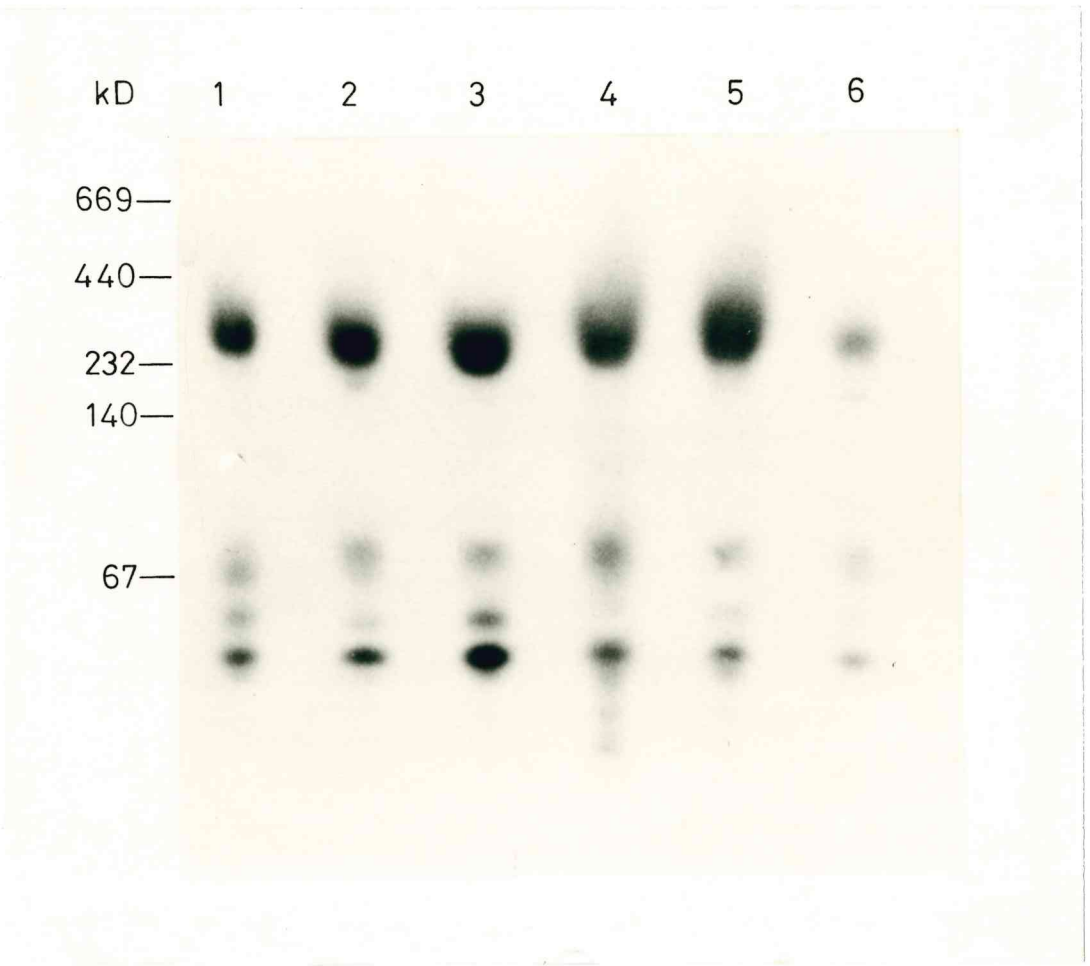
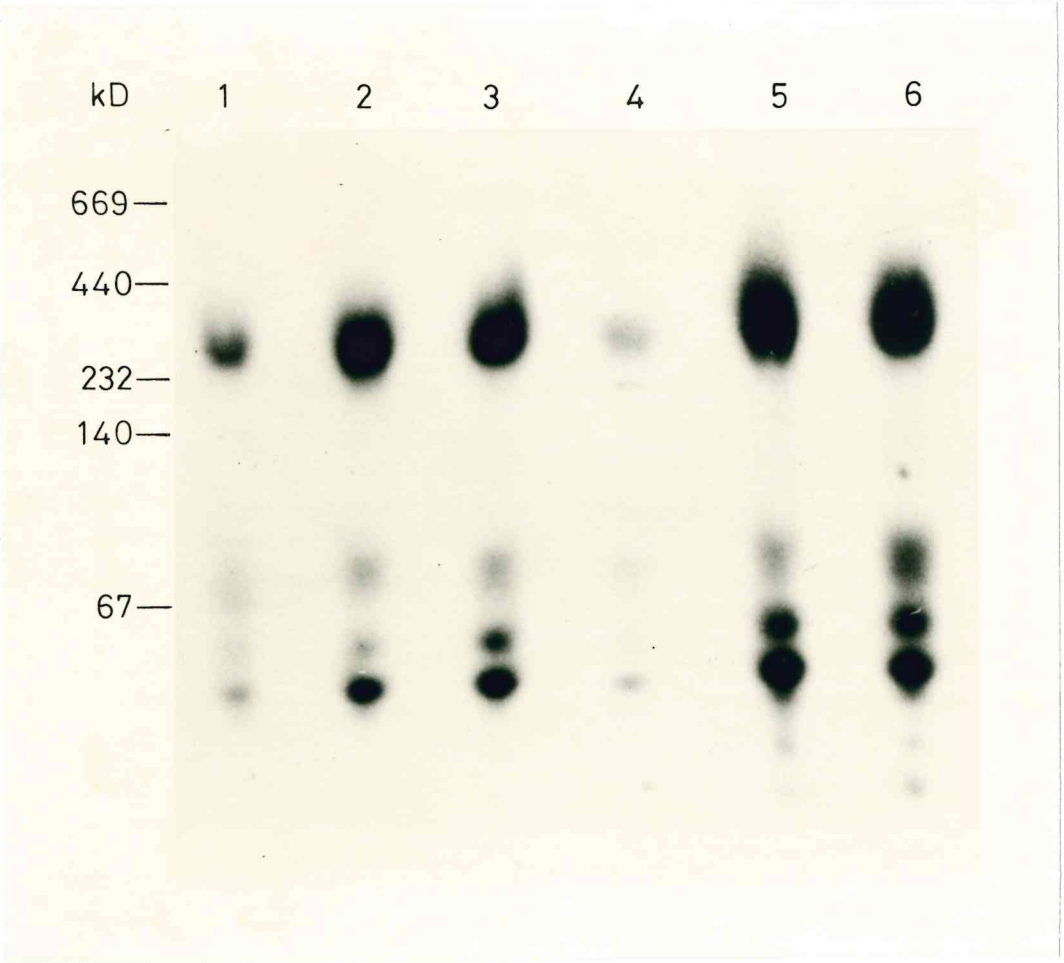

kD
1
2
3
4
5
6
669—
440—
232—
140—
67—

Fig. 30. **Native-PAGE of labelled midgut trypsins 72 h after bloodmeals.** Details are as in figure 23. **Lane 1**, unfed midgut; homogenates from tsetse fed on: **lane 2**, rat whole blood; **lane 3**, eland whole blood; **lane 4**, 50 % (v/v) eland RBCs in saline; **lane 5**, rat plasma; **lane 6**, eland plasma.


kD
1
2
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4
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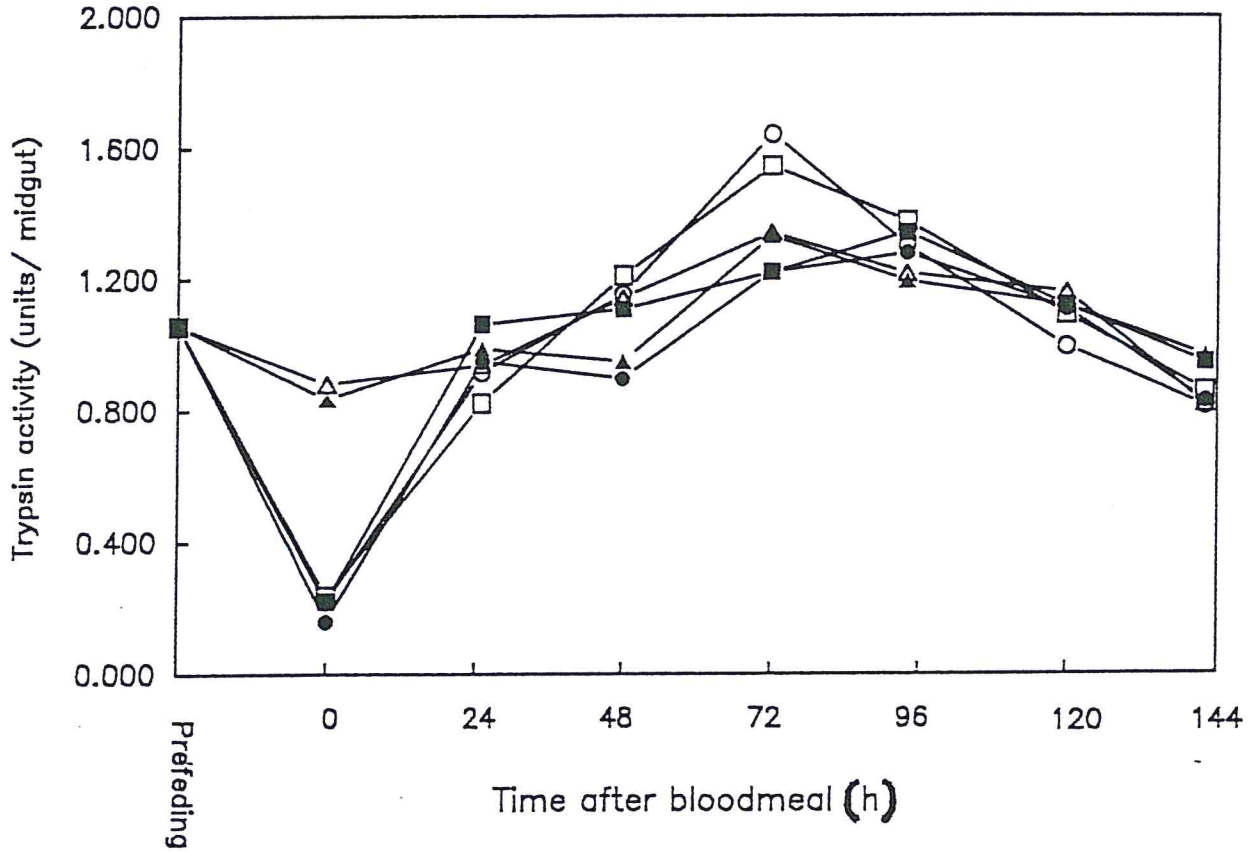
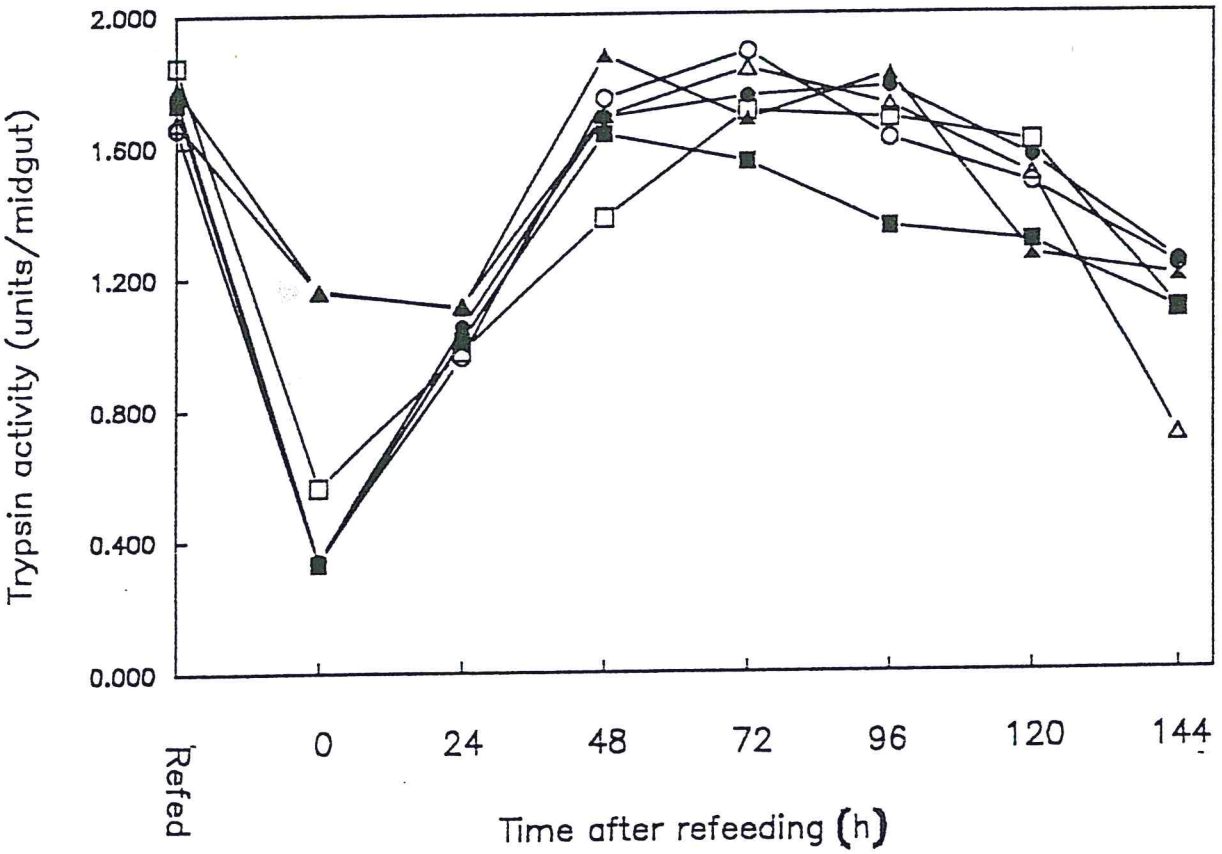
6
669
440
232
140
67

Fig. 31a. **Midgut trypsin activity in teneral *Glossina m. morsitans* after feeding on different bloodmeals.** Tsetse were fed on the following: rat whole blood (○); eland whole blood (●); 50 % (v/v) rat RBCs in saline (△); 50 % (v/v) eland RBCs in saline (▲); rat plasma (□) and eland plasma (■). At the indicated time points, 6 tsetse were dissected from each group and homogenized in 0.1 M Tris-HCl buffer, pH 7.8. Trypsin activity was determined in the supernatants after carrying out the appropriate dilutions. Each point represents the mean activity of 3 determinations.

(a)


2.000
1.600
1.200
0.800
0.400
0.000
Trypsin activity (units/ midgut)
Prefeding
0
24
48
72
96
120
144
Time after bloodmeal (h)

(b)


Trypsin activity (units/midgut)
2.000
1.600
1.200
0.800
0.400
0.000
Refed
0
24
48
72
96
120
144
Time after refeeding (h)

4.2 Discussion

4.2.1 Stimulation of trypsins by rat and eland blood and blood fractions

The present study has clearly demonstrated that teneral *Glossina morsitans* midgut homogenates contain substantial trypsin-like activity. The activity was initially inhibited by the ingested bloodmeal and later stimulated to new levels peaking around 48-72 h. Pre-bloodmeal trypsin activity in *Glossina* was previously reported by Langley (1967) and his finding agree with results of this study. This result shows that in teneral tsetse trypsin exist before a bloodmeal and that the meal enhances secretion by stimulating *de novo* synthesis. Pre-bloodmeal trypsin activity has also been reported in other haematophagous insects such as the Anopheline mosquitoes (Horler and Breigel, 1995; Muller *et al.*, 1995) and *Stomoxys* species (Moffat and Lehane, 1990). In *Stomoxys calcitrans*, for example, substantial amount of digestive proteases has been reported in unfed midguts packed in storage vesicles, and is rapidly released after a bloodmeal (Moffat and Lehane, 1990; Wood and Lehane, 1991). Based on pre-bloodmeal enzyme activity, it appears that trypsin induction in *Glossina* is closely related to that of *Anopheles* and *Stomoxys* but may be quite different from that of *Aedes*.

The mechanisms involved in the bloodmeal stimulated secretion of trypsin activity in the midgut of tsetse and other haematophagous insect is still not well understood. In *Aedes aegypti*, for example, bloodmeal stimulates trypsin secretion in two phases namely, the early and late trypsins (Barrillas-Mury *et al.*, 1995). The early trypsins are secreted 2-3 h after bloodmeal and are insensitive to transcriptional inhibitors given with a bloodmeal suggesting that their mRNA is synthesized prior to bloodmeal then translated immediately after bloodmeal (Felix *et al.*, 1991). On the other hand, secretion of the late trypsins begins about 8 h after bloodmeal, peaking around

28-30 h. Unlike early trypsins, the late trypsins are sensitive to transcriptional inhibitors given with a bloodmeal suggesting that their mRNA is transcribed after the meal (Felix *et al.*, 1991). Neither the early nor the late trypsins in *Aedes* were detected in homogenates of unfed female mosquitoes, suggesting that they are translated *de novo* following a bloodmeal (Graf and Briegel, 1989). This is in contrast to *Glossina morsitans* trypsin activity which is present in the tenerals and a bloodmeal just stimulates their release and enhances further secretion.

In *Anopheles* midguts, Horler and Briegel (1995) have demonstrated the presence of a constitutive and inducible trypsin-like activity. The synthesis of the *Anopheles* inducible trypsins has been shown to be stimulated only by a bloodmeal (Horler and Briegel, 1995). In contrast, the constitutive trypsins are present in *Anopheles* midgut even before a bloodmeal. They are synthesized and retained in the midgut epithelial cells and released after a bloodmeal (Horler and Briegel, 1995). As much as one third of the peak activity was reported in *Anopheles albimanus* females that had never taken a bloodmeal (Horler and Briegel, 1995). However, the authors did not analyse the trypsin isoenzymes involved therefore, it is not known whether the inducible trypsins represents an enhanced secretion of the same isozymes as the constitutive ones or different isomers are involved. From the present study, there was no evidence of early and late trypsins in *Glossina m. morsitans* for two reasons. First, the four trypsin isoenzymes observed in the teneral state were still the ones expressed in later stages of bloodmeal digestion except for the differences in signal intensities. Second, the early trypsins in *Aedes* were released 2-3 h after bloodmeal and not before or immediately after the meal. The differences in trypsin isoenzymes and activities soon after the bloodmeals is primarily due to inhibition by plasma and not as a result of induction. Further studies on mRNA synthesis prior and after bloodmeals may

prove useful in establishing whether early and late trypsins exists in *Glossina*. Such studies would be useful in confirming the results obtained by isoenzyme studies presented in this work. Since *Glossina morsitans* shows trypsin activity in teneral state which is stimulated to peak levels after bloodmeal, the mechanism of trypsin release resembles that of *Anopheles*. Thus, the trypsin activity in *Glossina morsitans* before and after a bloodmeal is comparable to the constitutive and inducible trypsins, respectively, in *Anopheles*.

The late and the inductive trypsins in *Aedes* and *Anopheles* mosquitoes, respectively, are important in bloodmeal digestion (Barillas-Mury *et al.*, 1995; Horler and Briegel, 1995). However, the role of the early and constitutive trypsins in *Aedes* and *Anopheles*, respectively, is still not clearly established. Barrillas-Mury *et al.* (1995) showed that the early trypsins may be involved in regulating the synthesis of the late trypsins in *Aedes aegypti*. On the other hand, the *Anopheles* constitutive trypsins may be important for bloodmeal digestion (Horler and Briegel, 1995) since it is known to ingest multiple bloodmeals as a strategy to optimize fecundity (Briegel and Horler, 1993). However, the role of these trypsins in parasite development in their respective vectors, particularly in midgut establishment remains to be clearly established.

Four trypsin isomers, M_r~256, 56, 40 and 30 kD were detected in native-PAGE using a tritiated-DFP in teneral *G. morsitans* midgut homogenates. Immediately after bloodmeal, the isomers, M_r-56, 40, and 30 kD disappeared in midgut homogenates from tsetse that were fed on either whole blood or plasma but the same isomers were only slightly inhibited in homogenates from tsetse that fed on rat or eland 50% (v/v) RBC suspension in saline. This clearly indicated that the initial inhibition of trypsin-like activity was mainly due to inhibitor(s) in the plasma fraction. Using tritiated DFP, Graf and Briegel (1985) detected five major trypsin bands,

M_r~26.7, 28.5, 29.7, 31.2 and 32 kD on SDS-PAGE in *Aedes aegypti* midguts 24 h after bloodmeal. In *Aedes*, peak activity is about 20-30 h after bloodmeal (Briegel and Lea, 1975), therefore the 5 bands were expressed within the peak activity. Using a similar assay system, Borovsky and Schlein (1988) also showed the presence of several trypsin isozymes on native-PAGE of *Culex* and *Aedes* mosquitoes but the molecular sizes were not reported. In contrast to the present study, the earlier workers analysed trypsins only at peak activity and therefore it is not possible to make a complete comparison with the results of the present study.

Like many blood clotting factors, activated thrombin is sensitive to DFP and can result in artifacts. In the present study, it is unlikely that there were any artifacts as a result of activated thrombin for two reasons. First, in the 24-72 h midgut the factor would be inactivated through proteolytic digestion. Second, in the earlier hours (0-20 h) after bloodmeal when thrombin is active, additional isomers other than the four present in teneral state were not observed. This suggested that no artifacts originated from such contaminations.

At peak activity (48-72 h), some minor bands, $M_r < 30$ kD were observed in midgut from tsetse that were fed on plasma but not whole blood or RBC. Earlier studies on mosquitoes showed that the quality and quantity of protein in bloodmeal was an important factor in determining the quality and quantity of trypsins released (Briegel and Lea, 1975; Graf and Briegel, 1989; Felix *et al.*, 1991; Noriega *et al.*, 1994). In *Aedes aegypti*, insoluble glutaldehyde fixed erythrocyte ghosts were shown to induce delayed as well as reduced trypsin activity compared to bovine serum albumin which stimulated trypsin just like whole blood (Felix *et al.*, 1991). On the other hand, small peptides in form of neutralized liver digests failed to induce any activity until 8-10 h after bloodmeal (Felix *et al.*, 1991). According to Gooding (1974), washed

beef erythrocytes failed to stimulate midgut trypsin activity in *Glossina morsitans*. In contrast to his finding, this study has clearly showed that washed RBCs are also capable of stimulating trypsin activity just like whole blood. Gooding (1974) also showed a correlation between posterior midgut trypsin activity and the amount of protein ingested. This study did not, however, attempt to correlate trypsin activity to the quantity of protein fed to tsetse. Mihok *et al.* (1995) reported that midgut trypsin activities in tsetse 48 h after bloodmeal were not significantly different whether samples originated from goat, buffalo or eland. Although Mihok *et al.* (1995) did not examine trypsin activity as a function of time, their results are in support of the findings of this study. Details on how bloodmeals influences the quality and quantity of the trypsins released are still not well established and requires further investigations.

4.2.2 Relationship between trypsins and trypanosome transformation

In vivo and *in vitro* studies on the transformation of bloodstream *Trypanosoma brucei* into midgut forms in *Glossina m. morsitans* suggested that the process is mediated by some factors present in midgut in the early hours after a bloodmeal (Imbuga *et al.*, 1992a). Morever, the demonstration that teneral gut homogenates added to parasitised rat blood stimulates transformation (Imbuga and Osir, personal communication) is in support of this observation. This study analysed trypsin isoenzymes released in tsetse midgut by rat and eland host blood/ blood components using tritiated-DFP between 0-72 h in native-PAGE. This was carried out to identify trypsin isoenzymes involved in bloodstream trypanosome transformation. Isoenzymes stimulated by rat and eland whole blood were the same despite the fact that the former supported while the latter inhibited transformation (Nguu *et al.*, 1996). The initial inhibition of the activity

and isoenzymes by the ingested whole blood possibly represents a natural adaptation to suppress protease mediated trypanosome destruction thereby increasing their chances of establishment in *Glossina*. Inhibition of *Aedes aegypti* trypsin activity by serum was earlier reported (Huang, 1971). Huang (1971) further demonstrated that sera from different animal hosts show variations in their capacities to inhibit midgut trypsins. The fact that the M_r~56, 40 and 30 kD isomers were completely inhibited during the period when transformation occurs suggested that the isomers are not important in this process but would be crucial in lysis and bloodmeal digestion. The 30 kD trypsin isomer that appeared in midguts from tsetse that were fed on rat whole blood at 0 h is possibly due to incomplete inhibition by the time of dissection since this was carried out immediately after feeding starting with the rat blood fed midgut.

Since midgut trypsins are implicated in the transformation of bloodstream form trypanosome into procyclics (Yabu and Takayanagi, 1988), this suggests that the four trypsin isomers are important in this process. A chimeric molecule with both trypsin and lectin activity, native M_r~61 kD, was isolated from *Glossina longipennis* midgut homogenates (Osir *et al.*, 1995). This molecule has been shown to stimulate trypanosome transformation from bloodstream to procyclic forms *in vitro* (Osir and Imbuga, unpublished data). Antibodies against this molecule showed cross-reactivity only to midgut homogenates from other *Glossina* species suggesting that it is specific to tsetse. Based on molecular size, the trypsin-lectin complex appears to be closely related to the M_r~56 kD isomer. However, since the M_r~56 kD isomer was also inhibited during the period when transformation is stimulated, then it is not clear what role it plays in the transformation process. It is possible that the brief encounter of the trypanosomes with these trypsins before their total inhibition provides the required signal for transformation

such that their subsequent inhibition is, essentially, of no consequence.

There were no apparent differences in trypsin isozymes observed in midguts homogenates from tsetse that had fed on rat or eland plasma despite the fact that the latter contains inhibitors for trypanosome transformation (section 3.7). Since trypsin isoenzymes elicited by rat and eland whole blood were the same, the eland plasma inhibitor for trypanosome transformation may be acting through mechanism(s) other than by trypsin inhibition.

CHAPTER 5

5.0 GENERAL DISCUSSIONS AND CONCLUSIONS

The objective of the present study was to understand the role of host blood in the transformation of bloodstream trypanosome into procyclic forms. This study has gone some way towards identifying some of the crucial factors in host blood that influences this process.

In the development of the African trypanosomes, the metacyclic forms represents the final developmental stage in the tsetse vector. The successful development of trypanosomes to this stage is a complex process in which many factors are involved (Maudlin, 1991). Each developmental stage requires stage-specific growth conditions (Vickerman, 1985).

The transformation of bloodstream trypanosomes to procyclic forms takes place in tsetse midgut in the presence of the host blood meal (Roditi *et al.*, 1989; Ziegebaur *et al.*, 1990) but it has not been previously established how it influences this process. This study has demonstrated that blood is important in the transformation of bloodstream trypanosomes to procyclic forms. Preliminary investigations in this work using blood samples from rat, buffalo, goat and eland hosts showed that rat and eland blood supported and inhibited transformation, respectively. Therefore, the two host blood samples were suitable choices in further studies to understand the influence of host blood in this process. Plasma and serum fractions from both hosts did not support the transformation process. In addition, eland plasma fraction also inhibited the process and this was the reason for the very low transformation rates using eland blood samples.

That host blood differs in their ability to support infections in tsetse has already been reported (Mihok *et al.*, 1991, 1993; Moloo, 1981, 1984). The results of the present study makes it possible to link transformation to infection prevalences, previously reported in tsetse fed on

infective meal in the respective host blood. The inhibition of trypanosome transformation from bloodstream to procyclic forms in tsetse that feeds on infective eland blood could be the main factor responsible for the low infection prevalence reported in tsetse taking such a meal (Mihok *et al.*, 1993).

Blood feeding arthropods acquire various parasites from vertebrate hosts but its only a restricted number of insect hosts that successfully transmit such parasites (Lehane, 1991). The fact that tsetse is the only known transmitter of trypanosomes is example of a specific interaction that promotes development of the parasite in that host where others cannot develop. From a biochemical viewpoint, a delicate balance of factors enables the trypanosomes to develop only in tsetse and not in other haematophagous insects. Several factors including midgut trypsins (Yabu and Takayanagi, 1988) and lectins (Maudlin and Welburn, 1988a,b) have been implicated in the transformation of bloodstream-form trypanosomes to procyclics. Other factors are the tricarboxylic cycle intermediates, cis-aconitate and citrate (Brun and Jenni, 1987). Variations in the levels of these factors, either individually or in combination, may have a profound influence on the ability of a given tsetse species to successfully transmit a given trypanosome species.

Whereas trypsin has been shown to stimulate trypanosome transformation (Yabu and Takayanagi, 1988), there is a general lack of knowledge on the mechanisms involved. In this study trypsin activity and isozymes stimulated by various fractions of rat and eland host blood were investigated to elucidate their role in transformation. However, a clear relationship of the observed transformations and trypsins under the influence of the different blood fractions could not be easily deduced. The significance of teneral trypsin activity in the four isomers (Chapter 4) on trypanosome transformation is yet to be established. If trypsin plays a role in transformation as earlier indicated, then the role the four isomers in this process is not clear since three of the isomers, M_r~56, 40 and 30 kD, were inhibited in midguts from tsetse that fed on either rat or eland whole blood but homogenates of tsetse fed on the latter are still capable of supporting

transformation. However, it could be that only a brief interaction of the trypanosomes with one or more of these isomers before they are inhibited by the blood provides the nessessary stimuli for transformation. Furthermore, since trypsin activities and isoenzymes stimulated by rat and eland blood were the same, this further suggests that differences in their ability to support transformation is not due to trypsin stimulation. This show that the interaction of trypanosomes and trypsins in the transformation process is complex and requires further clarification.

From this study, the following conclusions can be drawn on the role of blood in trypanosome transformation from bloodstream into procyclic forms:

1. Blood is important for trypanosome transformation.
2. Host blood differ in their ability to support this process.
3. Red blood cell fraction is crucial for the transformation process.
4. Plasma or serum fractions do not support transformation.
5. Eland blood does not support the transformation process due to a highly labile proteinaceous inhibitor in plasma.
6. The eland inhibitor is insoluble above 50% saturated ammonium sulphate and has a molecular weight higher than 14 kD as determined by dialysis.
7. The amino acids, proline and glutamine which are the most abundant bloodmeal digestive products in tsetse gut and haemolymph, appear not to support the transformation process.
8. Teneral tsetse gut homogenates has trypsin activity in four isomers of approximate M_r~256, 56, 40 and 30 kD.
9. Blood and plasma show an initial inhibition of trypsin activity and isoenzymes in tsetse midguts before a stimulatory phase. The inhibition was complete in the M_r~56, 40 and 30 kD isomers, while it was partial in M_r~256 isomer.
10. While the rat and eland blood showed extreme differences in their ability to support the transformation process, trypsin activities and isozymes stimulated were the same.

5.1 Future research projects based on this work

The demonstration that RBCs constitutes an important stimulatory factor for transformation of trypanosomes from bloodstream to procyclics forms is a significant result. Further work is required to elucidate molecules in RBCs that are responsible for stimulating/supporting transformation. The eland plasma inhibitor for trypanosome transformation needs to be isolated in an active form so that its other properties and mode of action can be studied. The presence of four trypsin isoenzymes in teneral midguts is interesting. Further attempt should, therefore, be made to elucidate the role of each of these isomers particularly in trypanosome transformation. This could possibly be done by selective inhibition of the isomers. Knowledge of the mode of action of the eland plasma inhibitor and the role of the individual trypsin isomers could be valuable in opening up new avenues that may be exploited in combating trypanosomiasis.

6.0 *References*

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