DOCTOR OF PHILOSOPHY

A study of the midgut (reservoir zone) and haemolymph lectins of the stable fly, Stomoxys calcitrans.

Abdally, Mohammed H.

Award date:
1997

Awarding institution:
Bangor University

A study of the midgut (reservoir zone) and haemolymph lectins of the stable fly, *Stomoxys calcitrans*.

A study of the midgut (reservoir zone) and haemolymph lectins of the stable fly, *Stomoxys calcitrans.*

A thesis submitted to the University of Wales in candidature for the degree of Philosophiae Doctor of the University of Wales.

By Mohammed H. Abdally B.V.S. (1985), M.Sc. (1990)

To

KING FAHD IBN ABDUL AZIZ AL-SAUD, the Custodian of the two Holy Mosques and the man who established health and education policies in Saudi Arabia

and to

His R.H. PRINCE ABDULLAH AL-FAISAL IBN ABDUL AZIZ AL-SAUD whose kindness enabled me to undertake my education

and to

my wife, SALWA M.Y. AL-GURASHI, our children and my brother KHALIL H. ABDALLY, whose kindness and support has enabled me to complete my work.

SUMMARY

Although it is sympatric with tsetse flies, *Stomoxys calcitrans* is not a biological vector of trypanosomes. It is known that haemolymph (HL) and midgut reservoir zone (RH) lectins regulate parasitic infections in some dipteran insects. Agglutinins (lectins) were detected in HL and RH from unfed stable flies (maximum titre 2^{-6}). Increased haemagglutination activity resulted post-feeding (maximum titre 2^{-16} - 2^{-18}). Optimum titres varied according to agglutinogen type and mammalian blood source. Rabbit erythrocytes produced the highest haemagglutination titres followed by human group B, human group O, horse, human group A, human group AB and sheep. *Stomoxys* haemagglutination activity was found to be 1.5 - 2.5 times stronger than that of *Glossina*.

Whole blood-fed flies produced the highest titre (2^{-18}), compared to glucose-fed insects, against rabbit erythrocytes. Anti-*Trypanosoma brucei brucei* titres ranged from 2^{-6} - 2^{-7} in both tissues. Similar results were obtained with *Leishmania hertigi* and *Crithidia fasciculata*.

Purification of the samples was performed in order to draw conclusions with confidence regarding the physico-chemical properties of the agglutinins (lectins) and in order to determine the molecular weight of the agglutinins. Protein contents of HL and RH samples of flies aged < 12 hours to 3 days were determined. They were 25 - 28 mg/ml and 6.4 mg/ml respectively. Protein contents increased with age reaching 32 mg/ml for HL and 7.2 mg/ml for RH at day 14 post-emergence (p.e.). The contents then started to decrease reaching 22 mg/ml for HL and 5.6 mg/ml for RH at day 28 p.e. Purified lectins constitute 4.3% of the total protein contents in RH samples (having molecular weights of 26,302 Da, 16,218 Da and 14,028 Da) and, approximately twice, 9.47% of the total protein contents, in HL samples (having similar molecular weights of 28,300 Da, 16,218 Da and 14,600 Da). HL and RH anti-parasite and anti-erythrocyte agglutinins (lectins) were basic glycoproteins in nature, calcium ion dependent for activity, heat labile, freeze-thaw sensitive and required slightly acid to alkaline pH conditions for optimum agglutination. Lectins were specific for galactosyl and glucosyl moieties. *In vivo* sugar inhibition of RH lectin activity resulted in three-fold increased *S. calcitrans* mortalities post- *T.b. brucei* infection, compared to the controls, suggesting a lectin parasite-killing function. However, sugar inhibition of lectins did not lead to transformation of trypanosomes to procyclic forms or to infection of the fly.

CONTENTS

ACKNOWLEDGEMENTS

First and foremost, praise be to Allah, The Most Gracious, The Most Merciful and peace be upon his Messenger Mohammed (PBUH).

I am particularly grateful to Dr. M.J. Lehane (University of Wales, Bangor) and Dr. G.A. Ingram, Prof. P.S. Craig and other members of staff of the University of Salford for providing me with perceptive comments, constructive criticisms and help.

I appreciate the very useful information and cooperation I have received from Drs. I. Maudlin and S. Welburn and other staff members of the Tsetse Research Laboratory (University of Bristol).

My thanks are extended to Mr. M. Thomas (Bangor), Mr. R. Barker (Bristol) and Mr. D. Levin (Salford) for their assistance in helping me to establish my practical work. My thanks also to Miss Sally Anne Perks and Mr. Anthony Thompson for providing me with animal bloods and to Dr. Jibani and the Department of Haematology at Ysbyty Gwynedd, Bangor for providing me with human blood.

Special thanks to Miss Ann Pennel (Bangor) for culturing insects in the animal house and Mr. A. Davies for helping me in freezing and packing the samples to be transported with me to Bristol and Salford.

I am indebted to King Faisal University, the Saudi Arabian Cultural Bureau in London and departments and staff of the Royal Embassy of Saudi Arabia for their financial support and assistance and in particular, his Excellency Dr. Ghazi Abdul Rahman al-Gusaibi, Ambassador of Saudi Arabia.

I also thank Mr. J. and Mrs. S. Cannon for their perceptive comments, help and constructive criticisms and for improving punctuation and English grammar throughout my work.

I am indebted to Mrs. E. Jones for the typing of both draft and final manuscripts of my work.

Last but not least, my deepest gratitude is to my wife, Salwa and to our children, Kholode, Hussain, Sarah and Abdullah for providing me with an inspiring and conducive family atmosphere whilst in this country (G.B.).

General Introduction

The role of lectins in insect's immunity

The term lectin (from the latin legere, to pick out or chose) was first used to describe a class of proteins of plant origin which agglutinate cells and exhibit antibody-like sugar binding specificity (Boyed, 1962) Goldstein and Etzler (1983) were the first to define a lectin as "a carbohydrate-binding protein of non-immune origin which agglutinates cells or precipitates polysaccharides or glycoconjugates". Lectins have now been isolated from many different sources and their behaviours defined in different ways. They have been isolated from bacteria, fungi and animals as well as from plants (Sharon and Liss, 1972). For example, the bacterium *Pseudomonas aeruginosa* has galactosephilic and mannosephilic lectins which increase the growth rate and stimulate the phagocytic activity of *Tetrahymena pyriformis* (Gilboa-Garer and Sharabi, 1980).

Liener *et al.* (1986) mentioned Stillmark as the first (in 1889) to show that plant agglutinins, in this case ricin, could agglutinate erythrocytes and that the erythrocytes from different animals reacted differently. They also reported that Stillmark had isolated another toxin, cortin, (from *Cortontiglium*) which showed a different agglutination activity from that of ricin towards erythrocytes of the same animals.

Lectins have been used in parasitological investigations and have been shown to influence growth and survival of parasites. Pearlman *et al.* (1982) carried out a study to detect the difference in sensitivity to concanavalin A (Con A) mediated agglutination between *Leishmania tropica* and *L. donovani*. The two parasite strains were grown in either Panmede (P) or Mand (M) medium. They found that a strain of *L. tropica* (LRC-L32) was more sensitive to Con A mediated agglutination than a strain of *L. donovani* (LRC-L52). They concluded that the greater sensitivity of *L.*

tropica was due to either the presence of more available Con A receptor sites on the cell surface or a greater membrane fluidity resulting in more rapid clustering at the receptor sites (increasing cross-linking and agglutination). They also found that the inhibiting factor which reversed the sensitivity of *L. donovani* when grown in P and M medium was a glycoprotein fraction of lactalbumin hydrolysate. Rudin *et al.* (1989), in a study of the binding of lectins to *Trypanosoma rangeli,* found that Con A was the only lectin out of nine which bound to the surface of culture and vector forms of the parasite. However, it did not react with any midgut or salivary gland forms of the parasite. Another study was done to investigate the binding of lectins to the cell surface of different stages of three strains of *Trypanosoma cruzi* (the causative agent of Chagas' disease). It was found that Con A was the only lectin amongst eight to bind to the cell surface of amastigotes, trypomastigotes and epimastigotes (Araugo *et al.*, 1980)

The above review indicates that lectins have been widely studied since the time of their discovery and much of this work has centered on their ability to bind to the surface of erythrocytes, other animal cells and parasites. This characteristic property attracted biologists, especially entomologists, to study invertebrate defence reactions towards pathogenic organisms ingested within food.

To understand the invertebrate immunological system it is useful to compare it to the more widely studied vertebrate system. Generally, the immune system of a vertebrate animal is composed of two major divisions, the innate and adaptive systems (Roitt *et al.*, 1989). In invertebrates, particularly insects, the primary physical defences

(barriers) to invading pathogens (e.g. bacteria or parasites) are the cuticle (the rigid exoskeleton) and the peritrophic membrane (which surrounds the food bolus and therefore protects the midgut epithelium). These physical barriers are analogous to the skin and mucosa of man and animals and are part of the innate system of an insect. Should pathogens penetrate these physical structural barriers and enter the haemocoel, the secondary haemolymph defences will take place by both cellular and humoral responses (Dunn, 1986; Lackie, 1988).

The cellular defence responses are performed by the circulating haemocytes within the haemolymph. They are extremely efficient at removing foreign particles (e.g. bacteria, fungi, microfilariae and eggs of hymenopteran endoparasites) from the haemocoel by either phagocytosis, nodule formation or encapsulation (Dunn, 1986; Lackie, 1988). The haemocytes which are circulating within the haemolymph protect the insects from the foreign invaders by phagocytosis and encapsulation. This is analogous to the phagocytes and natural killer cells in vertebrates. The primary phagocytes in the haemolymph are granulocytes and plasmatocytes which phagocytose small particles such as bacteria and protozoal parasites. However, the larger particles are removed by both nodule formation and encapsulation (Dunn, 1986; Lackie, 1988). These two mechanisms are enhanced by humoral factors which may be elements of the prophenoloxidase cascade, antibacterial and antifungal molecules or haemolymph lectins (Lehane, 1991).

The humoral defence mechanism in insects lacks the immunoglobulins, but instead has a variety of lectins (agglutinins) with different specificities as discussed above. The

humoral response is inducible e.g. by wounding or inoculation of pathogens (Ingram *et al.*, 1984; Roitt *et al.*, 1989; Azumbuja *et al.*, 1986) or by invasion of foreign particles such as bacteria and endoparasites e.g. trypanosomes (Dunn, 1986; Lackie, 1988; Lehane, 1991). This is the adaptive immune system. Attacins and cecropins and their related molecules are the major characterised and sequenced inducible factors found in insects (Dunn, 1986; Roitt *et al.*, 1989). These immune factors have been found in blood-sucking insects. For example, Azambuja *et al.* (1986) investigated the antibacterial activity of the haemolymph of *Rhodinus prolixus* after inoculation with *Streptococcus mutans.* The level of activity reached a maximum 5-6 days after inoculation and then declined gradually. At the time of maximum activity destruction of the bacteria in the haemolymph was found to occur. The antibacterial activity was due to a protein of low molecular weight. This protein was also found to be heat stable, dialyzable and inactivated by trypsin treatment and it was concluded that the antibacterial activity was due to small proteins called cecropins.

Generally lectins have been detected in insect haemolymph by agglutination of erythrocytes (haemagglutination). A strong haemagglutination activity (titre 512) was reported in the haemolymph of both sexes of *Teleogryllus commodus* towards erythrocytes of human, pigeon, chicken, rat, cat, rabbit, horse, monkey and sheep (Hapner and Jermyn, 1981). The carbohydrate binding specificity of that agglutinin was detected by determination of the haemagglutination titre in the presence of potentially competing substances (inhibition tests). A decrease in titre meant that the inhibiting carbohydrate was related to the erythrocyte binding receptors. Jurenka *et al.* (1982) detected the same activity in the haemolymph of seventeen species of

grasshopper (*Acrididae*) from 4th instar and throughout the adulthood of both sexes. Thirteen species showed positive results and gave agglutination titres in the range 2-64. Females showed slightly more activity than males and the greatest activities were shown by haemolymph of *Melanoplus bivattatus* and *M. sanguinipes*. The haemolymph of *M. sanguinipes* was found to agglutinate the erythrocytes of rabbit, calf, human (all ABO types), guinea pig, mouse, chicken, cat, pig and sheep as well as the protozoan *Nosema locustae* (grasshopper pathogen). Hapner (1983) reported that twenty-four individual grasshopper specimens contained a similar broad-spectrum haemolymphatic haemagglutination activity. Of nine types of erythrocytes tested, the highest titres were found in human (ABO) and rabbit erythrocytes. Lysates of heads, hind and midguts of the sandfly, *Phlebotomus papatasi* (both sexes) were found to contain lectins (or lectin-like molecules) which agglutinated human erythrocytes (ABO) (H) and the promastigotes of *Leishmania aethiopica, L. major* and *L. donovani*. However, the midgut extract haemagglutination activity towards human 'O' Rhesus positive blood cells was reported to be inhibited by two dissaccharides: trehalose and turanose (Wallbanks *et al.*, 1986).

Another experiment was carried out by Ingram *et al.* (1984) to investigate the effect of cell-free haemolymph of the locust, *Schistocerca gregaria* and the cockroach, *Periplaneta americana,* on the trypanosomatid flagellates, *Trypanosoma brucei* and *Leishmania hertigi*. They found that the parasites were agglutinated at titres of 2^{-4}-2^{-13}, the agglutinins were protein or glycoprotein in nature, and that the agglutination was not dependent on the presence of Ca^{2+} or Mg^{2+}. They also found an increase in activity after inoculation of the parasite into the insects. A single dose from

a suspension in Cunningham's medium of either 10^9 *T. brucei* or 10^9 *L. hertigi* cells was given by intrathoracic injection to cockroaches (dose of 50µl) and locusts (dose of 100µl). In locusts, the activity increased from day 1 post-injection, reached peak titre on day 4 and fell to normal values by day 7. However, in cockroaches a latent period of 3 to 4 days was noted following injection, the activity increasing suddenly to a maximum on day 5 and returning to normal by day 7. It was concluded that the refractoriness of the insects to the parasites might be due to rapid agglutination following inoculation. From the above review it can be seen that lectins (agglutinins) play an important role in the immunology of insects and that the response varies between species.

This work is concerned with the role of lectins in refractoriness of the stable fly, *Stomoxys calcitrans,* to trypanosomatid flagellates (especially trypanosomes). Although *S. calcitrans* has a worldwide distribution and inhabits the same areas as tsetse flies (*Glossina* spp.), it only mechanically transmits trypanosomes. *T. brucei, T. vivax, T. congolense* and *T. rhodesiense* are transmitted biologically by *Glossina* species *e.g. G. morsitans* (Kettle, 1984; Soulsby, 1986; Service, 1986; Lehane, 1991). S. *calcitrans* is the intermediate host of the nematodes *Habronema majus* (a horse stomach worm) and *Setaria cervi* (a parasite of cattle) (Kettle, 1984). What makes it a biological vector of the latter parasites, but not of trypanosome parasites? What is behind the susceptibility to *Trypanosoma* species infection of *Glossina* species?

Both *Stomoxys* and *Glossina* species are dipterans, and have type II peritrophic membranes (Lehane, 1991). The peritrophic membrane (p.m.) functions as a physical barrier (a primary barrier) which separates the blood contents and parasites from the midgut epithelial cells (Dunn, 1986; Lehane, 1991). However, trypanosomes were found to penetrate the p.m. 9-11 days after ingestion of infective blood, indicating that this membrane is not a physical barrier to the penetration of the haemocoel of infected insects by trypanosomes (Lehane and Msangi, 1991). It is clear that there is some fundamental but unknown difference between the two species determining their relations with trypanosomes. Lectins are the possible key to understanding this difference.

Lectins are found in the haemolymph of *Glossina m. morsitans* and have anti-parasitic agglutination activity against *T. brucei, T. vivax* and *T. congolense in vitro* (East *et al.*, 1983). Agglutination activity was also found in the haemolymph, midgut and hindgut of *Glossina m. morsitans* and *G. austeni* against *T. brucei* and erythrocytes of several animal species. Agglutinins (lectins) were noted to have a selective reactivity against D(+)-glucosamine (Ibrahim *et al.*, 1984; Molyneux *et al.*, 1986). Haemagglutination activity was found against human erythrocytes of ABO(H) blood groups *in vitro* by using the haemolymph and gut extracts of *G. m. morsitans, G. palpalis gambiensis* and *G. tachinoides* (Ingram and Molyneux, 1988) and haemolymph of *Glossina fuscipes fuscipes* (Ingram and Molyneux, 1990).

Msangi (1988, unpublished) used extracts of whole gut, peritrophic membrane and midgut remains in order to investigate the haemagglutination activity towards human

erythrocytes of AB Rh negative blood group. He found no activity in the extracts of whole gut of the newly emerged *G. m. morsitans* flies and only traces of agglutination were observed in flies 3-8 days post-emergence. However, activity increased to 50%(2+) in flies aged fourteen days. A comparison of the lectin activity of tsetse flies with that of stable flies may help us understand the difference in haemagglutination activity between the two species and thus the vectorial capacity.

Gray (1990, unpublished) reported that midgut extract from very young adult *Stomoxys calcitrans* (0 days) had activities with titres (the end point of dilutions where no agglutination was seen) ranging between 32^{-1} and 128^{-1}, that the greatest activity was in flies aged 4 to 8 days (titration range 128^{-1}-256^{-1}), and that the activity decreased slightly when flies reached 12-14 days post-emergence (titre was 128^{-1}).

Maudlin (1982) carried out an experiment to investigate the genetic basis of trypanosome susceptibility of tsetse flies. The parental stock of *Glossina m. morsitans* taken from a randomly bred colony (maintained for 14 years) was infected one day post-emergence with SBM (synthetic balanced medium consisting of equal parts of washed pig red cells, foetal calf serum and a proline - containing balanced salts solution) containing c. $4x10^6$ trypanosomes (*T. congolense*) ml^{-1}. Two days later they were fed on clean SBM and thereafter maintained on defibrinated pig blood (feeding was through a silicone membrane). Males were infected in advance of females and a determination of their phenotypes was done by probing on warmed slides 21 days post-infection for trypanosomes. Then they mated with three day-old females of unknown phenotype which were being maintained individually. Their offspring were

collected and maintained in the same manner except that some flies were fed 2-3 days post-emergence and all the flies were fed on SBM containing c. 0.5-2.5x10^6 trypanosomes ml^{-1} followed by a feed on clean SBM. Maudlin found that, of the 172 flies which were raised as parental stock (90 females and 82 males), 17.5% (15 females and 15 males) proved to be susceptible and all developed mature infections involving the hypopharynx. Then a total of 249 F_1 progeny (which were obtained by raising fifty-three families from the above parental stock by single pair matings) were divided into four classes according to parental phenotype.

He concluded from statistical analysis of this data that maternal effects were determining infection rates of *T. congolense* in *Glossina m. morsitans.* From the data it was not possible for him to distinguish between maternal inheritance due to cytoplasmic factors and maternal effects related to maternal nutrition. The latter is an important consideration in female tsetse flies in which eggs hatch and larvae are retained in the uterus for about 9 days, being fed on fluid produced by milk glands prior to larviposition (Kettle, 1984). Any maternal factor involved in producing susceptibility to trypanosome infection will be passed in this fluid to the larvae. This factor might be responsible for the low lectin activity in very young tsetse flies and might be affecting the role of lectins directly or indirectly.

Maudlin and Welburn (1987) investigated the effect of RLO's (Rickettsia-like organisms) on the role of lectins in the midgut. They cultured RLO's in mosquito cells and found that they produced chitinase capable of hydrolysing chitin to *glucosamine.* The latter was found to inhibit lectin activity. Maudlin and Welburn (1988b)

suggested that glucosamine was produced during the larval/pupal period in the RLO-infected flies, accumulating in the non-feeding larval midgut. This stored glucosamine would neutralize any lectins present, rendering the emerged fly susceptible to trypanosome infection at least at the first feed.

Ingram and Molyneux (1988) studied sugar specifities of haemagglutinins (lectins) against human ABO (H) RBC in the haemolymph of three *Glossina* species *(G. m. morsitans, G. palpalis gambiensis* and *G. tachinoides).* The lectins were found to be relatively heat-labile, human ABO (H) RBC non-specific lectins or lectin-like agglutinins with titres ranging from 2^{-9}-2^{-16}. They were found to exhibit wide heterogeneity in their sugar specificities on the surface of the human RBC used. Midgut haemagglutinins (titres 2^{-6} or 2^{-7}) were only found in *G. m. morsitans* exclusively against "SB" human RBC whilst hindgut extracts in all three *Glossina* species caused agglutination (titres 2^{-1}-2^{-7}) of most RBC used. Heat-labile, possibly protease but not trypsin haemolytic molecules were present in most gut preparations. Haemolymph agglutinin reactivities were directed mainly towards sorbose, trehalose, glucose, 2-deoxy-galactose and to a lesser extent the deoxy- [1-4] - and/or [1-6] - linked derivatives of glucose. The agglutination was occasionally minimally-inhibited by fructose, mannose, sucrose, turanose, stachyose and melezitose. Gut haemagglutinin specificities were less varied than those of the haemolymph agglutinins and were inhibited by glucose, galactose, mannose and their deoxy-aminated and N-acetylated derivatives. *G. m. morsitans* gut extracts were additionally inhibited by sorbose, sucrose, turanose, gluconic acid and methyl glucoside. Freezing (and

thawing) haemolymph and gut extracts of samples was seen to negate or reduce agglutinin and lytic activities.

Haemolymph samples from the same insects were subjected to enzyme, chemical and organic solvents, sodium periodate oxidation, and physico-chemical treatments in order to detect the nature of the haemolymph haemagglutinins and their physico-chemical properties (Ingram and Molyneux, 1993). A cross adsorption process was performed to detect the presence of haemolymph heteroagglutinins.

In this experiment human RBC were treated with various enzymes in order to determine the RBC surface haemagglutinin receptor sites. Haemolymph samples of the three *Glossina* species tested were found to possess multiple (hetero), glycoproteinaceous haemagglutinins which bind to human RBC surface glycoprotein/glycopeptide residues or with *G. m. morsitans* and *G. p. gambiensis* anti-O activity, glycolipid moieties. Variations in the physico-chemical properties of the lectins (with respect to relative heat-lability, susceptibility to dithiothreitol reduction, resistance to γ-radiation exposure and sensitivity to urea treatment) between the morsitans group *(G. m. morsitans)* and the palpalis group *(G. palpalis gambiensis* and *G. tachinoides)* were also found. In order to achieve optimum agglutination activities *G. p. gambiensis* haemolymph haemagglutinins required neutral to alkaline pH and Mg^{2+} ions whilst *G. tachinoides* and *G. m. morsitans* samples required acid and acid to neutral pH respectively and Ca^{2+}.

Abubakar *et al.* (1995) reported that samples of midgut homogenate from *G. m. morsitans* flies which had been fed twice had the highest agglutination activity against *T. brucei* followed by that from once-fed flies and then unfed flies (Ingram and Molyneux, 1993). A much lower sample concentration was required for agglutination of procyclic forms compared to bloodstream forms (trypomastigotes). The agglutination activities were inhibited by D-glucosamine and treatment of the samples with soya bean trypsin inhibitor abrogated agglutination of the bloodstream (trypomastigote) forms but not the procyclic forms. The activities were observed to be temperature-sensitive, with little activity being evident between 4^0C and 15^0C, and the activity being lost when the samples were heated to 60^0-100^0C. When a sample was separated by anion-exchange chromatography the agglutination activity co-eluted with trypsin activity at approximately 50% NaCl. These results suggested that a very close relationship exists between midgut trypsin-like enzymes and the agglutinin (lectin), particularly when the successful agglutination of bloodstream form trypanosomes requires protease activity. It was concluded that the enzyme might cleave off some parasite surface molecules thus exposing lectin binding sites.

Osir *et al.* (1995) reported that the blood meal induced lectin (agglutinin) in the midgut extracts of *G. longipennis* was found to agglutinate both bloodstream forms and procyclic forms of *T. brucei* as well as rabbit RBC. The activity was seen to be strongly-inhibited by D-glucosamine and weakly-inhibited by N-acetyl-D-glucosamine. Treatment of samples with soya bean trypsin inhibitor abrogated the agglutination of bloodstream forms but not procyclic forms. The acitivity was sensitive to temperatures above 40^0C but was unaffected by chelaters of metal ions.

In this study I will investigate the lectins of *Stomoxys calcitrans* to determine if any differences seen between *S. calcitrans* and tsetse can explain their different vectorial capacities for trypanosomes. Samples of reservoir extracts (RH) and haemolymph (HL) from stable flies (*S. calcitrans*) were tested *in vitro* in order to detect their agglutination activities against human (ABO) and animal RBC's and trypanosomatid flagellate parasites. These parasites were *T. brucei* (bloodstream and culture forms), *L. hertigi* and *C. fasciculata* (culture forms).

The characteristic properties of HL and RH agglutinins were investigated before and after purification of the samples. Sugar inhibition tests were also carried out with HL and RH samples in order to determine the concentration of inhibitor sugars to be used in the experiments of *in vitro* feeding of the infective blood from rats (which had been infected with *T. brucei* for 3 passages).

The experiment of *in vitro* feeding of the flies was performed to detect agglutinin (lectin) activity, to detect haemolysins inside the flies and to determine the time required for parasites to be found in the haemocoel and haemolymph or to be damaged by the fly's immune mechanisms. This information is needed in order to ascertain when a fly starts contributing the infection after ingestion of infected blood from man or animals to non-infected hosts. Such experiments have important implications for my country, Saudi Arabia, as live animals are imported from its nearest countries, particularly those of Africa, during the annual event of pilgrimage to Makkah.

Literature Review

INTRODUCTION

It is very important to investigate the role of *S. calcitrans* in the transmission of parasites (especially trypanosomes) of man and livestock in Saudi Arabia since little work has been published regarding this country. Saudi Arabia lies between the continents of Asia, Europe and Africa (see Fig.1). Parasites found naturally in Sudan, for example, also occur in Saudi Arabia because of the transport of livestock from Sudan to Saudi Arabia by sea (this transport taking 24 - 72 hours). Makkah, Medinah and other holy places make Saudi Arabia a central meeting place for many muslims. During Hajj (pilgrimage time), animals brought from different parts of the world, including Australia and some European countries, are slaughtered.

Most of Saudi Arabia is desert having insufficient food and water to raise livestock. Generally the Saudi Arabian climate is arid and influenced by north African subtropical high pressure. The occasional penetration of Mediterranean low pressure results in some rainfall mainly during the winter. Snowfall can be observed in the extreme north. The Mediterranean type climate is found in the northern and central regions. This permits the cultivation of crops such as cereals and vegetables.

The southern and the south-western parts of the country (particularly the Asir mountains) like North Yemen are affected by the south-western monsoon. Here, monsoonal weather supports rain-fed crops at medium to high altitudes. These include alfalfa, sorghum, millet, barley, wheat and maize.

In the south-east of the country lies the vast region of Rub'-al-khali (the empty quarter) where little or no rainfall occurs. However during short spells of rain, which occur once every few years, short grass and a few perennial shrubs grow in marginal regions. Some wild animals can be observed here too. Occasional spells of wind and heavy sandstorms (Shamal) cause sand dune drift affecting the vegetation of the narrow landstrips along the west coast of the Arabian Gulf.

Most of the wild animals and plants are well-adapted to the hot and arid conditions. Some groups of animals take refuge in cooler and more moist areas, invading the country during periods of rainfall and when temperatures are relatively low. Extreme climatic conditions and geographical isolation have led to the evolution of endemic animals and plants. They have remarkable physiological mechanisms enabling them to survive the harsh conditions. Adaptations have taken place in the life of the people who raise crops and breed animals suitable for the extreme environment. These domestic animals include camels, sheep, goats, horses, donkeys and a small number of cattle in the mountains of Al-hijaz and Asir (Siraj, 1985).

The desert is not generally a habitat conducive to the development and transmission of parasites. However, the Arabian camel (*Camelus dromedarius*), adapted to live there, harbours a large number of different parasites, especially helminthes. Systematic studies of the parasitic diseases of camels in Saudi Arabia are few. The first published work was concerned with the gastro-intestinal helminthes of these animals by El-Bihari and Kawasmeh (1980).

Systematic studies of the parasites of Saudi Arabian sheep and goat breeds (Najdi, Balady, Awasi, Nuaimi etc.) are few. The first work involved experimental infection of sheep with the camel stomach worm, *Haemonchus longistipes* (El-Bihari *et al.*, 1984).

Veterinary studies started only recently (in the 1970s) in Saudi Arabia. The first college of veterinary medicine was opened in King Faisal University in Al-Ahsa about 15 years ago. The college has a teaching veterinary hospital to train students. Cases are brought to the hospital from all over the eastern provinces of Saudi Arabia, other parts of the Kingdom, and from the Gulf states. The hospital has a number of diagnostic laboratories including a parasitological laboratory. Specimens from a variety of animal species are received from veterinarians working at the hospital, private farms and the local slaughter house.

To the best of my knowledge there is no published work concerning trypanosomiasis in Saudi Arabia. However, during pilgrimage time when there is movement of people and animals to Mecca from infected areas, disease may be transmitted mechanically by *Stomoxys* and other blood-sucking insects and be of medical and veterinary importance.

This work is concerned with the role of lectins in the refractoriness of stable flies (*S. calcitrans*) to trypanosomes and related parasite species. I will therefore briefly review the biology of these parasites, transmission of these parasites by their vectors,

their veterinary and medical significance and the role of the stable fly in disease transmission.

Trypanosomiases and Leishmaniases

The trypanosomiases are diseases of man and livestock whilst the leishmaniases are largely diseases of man. They are caused by parasitic flagellate *Protozoa*: superclass *Mastigophora*, order *Kinetoplastida*. Generally members of this order are recognized by the possession of a kinetoplast, a large mass of mitochondrial DNA contained within a very large mitochondrion (Kettle, 1984; Kreier and Baker, 1987). The economically important parasites are in the *Trypanosomatidae*, the only family in the suborder *Trypanosomatina* characterized by possession of a single flagellum. This flagellum may be free or attached, for much or all of its length, to the undulating membrane (Kettle, 1984; Soulsby, 1986; Kreier and Baker, 1987). Species in the two genera *Trypanosoma* and *Leishmania* assume various forms at different stages of their developmental cycles. Changes in morphology will be discussed in greater detail in a following section. Species of *Trypanosoma* and *Leishmania* are of economical importance, causing trypanosomiasis and leishmaniasis respectively.

Classification

Phylum: *Sarcomastigophora*

Subphylum: *Mastigophora*

Classes: *Phytomastigophorea* and *Zoomastigophorea*

Phytomastigophorea possess chromatophores which contain chlorophyll and are responsible for the synthesis of organic compounds from inorganic materials; their nutrition is holophytic. Consequently they are of no veterinary or medical significance (economical importance). However, in comparison, members of the class *Zoomastigophorea* lack chromatophores and feed in a holozoic manner. They are classified into several orders four of which are of veterinary and medical importance. These orders are *Kinetoplastida* (*e.g. Trypanosoma*), *Retortamonadidae (e.g. Cochlosoma*), *Diplomonadidae* (*e.g. Hexamita*), *Trichomonadidae* (*e.g. Trichomonas*). This work is concerned with the order *Kinetoplastida* which is divided into two suborders: *Bodonina* (members of which are free-living) and *Trypanosomatina.*

Suborder: *Trypanosomatina*

Family: Trypanosomatidae

Morphologically all are elongate, slender protozoa (at least at some stage of their life cycle) having a single nucleus and a kinetoplast. The latter is situated near the origin of the single anterior flagellum by means of which they swim actively in the blood of the infected host. Recognition of different forms of these organisms depends upon the position in the body of the kinetoplast and basal body and the course taken by the flagellum.

Some genera exist for part of their life cycle as nonflagellate or amastigote individuals. These genera are *Leptomonas, Herpetomonas, Crithidia, Blastocrithidia* and

Rhynchoidomonas. They are exclusively symbionts of insects and a few other invertebrates. They all inhabit the gut and are transmitted via the faeces, sometimes as encysted amastigote forms (Kreier and Baker, 1987).

Species of the genus *Phytomonas* (which resembles *Leptomonas*) inhabit plants (particularly succulents) and are transmitted by *Hemiptera* which feed on the plant juices. This genus has been recorded in different parts of the world, especially in the warmer regions (Kreier and Baker, 1987).

The genera *Leishmania, Trypanosoma* and *Endotrypanum* are parasites of vertebrates and almost all of their species inhabit blood-sucking invertebrates (usually insects or leeches) by which they are transmitted from one vertebrate host to another. Some of them are pathogenic to their vertebrate hosts but, with one possible exception (*T. rangeli*), there is no evidence to suggest that they have a detrimental effect on the invertebrate host (Kreier and Baker, 1987). The genus *Endotrypanum* is unique among the *Trypanosomatidae* because it lives inside the erythrocytes of its hosts (one of which is the sloth of South and Central America) and can be transmitted by sandflies (Kreier and Baker, 1987).

Genus: *Trypanosoma*

The trypanosomes inhabit the blood (and sometimes other tissues) of vertebrates and usually the gut of blood-sucking invertebrates. They exist as tryptomastigote forms for at least part of their life cycle in both hosts. They can be divided into two groups:

trypanosomes of non-mammals and trypanosomes of mammals. The latter group contains all the pathogens important to man and animals. These pathogens are all transmitted by arthropod vectors (except for *T. equiperdum* which causes a venereal disease in equines). Transmission can be of two types: cyclical or non-cyclical. The latter is essentially mechanical transmission in which the trypanosomes are transferred from an infected host to another by the interrupted feeding of biting insects, notably tabanids and *Stomoxys* (Service, 1986; Soulsby, 1986; Urquart *et al.*, 1987; Lehane, 1991). See Table 3.

In cyclical transmission the arthropod vector is a necessary intermediate host in which the trypanosomes multiply, undergoing a series of morphological transformations before becoming infective. This is also called biological transmission and, according to the route of exit of the trypanosomes, Hoare (1964) divided them into two major groups: *Stercoraria* (posterior station transmission) and *Salivaria* (anterior station transmission). In the former, developmental stages multiply in the gut and the metacyclic tryptomastigotes (the infective forms) accumulate in the hind gut and migrate to the rectum from where they are passed out with the faeces. The best example is *T. cruzi* the causative agent of Chagas' disease in South America which is transmitted by reduviid bugs (Soulsby, 1986; Urquart *et al.*, 1987). In the *Salivaria* (anterior station group) the developmental stage (e.g. epimastigotes) multiplies in the digestive tract and proboscis and the infective stage accumulates in the mouthparts or salivary glands so that the infection is transmitted when the vector takes a blood meal. This is the inoculative method of transmission. All the salivarian trypanosomes are

transmitted by tsetse flies and the main species are *T. congolense, T. vivax* and *T. brucei* (Kettle, 1984; Soulsby, 1986; Urquart *et al.*, 1987). See Table 3.

This thesis is concerned with the salivarians (anterior station development trypanosomes) and particularly African trypanosomiasis. Information about the economical (veterinary and medical) importance morphology, biology and life cycle of these trypanosomes will be given as will information about tsetse flies. Following this, the importance of stable flies (of worldwide distribution) in the transmission of African trypanosomiasis will be discussed as will the importance of the stable fly's contribution to the transmission of the disease in Saudi Arabia (especially during the holy session of pilgrimage to Makkah, the hajj).

African trypanosomiasis

There is no other disease which has affected the development of a continent more than trypanosomiasis has Africa. Tsetse flies infect 11 million km^2 south of the Sahara transmitting the disease to both man and his livestock, causing sleeping sickness in the former and nagana in the latter (see Fig. 2). Some 50million people are believed to be at risk from sleeping sickness, 20,000 new cases being reported anually. Nagana affects animal production and is therefore damaging to the economy of the endemic countries. The disease in cattle results in slow growth, weight loss, poor milk yield and an impaired ability to work as transport animals. Infertility, abortion and death add to the toll (Murray *et al.*, 1990).

The African trypanosomes and their life cycle

It is essential to have some knowledge of the intricacies of the life cycle of the trypanosomes in order to understand their biology, the development of immunity to them, the role of that in their pathogenicity, and finally, their role in the development of endemic areas. As previously mentioned, African trypanosomiasis is a spectrum of disease in humans and their domestic animals. It is caused by trypanosome species (*T. brucei, T. congolense* and *T. vivax*) which are transmitted by tsetse flies (*Glossina spp.*) from one mammal to another.

In the tsetse fly, the parasites undergo an elaborate cycle of development with characteristic division phases and morphological changes (see Fig. 3). This development will be addressed once the development in the mammalian host has been discussed.

In mammalian hosts:

When the tsetse fly feeds on a host the metacyclic trypanosomes are deposited (along with the discharged saliva) in the dermal connective tissue. Here, a local inflammatory reaction, the "chancre", develops. From the chancre the parasites enter the draining lymphatics and then the bloodstream. In the case of *T. congolense* the parasites multiply in the tissue of the chancre as a morphologically distinct phase before invading the bloodstream (Roberts *et al.*, 1969). Eventually all three cyclically-transmitted

species undergo a change in form here to emerge with the characteristic morphology of the dividing bloodstream stages in the life cycle.

The chancre phase completed, *T. congolense* and *T. vivax* remain largely as intravascular parasites, the former localizing in small blood vessels where it attaches to the endothelium. They can also be found in the lymphatics. The three species: *T. brucei, T. evansi* and *T. equiperdum* may secondarily escape from the bloodstream into the soft connective tissues and multiply in the tissue fluid (*T. equiperdum* is found principally in such tissues in its natural host). Invasion of the brain and cerebrospinal fluid occurs in chronic *T. brucei* group infections, thus the brain may be implicated as a source of relapse infections after chemotherapy (Jennings *et al.*, 1979; Poltera, 1985). Whitelaw and his collaborators (1988) reported that the central nervous system and aqueous humour of the eye were extravascular foci of *T. vivax* in goats and potential sources of relapse infections. Intracellular stages are absent from the life cycles of African trypanosomes but present in the life cycle of *T. cruzi* (Vickerman, 1985). It is well-known that all salivarian trypanosome infections in the natural hosts are characterized by an undulating parasitaemia. Each fall in trypanosome numbers (remission) corresponds to a destruction of a major antigenic type by the host's immune response and each ensuing increase in numbers (recrudescence) corresponds to a proliferation of trypanosomes of a different antigenic type (Soulsby, 1986; Vickerman *et al.*, 1993).

T. brucei is pleomorphic in the blood and multiplies by binary fission in the ascending parasitemia as a long, slender flagellate. It transforms into a non-dividing, short and

stumpy parasite when the parasitemia passes through crisis into remission (the life cycle is shown in Fig.1). These stumpy forms were reported to be more capable of surviving in the tsetse fly's blood meal and they initiate the cycle of development in the fly (Soulsby, 1986; Vickerman *et al.*, 1993). Although *T. evansi* morphologically resembles *T. brucei*, it rarely produces stumpy forms (Hoare, 1972). In fact, lack of pleomorphism is also a characteristic of *T. brucei* stocks which have been mechanically passaged through laboratory rodents (this was seen in infected rats and mice at Salford). *T. congolense* and *T. vivax* are less markedly pleomorphic than *T. brucei* and show differences from the latter species in their life cycle in the vector.

In the insect vector:

The majority of trypanosomes undergo cyclical development in an arthropod vector (Soulsby, 1986). Nevertheless, some biting flies such as *Stomoxys* (e.g. *S. calcitrans*) and *Tabanus* are only capable of transmitting the parasites mechanically. So, when they feed on an infected host, they become infective immediately and remain so only for a short time; they must feed on another host as soon as possible. *T. evansi* and *T. equinum* are solely transmitted in this way, while *T. equiperdum* is transmitted by coitus. In fact, even the cyclical trypanosomes such as the *T. brucei* group, *T. congolense* and *T. vivax* may be transmitted mechanically and possibly by stable flies (Soulsby, 1986).

In cyclical development, mammalian blood containing the trypomastigote forms is taken into the intestine of the arthropod and subsequent development depends on

whether anterior or posterior station development occurs. Only the former will be discussed in detail here.

Anterior station development (salivarian trypanosomes)

When tsetse flies feed on an infected host they ingest trypanosomes with blood or lymph. Thereafter the parasites lose their glycoprotein surface coat. In the case of *T. brucei* and *T. congolense* the parasites become elongated and multiply in the midgut to the trypomastigote stage which lasts for 10 days. Initially, the dividing forms in the midgut are broad with a kinetoplast midway between the nucleus and the posterior end. The long, slender forms are produced by days 10-11 and migrate backwards, enter the space around the peritrophic membrane and then penetrate the proventriculus, being found there within 12-20 days after infection (Soulsby, 1986). The parasites then migrate anteriorly to the oesophagus and pharynx and onwards to the hypopharynx and salivary glands (*T. brucei*) and proboscis (*T. congolense*). There they undergo a transformation in which they lose their typical trypomastigote form and acquire an epimastigote form. The latter is characterized by the kinetoplast being located just in front of the nucleus (Soulsby, 1986; Urquart, 1987). Within 2-5 days, after further multiplication of the epimastigote forms, they transform again into small, typically trypomastigote forms with a glycoprotein surface coat. These are the infective forms for the next hosts and are called metacyclic trypanosomes. They are small, stumpy forms resembling the stumpy forms found in the blood. The entire process takes at last 2-3 weeks after which the metacyclic trypanosomes are inoculated into the new host with saliva when the tsetse fly feeds (Soulsby, 1986; Urquart *et al.*,

1987; Vickerman *et al.*, 1993). Several thousand trypanosomes may be injected into the host with each bite (Soulsby, 1986).

The Mechanism of Infection

To understand the mechanism of infection it is essential to appreciate the factors influencing the relationship between wild animals, man and his livestock from the disease transmission point of view. Trypanosomiasis is basically an infection of wildlife in which the parasites have achieved a *modus vivendi*; the hosts are parasitemic for prolonged periods and generally remain in a state of good health. This is known as trypanotolerance. In contrast, the rearing of domestic livestock in endemic areas has always been associated with excessive morbidity and mortality. However, there is evidence to suggest that a degree of adaptation or selection has occurred in several domestic breeds. For example, in West Africa small humpless cattle, *Bos taurus* (N'dama), were seen to survive and breed in areas where trypanosomes were rife and where control measures were absent. Their resistance to the disease is not absolute; it may affect them particularly in terms of productivity (Soulsby 1986; Urquart, 1987). Indigenous breeds of sheep and goats in some areas of Africa are trypanotolerant but this may be partly due to the fact that they are unattractive hosts for *Glossina* species (Urquart *et al.*, 1987). There is no satisfactory explanation to account for the transition of the parasites from insect forms to vertebrate host forms. The bloodstream forms are trypomastigotes which possess a smooth electron-dense coat containing the variant surface antigens. This coat is lost during the course of cyclical development in *Glossina* spp. but it reappears in the

metacyclic trypanosomes in the salivary glands (Vickerman, 1972). Other differences between bloodstream and insect forms include variation in mitochondrial morphology. The long, slender bloodstream stages of *T. brucei* have mitochondria with sparse, short and tubular cristae, whereas the midgut stages in *Glossina* spp. have mitochondria with numerous plate-like cristae. The morphology of the mitochondria of the short, stumpy blood forms (which are taken up by the insect) is intermediate between the two (Vickerman, 1971). These morphological differences result in differences in metabolism. In the slender bloodstream forms, the prime source of energy for adenosine triphosphate (ATP) synthesis is from the catabolism of glucose to pyruvate (via the glycolytic pathway), the latter being excreted into the bloodstream. There are no functional cytochromes and hence oxygen (or glucose) consumption is unaffected by inhibitors of Kreb's cycle enzymes and sensitivity to cyanide is absent. In the insect forms, however, the active mitochondria catabolize glucose completely to carbon dioxide (the pyruvate being further metabolized through the tricarboxylic acid cycle). Cytochromes are present, respiration is inhibited by cyanide, and Kreb's cycle substrates are used (Trigg and Guttride, 1977). It is of interest to note that the bloodstream forms of some other species (e.g. *T. vivax* and *T. congolense*) have mitochondria with fully-functional cristae, therefore little difference in metabolism exists between the bloodstream and insect forms (Smyth, 1976).

Vickerman (1966) found that, when bloodstream trypanosomes were treated with some anti-trypanosomal drugs, some individuals lacking a kinetoplast (dyskinetoplastic trypanosomes) arose. It was thought that, when a mixed population containing these

forms was fed to a tsetse fly, only those possessing a kinetoplast would develop further in the fly.

The biochemical and morphological evidence is consistent with a partial switchover to aerobic metabolism in bloodstream forms in readiness for the change of environment which occurs when the parasites are taken up by a fly (Vickerman, 1971).

Some species and strains of trypanosomes can adapt to a novel species of host (abnormal host) via serial subpassages. This may be a short or prolonged process and is associated with increasing virulence for the new host. For example, the rat strain of *T. vivax* has evolved as a result of supplementing rats with sheep serum (mechanically by syringe), the dependence on sheep serum having been lost after 37 subpassages. These strains caused lethal infection in rats (Desowitz and Watson, 1953). A loss in the ability to infect the arthropod hosts and to undergo cyclical development is a result of this process and these are, in fact, features of the cyclical transmitted forms if they are transmitted continuously by mechanical means. The inability to undergo cyclical transmission cannot be fully-explained. However, the associated loss of polymorphism, especially the loss of the stumpy forms of *T. crucei*, may deplete the system of forms capable of infecting insects (Soulsby, 1986). When the organisms are passaged in an abnormal host, a loss of virulence for the normal host does not necessarily occur although it is a common phenomenon especially when the passage is by mechanical means. Thus cyclical maintenance in an abnormal host may preserve normal host infectivity for long periods. It was found that *T. rhodesiense* was passaged cyclically in sheep for 18.5 years without losing its infectivity for man.

Similar findings exist for its maintenance in antelope and monkeys (Ashcroft, 1959; Soulsby, 1986).

A detailed account of the developmental cycle of trypanosomes in *Glossina* species (e.g. *T. brucei* in *G. morsitans*) and factors influencing the infection rates in these species are mentioned within the information about tsetse flies. It is well-known that if a trypanosome is transmitted by one species of *Glossina* it will be transmitted by all *Glossina* species (Hornby, 1952). Hence, all species of *Glossina* which have been investigated appear capable of transmitting *T. vivax*, *T. congolense* and *T. brucei* groups. Factors determining which species of *Glossina* is prevalent are ultimately responsible for differences in transmission (see the *Glossina* spp. section). Climate and host species present are among these factors.

Glossinidae (Tsetse flies)

Classification

Tsetse flies are a well-defined taxonomic group previously included in the *Muscidae* as a separate family, *Glossinae*, or with the blood-sucking muscids in the *Stomoxinae*. Tsetse flies are now regarded as a separate family, the *Glossinidae* which contains only one genus, *Glossina* (Kettle, 1984).

The genus *Glossina* generally contains 23 living species (Jordan, 1995a). These species are assigned to three subgenera (*Glossina*, *Nemorhina* and *Austenina*), formerly referred to as *morsitans, palpalis* and *fusca* groups respectively (Kettle, 1984).

The subgenus *Glossina* contains five species four of which are *G. morsitans, G. pallidipes, G. swynnertoni* and *G. austeni.* The first two are of major economic importance and the last two of local significance (Kettle, 1984). For more information see Jordan (1995a).

Distribution and morphology

Presently species of *Glossina* are confined to the Afrotropical region (tropical and subtropical regions of Africa; latitude 5^0N to 20^0S). However, some evidence exists to indicate that *Glossina* species have occurred in the Nearctic (Kettle, 1984).

These tsetse are small to medium in size (6 - 11 mm long), all having distinct abdominal bands (with the exception of *G. austeni*). The distal segments of the hind tarsi are dark on the dorsal surface and the male claspers are distally swollen.

These tsetse species are commonly found in savanna, woodland and evergreen thickets with the exception of *G. austeni.* The latter is restricted to coastal and relict forests (Kettle, 1984). *G. morsitans* (see Fig. 4) is found throughout tropical Africa whilst other species of *Glossina* occur only in East Africa (see Table 3 and Fig. 2).

The subgenus *Nemorhina* contains five species, three of which play a major role as vectors of human trypanosomiasis. These species are *G. palpalis, G. fuscipes* and *G. tachinoides.*

These tsetse are a similar size to that of the species in the subgenus *Glossina.* The dorsum of the abdomen is dark brown with the exception of *G. tachinoides* (which has a banded abdomen like that of the *morsitans* type). The male claspers are not distally swollen but are joined by a membrane (Kettle, 1984).

This subgenus occurs in riverine and lakeside habitats, located mainly in West and Central Africa, extending eastwards to the shores of Lake Victoria. *G. tachinoides* is largely restricted to West Africa and *G. fuscipes* to Central Africa (Kettle, 1984).

The subgenus *Austenina* contains species of tsetse which are large (9 - 11 mm in length). Strong bristles are present on both the petropleuron and the sternopleuron. The male claspers are neither swollen distally nor joined by a membrane.

The species of this subgenus are forest-dwelling having negligible contact with man or his livestock. For this reason they are of little economic importance except when man moves his livestock into forested areas (Kettle, 1984; Lehane, 1991). See Table 3 and Fig. 2).

Life cycle

Tsetse flies are viviparous, giving birth to fully grown larvae. The ovaries of the female are each composed of two polytrophic ovarioles and, starting with the right ovary, produce ova alternately. Following ovulation a relict body remains in the ovariole which can be used to age the tsetse fly accurately during the first four cycles. At 25^{0}C the average life cycle lasts 40 days (Kettle, 1984).

The egg passes down the common oviduct to the uterus. Here the micropyle of the egg comes to lie opposite the spermathecal duct opening. The release of sperm is regulated by a spermathecal duct sphincter which is open at ovulation and closed during the rest of the breeding cycle (Kettle, 1984).

The female fly produces one larva at a time which grows within the uterus. The mouth of the larva is attached to a teat from which milk is obtained for nourishment. This milk is produced by uterine or milk glands (Kettle, 1984; Soulsby, 1986).

The larval posterior extremity which bears the stigmal plates, lies near the vulva. Two large respiratory lobes exist at the posterior end, each being perforated by about 500 spiracular openings. The larva is ovoid in shape and about 7 mm long when fully grown.

The gestation period lasts approximately ten days under suitable conditions. It is prolonged if food is scarce or in cold weather when the flies do not feed readily.

The larva is then deposited on soil where it wriggles down to a depth of about 2 cm. It turns into a pupa after 60 - 90 minutes.

Tsetse flies choose their breeding places carefully depending on various factors. For example, *G. palpalis* usually deposits its larvae not more than 25 m from water and about 1 m above water level in dry coarse sand or humus around tree trunks. Alternatively, the larvae are deposited in the forks of branches and cracks of bark up to 4 m above the ground. *G. morsitans* and *G. pallidipes* breed in loose sandy soil rich in humus in sheltered and well-drained areas near the game path where the flies abound. Pregnant females, soon to deposit larvae, are attracted to objects which provide shelter such as fallen tree trunks and slanting rocks. The larvae are deposited under these shelters. Shade is essential for the pupae even though they are buried in soil. If exposed to the sun's rays for a few hours per day, they will be killed (Bursell, 1970; Soulsby, 1986).

The posterior respiratory lobes persist with the last larval skin, the latter forming the puparium covering the brown or black pupa, and therefore give the pupa its characteristic appearance. The lobes vary from species to species and are used in pupal identification (Soulsby, 1986).

The pupa is approximately 6 - 7 mm long. The pupal period varies between species and with temperature but on average lasts about 35 days (range of about 17 - 90 days). The pupal period of *G. pallidipes*, for example, varies from about 31 days in the summer to about 92 days (may be 149 days or more) in the winter.

Control and factors affecting distribution

The bionomics of tsetse flies have been studied extensively for many years in order to obtain information for the development of effective and economical control measures (Kettle, 1984; Soulsby, 1986; Lehane, 1991).

Tsetse flies are found mainly in Central Africa extending from the southern boundaries of the Sahara and are confined to areas known as fly-belts. The limits of these well-defined areas are controlled by various factors such as altitude, moisture, vegetation and the presence of hosts (Kettle, 1984; Soulsby, 1986; Lehane, 1991).

For example, *G. palpalis* occurs mainly in areas drained by the Senegal, Niger and Congo rivers. *G. morsitans* occurs from Senegal to Ethiopia and extends southwards to Zimbabwe. *G. pallidipes* is essentially an East African species and its distribution ranges from the Republic of South Africa to Uganda and Kenya. *G. tachinoides* can tolerate higher temperatures than most other species and is therefore found in hot regions such as northern Nigeria (Ford, 1970; Potts, 1973; Kettle, 1984; Lehane, 1991).

Atmospheric humidity, temperature and the presence of shade have an important bearing on the life of the fly. Different tsetse species vary in their response to these factors hence each species tends to be associated with a particular type of vegetation.

In poor seasons (when vegetation and water supplies are diminished) flies are restricted to certain areas known as "primary fly centres" or permanent haunts. They migrate outwards from these along suitable water courses to temporary haunts during favourable seasons. *G. palpalis*, for example, will ascend to the upper limits of rivers in rainy seasons and descend when dry conditions set in (Glasgow, 1970; Kettle, 1984; Soulsby, 1986; Lehane, 1991).

Tsetse hosts

All tsetse flies feed on vertebrate blood, some host species being more preferable than others (see Table 3). The prevalence of flies is therefore dependent on the number and suitability of hosts. *G. palpalis* thrives best on the blood of warm-blooded animals and is said to prefer that of humans. However, it can also feed on the blood of cold-blooded animals such as the crocodile. It is generally agreed that this species is not dependent on big game for its existence (Soulsby, 1986). *G. morsitans* feeds on any large mammal or bird but is not able to exist permanently where big game or livestock are absent; when rinderpest killed out the big game of the Transvaal, *G. morsitans* disappeared from this region. *G. pallidipes* is also dependent on big game or cattle for its existence but warthogs are an additional host for both species (Soulsby, 1986).

Tsetse feeding

The flies feed approximately every three days depending on temperature and humidity. Most species are active in the forenoon and afternoon, disappearing during the hottest hours of the day. Some flies such as *G. brevipalpis* are nocturnal in their habits and feed on moonlit nights whilst others may attack hosts around a campfire at night. Rain or windy weather causes the flies to remain in shelters (Soulsby, 1986).

When the fly engorges, its abdomen becomes very distended. After leaving the host it settles on a log, tree bark, or the underside of a leaf. A drop of dark fluid is voided through the anus followed by several drops of clear fluid and sometimes a drop of fresh blood.

The females do not feed towards the end of the gestation period but remain in shelters. Unsuitable food or starvation may cause the female to abort (Bursell, 1970).

Diagnosis of trypanosomiasis

The simplest method by which vertebrate trypanosome infections can be diagnosed is by preparing thin films of peripheral blood stained with Giemsa's stain and examining them at a magnification of x500 - x1000. Alternatively, a fresh drop of blood (mounted on a slide beneath a coverslip) can be examined at a magnification of approximately x400 for motile trypanosomes (Kreier and Baker, 1987; Cheesbrough, 1991) (see Table 2). If the parasites are scanty, stained thick blood films can be made.

Concentration of the parasites can be achieved by centrifugation of blood in a heparinized capillary tube (the microhaematocrit centrifugation buffy coat technique), the trypanosomes accumulating just above the red blood cells. The tube is then scored and broken and the serum at that point, the buffy coat, removed for diagnostic purposes (Kreier and Baker, 1987; Cheesbrough, 1991).

The most sensitive method of diagnosis for most *Trypanosoma* species involves inoculation of aseptically-collected blood into blood-agar cultures. If trypanosomes are present they will multiply as epimastigotes. The salivarian trypanosomes are difficult to cultivate in this way; a special medium is required (Kreier and Baker, 1987).

T. brucei gambiense and *T. b. rhodesiense* can be very sparse in the peripheral blood of infected persons. They are often found more readily in the "juice" obtained by puncturing lymph glands (Kreier and Baker, 1987; Cheesbrough, 1991).

African trypanosomes can be detected by the miniature anion exchange centrifugation (MAEC) technique (as seen in the separation of *T. b. brucei* from rat blood in Chapter 1). This technique is generally thought to be the most sensitive for the detection of *T. b. rhodesiense* and *T. b. gambiense*. However, it is an expensive technique. Its use is therefore only recommended when the parasites cannot be found by alternative methods (Cheesbrough, 1991).

Control and Prevention of Trypanosomiasis

Complete control of trypanosomiasis by chemotherapy is not possible as drug-resistant strains exist. The control of *Glossina* offers a more long term control measure (Soulsby, 1986).

Jordan (1978) reviewed control techniques for tsetse flies. Almost all of these depend on the use of insecticides in conjunction with other methods such as bush-clearing (effective against *G. palpalis* which requires much shade) and the slaughter of game. Removal of antelope helps control *G. morsitans*, a species which prefers this host to other big game. It fails to control *G. pallidipes* probably because this species feeds on additional smaller hosts besides big game such as bush pig and bush buck.

Fly screens and other similar devices are designed to prevent flies gaining access to their hosts' shelters (houses and farm buildings). They can be used in conjunction with other control methods (Soulsby, 1986).

The majority of tsetse control programmes rely on insecticide use. Persistent insecticides such as DDT and dieldrin applied from the ground offered effective control (Soulsby, 1986; Service, 1986). For example, in the Sudan and the savanna vegetation zone of Nigeria, 2.5% DDT wettable powder applied in the dry season achieved eradication of *G. submorsitans, G. tachinoides* and *G. palpalis* (Soulsby, 1986). The frequency of application of these insecticides depends largely on local conditions such as rainfall and the rate of growth and spread of fresh untreated vegetation (Soulsby, 1986; Service, 1986).

Non-persistent insecticides applied from the air (often by fixed-wing aircraft) at repeated intervals (5 - 6 times) coinciding with pupal period length, give effective control. Endosulfan and isobenzan are examples of non-persistent insecticides. The former (as an ultra-low-volume formulation) is used most extensively (Soulsby, 1986; Service, 1986). In fact high degree of *Glossina* control can be achieved by target and trapping methods especially as they are cheap and cause virtually no environmental contamination. For example cloth screens are cheap and can be used by local communities (Jordan, 1995a and b). For more information about these methods see Jordan (1995a and b).

The stablefly, *Stomoxys calcitrans*

The stablefly, *Stomoxys calcitrans* (see Fig. 5), is a worldwide (cosmopolitan) haematophagous fly in the family *Muscidae*. It can be recognized by the four dark, longitudinal stripes (vittae) on its grey thorax (see Fig. 5). The lateral ones are narrow and interrupted at the suture (Kettle, 1984; Soulsby, 1986). Its abdomen is shorter and broader than that of the housefly with three dark spots on each of the second and third segments (Kettle, 1984; Soulsby, 1986). Generally the pattern is similar to that found in *Musca domestica* but the proboscis of the stablefly is prominent, directed horizontally forwards and has small labella. The M_{1+2} vein curves gently forwards and the R_5 cell is open, ending at/or behind the apex of the wing (Soulsby 1986).

Life Cycle

The female's eggs are creamy-white, 1mm long, and each bears a longitudinal groove on one side. A fly normally lays batches of less than 20 eggs (but sometimes as many as 50-100). The eggs are laid in horse manure, compost pits, decaying and fermenting piles of vegetable matter, weeds, cut grass or hay. These flies very rarely lay their eggs in human or animal faeces unless the faeces are mixed with hay or straw (Soulsby, 1986; Service, 1986). Eggs hatch within 1-4 days and the resultant larvae are creamy-coloured maggots (resembling those of houseflies). They can be recognized by the widely-separated arrangement of posterior spiracles and thus can be differentiated from *Musca* and *Muscina* which have more closely-positioned spiracular plates. The maggots are saprophagous, approximately round in outline, lack a peritreme, and the S-shaped spiracles are widely-separated from each other (Service, 1986). A maggot undergoes three moults before forming the pupa (Kettle, 1984). At 26.7^{0}C the egg stage lasts 23 hours and the three instars 23 hours, 27 hours and approximately 7 days respectively (Kettle, 1984). Larvae prefer a high degree of moisture for development and are therefore found mostly in wet mixtures of manure and soil (or straw) and also in vegetable matter at an advanced stage of decay. Generally the larval period lasts about 6-10 days but, in cooler weather or when there is a shortage of food, it can be prolonged to 4-5 weeks or more (Service, 1986). The larvae then migrate to drier areas and bury themselves in the soil prior to pupation. The puparium is dark brown and resembles that of the housefly but it can be distinguished from it by the fact that its posterior spiracles are widely-separated. The puparial stage lasts 5-26 days and the whole life cycle (from egg-laying to adult

emergence) may last from 12 to 58 days depending on temperature (Service, 1986; Kettle, 1984). In tropical areas the stableflies breed continuously throughout the year whilst in more temperate climates they pass through cooler months as larvae or puparia. Adults may survive winters in warm stable or buildings, feeding intermittently during the cooler months (Service, 1986).

Feeding

An understanding of the habits of *S. calcitrans* helps in appreciation of its economical importance. The flies are most abundant in Summer and Autumn, living about a month under natural conditions. They prefer a fairly strong light and are therefore not found in dark houses or stables. They only enter buildings when the weather is cold or rainy. Although swift fliers, they do not travel long distances. Both males and females are haematophagous (blood-suckers), attacking man, horses, cattle and other animals including birds and reptiles (Kettle, 1984; Soulsby, 1986; Service, 1986; Lehane, 1991). Each blood meal lasts approximately 3 - 4 minutes in which time the fly often changes its position and/or host (Soulsby, 1986). Feeding occurs by day, peaks of activity commonly occuring in the early morning and late afternoon. Individuals may feed more than once a day, the average blood meal being 25.8 mg, approximately three times the average 8.6 mg bodyweight (Kettle, 1986). Similar figures were obtained in practical work (see Chapter 4).

S. calcitrans flies are cosmopolitan synanthropes, worrying man and his animals. They are therefore of considerable medical and economic importance. They attack the ankles of man and the belly, lower body and limbs of domestic stock (particularly cattle

and horses). In cattle, milk yield may be reduced by 25% (or as much as 40 - 60%). In wild animals, behaviour can be affected. It was reported that a severe outbreak of *S. calcitrans* in the Ngorongoro Crater in Tanzania in 1962 led to a behavioural change in lions, causing them to climb trees in order to avoid being bitten (Kettle, 1984).

Stable flies are persistent biters, often engaging in interrupted feeding, and feeding more than once per day. They are therefore fit to be mechanical vectors of blood-dwelling pathogens and contribute to the spread of trypanosomiasis (Kettle, 1986; Service, 1986; Soulsby, 1986; Lehane, 1991). *S. calcitrans* is the intermediate host of nematode worms including *Setaria cervi* (a parasite of cattle) and of several *Habronema* species of horse parasites (Kettle, 1984; Soulsby, 1986).

S. calcitrans mechanically transmits *Trypanosoma evansi* (which causes surra in equines and dogs) and *T. equinum* (which causes mal de caderas of equines, cattle, sheep and goats). It may also mechanically transmit *T. b. gambiense* and *T. b. rhodesiense* (which cause African trypanosomiasis of man) and *T. brucei* and *T. vivax* (which cause nagana in cattle, sheep, goats and equines) (Kettle, 1984; Soulsby, 1986; Service, 1986; Lehane, 1991).

S. calcitrans has been implicated in the transmission of other pathogenic organisms such as polio virus, equine infectious anaemia, anthrax and fowl pox (Lehane, 1991).

Control

The stable fly is most troublesome in localities where suitable breeding places are readily found. Control measures should therefore be directed towards these areas. It involves the regular removal of moist bedding, hay and faeces from stables and yards, and the removal of food waste from feeding troughs. As larvae can grow on decaying material mixed with urine and faeces, it is important to prevent the accumulation of heaps of weeds, grass cuttings and vegetable refuse (Soulsby, 1986; Service, 1986).

Control by insecticides is similar to that used for houseflies. Small aerosol dispensers sold commercially are commonly used in homes as space-sprays to kill flies. Most of these contain knock-down insecticides such as 0.5% dichlorvos (DDVP) or 0.1 - 0.2% pyrethrins synergised with piperonyl butoxide. However, aerosol sprays have virtually no residual effect and must therefore be used repeatedly in order to achieve control. They do little to alleviate the source of fly nuisance and are costly (Service, 1986).

Residual insecticides include 3% malathion, 1% dimethoate, 0.5 - 1.0% fenchlorphos (Ronnel), 1 - 2% tetrachlorvinphos (Rabon) and 1% propoxur (Baygon). Internal wall-applications remain effective for 1 - 2 months depending on local factors. External wall application may also be carried out. Their duration of effectiveness depends on factors such as rainfall (Service, 1986).

Organophosphates can be applied in animal housing as liquid or dry bait formulated with an attractant such as sugar. They are placed in areas where flies congregate

(window sills, ledges, gutters *etc.*) but where animals cannot gain access to them. Compounds such as DDVP, diazinon, malathion and neguvon can be used in this manner. Resin strips impregnated with DDVP or other similar organophosphates are used extensively for housefly control and are also used against stable flies. However, they are not effective where there is intense air movement (Soulsby, 1986; Service, 1986). Insecticides can also be applied inside and outside the breeding sites of the stable flies as sprays. This is done at weekly intervals (Soulsby, 1986; Service, 1986).

TABLE 1 Geographical distribution, vertebrate hosts, and transmission of salivarian trypanosomes.*

Species	Geographical distribution	Main vertebrate hosts			Transmission	
		wild	domestic	laboratory	cyclical	noncyclical
T.v. vivax	West Central, and East Africa	waterbuck, reedbuck, eland, giraffe, bushpig	cattle, sheep, goats, horses, donkeys (often pathogenic)	normally none (rats with serum supplement)	*Glossina* spp.	biting Diptera
T.v. viennei	Mauritius, South America	unknown	As for *T.v.vivax*	normally none	none	biting Diptera
T. uniforme	West Uganda, East Congo (Tanzania and Zululand rarely)	antelope	cattle, pigs (rarely if ever pathogenic)	normally none	*Glossina* spp.	biting Diptera
T. congolense	West Central and East Africa	many antelope, giraffe, eland, wildebeest	cattle, sheep, horses, donkeys, pigs, dogs, rarely camels (often pathogenic)	rodents (not all strains are infective; pathogenicity varies)	*Glossina* spp.	biting Diptera (less important)
T. simiae	West, Central, East, and parts of South Africa	warthog	pigs (very pathogenic)	*Cercopithecus* monkeys (very pathogenic) and splenectomized rabbits	*Glossina* spp.	biting Diptera (less important)
T. suis	Tanzania, Rwanda-Burundi, Zaire?	warthog	pigs (pathogenic to young)	none known	*Glossina* spp.	
T.b. brucei	West, Central, East, and parts of South Africa	many antelope, warthog, wildebeest	camels, horses, donkeys, dogs (fatal); cattle, pigs, sheep, goats (may be pathogenic but more chronic)	rodents, rabbits, monkeys (pathogenic)	*Glossina* spp.	biting Diptera (unimportant)
T. brucei "rhodesiense"	Central and East Africa	bushbuck, other antelope	cattle, others? MAN (pathogenic)	As for *T.b. brucei*	*Glossina* spp.	biting Diptera (unimportant)
T.b. gambiense	West and Central Africa	?	pigs and other domestic mammals, MAN (less pathogenic but more chronic than *"rhodesiense"*)	As for *T.b. brucei* (less pathogenic)	*Glossina* spp.	biting Diptera (unimportant)
T.e. evansi	North Africa, Asia, South China, Ptilipines, Maruitius, Central and South America (eliminated from North America and Australia)	tapir (in America), deer (in Mauritius), vampire bat (South America)	camels, horses, donkeys, mules (often pathogenic0); cattle, water buffalo, sheep, goats, dogs, Indian elephant	rodents (pathogenic)	none	biting Diptera

TABLE 1 continued

Species	Geographical distribution	Main vertebrate hosts			Transmission	
		wild	domestic	laboratory	cyclical	noncyclical
T.e. equiperdum	South America mainly, USSR, Iran and probably North and South Africa, (previously also Europe, India, North America)	none	equines (pathogenic)	rabbits (intratesticular or intrascrotal); dogs (some strains)	none	coitus
T. equinum	South America (and ? Sudan)	vampire bat and capybara (in South America)	horses (pathogenic); donkeys, mules (less pathogenic); cattle, sheep, goats, pigs (chronic)	rodents (pathogenic)	none	biting Diptera

* From Kreier and Baker (1987).

FIG. 1. Map of Saudi Arabia

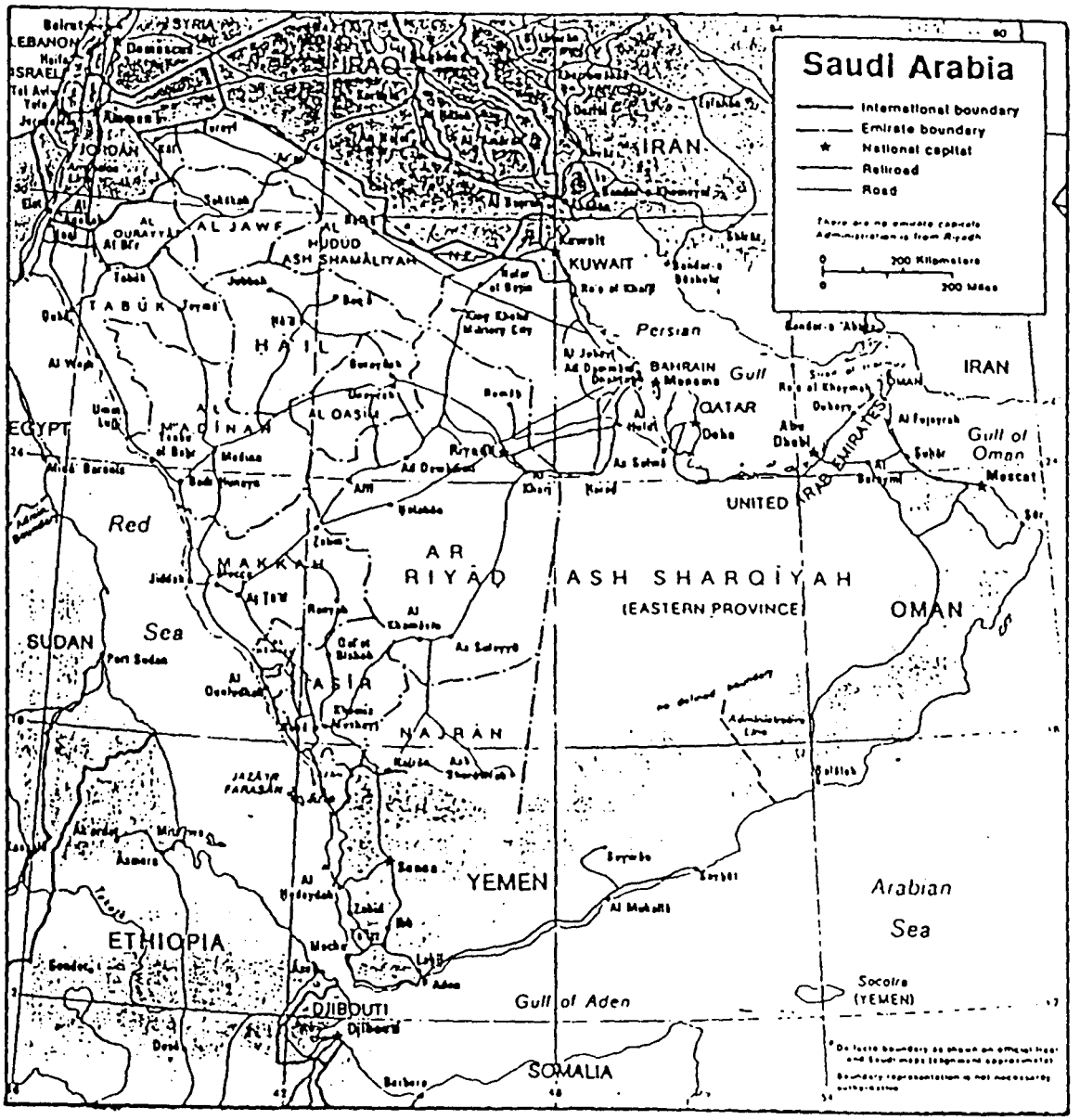


FIG. 2. Distribution maps for the three major groups of tsetse in Africa - (a) *Glossina fusca* group, (b) *Glossina palpalis* group, (c) *Glossina morsitans* group [taken from Jordan (1986)].

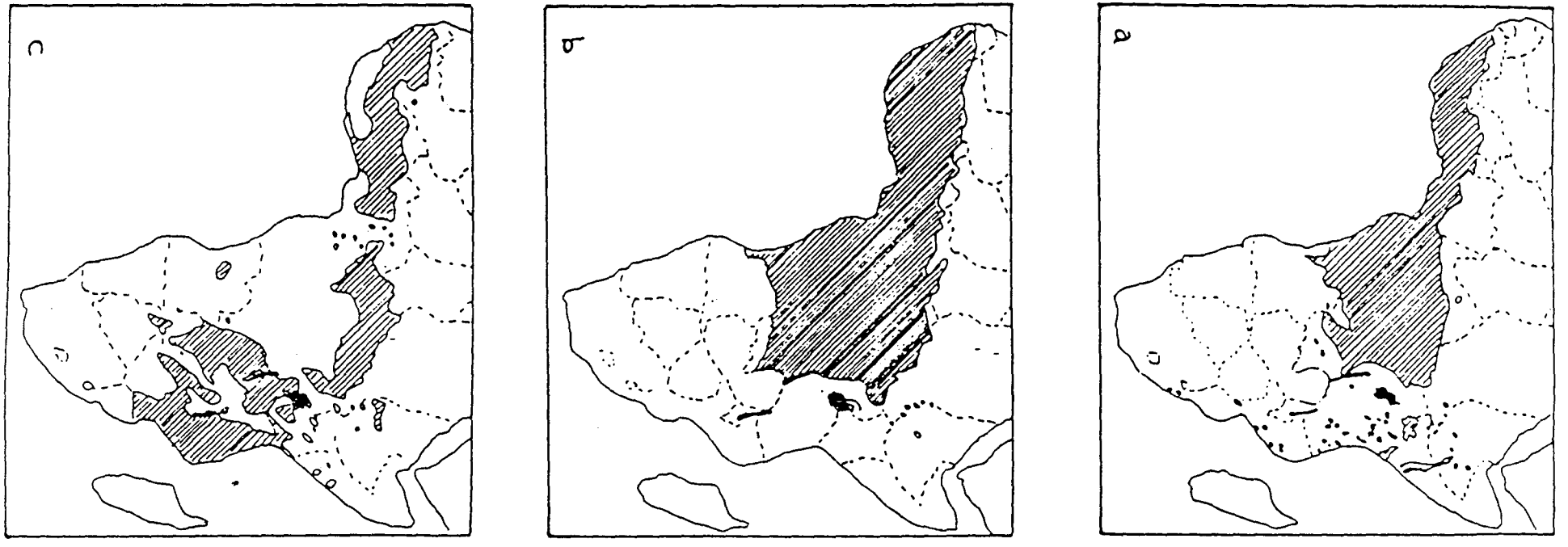
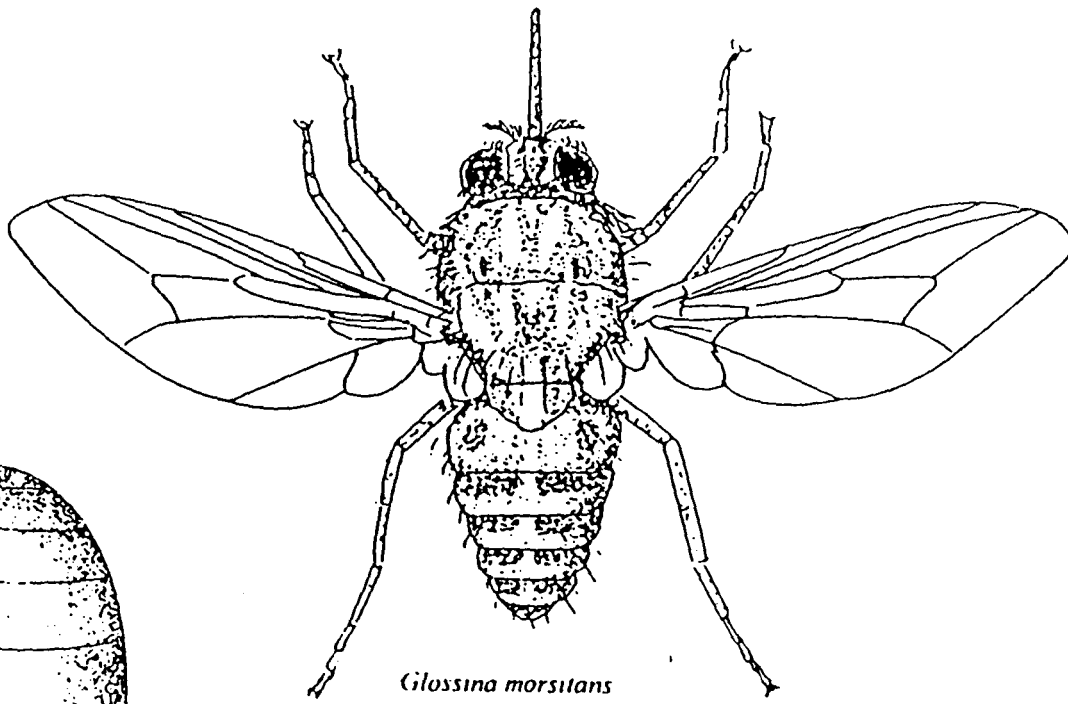
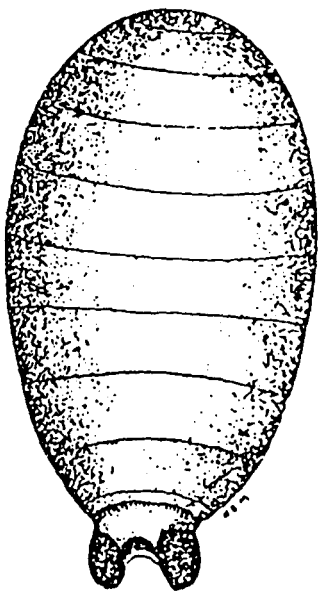


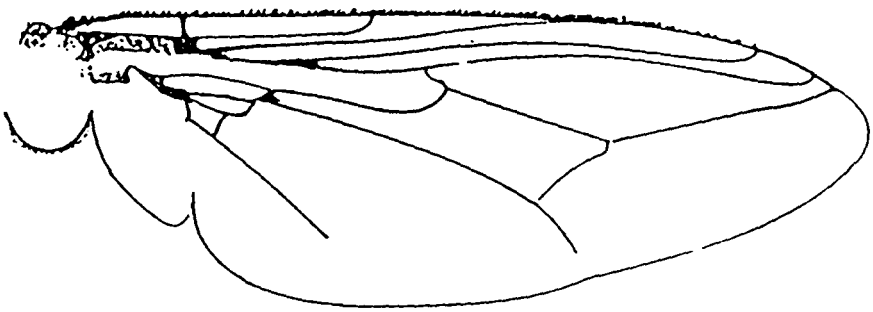
FIG. 3. Morphology of *Glossina* species



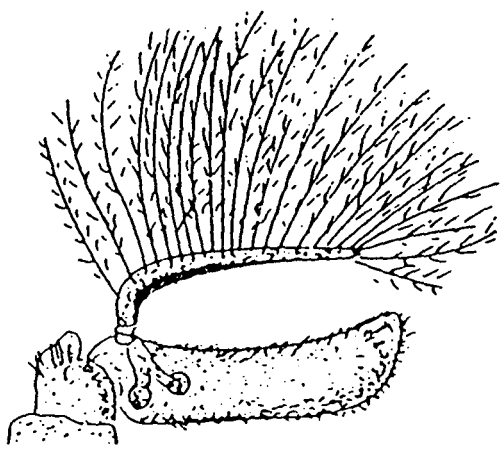
Glossina morsitans



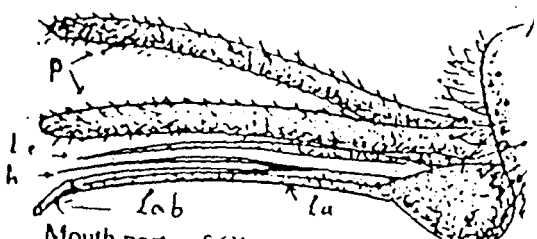
Pupa of *Glossina pallidipes*



Wing of *Glossina pallidipes*



Antenna of *Glossina pallidipes*

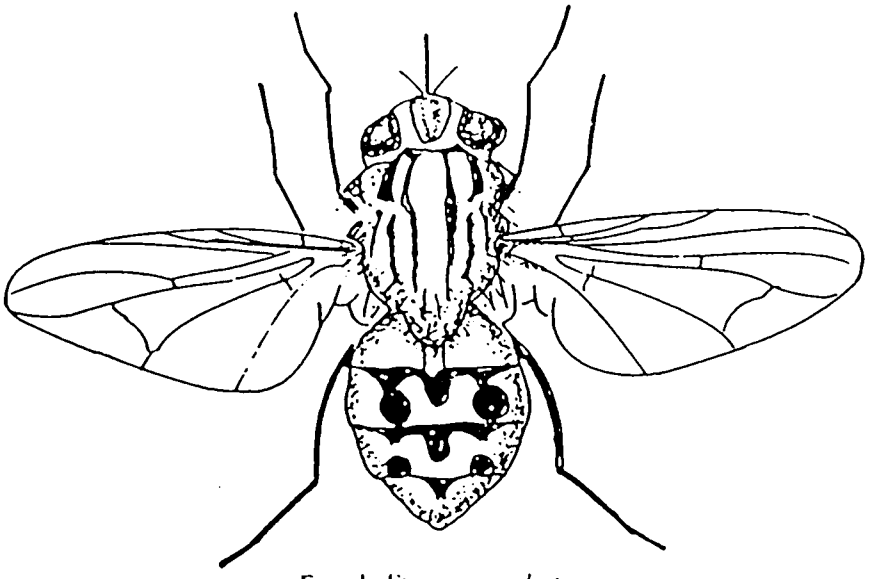


Mouth parts of *Glossina pallidipes*

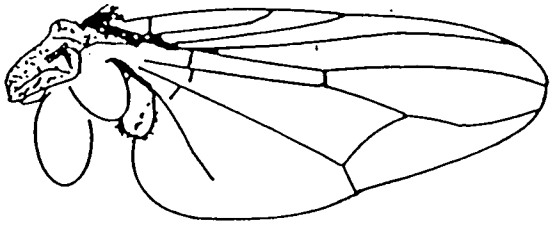
h	hypopharynx
la	labium strengthened by mentum
le	labrum-epipharynx
lab	labellum with enlarged medial view showing armature
p	palps

(Redrawn from Soulsby, 1986)

FIG. 4. **Morphology of *Stomoxys calcitrans***



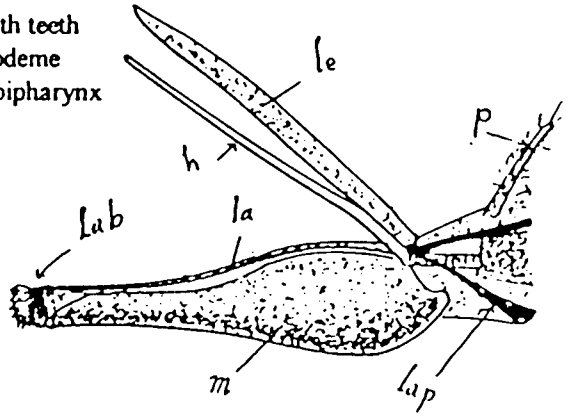
Female *Stomoxys calcitrans*

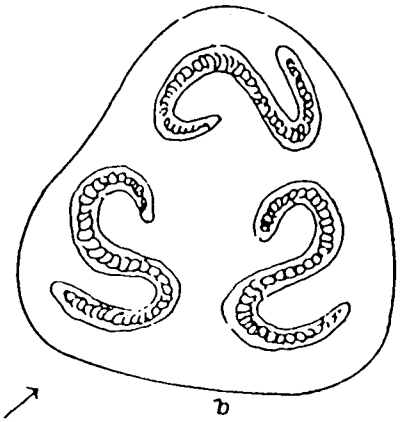


Wing of *Stomoxys calcitrans*

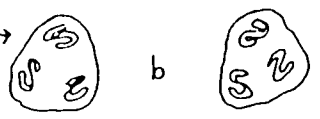
Mouth parts of *Stomoxys calcitrans*

h	hypopharynx
la	labellum
lab	labella with teeth
lap	labral apodeme
le	labrum-epipharynx
m	ment
p	palp





Stomoxys calcitrans a, stigmal plate (enlarged); b, Relative distance between stigmal plates.



(Redrawn from Soulsby, 1986)

Chapter 1

In vitro Studies of the Agglutination Activity of Haemolymph and Reservoir Extracts from Adult *Stomoxys calcitrans.*

ABSTRACT

The activity of haemolymph (HL) and reservoir (RH) fractions of *S. calcitrans* against mammalian erythrocytes (RBC from man [ABO], rabbit, horse and sheep) and trypanosomatid flagellates (*Trypanosoma brucei, Leishmania hertigi* strains 42 and 43, and *Crithidia fasciculata*) was studied.

Anti-erythrocyte agglutination activity was observed. The highest titres were observed with rabbit RBC followed by human group B, human group O, horse, human group A, human group AB and sheep.

Anti-parasite agglutination was also observed. It followed a similar pattern to that of haemagglutination, the activity against all three parasites increasing with age in the case of both HL and RH samples. Maximum activity was observed on days 6 - 7 post-emergence (p.e.) and a decrease in activity against all three parasites was observed on day 14 p.e.

Fly age and food type were found to have a significant influence on RH and HL agglutinin (lectin) production. Fractions from flies fed on 5% glucose solution and on human, sheep and pig blood were tested against rabbit RBC. Results showed that samples collected from flies fed on whole blood had higher activity than those from flies fed on 5% glucose solution. Flies fed on glucose died on day 21 p.e. whilst those fed on whole blood lived for 28 -30 days p.e.

INTRODUCTION

Insects display a diverse array of defence mechanisms to counteract the wide spectrum of potential pathogens in the surrounding environment (Lackie, 1988). Both cellular (haemocyte-mediated) and non-cellular (humoral) haemolymph defence responses are used against naturally-occurring pathogenic organisms such as bacteria and parasites (Ingram and Molyneux, 1988). The functions of the cell-mediated immune system are phagocytosis, nodule formation and encapsulation (Ingram and Molyneux, 1988; Lehane, 1991).

The circulatory system of an insect comprises an open haemocoelic space (without an endothelial lining) through which the haemocytes - invertebrate blood cells - can circulate freely (Lackie, 1988). To generalise, these cells include prohaemocytes (small cells commonly forming a small percentage of the total blood cell count), granular cells (making up 25%-60% of the total blood cell count) which may be involved in the coagulation of insect blood (haemolymph), and plasmatocytes (making up about 25-60% of the total blood cell count). The latter are amoebic in nature and involved in phagocytosis and encapsulation responses. Cystocytes (small cells distinguished from prohaematocytes by their granular appearance) are particularly abundant, accounting for 40-60% of blood cells). Spherulocytes (characterised by 1-3μm inclusions in the cytoplasm) account for less than 5% of blood cells. Oenocytoids (large cells forming 1-2% of blood cells) are believed to be involved in

melanisation because they possess prophenoloxidase system components (Lehane, 1991).

In this investigation attention was paid to the humoral defence mechanisms of *S. calcitrans.* In particular, the role of agglutinins (lectins) in the haemolymph and midgut extracts of the fly in the resistance to infection with the trypanosomatid parasites, *Trypanosoma brucei, Leishmania hertigi* and *Crithidia fasciculata* was studied.

Lectins (defined as proteins that possess the ability to agglutinate erythrocytes (RBC) and other cells) occur in plants, animals and micro-organisms (Sharon and Liss, 1972). Some lectins were reported to be specific in their reactions with human blood groups (ABO) and have been used in blood-typing and investigations of the chemical basis of blood group specificity (Sharon and Liss, 1972).

Agglutination activity in American cockroaches *(Periplanata americana)* and locusts *(Schistocerca gregaria)* is considered to be part of the insect's immune response (Whitcomb *et al.,* 1974; Lackie, 1981). Parasite agglutinins were detected in the haemolymph of these insects and are active against *T. brucei* and *L. hertigi* at titres of 2^{-4}-2^{-13} (Ingram *et al.,* 1984).

Haemagglutination activity by the haemolymph of Acrididae (grasshopper) was also demonstrated against human (ABO), guinea pig, mouse, chicken, cat, pig and sheep RBC by Jurenka *et al.* (1982). They reported that the activity was continuously

present in male and female insects from 4th instar and throughout adulthood, the females having slightly more activity than the males. Rabbit RBC were the most strongly-agglutinated whilst sheep and chicken cells were the least strongly agglutinated. In another study (of the same insects), haemagglutination activity was tested against nine types of RBC. Of these nine, human (ABO) and rabbit RBC showed the highest titres (Hapner, 1983).

Wallbanks *et al.*, (1986) found that lectins or lectin-like molecules from lysates of heads, hind and midguts of male and female sandflies (*Phlebotomus papatasi)* were capable of agglutinating human RBC type ABO(H), promastigotes of *Leishmania aethiopica, L. major* and *L. donovani* but not *L. hertigi* promastigotes or *Crithidia fasciculata* choanomasigotes. They also found that the agglutination of human RBC group O (Rh+) was inhibited by two disaccharides, trehalose and turanose. It is clear that lectins are found in vector and non-vector insects and they may play a very important role in resistance against pathogenic organisms.

Ingram and Molyneux (1988) reported that relatively heat-labile, human ABO(H) blood group non-specific lectins or lectin-like agglutinins were detected in the haemolymph of *Glossina morsitans morsitans, G. palpalis gambiensis* and *G. tachinoides* (titres ranged from 2^{-9}-2^{-16} in haemolymph and from 2^{-1}-2^{-7} in hindgut). Only midgut extracts of *G. m. morsitans* were found to agglutinate AB erythrocytes exclusively (2^{-1}-2^{-7}).

Ibrahim *et al.* (1984) reported that calf, guinea pig and chicken RBC were agglutinated by haemolymph and extracts of mid and hindguts of *G. austeni* (titres reached 2^{-6}) whilst *T. brucei* was only agglutinated by the mid and hindgut extracts to give maximum titres of 2^{-7} and 2^{-8} respectively. In an earlier study, Croft *et al.* (1982) reported that the mortality of cultured procyclic forms of *T. brucei brucei* was greatly increased when incubated *in vitro* with the haemolymph of teneral and non-teneral male and female *G. m. morsitans* from 1-2 hours at 27°C. The maximum titre was 2^{-9} which was reduced after the treatment of the haemolymph with enzymes. However, very little effect was seen against *T. dionisii, Crithidia fasciculata* and *L. hertigi.*

Parasitologists have paid considerable attention to the lectins due to the realisation that they may be involved in host-parasite vector interactions particularly in such important disease vectors as *Glossina spp.* (Maudlin and Welburn, 1987) and *Rhodinus prolixus* (Pereira *et al.*, 1981). Maudlin and Welburn (1988b) concluded that, as well as acting as a protective agent, midgut lectins could trigger the maturation of those procyclic trypanosomes able to withstand their defence mechanism and that the differences between species and strains may reflect strain-specific variations in the number of lectin binding sites. They also suggested that susceptibility to trypanosome infection in tsetse was mediated by midgut lectins.

Welburn *et al.* (1989) reported procyclic trypanosomes were killed by cell lysis which might be a result of membrane damage by lectins. Later, more evidence for the involvement of trypsin or trypsin-like enzymes in trypanosome differentiation and lysis was reported (Imbuga *et al.*, 1992). The involvement of lectins in the action of those

enzymes was considered since lectins and trypsins were reported to be closely related (Osir *et al.*, 1993). Evidence supporting this relationship was produced by Abubakar and his collaborators (1995) when they found that agglutination of bloodstream form parasites was abrogated by soybean trypsin inhibitor. They also found that agglutinins (lectins) from the midgut of *Glossina m. morsitans* were capable of agglutinating *Trypanosoma brucei.* Midgut extracts from flies fed twice had the highest agglutination activity (2^{-12}) followed by flies fed once (2^{-11}) and teneral (unfed) flies had the lowest titres (2^{-1}).

This chapter is an introductory investigation of the haemagglutinins in the haemolymph (HL) and reservoir zone of the midgut (RH) of stable flies *(Stomoxys calcitrans)* of different ages, sexes and nutritional states. Agglutination activity was determined against human (ABO), rabbit, sheep and horse RBC, and procyclic culture forms of trypanosomatid haemoflagellate parasites (*T. brucei, L. hertigi* and *C. fasciculata).*

MATERIALS AND METHODS

Maintenance of the Flies

Flies were obtained from self-supporting colonies maintained in the laboratory at 25-28^{0}C in a 12 hour light/dark cycle. Larvae were reared in a mixture of 1 litre of each of bran, hardwood chips and molichop (Spillers Molassed Chaff Complementary Compound Feeding Stuff for horses consisting of straw and molasses: protein 6.5%, oil 0.8%, ash 10.5%, fibre 26% and moisture 24%), to which was added 1 tablespoon of malt extract, 15 yeast tablets, half a litre of milk powder and two litres of water. The mixture was kept in plastic fish tanks 13"x 10"x 8" (covered with 13.5"x 10" wooden framed cotton lids) for about 3 days before it was used. It was mixed well and then stirred daily to ensure that it was moist but did not contain pools of liquid. Approximately 1000 *Stomoxys calcitrans* eggs were added to each tank. The tanks were left undisturbed for 10-14 days or until the pupae were seen. Flies were fed on cotton wool swabs soaked in heparinised pig blood. Experimental flies were fed on heparinised blood (human, sheep and pig) and 5% glucose solution when they were 2 days post-emergence (p.e.). They were fed once a day until they were 30 days p.e.

Collection of Haemolymph

The collection was done according to Ingram and Molyneux (1988). Samples of haemolymph were obtained from unfed flies 6, 12, 24 and 48 hours p.e. Samples were then collected from fed flies 24 hours after the meal. Haemolymph was collected via a 5 microlitre capillary tube (CAMLAB Serving Science) from the dorsal sinus after puncturing the cervical membrane at the junction of the cervix and the throat. About 0.2-2.0µl of haemolymph was collected from each fly depending on the meal type, time since feeding and the age. Usually 40-50 flies were bled to collect about 40-80µl haemolymph. To that a small amount (approximately 0.05mg) of saturated phenylthiocarbamide was added in order to prevent activation of the prophenoloxidase cascade. Activation of these proteins may cause false RBC agglutination (Ingram and Molyneux, 1988 and 1993). The samples were collected in 10µl aliquots and kept in an ice bath. They were then centrifuged at 12,000rpm for 2-3 mins. After that the cell free haemolymph was separated into another 10 µl aliquot, labelled and stored at -20^{0}C until required.

Reservoir Extraction

Flies were held on ice prior to dissection. The reservoir zones of the midgut (see Fig. 3) were carefully removed from 40 dissected flies of both sexes at each sampling time. They were placed in 1 ml of cold phosphate buffer saline (PBS), pH 7.4, which was kept in an ice bath until the dissection was complete. In these experiments, reservoirs were homogenised and used in agglutination assays because these tissues were found

to have the strongest agglutination activity without causing haemolysis of the RBC (pilot experiment, see appendix 1). Tissues were washed twice in two changes of cold PBS and then homogenised in chilled PBS using a 1 ml homogeniser (UNIFORM Jencons, England). Gut homogenates were stored in lml aliquots and left at 4°C for 3-4 hours, after which the samples were resuspended, frozen and thawed three times at -20°C. They were then kept overnight at 4°C to permit tissue release of lectins. The gut extracts were then centrifuged at 6000rpm for 5 mins after which the supernatant was dispensed into another aliquot (1 ml), labelled and stored at -20°C until required. The concentration of this homogenate was adjusted to 40 reservoirs/ml. This method followed that of Ingram and Molyneux (1988).

Agglutination assays

The required volumes of blood (2-5 ml) were withdrawn aseptically from stocks of fresh rabbit and human (A, AB, B and O) blood. Rabbit blood was obtained via syringe (containing anti-coagulant) from the ear vein. Human blood was donated by Gwynedd Hospital. Samples were tested for the presence of rouleaux formation and intra-erythrocytic haemoglobin crystals. Rouleaux formation is found in the thicker regions of blood films and wet preparations of whole blood. It appears as a face to face superimposition of the cells into stacks of various lengths and can be mistaken for agglutination. Intra-erythrocytic haemoglobin crystals can disrupt the spreading of the blood film and the red cells can appear agglutinated (Hawkey and Dennet, 1989). Samples which showed the above abnormalities were rejected.

The erythrocyte suspensions were prepared by treating the samples according to the method described by Kabat (1961). Each blood sample was centrifuged (10 - 15 minutes) and the supernatant was discarded. Sedimented cells were washed in 5-10 volumes of isotonic PBS, pH 7.4, 3-5 times (until the supernatant appeared very clear). The washed erythrocytes were then resuspended in approximately 19 volumes of PBS, pH 7.4, in order to achieve an approximate 5% suspension. One ml of the suspension was lysed with 14ml of 0.1% aqueous solution of anhydrous sodium carbonate. The optical density (OD) of the clear lysate was measured against a water blank using a Beckman spectrophotometer/Ultraspec II with a 1cm cuvette at wavelength of 541nm. An OD of 0.7 corresponds approximately to 1 x 10^9 RBC per ml of 5% cell suspension. The original suspension was then adjusted to 5% using the Kabat's formula:

Final volume (V_f) = Initial Volume (V_i) x absorbence (OD)/0.7.

Occasionally glutaraldehyde-stabilised human (ABO) erythrocytes were obtained from Sigma (because it was difficult to obtain fresh human blood samples). These samples were resuspended in 0.1M PBS, pH 7.4, to make a 5% suspension according to the instructions of Sigma. These standardised erythrocyte suspensions were kept in labelled, well-stoppered flasks in the refrigerator at 4^0C until required. 0.1% suspensions were prepared at the time of the assay. Pilot experiments showed that there was no significant difference in agglutination using human fresh blood and blood obtained from Sigma (see Appendix 1).

Doubling dilutions of haemolymph (HL) and reservoir homogenates (RH) was performed with PBS in microtitre plates. In the first experiment the lectin concanavalin A (Con A) (from *Canvalia ensiformis*, obtained from Sigma), was used as a positive control (Msangi, 1988). Con A was found to agglutinate 2% fresh human erythrocytes in 0.1M PBS, pH 6.8, at 25 mg/ml (Sigma). In the lab it was found to agglutinate 0.1% suspensions of rabbit erythrocytes and human (ABO), sheep and horse erythrocytes in 0.1M PBS, pH 7.4. Equal volumes of the adjusted erythrocytes (ER) were added to all wells. The plates were then incubated at 25-26^{0}C for two hours and examined for haemagglutination.

The degree of agglutination was usually assessed using a seven score relative scale according to Cunningham and Vickerman (1962) and Ingram and Molyneux (1988). 100% agglutination (3+), 75%(3+/2), 50%(2+/1), 25%(2+/+), 10-20%(1+), trace (tr) and no agglutination (0). The dilutions which just failed to give visible agglutination were regarded as the end points (titres). They were expressed as $-\log_2 2^{-n}$. Controls comprised PBS alone against each sample and all experiments were done in duplicate. If a significant difference occurred between duplicates, the experiment was repeated. To make sure that the agglutinations were not due to the presence of naturally-occurring abnormalities (rouleaux formation and/or intra-erythrocytic haemoglobin crystals) and/or lysins against the RBC types, fresh normal serum from rabbit, sheep, pig and bovine foetus was also used as a control (results in Appendix 1). These experiments were performed with the assumption that the agglutinations were not due to the antibodies present in the blood meal ingested by the flies.

RH and HL samples were obtained from unfed flies (6 hours, 12 hours, 1 day and 2 days) and pig blood fed flies (aged 3, 4, 5, 6 and 7 days). Haemagglutination activities due to HL and RH samples were compared to Con A (positive control). Assays were carried out in 96 well plates (Dynatech). The first well from every sample row was used for the neat sample. In these assays dilution started from 2^{-1} (well 2) to 2^{-11} (well 12) for each sample. 50µl of PBS was added to all the wells in the rows except well 1 in rows A, B, D, E, G and H in which 10µl of the sample (neat) was added. Doubling the dilutions of the samples was then performed by the transference of 5µl of the neat sample from well 1 to the diluent (PBS) in well 2. The solution was mixed thoroughly. After changing the tip, 5µl of this diluted solution was transferred to the next 5µl of the diluent (PBS) in well 3 and the process was repeated up until well 12. This process was performed for all samples. Every sample of HL and RH was tested in a single plate alone. The plates were covered and incubated at 25-26^{0}C for 2 hours after which the haemagglutination activity was determined.

Investigation of the types of RBC most susceptible to haemagglutination.

The above experiments were repeated with erythrocytes (RBC) from sheep, horse and man (types A, B, AB and O) in order to detect the RBC types most sensitive to agglutination by HL and RH.

The Influence of Sex, Age, Nutrition and Rickettsia-like organisms (RLOs) on the Production of HL and RH Agglutinins (Lectins) in *S. calcitrans*.

Flies (aged 6 and 12 hours, 1, 2, 3, 4, 5, 6, and 7 days) were sexed after mating. Samples of HL and RH were obtained from them and tested, using the techniques described above with rabbit, sheep, horse and human (ABO) RBC. The results were analysed using the chi-square test.

Flies were fed on 5% glucose solution or whole blood from pig, sheep or man. Flies were divided into 4 groups (about 200 flies in each) and all groups were maintained at 26-27^{0}C for a period of about 4 weeks during which time sample flies were collected. Samples were collected from unfed flies 6 hours, 12 hour, 1 day and 2 days post-emergence. Flies were fed 2 days post-emergence and samples of HL and RH were then collected daily to day 30. Samples were held at -20^{0}C until required. In this experiment only rabbit RBC's were used and there was no positive control. This experiment was repeated twice for both HL and RH samples obtained from flies fed on pig, sheep and human blood and 5% glucose solution. Protein contents of HL and RH samples from flies fed on whole blood (sheep) and 5% glucose solution were determined (see Chapter 3 for materials and methods).

An experiment to detect the presence of Rickettsia-like organisms (RLO's) in *S. calcitrans* was carried out because their presence may affect lectin activity and consequently infection of the fly with trypanosomes. This experiment was performed according to Welburn (1991). An identical experiment was performed with *Glossina m. morsitans* in order to make a comparison with *S. calcitrans*.

Haemolymph was collected from teneral and fed flies by puncturing the fly head in the fons region and gently squeezing the fly thorax. Samples were collected from both *S. calcitrans* and *G. m. morsitans* in order to match the RLO's. Samples were collected via sterile microcapillaries and placed on 10-well microscope slides to air-dry.

Midguts were dissected from teneral flies of *S. calcitrans* and *G. m. morsitans* and each one was homogenised in a 20µl aliquot of PBS, pH 7.4, (using an electric homogeniser). The 20µl midgut suspension of each fly was then distributed over an area of 2 cm^2 on a microscope slide.

Individual *S. calcitrans* and *G. m. morsitans* pupae were washed and homogenised in 100µl sterile phosphate buffer saline (pH 7.4) using glass/glass homogenisers. 20µl of each pupal suspension was then distributed over an area of 2 cm^2 on a microscope slide.

All the above tissue samples were air-dried. The dried preparations of haemolymph, midgut and pupae were then heat fixed and stained for 12 minutes with Ziehl Neilson carbol fuchsin in distilled water (ratio 1:10). The slides were rinsed with tap water, decolourised with 1% aqueous acetic acid for 5 -10 seconds and then rinsed in tap water for a second time. They were then counterstained with 2% malachite green for

1 minute. Slides were well-rinsed and air-dried after which they were ready for microscopy.

Haemagglutination of trypanosomatid flagellates.

Procyclic forms of *T. brucei* (ANTA serodome obtained from EATRO 112/LUMP581) were cultivated *in vitro* (according to Ingram *et al.*, 1984) using Cunningham's medium, (according to Cunningham, 1977) supplemented with 10% heat-inactivated foetal calf serum at 26^{0}C. The parasites were sub-cultured weekly (according to Ingram *et al.*, 1984).

Bloodstream forms of *T. brucei* were also required for this work so these parasite forms were maintained in mice and rats. These animals were infected by intraperitoneal inoculation of 1-5x10^6 parasites (trypomastigotes). Parasitaemia was checked every day until it reached about 1.5 - 2x10^8 parasites/ml. The infected animals were then anaesthetised with ether. After exposing the chest cavity, the parasites were obtained by drawing the infected blood by cardiac puncture into a syringe moistened with heparin. The parasites were counted using a haemocytometer. In general, about 5-8ml of infected heparinised blood containing about 1-2x10^8 parasites/ml was obtained.

L. hertigi (Liverpool strain LV43) and *C. fasciculata* (Liverpool strain LV116) were used. They were maintained in human blood-nutrient agar slopes with an overlay of Locke's solution (according to Ingram *et al.*, 1984). The culture media were

inoculated with *L. hertigi* or *C. luciliae* at 21^{0}C for 10 days after which time the parasites were sampled and counted (Ingram *et al.*, 1984).

Preparation of the Parasite Suspensions for the Agglutination Assays:-

Parasites were cleaned from their media by washing in PBS after which they were resuspended in the same buffer. In the case of *T. brucei*, parasites were prepared in 0.5ml of Cunningham's medium drawn from the culture flask aseptically by syringe. The medium was then put in a centrifuge tube and PBS added to the 3ml mark. The tube was centrifuged at 1000rpm for 10 mins after which the supernatant was removed. Another 3ml of PBS was added and the tube was centrifuged again at 1000rpm for 10 mins. The supernatant was removed again. This process was performed 3 times until it was clear that there was no parasite waste (or medium contents) which may affect the results of the agglutination tests. In the case of *L. hertigi* and *C. fasciculata*, 0.2ml of the culture medium was withdrawn and the same process was performed. The number of parasites/suspension was counted using the haemocytometer. Parasites were isolated from the whole blood by using a Diethylaminoethyl (DEAE) cellulose column for removal of blood cells and platelets. The column was prepared according to the method of Mercado and Katusha (1979). The infected rat blood, however, was diluted 1:3 in PBS, pH 7.4, and used directly without separation of RBC and platelets from it. The separated parasites were then washed twice in PBS, suspended in 3ml PBS, pH 7.4, and the parasite number per ml of the suspension was counted using the haemocytometer.

Agglutination of trypanosomes

Doubling dilution of HL and RH was performed using PBS, pH 7.4, in microtitre plates (as described above). 5µl of a parasite suspension (3.0-5.5x10^6 cells per ml) were added to each well. Plates were then incubated at 25^{0}C for 3 hours. All agglutination tests were performed in duplicate with controls consisting of parasites and PBS alone. End point titres were scored as those dilutions which just failed to give visible agglutination. Every titre was expressed as its -$\log_2 2^{-n}$ value. The degree of agglutination was assessed using the seven score relative scale previously mentioned. Trypanosome agglutination activity trials were performed for HL and RH samples from unfed flies (aged 6, 12 and 24 hours and 2 days post-emergence) and from sheep blood fed flies (aged 3, 4, 5, 6, 7 *(T. brucei* blood form assays stopped here) 8, 9, 10, 11, 12, 13 and 14 days) of both sexes.

RESULTS

Most susceptible RBC types

Human (ABO), sheep, rabbit and horse RBCs were used in these assays and the results are shown in Table 1.1. It is clear from the results that the haemagglutination activity increases with age. Rabbit RBC are the most sensitive cells followed by human RBC group B, human RBC group O, horse, human A and AB and lastly sheep RBC.

Effect of Sex

Flies were being sexed and separated 6 hours to 2 days post-emergence (unfed and non-mated) and 6h to 3-7 days post-emergence (fed and mated). Samples were collected from the flies. In general, results were the same as those for samples collected from mixed flies. There was no clear evidence to suggest that sex has an influence on HL and RH agglutinin production in *S. calcitrans* ($p > 0.05$) (see Table 2).

Presence of RLO's

No RLO's were seen in any of the samples from stable flies but RLO's were observed in all *G. m. morsitans* samples.

Effect of age and nutrition

In general, flies fed on whole blood (from animals and man) had maximum activity by the second week post-emergence (14 days). This activity decreased towards the end of the fly's life (28-30 days), the titres returning to the same level of those of unfed flies (Figs. 1.2 and 1.3). However, in flies which fed on 5% glucose solution, maximum activity was reached by days 8-10 (titres 2^{-15}-2^{-16}). It then decreased, the titres returning to the same level of those of unfed flies by days 17-18 (2^{-8}). The flies then started to die and at day 20 had the same titres as flies aged 12 hours post-emergence (Figs. 1.2 and 1.3). The average protein contents of HL and RH samples from flies fed on whole blood were 26.3 mg/ml and 6.4 mg/ml respectively. The average protein contents of HL and RH samples from flies fed on 5% glucose solution were 20.5 mg/ml and 3.3 mg/ml respectively (details of the results are given in Chapter 3).

Agglutination of parasites

Results of the agglutination of the bloodstream form *of T. brucei* and culture forms of *T. brucei*, *L. hertigi* and *C. fasciculata* with both samples are shown in Table 3. Generally the same performance was seen in all three parasites. In the case of bloodstream form *T. brucei,* the activity started at 6 hours post-emergence (2^{-2} for both samples), increasing with age until it reached peak activity (2^{-15}) at day 8 (in the case of RH) and at days 10 and 11 (in the case of HL). The activity then started to

decline at day 9 (2^{-13}) in the case of RH, reaching 2^{-10} by the end of the second week. A similar pattern of activity was seen in the case of HL.

In culture form parasites almost the same mode of activity was seen. The activity started at 2^{-1}-2^{-2} (6 hours post-emergence) for the three parasites (both RH and HL samples) to reach the peak at day 11 (2^{-14}-2^{-15}) in the case of *T. brucei* (RH and HL), *L. hertigi* (RH) and *C. fasciculata* (RH). The peak (2^{-15}) was at day 12 for *C. fasciculata* (HL) and the peak (2^{-14}) was at day 13 for *L. hertigi* (HL). By day 14 the activity had declined to 2^{-11}-2^{-13}.

DISCUSSION

In this work the potential role of lectins in the refractoriness of the stable fly, *S. calcitrans*, to trypanosomes was investigated. Although this fly has a worldwide distribution and inhabits the same areas as tsetse flies *Glossina* spp. (Kettle, 1984; Service, 1986; Soulsby, 1986; Lehane, 1991), it only transmits trypanosomes by mechanical means. However, the stable fly *S. calcitrans*, is the biological vector of the nematode *Habronema majus* (a horse stomach worm) and *Setario cervi* (a parasite of cattle) (Kettle 1984; Soulsby, 1986; Lehane, 1991). It has also been implicated in the transmission of other pathogenic organisms *e.g.* polio virus, anthrax and fowlpox (Lehane, 1991). What makes this insect a biological vector of the latter organisms, but not of the trypanosome parasites? What are the differences between *Glossina* spp. and *Stomoxys* which make the former susceptible to *Trypanoma spp.* infection and the latter not susceptible? Both vectors are haematophagous dipterans and both have type II peritrophic membranes (Lehane, 1991). Maximum agglutination activity was observed with homogenates taken only from the reservoir zone of the midgut. Whole midgut (MG) extracts containing the opaque and lipoid zones (see Fig. 1.1) caused less agglutination than the reservoir zone. Whole midgut extracts caused haemolysis of the RBC whereas reservoir zone extracts did not (see results in Appendix 1). This is not surprising as there have been other reports of haemolysis occurring with the use of whole midgut extracts (Spates and Deloach, 1980; Spates, 1981).

Lehane (1976) reported that the opaque zone midgut cells of *S. calcitrans* were responsible for the production and release of proteolytic digestive enzymes into the gut

lumen via membrane bound vesicles (MBV). Recently Blakemore *et al.* (1993) reported that the trypsin enzyme in the opaque zone was stimulated by cyclic AMP but not cyclic GMP.

The peritrophic membrane (p.m.) functions as a physical barrier (a primary barrier) which separates blood contents and parasites from the midgut epithelial cells (Dunn, 1986; Lehane, 1991). However, it was reported that trypanosomes can penetrate the peritrophic membrane 9 - 11 days after ingestion of infective blood (Ellis and Evans, 1977). This indicates that the peritrophic membrane is not a physical barrier to the penetration of trypanosomes to the haemocoel of the infected insects (Lehane and Msangi, 1991).

Trypanosomatidae may be able to penetrate the membrane by secreting chitinases (Schlein *et al.*, 1992). Billingsley (1994) suggested that anti-vector vaccines offer tremendous potential for disease control either directly by reducing the vector fitness or indirectly by affecting transmission to it. Schlein and Jacobson (1994) found that haemoglobin or blood in the growth medium of *Leishmania major* inhibited the formation of the infective promastigotes and the secretion of chitinases. I assume that the blood which was introduced to the growth medium of the parasite (*L. Tropica*) may contain some antibodies against the parasite secreted chitinases. This may explain the suggestion of Billingsley (1995) which said immunization with crude vector or parasite preparations can result in isolation of vaccines which are very effective but often with unknown function.

Lectins play a very important role as humoral defence factors in the immune mechanisms of insects (Dunn, 1986; Lackie, 1988). They were reported to be found in the haemolymph of *G. m. morsitans* and had anti-parasitic agglutination activity against *T. brucei, T. vivax* and *T. congolense in vitro* (East *et al.*, 1983). The agglutination activity was reported also in the haemolymph mid and hind guts of *G. m. morsitans* and *G. austeni* against *T. brucei* and erythrocytes (RBC) of several animal species. Lectins (agglutinins) were found to have a selective reactivity against D(+)-glucosamine (Ibrahim *et al.*, 1984, Molyneux *et al.*, 1986). Haemagglutination activity was reported in gut and haemolymph extracts of *G. morsitans, G. palpalis gambiens*is and *G. tachinoides* (Ingram *et al.*, 1988) and haemolymph of *G. fuscipes fuscipes* (Ingram and Molyneux, 1990) against human erythrocytes (RBC) of ABO(H) blood groups.

Lehane and Msangi (1991) studied extracts of whole gut, peritrophic membrane and midgut remains in order to investigate the haemagglutination activity towards human erythrocytes of AB Rh -ve blood group. They found that there was no activity in the extracts of whole gut of newly-emerged *G. m. morsitans* flies and only a trace of agglutination was observed in flies aged 3-8 days post-emergence (p.e.) (titres neat -2^{-3}) whilst flies aged 14 days p.e. had the activity of 50% (2+) and titres ranged neat -2^{-8}. The same RBC type was found to be agglutinated by midgut (MG) extracts of *G. m. morsitans* (titre 2^{-7}), *G. palpalis gambiensis* (titres 2^{-3}-2^{-5}) and *G. tachinoides* (titre 2^{-3}) (Ingram and Molyneux, 1988).

Comparing the lectin activity of tsetse flies to that of stable flies, *S. calcitrans,* we found that the human RBC group AB gave a very weak (trace) agglutination to neat RH extract from stable flies aged 6h. This increased with age to reach 1+ at 12h and 2+ at 1 day p.e. with titres of 2^{-3}-2^{-4}. It continued to increase until the titre reached 2^{-8}-2^{-9} at days 6 and 7 (p.e.) for flies of both sexes.

Gray (1990, unpublished) found that very young *S. calcitrans* flies (0 days) had activities against rabbit RBC with highest titres ranging from 2^{-5}-2^{-7}. The greatest activity was seen in flies aged 4 - 8 days with titres 2^{-7}-2^{-8}. He reported that the activity decreased slightly when flies reached days 12-14 p.e. (titre 2^{-7}). In our work rabbit RBC were found to be agglutinated by MG extracts from unfed flies 6h and 12h p.e. with activity 1+ (titres neat -2^{-2}). The titres reached 2^{-5}-2^{-7} for both sexes and the activity increased to 2+ at day 1 p.e. with titres 2^{-6}-2^{-7} for both sexes. The activity continued to increase with age to 2+-3+ (neat to 2^{-7}), the highest titres ranging from 2^{-6}-2^{-15} at days 1-7 p.e..

Comparing our results to those of Gray (1990, unpublished) revealed some variations in the results despite the samples being from the same insects against the same RBC type. These variations might be due to differences in the way the insects were maintained and prepared for dissection. Gray (1990, unpublished) collected flies every 4 days, whilst here, flies (6h, 12h, 1 day and 2 days p.e. (unfed)) were dissected every day. Gray (1990, unpublished) reported that the insects were fed on 5% glucose solution for 2 days in order to flush any residual blood from the gut. This procedure might have washed out some of the lectins or diluted them or altered their

rate of secretion, thereby affecting the titres. In our work, flushing the gut with 5% glucose solution was not performed. Unfed flies were dissected once collected whilst fed flies were kept in the lab for 24 hours after the last feeding (in a previous experiment we had noticed that *S. calcitrans* needs 24-36 hours for remains of the blood meal to be cleared from the reservoir region; see appendix - pilot experiment).

Gray's results were approximately the same as those of agglutination of rabbit RBC by MG extracts from flies fed on 5% glucose solution. The activity increased with age until it reached maximum activity at day 5 (2^{-13}) and day 9 (2^{-11}). It then started to decrease from day 11 to reach the same titration of day 2 (2^{-8}), decreasing further until it reached the neat by day 20 (in which only a few cells were agglutinated (trace[tr]). More details about this are found in the discussion of nutritional factors. In general, the pattern of the activities was almost the same.

Now, let us compare our RH and HL sample results from *S. calcitrans* with those of MG and HL samples from *Glossina spp.* against human RBC (ABO). Ingram and Molyneux (1988) performed the haemagglutination activity experiment by using samples of haemolymph and extracts of mid and hind guts of *G. m. morsitans, G. palpalis gambiensis* and *G. tachinoides* against human RBC, Rhesus positive (Rh+) and negative (Rh-). They reported that extracts of midgut from *G. tachinoides* and *G. palpalis gambiensis* caused haemolysis of all the RBC types tested with titres ranging from 2^{-3} to 2^{-5} in the former and from 2^{-5} to 2^{-9} in the latter. No haemagglutination was seen. They also reported that *G. m. morsitans* lysin activity values ranged from 2^{-4} to 2^{-8} and that haemagglutination occurred with "AB" RBC.

The neat (undiluted) hindgut samples of *G. m. morsitans* agglutinated group B rh+ to a degree of 1+ (10-20%) whilst tr agglutination was observed with B rh-. Weak agglutination (tr-1+) was present with O rh+ RBC. They noticed that the remainder of blood types were agglutinated to a degree of 3+/2+ (75-100%). In both *G. palpalis gambiensis* and *G. tachinoides* hind gut extracts, tr agglutination of B rh- and 3+/2+ (75%) of B rh+ were found. The workers reported that only the hindgut of *G. tachinoides* had agglutinated both A rh+ at a degree of 3+ (100%) and A rh- (tr).

In my work only reservoir extracts (RH) of *S. calcitrans* were used because these were found to exhibit maximum agglutination activity without erythrocyte haemolysis. After rabbit RBC, human RBC group B were seen to be the most sensitive cells to agglutination with both HL and RH samples followed by human RBC group O and horse RBC. Human RBC groups A and AB and sheep RBC were the least sensitive cells. The activity started in flies aged 6-12h p.e. at 1+ (with titres reaching 2^{-5}-2^{-6}). The activity increased with age, reaching the degree of 2+ at day 1 p.e. with titres 2^{-6}-2^{-7}. It continued to increase until the maximum titre of 2^{-13}-2^{-15} was reached at about 2 weeks p.e.

Ingram and Molyneux (1988) reported that there were no differences in the degree of agglutination in any of the haemolymph samples from the three tsetse fly species examined. No marked specificity towards any one of the human RBC types was found. They also reported that titre values and ranges were very similar in the cases of *G. m. morsitans* and *G. palpalis gambiensis* whereas higher values were obtained for *G. tachinoides*. The titres were 2^{-15} (*G. m. morsitans* against A rh-), 2^{-16} (*G. tachinoides* against O rh-).

The flies had been fed on rabbit marginal ear veins 3 times a week but were not fed for 24h before haemolymph sampling or for 6 days prior to removal of the gut tissues. However, we fed flies daily from 2 days p.e and flies were not fed for 24 hours before haemolymph sampling or removal of the gut tissues.

Our results varied with age whilst their results did not take age into account. However, it can be predicted that flies used for haemolymph sampling were about 7-9 days p.e. and flies used for gut extracts were dissected at approximately 13-15 days p.e.. In our experiment the average protein contents of *S. calcitrans* HL and RH samples were found to be 26.3 mg/ml and 6.4 mg/ml respectively and in unfed *Glossina* spp. these were 28.5 mg/ml and 18.2 mg/ml respectively. Information concerning protein contents of *Glossina* spp. HL and MG samples was not provided by any other worker. Therefore comparing results we can see that *S. calcitrans* had more activity against all the human RBC (ABO) regardless of the Rhesus factor. Animal RBC from rabbit, horse and sheep were used in this work, the highest activities of both samples of HL and RH being against rabbit RBC. Performance of RH agglutination against rabbit RBC was discussed above when it was compared with that of Gray's results (1990, unpublished). Almost the same mode of activity was obtained by the haemolymph against rabbit RBC. The highest titration was seen at days 5(2^{-13}), 6(2^{-14}), 7(2^{-16}), the activity continuing to increase until it reached the maximum at days 9-16 with titres ranging between 2^{-16}-2^{-18} (for haemolymph from flies fed on whole blood). Only rabbit RBC were used to detect the activities of flies more than 7 days (p.e.) in order to determine the effect of age and nutrition. Horse RBC had the same mode of activity as that of rabbit RBC but the titration started from 2^{-3} (6-12h p.e.)

and increased with age until it reached 2^{-11}-2^{-12} (6 and 7 days p.e.) The sheep RBC had the same mode of activity, the highest titration being reached at days 6 and 7 (2^{-6}-2^{-7}). Sheep RBC were found to be the least sensitive indicators for both HL and RH sample lectins from *S. calcitrans* (Table 1). The most sensitive indicators were rabbit RBC > human RBC group B > human RBC (group O) > horse RBC > human group AB > human group A (Table 1). Rabbit RBC were therefore selected for the determination of the effect of age and nutrition on HL and RH agglutinin production. They were also used as an indicator in the experiments with trypanosmatid flagellates subjected to agglutination with HL and RH.

Despite the reduction in the activities with age, flies which fed on whole blood (types mentioned above) showed lower mortality than flies fed on 5% glucose solution. Some blood-fed flies were seen alive at days 30-32 whilst the flies fed on glucose solution were found dead at days 20-21.

It can be concluded from this experiment that agglutination activity started in young flies (6-12h p.e.) in both samples and despite the starvation of the flies (unfed until day 2) the activity increased with age. There was a strong correlation between activity and nutritional status (and type of food). It is clear that flies fed on whole blood can survive for their expected life span, about one month according to Soulsby (1986).

Welburn *et al.* (1989) reported that increasing the period of starvation before infection increased the susceptibility to trypanosome infection of non-teneral flies. Teneral flies showed little agglutination activity *in vitro* suggesting that the lectin is produced in

response to the blood-meal. It was reported that feeding flies before infection abolished the differences in rate of trypanosome killing found between teneral susceptible and teneral refractory *G. m. morsitans.* Welburn *et al.* suggested that the maternally-inherited susceptibility to trypanosome infection (reported by Maudlin, 1982) was a phenomenon found only in teneral flies.

In this work, both samples of HL and RH were found to agglutinate the procyclic trypanosmatid flagellate parasites; *T. brucei, L. hertigi* and *C. fasciculata in vitro.* In the case of *T. brucei,* the agglutination started 6h-2 days p.e. (unfed flies) with a degree 1+ to trace (titres 2^{-1}-2^{-6}). They continued to increase with age until the maximum activity was reached at days 10-13 (2^{-14}-2^{-15} for HL) and days 10-12 (2^{-13}-2^{-14} for RH). The acitivity then declined to give the titres of 2^{-13}-2^{-12} at day 13 (for HL) and day 14 (for RH). Almost the same mode of activity was seen in the case of bloodstream form *T. brucei.* The highest titration was seen at days 9-11 (2^{-14}-2^{-15}) for HL and days 8-11 (2^{-13}-2^{-14}) for RH.

These results support the suggestion of Welburn *et al.* (1989) that lectin was produced in response to the blood meal. Croft *et al.* (1982) reported that there was an anti-trypanosomal factor in the haemolymph of *Glossina* spp. They examined the reduction of the movement of the parasites after 1h and 2h of incubation of the haemolymph of teneral and non-teneral flies with the procyclic (cultured forms) of *T. brucei, T. dionisii, L. hertigi* and *C. fasciculata.* They found that motility of *T. brucei* decreased at 2^{-1} and increased with an increase in titrations until it reached 5+ (the maximum motility of parasites in the culture medium) at titre 2^{-8} after 1h and 2^{-9}

after 2h in the case of male teneral flies. The highest motility was reached at 2^{-10} for both periods in the case of female teneral flies. In the case of inactivated haemolymph (both sexes), the maximum motility was seen at 2^{-4} for both periods. Maximum motility was seen at 2^{-10} for both periods in the case of female non-teneral flies and at 2^{-9} in the case of teneral males. After incubating the parasite with heat-inactivated haemolymph from non-teneral female flies for 2h the maximum motility was seen at 2^{-4}. In the case of *T. dionisii* incubated with *G. m. morsitans* haemolymph (for 1h), the maximum motility was seen at 2^{-10} and 2^{-9} for teneral and non-teneral flies respectively. In the case of *C. fasciculata* incubated with haemolymph for 2h, the maximum motility was seen at 2^{-6} for both teneral and non-teneral fly samples. *L. hertigi* was incubated with non-teneral fly haemolymph for 2h and showed the maximum motility at 2^{-5}.

A similar experiment was done by East *et al.* (1983) using *T. brucei, T. congolense* and *T. vivax*. They found that, after incubating bloodstream forms of *T. vivax* and *T. congolense* with haemolymph at a dilution of 2^{-8} for 30 mins., no movement of any trypanosome was observed. They also reported that, after incubation of procyclic *T. brucei* with haemolymph of *T. congolense*-susceptible flies for 1 and 2h, the maximum motility was seen at 2^{-10} and 2^{-11} respectively. It was concluded that invasion of these three salivarian sub-genera into the haemocoel could be controlled to prevent an excessive and potentially lethal infection from developing in the haemolymph.

Ibrahim *et al.* (1984) reported that the procyclic form of *T. brucei* was agglutinated by mid and hindgut extracts of *G. austeni.* The highest titres were 2^{-7} (midgut) and 2^{-8}

(hindgut). They also reported that RBC from calf, guinea pig and chicken were agglutinated by haemolymph, mid and hindgut extracts and that the results were approximately the same at a dilution of 64.

From the above it can be seen that insects have anti-parasite factors to prevent or control the invasion of pathogenic organisms. These factors could be affected by age, sex, tenerality of the insects and RLO's.

RLO's were found in *Glossina* spp. These organisms were found to produce the enzyme chitinase which can hydrolyse insect chitin to glucosamine. This glucosamine production in pupae is thought to be responsible for increased susceptibility to trypanosome infection in RLO-infected tsetse flies (Maudlin and Welburn, 1988a). Since RLO's are not found in *S. calcitrans*, this might be the reason why they show greater resistance to trypanosome infection compared to *Glossina* spp.

From my work with stable flies, *S. calcitrans,* it can be concluded that the anti-parasite factors are lectins. Lectin production is induced by feeding the insects and it is affected by food type and age. I can also conclude that production of haemolymph and reservoir lectins depends on the nutritional status of the flies. This is supported by the work of Abubakar *et al.* (1995) who found that the highest agglutination activity (against *T. brucei*) was seen in extracts from flies fed twice (2^{-12}), followed by flies fed once (2^{-11}), and the lowest titre (2^{-1}) was seen in teneral flies.

Further work in Chapters 2, 3 and 4 deals with the characterisation of these lectins and factors influencing their activity *in vitro* (Chapter 2). I also attempted to purify these lectins and determine their relative molecular weights (Chapter 3). All samples used in the next chapter were collected from blood fed flies aged 1-2 weeks p.e.

TABLE 1.1

Haemagglutination titres in HL and RH samples of *S. calcitrans* against human (ABO), rabbit, sheep and horse RBC.

(Each ml of reservoir homogenate contains 40 guts and each 80µl haemolymph sample was obtained from approximately 40 flies.)

	A		B		AB		O		Rabbit		Sheep		Horse	
Fly Age	RH	HL	RH	HL	RH	HL	RH	HL	RH	HL	RH	HL	RH	HL
6h	2^{-1}	2^{-2}	2^{-5}	2^{-5}	2^{-2}	2^{-2}	2^{-3}	2^{-3}	2^{-4}	2^{-5}	2^{-2}	2^{-1}	2^{-2}	2^{-3}
12h	2^{-2}	2^{-2}	2^{-6}	2^{-6}	2^{-2}	2^{-2}	2^{-4}	2^{-4}	2^{-5}	2^{-6}	2^{-2}	2^{-2}	2^{-2}	2^{-4}
1d	2^{-3}	2^{-5}	2^{-7}	2^{-7}	2^{-3}	2^{-3}	2^{-6}	2^{-5}	2^{-6}	2^{-7}	2^{-3}	2^{-2}	2^{-3}	2^{-5}
2d	2^{-4}	2^{-6}	2^{-9}	2^{-9}	2^{-4}	2^{-4}	2^{-7}	2^{-6}	2^{-7}	2^{-8}	2^{-4}	2^{-3}	2^{-4}	2^{-7}
3d	2^{-6}	2^{-7}	2^{-11}	2^{-11}	2^{-5}	2^{-5}	2^{-7}	2^{-8}	2^{-10}	2^{-10}	2^{-4}	2^{-3}	2^{-4}	2^{-8}
4d	2^{-6}	2^{-8}	2^{-12}	2^{-12}	2^{-5}	2^{-5}	2^{-8}	2^{-8}	2^{-12}	2^{-11}	2^{-5}	2^{-4}	2^{-5}	2^{-9}
5d	2^{-7}	2^{-8}	2^{-12}	2^{-12}	2^{-6}	2^{-6}	2^{-10}	2^{-9}	2^{-13}	2^{-12}	2^{-6}	2^{-5}	2^{-6}	2^{-10}
6d	2^{-8}	2^{-9}	2^{-13}	2^{-13}	2^{-7}	2^{-7}	2^{-11}	2^{-11}	2^{-14}	2^{-16}	2^{-6}	2^{-6}	2^{-6}	2^{-11}
7d	2^{-8}	2^{-10}	2^{-14}	2^{-14}	2^{-8}	2^{-8}	2^{-12}	2^{-12}	2^{-15}	2^{-16}	2^{-7}	2^{-6}	2^{-7}	2^{-12}

TABLE 1.2

Haemagglutination titres in HL and RH samples of male (M) and female (F) *S. calcitrans* against human (ABO), rabbit, sheep and horse RBC at different ages post-emergence.

	HUMAN R B C																ANIMAL R B C											
	A				B				AB				O				Rabbit				Horse				Sheep			
	RH		HL		RH		HL		RH		HL		RH		HL		RH		HL		RH		HL		RH		HL	
Age	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
6h	2^{-1}	2^{-1}	2^{-3}	2^{-4}	2^{-3}	2^{-4}	2^{-5}	2^{-5}	2^{-3}	2^{-3}	2^{-2}	2^{-2}	2^{-3}	2^{-3}	2^{-3}	2^{-3}	2^{-4}	2^{-5}	2^{-5}	2^{-5}	2^{-3}	2^{-3}	2^{-4}	2^{-3}	2^{-2}	2^{-2}	2^{-2}	2^{-2}
12h	2^{-3}	2^{-3}	2^{-5}	2^{-5}	2^{-7}	2^{-5}	2^{-6}	2^{-6}	2^{-4}	2^{-4}	2^{-4}	2^{-4}	2^{-5}	2^{-5}	2^{-5}	2^{-5}	2^{-6}	2^{-6}	2^{-6}	2^{-7}	2^{-3}	2^{-5}	2^{-5}	2^{-3}	2^{-3}	2^{-3}	2^{-3}	2^{-2}
1d	2^{-4}	2^{-4}	2^{-6}	2^{-6}	2^{-8}	2^{-6}	2^{-7}	2^{-7}	2^{-5}	2^{-5}	2^{-5}	2^{-4}	2^{-7}	2^{-7}	2^{-7}	2^{-7}	2^{-6}	2^{-6}	2^{-7}	2^{-9}	2^{-4}	2^{-6}	2^{-7}	2^{-4}	2^{-4}	2^{-3}	2^{-3}	2^{-3}
2d	2^{-4}	2^{-4}	2^{-7}	2^{-6}	2^{-10}	2^{-7}	2^{-9}	2^{-9}	2^{-6}	2^{-6}	2^{-6}	2^{-5}	2^{-8}	2^{-8}	2^{-8}	2^{-9}	2^{-7}	2^{-7}	2^{-9}	2^{-9}	2^{-6}	2^{-7}	2^{-8}	2^{-6}	2^{-4}	2^{-4}	2^{-4}	2^{-4}
3d	2^{-6}	2^{-6}	2^{-8}	2^{-7}	2^{-12}	2^{-8}	2^{-11}	2^{-9}	2^{-6}	2^{-6}	2^{-6}	2^{-6}	2^{-9}	2^{-10}	2^{-10}	2^{-10}	2^{-10}	2^{-7}	2^{-10}	2^{-10}	2^{-7}	2^{-8}	2^{-9}	2^{-7}	2^{-5}	2^{-4}	2^{-5}	2^{-4}
4d	2^{-6}	2^{-6}	2^{-8}	2^{-7}	2^{-13}	2^{-10}	2^{-12}	2^{-10}	2^{-7}	2^{-6}	2^{-6}	2^{-6}	2^{-9}	2^{-11}	2^{-10}	2^{-11}	2^{-11}	2^{-8}	2^{-12}	2^{-11}	2^{-8}	2^{-9}	2^{-9}	2^{-7}	2^{-5}	2^{-5}	2^{-5}	2^{-5}
5d	2^{-7}	2^{-7}	2^{-8}	2^{-8}	2^{-14}	2^{-12}	2^{-12}	2^{-12}	2^{-7}	2^{-7}	2^{-7}	2^{-7}	2^{-11}	2^{-11}	2^{-11}	2^{-11}	2^{-12}	2^{-9}	2^{-13}	2^{-12}	2^{-9}	2^{-9}	2^{-9}	2^{-9}	2^{-6}	2^{-5}	2^{-6}	2^{-5}
6d	2^{-8}	2^{-8}	2^{-8}	2^{-8}	2^{-14}	2^{-14}	2^{-13}	2^{-15}	2^{-7}	2^{-7}	2^{-7}	2^{-7}	2^{-12}	2^{-12}	2^{-11}	2^{-12}	2^{-14}	2^{-14}	2^{-14}	2^{-13}	2^{-10}	2^{-10}	2^{-10}	2^{-10}	2^{-6}	2^{-6}	2^{-6}	2^{-6}
7d	2^{-9}	2^{-9}	2^{-9}	2^{-9}	2^{-15}	2^{-15}	2^{-15}	2^{-15}	2^{-8}	2^{-8}	2^{-8}	2^{-8}	2^{-12}	2^{-12}	2^{-12}	2^{-12}	2^{-15}	2^{-16}	2^{-16}	2^{-16}	2^{-11}	2^{-11}	2^{-11}	2^{-11}	2^{-7}	2^{-7}	2^{-7}	2^{-7}

TABLE 1.3

Haemagglutination titres in HL and RH of blood-fed stable flies, *S. calcitrans*, against bloodstream forms of *T. brucei* and culture forms of *T. brucei*, *L. hertigi* and *C. fasciculata* at different ages post-emergence.

Fly Age	Bloodstream forms *T. brucei*		Culture forms					
			T. brucei		*L. hertigi*		*C. fasciculata*	
	RH	HL	RH	HL	RH	HL	RH	HL
6h	2^{-2}	2^{-2}	2^{-1}	2^{-1}	2^{-1}	2^{-2}	2^{-2}	2^{-2}
12h	2^{-2}	2^{-3}	2^{-2}	2^{-2}	2^{-2}	2^{-4}	2^{-4}	2^{-4}
1d	2^{-3}	2^{-3}	2^{-4}	2^{-3}	2^{-4}	2^{-6}	2^{-5}	2^{-6}
2d	2^{-4}	2^{-5}	2^{-6}	2^{-6}	2^{-7}	2^{-7}	2^{-6}	2^{-7}
3d	2^{-5}	2^{-7}	2^{-6}	2^{-6}	2^{-7}	2^{-8}	2^{-7}	2^{-7}
4d	2^{-6}	2^{-8}	2^{-7}	2^{-7}	2^{-7}	2^{-9}	2^{-8}	2^{-8}
5d	2^{-8}	2^{-10}	2^{-7}	2^{-8}	2^{-8}	2^{-9}	2^{-9}	2^{-9}
6d	2^{-10}	2^{-11}	2^{-8}	2^{-8}	2^{-9}	2^{-9}	2^{-9}	2^{-9}
7d	2^{-11}	2^{-11}	2^{-9}	2^{-9}	2^{-9}	2^{-9}	2^{-10}	2^{-10}
8d	2^{-15}	2^{-13}	2^{-10}	2^{-10}	2^{-10}	2^{-10}	2^{-11}	2^{-11}
9d	2^{-13}	2^{-14}	2^{-10}	2^{-11}	2^{-10}	2^{-11}	2^{-12}	2^{-12}
10d	2^{-13}	2^{-15}	2^{-12}	2^{-14}	2^{-12}	2^{-11}	2^{-12}	2^{-13}
11d	2^{-13}	2^{-15}	2^{-14}	2^{-15}	2^{-14}	2^{-12}	2^{-14}	2^{-14}
12d	2^{-12}	2^{-13}	2^{-13}	2^{-15}	2^{-13}	2^{-13}	2^{-14}	2^{-15}
13d	2^{-11}	2^{-13}	2^{-11}	2^{-14}	2^{-12}	2^{-14}	2^{-13}	2^{-13}
14d	2^{-10}	2^{-11}	2^{-11}	2^{-13}	2^{-11}	2^{-12}	2^{-12}	2^{-12}

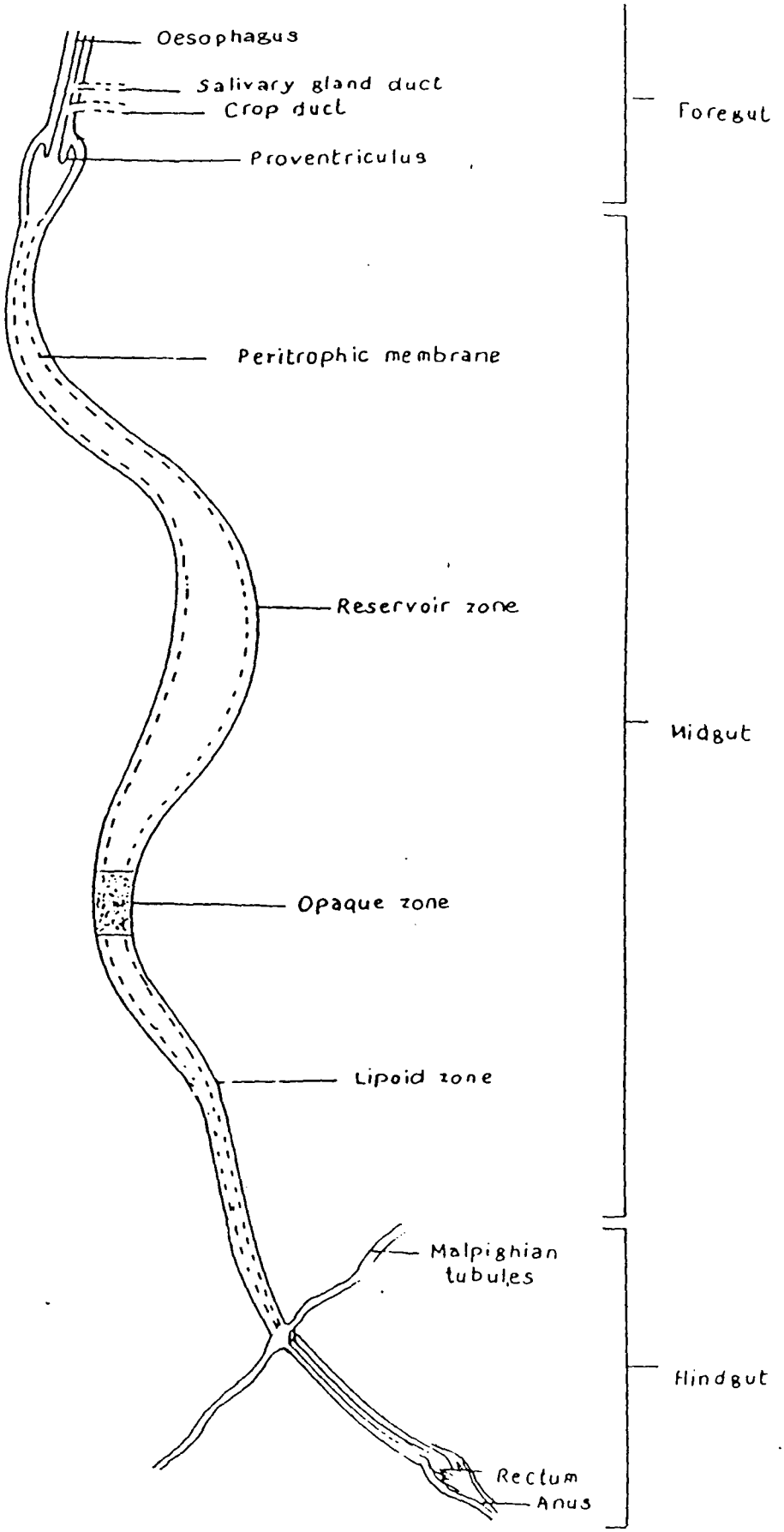


FIG. 1.1. The alimentary canal of *Stomoxys calcitrans* (redrawn from Blakemore, 1994).

FIG. 1.2.

Temporal kinetics of midgut homogenate (RH) haemagglutination activity in blood (pig, sheep, human)-fed and 5% glucose-fed stable flies, *S. calcitrans*. Haemagglutinations are expressed as $-\log_2 2^{-n}$ values. The insects were given their blood meals 2 days post-emergence and daily thereafter.

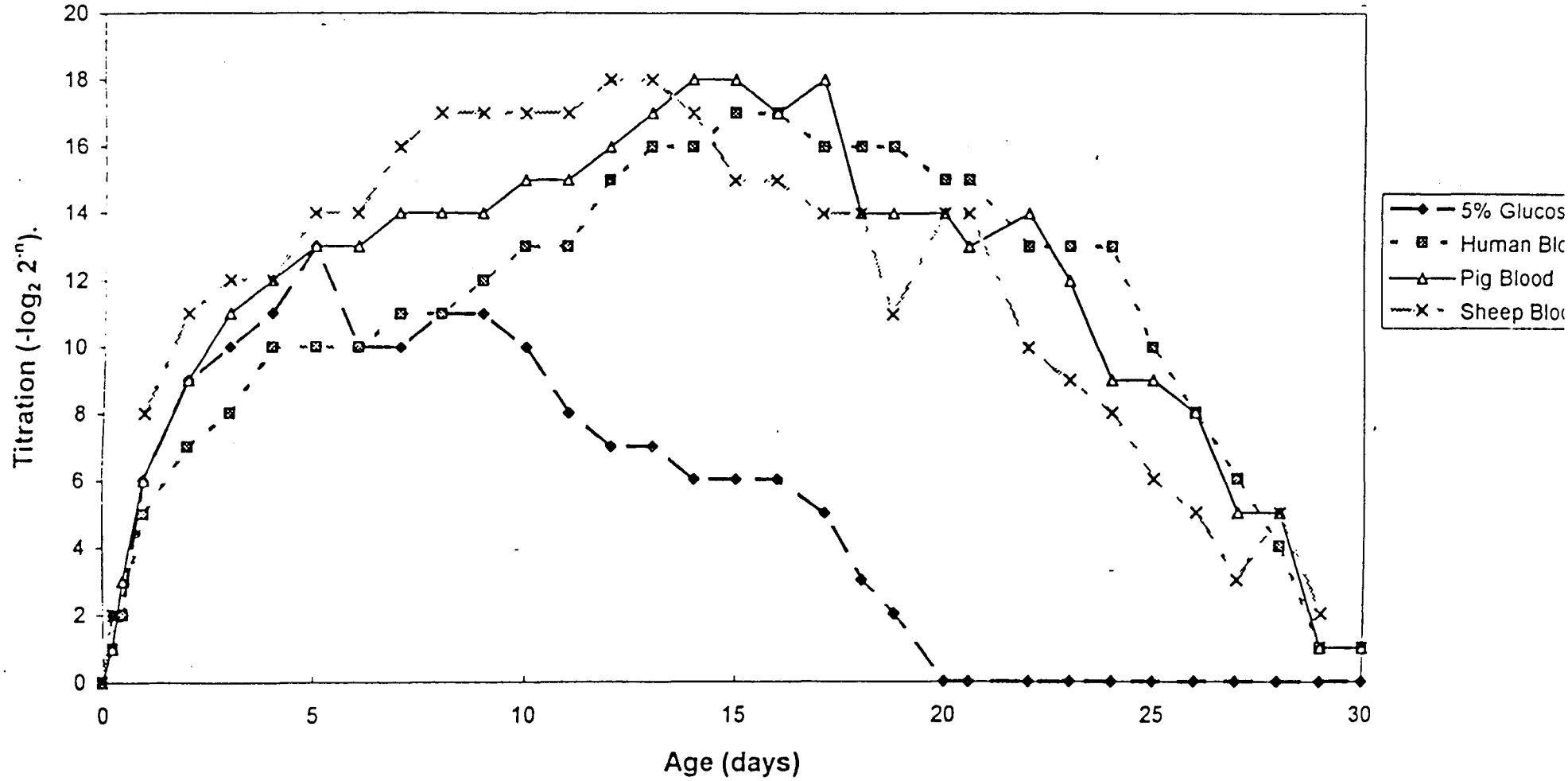


FIG. 1.3.

Temporal kinetics of haemolymph (HL) haemagglutination activity in blood (pig, sheep, human)-fed and 5% glucose-fed stable flies, *S. calcitrans*. Haemagglutinations are expressed as $-\log_2 2^{-n}$ values. The insects were given their blood meals 2 days post-emergence and daily thereafter.

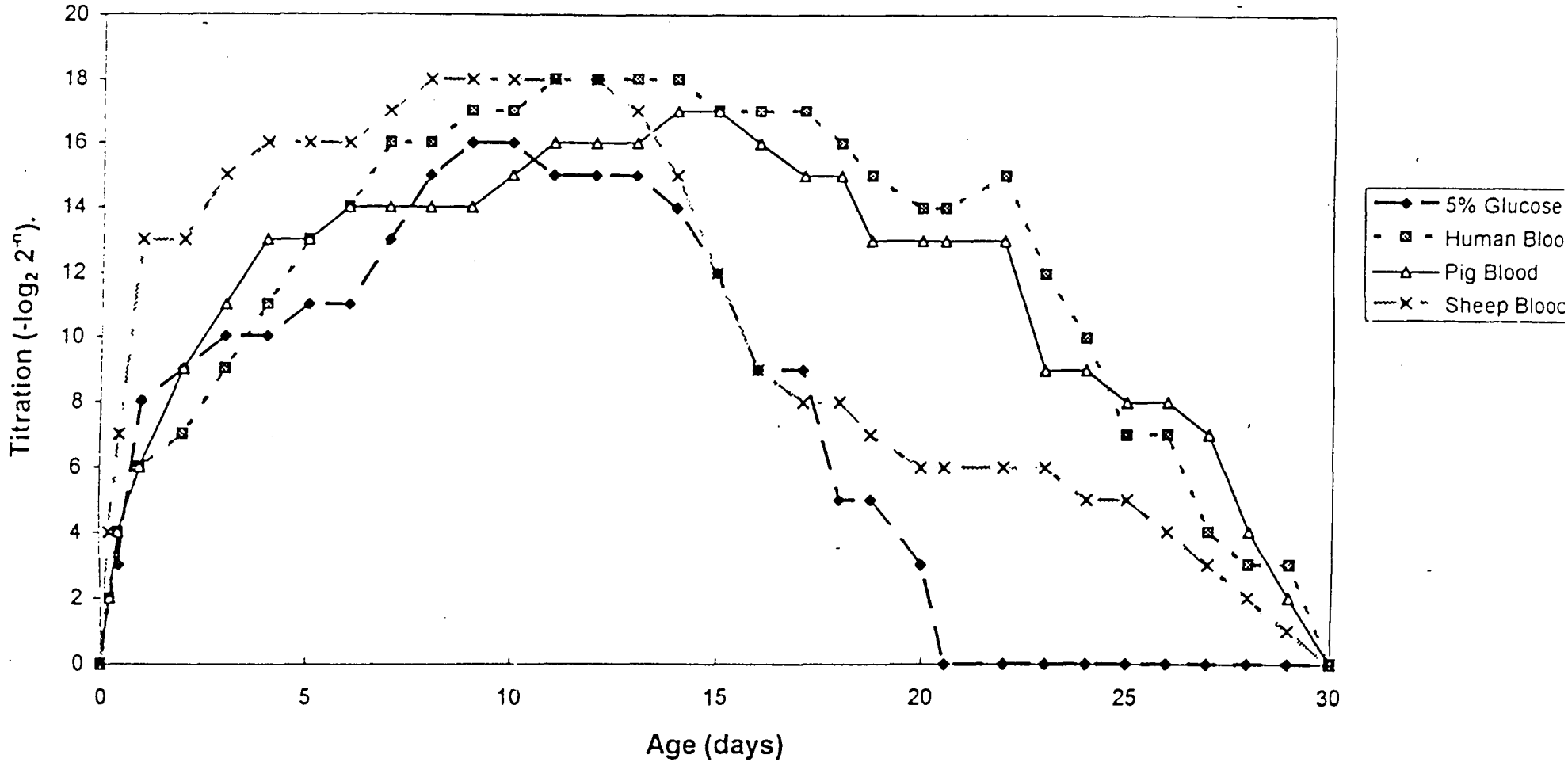


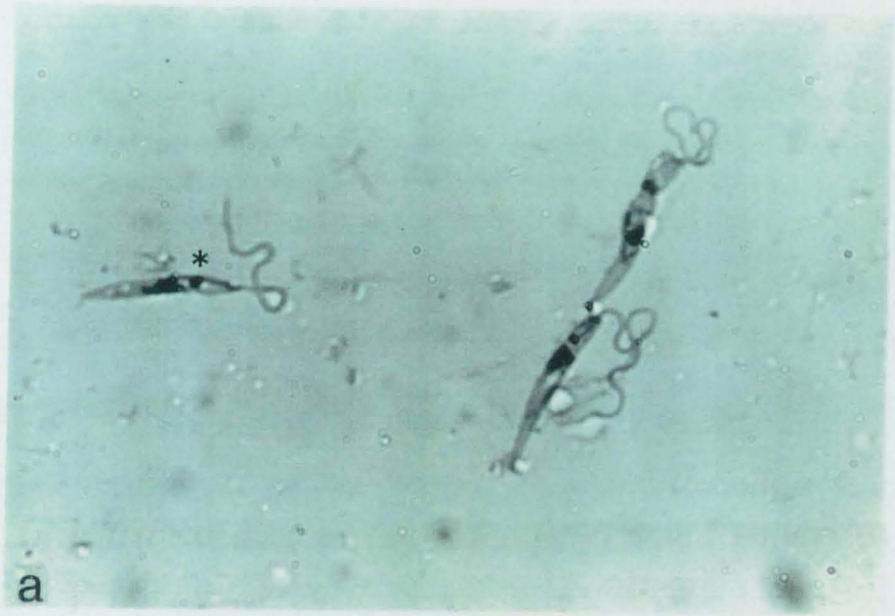
PLATE 1 a and b are Giemsa stained slides of *T. brucei.*

a) The parasite in Cunningham's medium (1977).

b) The parasites stained after being agglutinated with HL.

Note * The kinetoplast and the axoneme are at the anterior end of the body of the trypanosome.

Ø trypanosomes are stuck together (agglutinated).



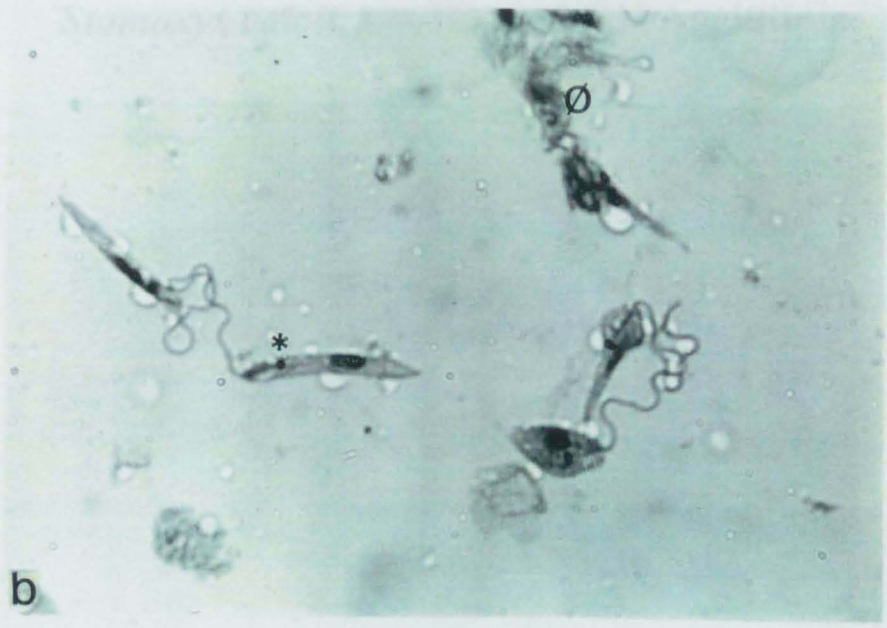


Plate 1

Chapter 2

In Vitro Studies of the Characteristic Properties of *Stomoxys calcitrans* HL and RH Agglutinins

ABSTRACT

Stomoxys calcitrans haemolymph (HL) and reservoir homogenate (RH) samples possess multiple, glycoproteinaceous anti-human (ABO) and anti-animal RBC haemagglutinins which also act as anti-parasitic agglutinins against *Trypanosoma brucei* (bloodstream and culture forms), *Leishmania hertigi* and *Crithidia fasciculata* (culture forms). They bind to cell glycoprotein/glycopeptide residues. No significant differences were found in the physico-chemical properties between HL and RH agglutinins against any of the above agglutinogens. These agglutinins (lectins) are heat-labile, susceptible to dithiothreitol (DTT) reduction, oxidised by $NaIO_4$, and are sensitive to urea treatment. They require divalent cations (especially Ca^{2+} ions) and work best at alkaline pH.

INTRODUCTION

It is now well known that the defence mechanism of insects consists of 2 major parts. These are the cellular (haemocyte-mediated) and non-cellular (humoral) responses, active against a wide variety of pathogenic organisms such as bacteria (Gupta, 1986; Christensen and Nappi, 1988; Lehane, 1991). These responses clearly recognise self from non-self molecules (Kyaya, 1989; Lehane, 1991). The insect humoral immune mechanism consists of various substances which act as anti-bacterial agents such as attacins, cecropins, diptericins, phormicins, sarcotoxins, sapesin, lysozyme, defencins and phenol oxidase (Boman and Hultmark, 1987; Dunn, 1990; Ingram and Molyneux, 1990) and anti-parasitic agents such as agglutinins (lectins). The latter are found in haemolymph, mid and hindguts of tsetse flies (*Glossina* spp.) where they are active against trypanosomes (Ibrahim *et al.*, 1984; Maudlin and Welburn, 1988a and b; Welburn *et al.*, 1989).

Ibrahim *et al.* (1984) were the first to report agglutination acivity in *Glossina* spp. They found agglutination activity of haemagglutinins and parasite agglutinins from haemolymph, mid and hindgut extracts of *Glossina austeni* against calf, guinea pig and chicken RBC, procyclic forms of *Trypanosoma brucei* parasites being agglutinated by mid and hindgut extracts only. Other trypanosomatids *(Leishmania hertigi* and *Crithidia fasciculata)* were not found to be agglutinated by the samples of the 3 fractions. This means that each agglutinin (lectin) has a special affinity for RBC, parasites and other cells which is reflected in the fact that each of these lectins has a specific inhibitor. The same workers found that the activities were specifically

inhibited by D+glucosamine. However, treatments with sialidase and trypsin had no effect.

Wallbanks *et al.* (1986) were the first to report haemagglutinin activity in sandflies (*Phlebotomus* spp.). They found haemagglutinins and parasite agglutinins in extracts from head, mid and hindgut of sandflies (against human blood ABO(H) and promastigotes of the parasites *Leishmania aethiopica*, *L. major* and *L. donovani* but not against *L. hertigi* or *Crithidia fasciculata* choanomastigotes. The agglutination activities of these haemagglutinins were inhibited by trehalose and turanose.

Hapner and Jermyn (1981) found haemagglutination activity against all the human (ABO) and some animal RBC in the haemolymph of both sexes of the adult cricket, *Teleogryllus commodus* (Walker). They noticed that human (ABO), chicken, pigeon and rat RBC were strongly-agglutinated, whilst those of sheep, cat and monkey were weakly-agglutinated. Horse and rabbit RBC were not agglutinated. It was also found that the activities of purified and unpurified haemagglutinin were inhibited by N-acetyl-neuraminic acid, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine but not by bovine serum albumin, trehalose, sucrose, D-glucuronic acid and several simple carbohydrates. The workers reported that haemagglutination activities with purified haemagglutinins were lost or diminished upon lyophilisation, dialysis, heating, freezing, EDTA and trypsin treatment. However, the frozen crude (unpurified) haemolymph retained its activity.

Jurenka *et al.* (1982) found that haemolymph of male and female Acrididae (grasshopper) was capable of agglutinating human (ABO) rabbit, calf, guinea pig, mouse, chicken, cat, pig and sheep RBC. Rabbit RBC were the most strongly-agglutinated whilst sheep and chicken were the least. They noticed that haemolymph from *Melanoplus sanguinipes* also agglutinated the protozoan *Nosema locustae* (a naturally-occurring pathogen of the grasshopper). The preabsorption of the haemolymph samples with different RBC types selectively reduced the activity suggesting the presence of multiple or heteroagglutinins. The activities were inhibited by glycoproteins, some simple carbohydrates and carbohydrate derivatives. Amongst them only mannose and its derivatives were found to be exclusively non-inhibitory. The workers noticed that the activity was stable in frozen haemolymph whilst it was negated by dialysis against water, and might partially recover in PBS or Tris buffer. Activity was negated by heat and EDTA treatments.

Hapner (1983) found haemagglutination activity in twenty four individual grasshopper specimens against nine RBC types. Amongst these nine, human ABO and rabbit RBC showed the highest titres. Although titre values were noticed to differ between individual insects, the agglutination specificity towards different RBC was similar. Hapner attempted to inhibit the haemagglutination of human ABO type O RBC (with an individual insect's haemolymph) with 41 compounds (carbohydrates, carbohydrate derivatives, alcohols and chelating agents). He observed that haemolymph from an individual grasshopper contained complex heteroagglutinin activity similar to that found in haemolymph pooled from several insects. This was confirmed by

determination of the minimal effective inhibitor concentrations and it was primarily directed towards galactose, glucose and related α-linked glycosidic derivatives.

Ingram *et al.* (1984) studied the behaviour of the trypanosomatid flagellates *Trypanosoma brucei* and *Leishmania hertigi* in the presence of cell-free haemolymph samples from the locust, *Schistocerca gregaria,* and the cockroach, *Periplaneta americana, in vitro.* They revealed the presence of parasite agglutinins with agglutination titres of 2^{-4}-2^{-13}. These agglutinins were subjected to physico-chemical treatments, their responses to these treatments indicating that they were protein or glycoprotein in nature. Agglutination was not dependent on the presence of Mg^{2+} or Ca^{2+}. It was partially affected by heat treatment below 65°C. At or above this temperature activity was found to be abrogated.

Sugar specificities of haemagglutinins (lectins) against human ABO (H) RBC were studied in the haemolymph of three *Glossina* spp., *G. m. morsitans, G. palpalis gambiensis* and *G. tachinoides* (Ingram and Molyneux, 1988). They were found to be relatively heat-labile, human ABO (H) RBC non-specific lectins or lectin-like agglutinins with titres ranging from 2^{-9}-2^{-16}. The workers observed that these haemagglutinins exhibited wide heterogeneity towards carbohydrate residues on the surface of human RBC. Midgut haemagglutinins (titres 2^{-6} or 2^{-7}) were only found in *G. m. morsitans* and these acted exclusively against "AB" human RBC. Hindgut extracts in all three *Glossina* spp. caused agglutination titres of 2^{-1}-2^{-7} to most RBC used. Heat-labile, possibly protease but not trypsin-haemolytic molecules were present in most gut preparations. Haemolymph agglutinin reactivities were directed

mainly towards sorbose, trehalose, glucose, 2-deoxy-galactose and to a lesser extent the deoxy, (1-4) - and/or (1-6) - linked derivatives of glucose. The agglutination was occasionally minimally-inhibited by fructose, mannose, sucrose, turanose, stachyose and melezitose. Gut haemagglutinin specificities were less varied than those of the haemolymph and were inhibited by glucose, galactose, mannose and their deoxy-aminated and N-acetylated derivatives. *G. m. morsitans* gut extracts were additionally inhibited by sorbose, sucrose, turanose, gluconic acid and methyl glucoside. Freezing and thawing haemolymph and gut sample extracts negated or reduced agglutinin and lytic activities.

Ingram and Molyneux (1993) subjected haemolymph samples to enzyme, chemical and organic solvents, sodium periodate oxidation and physico-chemical treatments in order to detect the nature of the haemolymph haemagglutinins and their physico-chemical properties. The crossed adsorption process was also performed to detect the presence of haemolymph heteroagglutinins.

Human RBC used were treated in experiments with various enzymes in order to determine the RBC surface haemagglutinin receptor sites. Ingram and Molyneux (1993) found that haemolymph samples from the three *Glossina* spp. possessed multiple (hetero), glycoproteinaceous haemagglutinins which bound to human RBC surface glycoprotein/glycopeptide residues or with *G. m. morsitans* and *G. p. gambiensis* anti-O activity, glycolipid moieties. They also found variations in the haemagglutinin's physico-chemical properties between the three species (*G. m. morsitans, G. p. gambiensis* and *G. tachinoides*) particularly with respect to relative

heat-lability, susceptibility to dithiothreitol reduction, resistance to γ-radiation exposure and sensitivity to urea treatment. *G. p. gambiensis* haemolymph haemoagglutinins required neutral to alkaline pH and Mg^{2+} ions whilst *G. tachinoides* and *G. m. morsitans* samples required acid to neutral pH and Ca^{2+} ions in order to achieve optimum agglutination activities (for more information about insect lectins see Appendix 2).

Abubakar *et al.* (1995) reported that samples of midgut homogenate from *G. m. morsitans* flies which were fed twice had the highest agglutination activity against *T. brucei* followed by that from once fed flies and then the unfed flies. Agglutination of procyclic forms of the parasites required a much lower concentration of the sample than that of bloodstream forms (trypomastigote). The agglutination activities were seen to be inhibited specifically by D-glucosamine and treatments of the samples by soybean trypsin inhibitor abrogated agglutination of the bloodstream forms (trypomastigotes) but not the procyclic forms. The activities were temperature-sensitive with little activity being evident from 4 - 15^{0}C. The activity was lost when the samples were heated to 60^0-100^{0}C. When the sample was separated by anion-exchange chromatography the agglutination activity co-eluted with trypsin activity at approximately 50% NaCl.

It was suggested from these results that there was a very close relationship between midgut trypsin-like enzymes and the agglutinin (lectin) particularly in the light of the fact that successful agglutination of bloodstream form trypanosomes require protease

activity (Abubakar *et al.*, 1995). They concluded that it might be that the enzyme cleaves off some parasite surface molecules, thus exposing the lectin binding sites.

Osir *et al.* (1995) reported that the blood meal-induced lectin (agglutinin) in the midgut extracts of *G. longipennis* was found to agglutinate both bloodstream forms and procyclic forms of *T. brucei* as well as rabbit RBC. The activity was seen to be strongly-inhibited by D-glucosamine and weakly-inhibited by N-acetyl-D-glucosamine. Treatments of samples with soya bean trypsin inhibitor abrogated the agglutination of bloodstream forms but not the procyclic forms. The activity was sensitive to temperatures above 40^{0}C but was unaffected by chelaters of metal ions. In this chapter experiments were performed to characterize *S. calcitrans* reservoir (RH) and haemolymph agglutinins (lectin) to permit a comparison with the lectins of the trypanosome-susceptible *Glossina* spp.

MATERIALS AND METHODS

1. Maintenance of the Flies

Stable flies were obtained from colonies maintained in the insect house at U.W., Bangor at 25-28^{0}C in a 12 hour light/dark cycle. Details of the maintenance are given in Chapter 1.

2. Collection of RH and HL samples

Samples of HL and RH used for experiments in this chapter were obtained from blood-fed flies aged 2 weeks p.e. in order to eliminate any variations which might have arisen during the *S. calcitrans* life-cycle. Collection of samples is described in Chapter 1.

3. Preparation of Rabbit RBC and Parasites

As described in Chapter 1.

4. Performance of the Agglutination Assays

As described in Chapter 1.

5. Cross Adsorption Process

This experiment was carried out to detect the presence of heteroagglutinins (i.e. multiple lectins) in the HL and RH. Rabbit erythrocytes and parasites (*T. brucei, L. hertigi* and *C. fasciculata*) were washed in PBS (pH 7.4) and centrifuged at 1000rpm for 5 mins. Then equal volumes of the packed cells and a sample of HL or RH were added together and incubated at room temperature (25-26^{0}C) for 45 mins. The mixtures were then centrifuged, supernatants removed and readsorbed with another equal volume of packed cells (RBC or parasites). This process was performed in quadruplicate. The final supernatants were examined against 0.1% RBC suspension and against each of the parasite suspensions in order to examine agglutination activity. Controls were performed using adsorbed RBC or parasites plus either non-adsorbed samples or PBS. This method was done according to Ingram and Molyneux (1993).

6. Enzyme-Treated RBC

In order to tentatively determine the nature of the rabbit RBC surface receptor sites of RH and HL agglutinins, the cells were treated with various enzymes. The enzymes used were trypsin (4mg/ml), chymotrypsin (4mg/ml), pepsin (4mg/ml), pronase (4mg/ml), papain (3mg/ml), neuraminidase (2mg/ml), α and β-glucosidase (2mg/ml), α and β-galactosidase (2mg/ml), mannosidase (2mg/ml), and fucosidase (2mg/ml).

The process was performed by adding equal volumes of 1% RBC suspensions and appropriately-buffered solutions of each of the enzymes to RH and HL samples. The mixtures were then incubated at 37^{0}C for 30 mins after which the treated cells were thoroughly washed in PBS and adjusted to 0.1% suspensions. Controls were performed using cells incubated with heat-inactivated enzymes and untreated cell suspensions added to PBS only. The method was performed according to Ingram and Molyneux (1993).

7. Sugar Specifities of Anti-parasite and Anti-erythrocyte Agglutinins (lectins) in HL and RH of stable fly, *S. calcitrans*:-

(a) Investigating the inhibitor sugars.

The agglutination activities of the parasites and RBC were inhibited by a range of carbohydrates and glycoproteins (obtained from Sigma Chemical Co. Ltd) which are listed in Table 1. Stock solutions of sugars (600mM), polysaccharides (10mg/ml) and glycoproteins (20mg/ml) were prepared in PBS (pH 7.3). After doubling the dilutions of samples (HL and RH) with PBS, the carbohydrates or glycoproteins were added to each well at the above initial concentrations to give final concentrations of 300mM, 5mg/ml and 10 mg/ml respectively. The plates were then incubated at 20^{0}C for 1 hour (according to Ingram and Molyneux, 1988). Equal volumes of adjusted suspensions of rabbit RBC and parasites (3-5.5 x 10^3 cells/ml) were added. Then the plates were further incubated at 20^{0}C for 1.5 hours. Inhibition of agglutination was measured as the reduction in endpoint titres when compared to control

(samples of HL and RH) RBC plus PBS substituted for inhibitor and RBC with either PBS or inhibitor alone (according to Ingram and Molyneux, 1988). Each sample (HL and RH) was tested with each inhibitor 3 times. Degrees of inhibition (D1) were scored according to the scale of Ingram and Molyneux (1988); thus a reduction in titre by 2 wells was scored as 1+, 3 wells as 2+, 4 wells as 3+, 5 wells as 4+ and 6 or more wells as 5+. Total inhibitions (100%) were scored as 6+. Inhibition of one well was denoted by ± and no inhibition was denoted by -.

TABLE 2.1

Carbohydrates and Glycoproteins Used as Inhibitors of Agglutination Activity

Monosaccharides

D(+)-Glucose
D(+)-Galactose
D(+)-Mannose
D(+)-Xylose
D(-)-Xylose
D-Mannoheptulose (Manhept)
L(-)-Arabinose
L(-)-Sorbose
β-D(-)-Fructose
D(-)-Ribose

Deoxy Sugars

2-Deoxy-D-ribose (2-dRib)
2-Deoxy-D-glucose (2-dGlc)
6-Deoxy-D-glucose (6-dGlc)
2-Deoxy-D-galactose (2-dGal)
6-Deoxy-L-mannose (=α-L-rhamnose)
6-Deoxy-L-mannose (=α-L(-)-fucose)
6-Deoxy-L-galactose (=α-D(+)-fucose)

Amino Sugars

D(+)-Galatosamine (GalN)
D-Mannosamine (ManN)
N-Acetyl-D-glucosamine (GlcNAC)
N-Acetyl-D-galactosamine (GalNAC)
N-Acetyl-β-D-mannosamine (ManNAC)
N-Acetylneuraminic acid (NeuAC)

Sugar Acids and Amids

D-Glucuronic acid
D-Galacturonic acid
D-Gluconic acid (GlcA)

Glucuronamide (Gluc Amide)

Methyglycosides
β-Methyl-D-glucoside (MeGlc)
2-Hydroxymethyl-phenyl-β-D-glucose (=salicin)
6,7-Dihydroxycoumarin-G-glucoside (-aesculin)
α-Methyl-D-mannoside (MeMan)

Oligosaccharides
Maltose [α-D-Glu(1-4)D-Glu]
D(+)-Trehalose [α-D-Glu(1-1)α-D-Glu
α-Lactose[β-D-Gal(1-4)D-Glu]
Sucrose [α-D-Glu(1-2)β-D-Fru]
D(+)-Turanose{α-D-Glu(1-3)D-Fru]
D(+)-Melezitose[α-D-Glu(1-3)β-D-Fru(2-1)α-D-Glu]
Raffinose[α-D-Gal(1-4)α-D-Glu(1-2)β-D-Fru]
Stachyose[α-D-Gal(1-6)α-D-Gal(1-6)α-D-Glu(1-2)β-D-Fr]
D-(+)-Melibiose[α-D-Gal(1-6)D-Glu]
β-Gentiobiose[α-D-Glu(1-6)D-Glu]

Polysaccharides
Chitin
Glycogen
Xylan
Mannan
Arabinogalactan (Arab Gal)

Glycoproteins

Bovine α-globulins	
Fetuin	β-Glucosidase
α-Amylase	Human chorionic gonadotrophin
Mucin	β-lactoglobulin
Albumin	Invertase
Casein	Glucose oxidase

(b) Minimum Inhibitor Concentration Determination

In this experiment only inhibitors which had previously been found to cause inhibition were used. The haemagglutinin titres of pooled samples of HL or RH against RBC or the parasites were evaluated and the samples were adjusted with PBS to the minimum titre values giving 100% agglutination (usually 2^{-6} and 2^{-7} for HL and RH respectively). The doubling of serial dilutions of each inhibitor was performed using PBS to give final concentrations of 150, 75, 37.5, 9.4, and 4.7 mM. To each dilution the adjusted samples of HL or RH were added and the plates were incubated at 20°C for 1 hour. Suspensions of RBC (0.1%) and parasites (3-5.5x10^6 cells/ml) were added and the plates were then incubated at 20°C for 1.5 hours. The degree of agglutination was then assessed. The minimum inhibitor molarity was recorded as that which caused a reduction in agglutination from 100% to 10-20% or less (as compared to the appropriate controls).

8. Determination of the Nature of HL and MG Agglutinins

(a) Enzyme treatments of HL and RH:-

This experiment was carried out to determine the possible chemical nature of the HL and RH agglutinins. Samples of HL and RH were treated with various enzymes. Solutions with enzyme concentration of 1mg/ml were prepared in PBS with different pH. These enzymes were chymotrypsin (pH 7.8), trypsin (7.5), pronase (7.2), pepsin (4.5), neuraminidase (5.5), lipase (7.7), ribonuclease and deoxy-ribonuclease (5.0), α and β-galactosidase (7.4)), α and β-glucosidase (7.4), α-L-fucosidase (7.4), and α-mannosidase (7.4). To each sample of HL or RH was added an equal volume of each of the above enzymes and the mixtures incubated at 37°C for 1 hour. The enzymatic action was then stopped by addition of 0.1 mg/ml of phenylmethyl sulfonyl fluoride to each. This product and the above enzymes were obtained from Sigma. Then the HL and RH titres were determined and the controls were performed using untreated samples, PBS, heat-inactivated enzymes, enzyme with inhibitor, and enzyme inhibitor alone plus RBC and parasite suspensions.

(b) Organic Solvents and Chemical Treatments:-

Integrated with enzyme treatment studies, the samples were further subjected to various lipid solvents and protein precipitation reagents. Equal volumes of the samples (HL and RH) were diluted 1:4 with PBS, ethanol, chloroform, chloroform/ethanol (2:1V/V). The mixtures were incubated at 5°C for 16

hours after which they were centrifuged at 4000rpm for 15 mins. Then the aqueous phase was treated for agglutination. The samples were also extracted with xylene and acetone by using 4 volumes of solvent to 1 volume of diluted sample. The aqueous and solvent fractions were then separated by centrifugation (4000rpm for 15 mins). The aqueous fractions of the samples were then tested for agglutination. Either 0.9M trichloroacetic acid (in PBS, pH 7.4) or 0.75M phenol (in PBS, pH 7.4) was added to an equal volume of each sample. The resultant mixtures were incubated at 10^0C for 2 hours. The aqueous phase of the phenol extraction was washed with ether. All samples were then centrifuged (4000rpm for 15 mins.) and the supernatants examined for agglutination. Controls were performed with samples alone, PBS alone, and each of the reagents above plus RBC (0.1%) and parasites.

(c) Sodium Periodate Oxidation ($NaIO_4$)

Samples of HL and RH were subjected to sodium periodate ($NaIO_4$) oxidation. Equal volumes of the samples and 30mM $NaIO_4$ were added together and the mixtures were incubated in the dark for 24 hours at 5^0C after which the samples were tested for agglutination. Controls were performed using untreated samples, PBS, $NaIO_4$ alone plus RBC and parasites.

9. Physico-Chemical Treatments:

In the following experiments the samples (HL and RH) were subjected to selected physico-chemical treatments in order to determine their effects on the agglutination activity of treated samples against RBC and parasites.

(a) Presence of Metal Ions

Certain divalent cations (i.e. Ca^{2+} or Mg^{2+}) are essential for the agglutination of cells by HL and RH agglutinins (Ingram and Molyneux, 1993). Samples were incubated for 16 hours at 10^0C with an equal volume of 1.5mM ethylenediaminetetraacetic acid (EDTA) [which chelates all divalent cations] and 1.5mM EGTA ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid [which chelates only Ca^{2+} ions] in PBS (pH 7.4). In this experiment doubling serial dilutions of untreated samples, EDTA- and EGTA-treated samples was done using PBS. Additional EGTA-treated sample dilutions were similarly prepared in PBS containing excess Mg^{2+} ions. Doubling dilutions of samples was also done using PBS containing $MgCl_2$ and $CaCl_2$ (which were also added to untreated samples and EDTA-treated samples). Controls were made with untreated samples alone with EDTA, EGTA, $MgCl_2$ and $CaCl_2$ plus RBC and parasites. Removing EDTA and EGTA from treated samples by dialysis was not possible in these very small amounts. For this reason agglutination tests were performed in the presence of excess divalent cations (according to Ingram and Molyneux, 1993).

(b) Dithiothreitol (DTT) Reduction and Exposure to Urea

DTT splits disulphide linkages whilst urea ruptures the hydrogen bonds in protein and glycoprotein molecules (Ingram and Molyneux, 1993). In this experiment samples of HL and RH were incubated for 1.5 hours at 30^{0}C with equal volumes of 10mM DTT dissolved in 10mM Tris-HCl (containing 150mM NaCl and adjusted to pH 8). The samples were also incubated with 8M urea in PBS (pH 7.6). The actions of DTT and urea were inhibited by addition of 150mM iodoacetamide (Sigma) before the agglutination tests. Controls were performed using untreated samples, PBS, urea and DTT with and without iodoacetamide, and iodoacetamide alone plus RBC and parasites.

(c) Variations in Buffer pH

In this experiment, PBS was adjusted to pH values of 2.2, 3, 3.6, 4, 5, 5.6, 6, 7.0, 7.4, 8, 9 and 10 for use in the double dilution series of the tested samples of RH and HL against RBC and parasites. Controls were samples diluted in PBS (pH 7.4), PBS (pH 7.4) alone, and each of the above adjusted buffers.

(d) Effect of Temperature

Heat labilities of HL and RH samples were studied in order to determine the lowest and highest temperatures at which the samples were active. In this experiment, HL and RH samples were frozen at -20^{0}C and thawed 8 times.

They were then exposed to 0, 5, 10 and 15^{0}C (for 45 mins) and heated at 25, 35, 50, 65, 80 and 100^{0}C in water baths (for 45 mins). The treated samples were then examined for agglutination activities. Controls were performed using untreated samples together with PBS plus RBC and parasites.

RESULTS

1. Heteroagglutinin assay

In all cases, preadsorption of HL and RH with each of the four agglutinogens resulted in total removal of agglutinins against the adsorbing agglutinogen and a reduction in the agglutination activity of the non-adsorbing ones in both samples. Adsorption of both samples with rabbit RBC resulted in removal of the haemagglutinins against them and reduced the activity against the parasites about 6-8 wells for each (see Table 2.2). Adsorption of HL and RH with each parasite reduced the activity against the RBC 7-8 wells and against other parasites 8-9 wells for both samples.

2. Enzyme treatment of rabbit RBC

The results of enzyme treatment are shown in Table 2.3. Treatment of rabbit RBC with neuraminidase increased the activity against them up to 8-fold titre end points for both HL and RH. The same results were seen in the case of treatment with trypsin and papain. Treatment of RBC with α-galactosidase resulted in an 8-fold titre decrease whilst treatment with β-galactosidase resulted in 4-fold reduction for both HL and RH. However, treatment with β-glucosidase gave a 2-fold titre reduction and no change was seen in the case of treatment with α-glucosidase (see Table 2.5).

3. Carbohydrate Inhibition

Results of carbohydrate inhibition of agglutination are given in Table 4a. In the case of the monosaccharides the most effective inhibitors were galactose and mannose (5+) followed by sorbose, manheptalose and arabinose (1-2), then xylose and ribose (1+) for both RBC and parasites. Inhibiting the activity with deoxy and acetylated sugars showed that D(+)- glucosamine was the most potent inhibitor sugar (5+ to 6+) followed by D(+) mannosamine (5+), N-Acetyl-D-glucosamine (5+), N-Acetyl-D-galactosamine (5+), D(+)-galactosamine (4+), N-Acetyl-Neuraminic Acid, and 2 deoxy-D-galactose (2+). Rhamnose showed 2+ in the case of HL *vs.* RBC and 1+ in the case of MG *vs.* RBC and both samples *vs.* parasites. 6-Deoxy-L-mannose and 6-deoxy-L-galactose showed 1+ in the case of both samples against the four agglutinogens. No inhibition was seen with other sugars. Use of the sugar acids and amides, methylglycosides and oligosaccharides showed that D(+)-melibiose was the most potent inhibitor (5+) in the case of both samples against RBC and parasites. Inhibition of the activities with polysaccharides showed that mannan had degrees of inhibition of 2+ and 1+ against RBC for HL and RH activity respectively whilst it had a degree of inhibition of 1+ for both samples against parasites. The same mode of inhibition was seen with bovine γ-globulins whilst glycogen had 1+ of inhibition for HL *vs.* RBC and no inhibition in the case of RH *vs.* RBC or both samples *vs.* parasites. 1+ inhibition was seen for both samples in the case of fetuin, α-amylase, mucin, albumin, b-lactoglobulin and invertase for all the four agglutinogens.

Effect of enzyme treatment of the samples (HL and RH) on agglutination activity against rabbit RBC, *T. brucei*, *L. hertigi* and *C. fasciculata* was done with most inhibitor sugars (the minimum concentrations of inhibitor sugars are shown in Table 2.4b). It is clear that N-acetyl-D-glucosamine [Glc Nac] (with concentration of 0.29mM) is the most effective inhibitor sugar followed by D(+)-glucosamine (0.58mM) and D(+)-melibiose (0.58mM), then N-acetyl-D-galactosamine (1.17mM).

4. Effect of prior treatment of *S. calcitrans* HL and RH with enzymes on the titres of agglutination activity against rabbit RBC, *T. brucei, L. hertigi* and *C. fasciculata.*

(a) Various proteases

Prior chymotrypsin treatment of the haemagglutinin reduced the activity 64-fold in the case of HL and RH against rabbit RBC and 8-fold, 4-fold and 16-fold in the case of HL against *T. brucei, L. hertigi* and *C. fasciculata* respectively. The activity of RH was reduced to 16-fold for *T. brucei*, and 8-fold for *L. hertigi* and *C. fasciculata*. Trypsin reduced the activity against RBC 32 and 16-fold for HL and RH respectively ($P<0.05$). No significant reduction ($P>0.05$) was observed in the activities of both samples against any of the parasites. The activity was seen to be reduced by use of pronase 256-folds for both HL and RH against RBC ($P<0.05$) and the parasites ($P<0.05$). Pepsin reduced the activity of HL and RH against RBC up to

16-fold whereas the activities against the parasites were reduced 8-16 fold for both samples, (results are shown in Table 5).

(b) Lipase, nucleases and neuraminidase

In most of the activities there were no significant reductions ($P>0.05$). However, the use of neuraminidase treatment resulted in a reduction of up to 32 and 64-fold in the activity against RBC for HL and RH respectively. The same enzyme reduced the activities of HL against the parasites up to 32, 16 and 64-fold for *T. brucei, L. hertigi* and *C. fasciculata* respectively and the activities of RH to 128-fold against *T. brucei* and 64-fold against both *L. hertigi* and *C. fasciculata*. (See Table 2.5).

5. Chemical and organic solvent treatment

TCA and phenol treatment eliminated the activities of both samples against the four agglutinogens. No significant reduction was seen in the case of treatment of both samples with ethanol, ether, ether/ethanol and xylene. Chloroform treatment reduced the activity against the four agglutinogens in both samples to 512-fold and 256-fold for RBC and *T. brucei* respectively and 64-fold for both towards *L. hertigi* and *C. fasciculata*. Chloroform/ethanol treatment of the samples reduced the activity by 4096-fold ($P<0.01$) in the case of HL against RBC and 1024-fold ($P<0.01$) for RH activities; both sample activities against

parasites were reduced up to 128-256 fold. The same reduction was seen with acetone. (Results are shown in Table 2.6).

6. **Effect of sodium periodate:-**

$NaIO_4$ caused reduction of the activity of HL and RH up to 128 and 64-fold respectively against RBC and up to 128-fold for both samples against the parasites (Table 2.7).

7. **Influence of divalent cations:-**

Compared to PBS controls, EDTA reduced the activity titres up to 512-fold ($P<0.05$) for both samples against rabbit RBC and 128, 64 and 32-fold in the case of HL against *T. brucei*, *L. hertigi* and *C. fasciculata* whereas a reduction of 64-fold was seen in the case of RH against the 3 parasites. EGTA reduced the activity titres against RBC up to 16 and 64-fold for HL and RH respectively and against the parasites up to 8-fold for both samples (except HL *vs. T. brucei* which was reduced to 16-fold). Almost the same results were seen in the case of addition of the Mg^{2+} ions to EGTA or EDTA. However, EDTA + Ca^{2+} ions showed the same activity as untreated HL and RH samples. Sample + PBS + Mg^{2+} + Ca^{2+} ions increased the activity titres up to 2-fold for both HL and RH against all the four agglutinogens and almost the same results were seen when the same ions were added + EDTA (Table 2.8).

8. Exposure of HL and RH to DTT and urea:-

A titre reduction range of 2 to 8-fold was seen after treatment of both samples with urea. Treatment of the samples with DTT resulted in reduction of activities up to 32 and 64-fold against RBC for HL and RH respectively whereas HL against parasites reduced activity up to 4, 16 and 8-fold for *T. brucei, L. hertigi* and *C. fasciculata* respectively. However, a reduction in activity of 4-fold was seen in the case of RH against the 3 parasites.

9. Variation in pH.

Optimum agglutination of rabbit RBC with both samples (HL & RH) occurred over a pH range of 5-9; pH levels of <5 and >9 caused reduction in activity up to 1024-fold ($P<0.05$) and 256-fold ($P<0.05$) respectively. The optimum agglutination of *T. brucei, L. hertigi* and *C. fasciculata* with both samples occurred over a pH range of 5-8. At pH levels of <5 and >8 a reduction in activity of up to 512-fold ($P<0.05$) and 256-fold ($P<0.05$) respectively was observed. (See Figures 2.2 and 2.3).

10. Effect of temperature

In the case of all four agglutinogens, optimum agglutination titres were found at temperatures ranging between 5 and 40^{0}C in both samples (HL & RH).

Heating both samples at 50^0C for 45 minutes resulted in reduction of agglutination with HL up to 2048-fold against RBC and 256-fold against *T. brucei* and *L. hertigi*. Reduction of the activity of RH agglutinin up to 512-fold against RBC and 64-fold against *T. brucei* and *L. hertigi* occurred after the same treatment. The reduction of the activities against *C. fasciculata* were seen to be up to 32-fold for both samples. The maximum reduction of the activities of both samples against the four agglutinogens were seen when the samples were incubated at 100^0C for 45 minutes with reductions up to 8192-fold in the activity against RBC ($P<0.05$) and 1024-4096-fold against the parasites ($P<0.05$). (results are shown in Table 2.8).

DISCUSSION

Maudlin and Welburn (1987) found that midgut extracts from *G. m. morsitans* flies, bred for refractoriness to infection with trypanosomes, showed significantly greater erythrocyte agglutination activity and were more trypanocidal *in vitro* than flies bred for susceptibility to trypanosome infection. They also found that feeding D+glucosamine with the infective feed increased the midgut infection rates with *T. congolense* and *T. brucei rhodesiense*. From this study they concluded that susceptibility to infection in tsetse was mediated by midgut agglutinins. The findings reported here support this since the reservoir region was seen to have higher activity than the thoracic and hindgut samples.

Ingram and Molyneux (1988) concluded from their inhibition studies with tsetse haemagglutinins that they were heterogeneous with regard to reactivity towards a diverse array of sugar moieties. The same conclusion could be drawn from my work with the exception of glucose and its deoxy derivatives. D(+)-glucosamine and melibiose were shown to be the most effective inhibitors of HL and RH agglutinins of *S. calcitrans*. Galactose and mannose simple sugars and their derivatives or moieties with other combinations were also found to be specific inhibitors. Glucose, fructose and sucrose were found to be non-specific inhibitors in this work. These sugars are found naturally in plants and might have been ingested as part of the flies' original diet prior to their development into adult blood-sucking flies. *Stomoxys,* unlike tsetse, will

still feed on plant juices. The stable fly, *S. calcitrans,* is one amongst a few species which are facultatively haematophagous (as adults) in the family *Muscidae* (Lehane, 1991).

From my results it was seen that haemolymph (HL) and reservoir (RH) agglutinins agglutinated all the RBC types and the parasites used. Only rabbit RBC's were treated with enzymes. The results of the use of proteolytic enzymes and glycosidase suggested the presence of potential glycoprotein or glycopeptide membrane receptors on the rabbit-RBC cell wall. This is supported by evidence of elevation of the titres of the activity of both samples when RBC's were treated with neuraminidase and proteolytic enzymes which may have partially digested the membrane integrated proteins or peptides, resulting in exposure of potential sugar residues involved in the lectin binding. The removal of N-glycolneuraminyl and N-acetylneuraminyl linkages from the RBC membrane neuraminic acid by neuraminidase could equally unmask cryptic acid galactosyl and glucosyl residues present on the RBC surface. Conversely, glycosidase usage decreases the activity titres suggesting this enzyme may cleave lectin binding sites from the surface of the RBC.

These findings imply that *Stomoxys* RH and HL agglutinin specificities are mainly directed towards α-D- and/or β-D-galactose residues, but to a lesser degree towards α-D- and/or β-D-glucose moieties on the RBC surface. Almost all these findings are similar to those of Ingram and Molyneux (1988 and 1993) who found that tsetse HL agglutinins have the same specificities except in case of *G. m. morsitans* and *G. p. gambiensis* anti-O agglutinins. The results of previous carbohydrate inhibition

experiments correlated well with those for the use of glycoside hydrolase-treated rabbit RBC's. However, different cellular membrane structural glycoconjugate orientations (caused by varied oligosaccharide chains, glycoproteins or glycolipids of RBC's and the trypanosomatid flagellates used in this work) may be stereochemically presented in various configurations. This could also account for the multispecific binding nature of *Stomoxys* lectins of HL and RH samples. This finding was also reported by Ingram and Molyneux (1993) in the case of tsetse lectins.

The limited effects of the nucleases, together with negation or marked reduction in the titres of the activities after treatment of HL and RH samples with phenol, TCA, chloroform, acetone or neuraminidase, suggest that the agglutinins (lectins) are not nucleic acids or polysaccharides but are more likely to be glycoproteins, lipoproteins, lipids or glycolipids. However, treatment of *Stomoxys* HL and RH samples with lipase and organic solvents (xylene, ether, phenol), which cause lipid hydrolysis and inactivation/denaturation respectively, had relatively little effect on agglutinins (lectin) activities. This finding would tend to eliminate a potential glycolipid or lipoprotein involvement for the activities. Thus the *Stomoxys* HL and RH agglutinins (lectins) are most likely to be protein or glycoprotein in nature. This is supported by the evidence for the glycosidic moieties of the samples which can be seen from the results of the sugar inhibition studies coupled with exposure to $NaIO_4$ or neuraminidase (which hydrolyses 2-, 3-, 2,5- and 2,8-N-glycolneuraminyl and N-acetylneuraminyl linkages). The agglutinin levels in HL and RH were seen to be decreased in neuraminidase-treated samples in all cases especially anti-rabbit RBC's. Treating the HL and RH samples with proteolytic enzymes gave significant decreases in all cases

(RBC's and the parasites). Both non-specific pronase and relatively specific pepsin break most peptide linkages, whilst trypsin and chymotrypsin are more specific in their actions. The use of pronase reduced the activity titres of both samples and pepsin to a lesser extent in case of RBC's and parasites, implying that *Stomoxys* HL and RH anti-RBC agglutinins and anti-parasite agglutinins possess peptide bonds and are therefore proteinaceous in nature. Evidence for this was reported with HL haemagglutinins of tsetse flies against human (ABO) RBC by Ingram and Molyneux (1990 and 1993).

Work with chymotrypsin suggests that HL and RH haemagglutinin peptide bonds contain several aromatic amino acid residues. The same property was reported in tsetse HL haemagglutinins (especially *G. m. morsitans* and *G. p. gambiensis* anti-AB RBC) indicating that they contain basic amino acid groups (lysine and arginine) which are preferentially split by trypsin. It is not surprising for *Stomoxys* haemagglutinin to have trypsin sensitivity since this property was reported for lepidopteran (Suzuki and Natori, 1983), dipteran (Ingram and Molyneux, 1990; McKenzie and Preston, 1992; Ingram and Molyneux, 1993) and orthopteran (Hapner and Jermyn, 1981; Stebbins and Hapner, 1985) agglutinins. There is a very high level of trypsin in *Stomoxys* midgut, 95% of which is found in the posterior midgut (Lehane, 1991) and it is therefore not surprising that peak lectin activity is found in the anterior, reservoir region, of the midgut.

The thermolability of *Stomoxys* HL and RH agglutinins against RBC and the trypanosomatid flagellate parasites with rise in temperature and influence of pH

(outside the normal physiological range) on their activities are also indicative of proteins or glycoproteins. Heating both samples of HL and RH of *Stomoxys* affects the agglutination activity. Increasing the temperature reduces the titres until boiling the samples at 100^{0}C almost negates the activities. In contrast, the tsetse *G. tachinoides* and *G. p. gambiensis* (active against human RBC group O and B respectively) were reported to be affected only slightly after heating the samples at 80^{0}C (Ingram and Molyneux, 1993). However, most insect HL agglutinins (lectins) are heat-labile (Komano *et al.*, 1980; Ingram and Molyneux, 1993; Ingram *et al.*, 1984).

The pH for maximum activities of *Stomoxys* HL and RH agglutinins against RBC's and the trypanosomatid flagellate parasites normally occurred within the range 7.0 - 7.4. Ingram and Molyneux (1993) reported that the HL agglutinin activities of tsetse flies occurred within the range 6.2 - 8.2 and, although their HL was slightly acidic the *G. tachinoides, G. m. morsitans* and *G. p. gambiensis* haemagglutinins required pH 5-6, 5-7 and 7-9, respectively for optimum agglutination. The difference in activity pH range between the *Stomoxys* and *Glossina* spp. may be due to physiological and feeding habit differences. *Stomoxys* can be maintained on sugar syrup *i.e.* glucose solution (5%) or wet dirty animal excreta as well as on blood sucked from animals. In comparison, the tsetse fly sucks blood only from man and animals through intact skin. Richards *et al.*, (1988) reported that optimum anti-rabbit RBC HL haemagglutinin activity occurred under alkaline pH values in stick insects *Extatosoma tiaratum*.

Treating the HL and RH samples with DTT lowered the agglutination activity titres in the case of the RBC and the parasites whilst urea reduced the activity noticeably against the above agglutinogens especially with the parasites. Almost the same results were reported by Ingram and Molyneux (1993). They concluded that tsetse HL haemagglutinins against human (ABO) RBC (especially group B and AB in *G. m. morsitans*) might contain higher amounts of sulphur-containing amino acids forming S-S bonds compared to a restricted number of H-H bonds that may be of greater significance in HL haemagglutinins structural configuration. This conclusion might be true for HL and MG agglutinin samples against RBC's and parasites.

The maximum activities of HL and MG agglutinins of *Stomoxys* against the RBC's and the parasites required Ca^{2+} ions more than Mg^{2+} ions. *G. p. gambiensis* was reported to require Mg^{2+} ions and both *G. tachinoides* and *G. m. morsitans* require Ca^{2+} ions for optimum HL haemagglutinin activity. However haemolymph Mg^{2+} ion levels in adult dipterans tend to be higher than those of Ca^{2+} ions (Sutcliffe, 1963). It was reported that in some insects Ca^{2+} ions and/or other divalent metal ions are essential requirements for normal HL haemagglutinin function (Stebbins and Hapner, 1985; Kubo and Natori, 1987) whereas in others they are not essential (Ingram *et al.*, 1984; Umetsu *et al.*, 1984). Since the HL and RH agglutinins of *Stomoxys* required Ca^{2+} ions for optimum activity like those of *G. tachinoides* and *G. m. morsitans* HL samples (Ingram and Molyneux, 1993), they may belong to the C-type class of animal lectins, so-termed because of their requirements of Ca^{2+} ions for functional activity (Drickamer, 1988). It is clear that cations (especially Ca^{2+} ions) are essential for activity and maintenance of the structural integrity of the *Stomoxys* HL and RH

agglutinin molecules. In view of the above findings, it is highly plausible that these molecules are proteins or glycoproteins, in keeping with findings in other insect studies (Hapner and Jermyn, 1981; Pendland and Boucias, 1986; Richards *et al.*, 1988). However we must take care in assuming this since the results were determined for non-purified samples of the *Stomoxys* HL and RH extracts. These may contain mixtures of enzymes, proteins and other organic materials capable of influencing the findings. In order to draw conclusions with confidence regarding physico-chemical properties of these agglutinins (lectins), studies using purified samples were carried out. These are described in the next chapter.

The insect lectins were found to react with free sugars and with sugars on the trypanosome surface (Jackson *et al.*, 1983; Mutharia and Pearson, 1987; Ingram and Molyneux, 1988 and 1991). Thus *Stomoxys* lectins (in this study) reacted with trypanosomatid flagellates; *T. brucei* (bloodstream and culture forms), *L. hertigi* (culture form) and *C.fasciculata* (culture form). This is noteworthy as tsetse lectins were reported to be involved in the regulation of parasite development within the midgut of the tsetse fly (Welburn and Maudlin, 1990).

A more interesting finding was reported by Abubakar *et al.* (1995) who partially purified tsetse MG lectin previously found to have trypsin activity. They reported that, when the midgut homogenate of *G. m. morsitans* was separated by anion-exchange chromatography, the agglutination activity co-eluted with trypsin activity at approximately 50% NaCl. They suggested that there was a very close relationship between the midgut trypsin-like enzyme and the agglutinins (lectins).

They also suggested that the lectin-binding sites might be exposed by the enzyme cleaving off some of the parasite surface molecules since successful agglutination of bloodstream form trypanosomes requires protease activity.

From our findings, it was seen that the activity increased after treating the rabbit RBC with trypsin and papain for both samples (RH and HL). Purification of the MG and HL samples from *S. calcitrans* was performed followed by cross-reaction and enzyme treatment of rabbit RBC, human (ABO) RBC and *T.brucei* (bloodstream forms and culture forms). In order to determine the most effective specific inhibitor sugars the inhibition test was done along with other physico-chemical tests (see Chapter 3). In the final discussion, more information, predictions and conclusions will be given.

TABLE 2.2

Mean Agglutination titres of *S. calcitrans* HL and RH against Rabbit RBC, *T. brucei* (Tb), *L. hertigi*(L.h) and *C. fasciculata*(C.f) after adsorbing the samples by each of them (cross adsorption process).

(n = 4, SE 0.0)

SAMPLES	HL				RH			
	RBC	T.b	L.h	C.f	RBC	T.b	L.h	C.f
Non-adsorbed sample	2^{-17}	2^{-14}	2^{-14}	2^{-15}	2^{-16}	2^{-14}	2^{-14}	2^{-15}
Adsorbed by RBC	0	2^{-6}	2^{-8}	2^{-9}	0	2^{-7}	2^{-8}	2^{-9}
Adsorbed by T.b	2^{-9}	0	2^{-5}	2^{-6}	2^{-8}	0	2^{-4}	2^{-6}
Adsorbed by L.h	2^{-8}	2^{-5}	0	2^{-5}	2^{-8}	2^{-4}	0	2^{-4}
Adsorbed by C.f	2^{-9}	2^{-4}	2^{-3}	0	2^{-9}	2^{-5}	2^{-5}	0

TABLE 2.3

Enzyme treatment of Rabbit erythrocytes

(a) **Effect of glycoside hydrolase treatments of rabbit erythrocytes on mean *S. calcitrans* HL and RH haemagglutination titres ($-\log_2$) (n = 4, SE 0.0)**

Treatment	HL	RH
Untreated RBC	17	16
α-galactosidase	14	13
β-galactosidase	15	14
α-Glucosidase	16	16
β-Glucosidase	15	14

(b) **Effect of neuraminidase or proteolytic enzyme treatment of rabbit erythrocytes on mean *S. calcitrans* HL and RH haemagglutination titres ($-\log_2$). (n = 4, SE 0.0)**

Treatment	HL	RH
Untreated Sample	16	15
Neuraminidase	19	18
Trypsin	18	18
Papain	19	18

TABLE 2.4a

Degree* of Inhibition (in descending order) of *S. calcitrans* HL and RH Agglutination of rabbit RBC, and *T. brucei* procyclic (T.b) and bloodstream forms (T.b^2) with carbohydrates and glycoproteins.

Carbohydrate Inhibitor	HL			RH		
	RBC	T.b^1	T.b^2	RBC	T.b^1	T.b^2
D(+)-Glucosamine	6+	6+	6+	6+	6+	6+
D(+)-Galactosamine	5+	5+	5+	5+	5+	5+
D(-)-Mannosamine	5+	5+	5+	5+	5+	5+
N-Acetyl-D-Glucosamine	5+	5+	5+	5+	5+	5+
N-Acetyl-D-Galactosamine	5+	5+	5+	5+	5+	5+
D(+)-Galactose	5+	5+	5+	5+	5+	5+
D(+)-Mannose	5+	5+	5+	5+	5+	5+
D(+)-Melibiose	5+	5+	5+	5+	5+	5+
6.7-Dihydrosycoumarine-6-Glucoside	3+	2+	2+	3+/2+	2+	2+
D-Galacturonic acid	3+/1+	2+	2+	3+	1+/2+	2+
2-Deoxy-D-Galactose	2+	2+	2+	2+	2+	2+
N-Acetyl-Neuraminic Acid	2+	2+	2+	2+	2+	2+
D-Manheptalose	2+	1+	1+	2+	1+	1+
2-Hydroxymethyl-phenyl-β-D-glucose	2+	1+	1+	2+	1+	1+
L(-)-Arabinose	1+/2+	1+	1+	2+	1+	1+
L(-)-Sorbose	1+/2+	1+	1+	1+/2+	1+	1+
D-Glucuronic acid	1+/2+	1+	1+	1+/2+	1+	1+
D-Glucurnamide	1+/2+	1+	1+	1+	1+	1+
β-Methyl-Glucoside	+1	1+	1+	2+	1+	1+
Mannan	2+	1+	1+	1+	1+	1+
Bovine γ-globulins	1+/2+	1+	1+	1+	1+	1+
D(+)-Trehalose	1+	1+	1+	2+	2+	2+
Rhamnose	2+	1+	1+	1+	1+	1+
D(-)-Ribose	1+	1+	1+	1+	1+	1+
6-Deoxy-L-Mannose	1+	1+	1+	1+	1+	1+
6-Deoxy-L-Galactose	1+	1+	1+	1+	1+	1+
α-Lactose	1+	1+	1+	1+	1+	1+
D(+)-Turanose	1+	1+	1+	1+	1+	1+

Table 2.4a continued	HL			RH		
Carbohydrate Inhibitor	**RBC**	**T.b[1]**	**T.b[2]**	**RBC**	**T.b[1]**	**T.b[2]**
β-Gentiobiose	1+	1+	1+	1+	1+	1+
Fetuin	1+	1+	1+	1+	1+	1+
α-Amylase	1+	1+	1+	1+	1+	1+
Mucin	1+	1+	1+	1+	1+	1+
Albumin	1+	1+	1+	1+	1+	1+
β-Lactoglobulin	1+	1+	1+	1+	1+	1+
Invertase	1+	1+	1+	1+	1+	1+
Glycogen	1+	No	No	No	No	No
D(+)-Glucose	No	No	No	No	No	No
D(+)-Xylose	No	No	No	No	No	No
β-D-Fructose	No	No	No	No	No	No
2-Deoxy-D-ribose	No	No	No	No	No	No
2-Deoxy-D-glucose	No	No	No	No	No	No
6-Deoxy-D-glucose	No	No	No	No	No	No
L(-)-Fucose	No	No	No	No	No	No
D(+)-Fucose	No	No	No	No	No	No
Maltose	No	No	No	No	No	No
Sucrose	No	No	No	No	No	No
D(+) Melezitose	No	No	No	No	No	No
Raffinose	No	No	No	No	No	No
Stachyose	No	No	No	No	No	No
Chitin	No	No	No	No	No	No
Xylan	No	No	No	No	No	No
Casein	No	No	No	No	No	No
β-Lactoglobulin	No	No	No	No	No	No
Human Chorionic Gonadotrophin	No	No	No	No	No	No
Glucose Oxidase	No	No	No	No	No	No

* Degrees of inhibition (D1) were scored according to the scale of Ingram and Molyneux (1988); thus a reduction in titre by 2 wells was scored as 1+, 3 wells as 2+, 4 wells as 3+, 5 wells as 4+ and 6 or more wells as 5+. Total inhibitions (100%) were scored as 6+. Inhibition of one well was denoted by ± and no inhibition was denoted by -.

TABLE 2.4b

The minimum concentration of some inhibitor sugars required to give 100% inhibition of agglutination activity against rabbit RBC, and bloodstream and culture forms of *T. brucei.*

Sugar	Minimum concentration
N-Acetyl-D-glucosamine (GlcNAC)	0.29mM
D(+)-Melibiose [α-D-Gal(1-6)D-Glu]	0.58mM
D(+)-Glucosamine (GlcN)	0.585mM
N-Acetyl-D-galactosamine (GalNAC)	1.17mM
D(+)-Galactose	4.7mM
D(+)-Mannose	9.4mM
D(+)-Galactosamine (GalN)	18.75mM
2-Deoxy-D-galactose (2-dGal)	37.5mM
D-Mannosamine (ManN)	37.5mM
N-Acetyl-β-D-mannosamine (MoNAC)	75mM
N-Acetylneuraminic acid (NeuAc)	75mM

TABLE 2.5

Effect of prior enzyme treatment of *S. calcitrans* HL and RH haemagglutinin on the titres of the agglutination activity against rabbit RBC, *T. brucei* (T.b), *L. hertigi* (L.h) and *C. fasciculata* (C.f) (n = 4, SE 0.0)

ENZYMES	HL				RH			
	RBC	T.b	L.h	C.f	RBC	T.b	L.h	C.f
Untreated	2^{-17}	2^{-13}	2^{-12}	2^{-14}	2^{-16}	2^{-14}	2^{-13}	2^{-13}
Chymotrypsin	2^{-11}	2^{-10}	2^{-10}	2^{-10}	2^{-10}	2^{-10}	2^{-10}	2^{-10}
Trypsin	2^{-12}	2^{-12}	2^{-12}	2^{-13}	2^{-12}	2^{-14}	2^{-13}	2^{-13}
Pronase	2^{-9}	2^{-7}	2^{-7}	2^{-7}	2^{-9}	2^{-7}	2^{-10}	2^{-9}
Pepsin	2^{-13}	2^{-10}	2^{-9}	2^{-10}	2^{-12}	2^{-10}	2^{-10}	2^{-10}
Lipase	2^{-16}	2^{-12}	2^{-12}	2^{-13}	2^{-16}	2^{-14}	2^{-13}	2^{-13}
Ribonulease	2^{-16}	2^{-11}	2^{-11}	2^{-13}	2^{-16}	2^{-14}	2^{-13}	2^{-13}
Deoxyribonuclease	2^{-17}	2^{-13}	2^{-12}	2^{-13}	2^{-16}	2^{-14}	2^{-14}	2^{-13}
Neuraminidase	2^{-12}	2^{-8}	2^{-8}	2^{-8}	2^{-11}	2^{-7}	2^{-7}	2^{-7}

TABLE 2.6

Effect of treatment of *S. calcitrans* HL and RH agglutinins with chemical and organic solvents when assayed against rabbit RBC, *T. brucei* (T.b), *L. hertigi* (L.h) and *C. fasciculata* (C.f) on mean agglutination titres ($-\log_2 2^{-n}$). (n = 4, SE 0.0)

	HL				RH			
TREATMENT	**RBC**	**T.b**	**L.h**	**C.f**	**RBC**	**T.b**	**L.h**	**C.f**
Untreated Sample	17	13	12	12	15	12	12	13
Ethanol	16	12	12	12	14	12	12	12
Ether	17	13	12	12	15	12	12	13
Chloroform	8	5	6	6	7	5	6	6
Chloroform/Ethanol	5	5	5	5	5	5	5	5
Xylene	17	13	12	12	15	12	12	12
Acetone	5	4	4	5	5	4	4	5
T.C.A	0	0	0	0	0	0	0	0
Phenol	0	0	0	0	0	0	0	0

TABLE 2.7

Influence of Divalent Cations (effect of EDTA & EGTA), Sodium Periodate ($NaIO_4$), Urea and DTT, on mean titres ($-\log_2 2^{-n}$) in *S. calcitrans* HL and RH anti-rabbit RBC, *T. brucei* (T.b), *L. hertigi* (L.h) and *C. fasciculata* (C.f) agglutination activity.
(n = 5, SE 0.0).

	HL				RH			
TREATMENT	**RBC**	**T.b**	**L.h**	**C.f**	**RBC**	**T.b**	**L.h**	**C.f**
Influence of divalent cations								
PBS	16	13	13	12	15	12	12	12
PBS/Mg^{2+}/Ca^{2+}	18	16	16	15	18	15	15	15
EDTA	7	6	7	7	6	6	6	6
EGTA	12	10	9	9	10	9	9	9
EDTA+PBS/Mg^{2+}	13	10	11	9	12	10	10	11
EDTA+PBS/Ca^{2+}	16	13	13	12	15	12	12	12
EGTA+PBS/Mg^{2+}	11	11	10	10	10	10	10	10
EDTA+PBS/MG^{2+}/Ca^{2+}	18	15	15	14	18	14	14	15
Effect of Oxidation with $NaIO_4$								
Untreated	17	13	13	14	16	12	12	13
Sample +$NaIO_4$	10	7	7	7	10	7	7	7
Effect of Urea & DTT								
Untreated	16	12	11	11	15	12	12	12
Urea	13	10	9	10	14	11	11	11
DDT	12	11	10	11	9	10	10	10

TABLE 2.8

Effect of Temperature, Freezing and Thawing on the Titres ($-\log_2 2^{-n}$) of agglutination activity of *S. calcitrans* HL and RH against Rabbit RBC, *T. brucei* (T.b), *L. hertigi* (L.h) and *C. fasciculata.* (n = 5, SE 0.0).

TEMPERATURE (°C) (in water bath for 45 min.)	HL				RH			
	RBC	T.b	L.h	C.f	RBC	T.b	L.h	C.f
Untreated	16	12	12	11	15	11	11	10
5°C	16	12	12	11	15	11	11	10
15°C	16	12	12	11	15	11	11	10
25°C	16	12	12	11	15	11	11	10
26°C	15	12	12	11	14	11	11	10
35°C	14	10	10	10	12	8	8	8
40°C	12	8	8	8	10	7	7	7
50°C	5	4	4	6	5	4	4	4
60°C	4	3	3	3	4	4	4	4
80°C	2	2	2	2	2	2	2	2
100°C	1	Neat	Neat	Neat	1	Neat	Neat	Neat
Freezing and Thawing								
Untreated	15	11	12	11	14	12	13	13
Frozen and Thawed Once	15	10	11	11	14	11	12	12
Frozen and Thawed Twice	14	10	10	10	13	11	11	11
Frozen and Thawed 3 times	14	9	9	9	13	10	10	10
Frozen and Thawed 5 times	10	8	8	8	10	8	8	8
Frozen and Thawed 8 times	8	6	6	6	8	6	6	6

Fig. 2.2. The effect of variations in PBS pH on *S. Calcitrans* HL titres ($-\log_2 2^{-n}$) against rabbit RBC, *T. Brucei* (T.b), *L. Hertigi* (L.h) and *C. Fasciculata*(C.f).

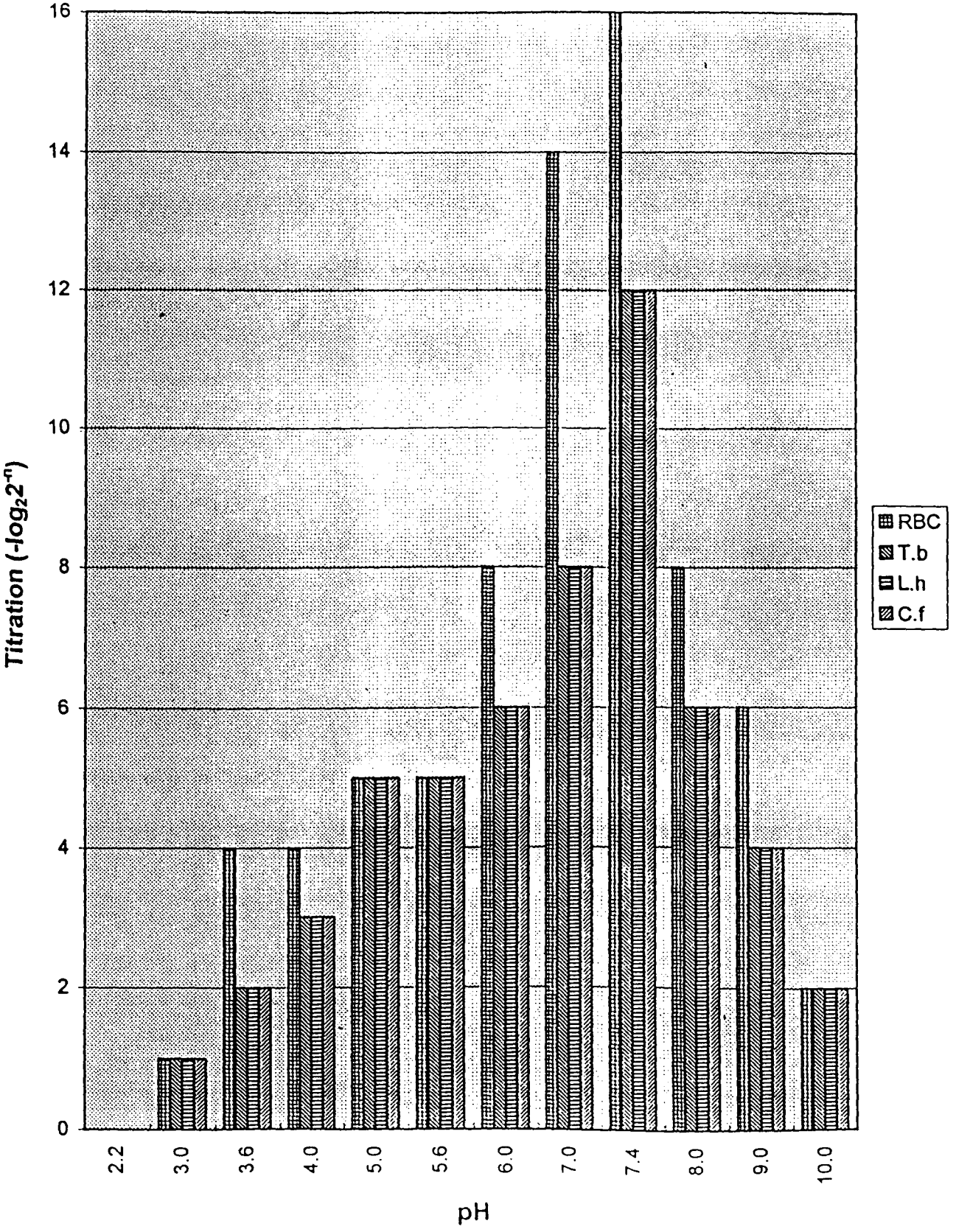
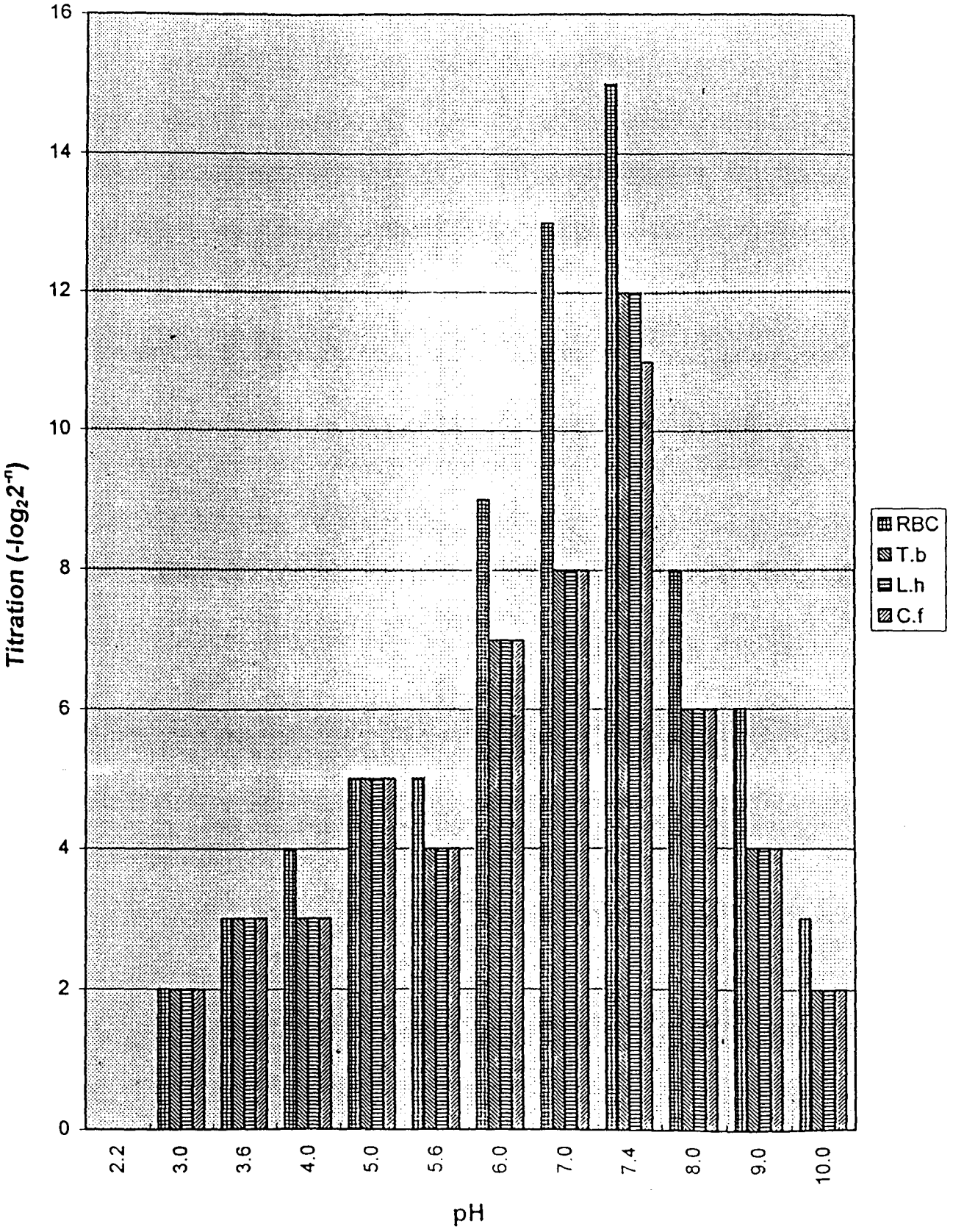


Fig. 2.3. The effect of variations in PBS pH on *S. Calcitrans* RH titres ($-\log_2 2^{-n}$) against rabbit RBC, *T. Brucei* (T.b), *L. Hertigi* (L.h) and *C. Fasciculata*(C.f).



Chapter 3

In Vitro Studies of the Characteristic Properties of the *Stomoxys calcitrans* Purified HL and RH Agglutinins

ABSTRACT

In the previous two chapters it was found that *S. calcitrans* haemolymph (HL) and reservoir (RH) extracts agglutinated animal and human (ABO) RBCs and trypanosomatid haemoflagellate parasites (procyclic *T. brucei*, *L. hertigi* and *C. fasciculata* and bloodstream form *T. brucei*). From results of physico-chemical and enzyme treatment of the samples, enzyme treatment of the RBCs and results of inhibition tests, these haemagglutinins (lectins) were found to be proteins or glycoproteins and heterogeneous in their specificity.

Purification of the samples was performed in order to study the physico-chemical properties of the agglutinins (lectins) and to determine their molecular weights.. The mean protein contents of HL and RH samples of flies aged < 12 hours to 3 days were determined to be 26.33 mg/ml (SE 0.9) and 6.4 mg/ml (SE 0.09) respectively. Mean protein contents increased with age reaching 32 mg/ml (SE 0.9) for HL and 7.2 mg/ml (SE 0.26) for RH at day 14 p.e. The contents then started to decrease reaching 22 mg/ml (SE 0.9) for HL and 5.6 mg/ml (SE 0.09) for RH at day 28 p.e. Purified lectins constitute 4.3% of the total protein contents in RH samples and 9.47% of the total protein contents in HL samples. In order to compare *Glossina* spp. and *Stomoxys* agglutination activity the protein contents of *G. m. morsitans* HL (30-50 mg/ml) and RH (16-20 mg/ml) and *G. palpalis* HL (28-45 mg/ml) and RH (15-18 mg/ml) were determined.

Molecular weights of the purified samples were determined using SDS-PAGE and the results showed 3 bands in each sample. These bands have relative molecular weights of 26,302, 16,218 and 14,028 daltons for HL and 28,300, 16,218 and 14,600 daltons for RH. In general, results of agglutination of RBCs and parasites were the same in both purified and crude HL and RH samples.

INTRODUCTION

Insects display a diverse array of defence mechanisms to counteract the wide spectrum of potential pathogens present in the environment. These defence mechanisms include cellular and humoral immunity (Lackie, 1988; Lehane, 1991; Ingram and Molyneux, 1991). Dipteran haemolymph and midgut extracts possess anti-parasitic agglutinins and haemagglutinins. In some cases these are inducible molecules (carbohydrate-binding proteins or lectins) which appear to participate in immune reactions, having an opsonic role in phagocytosis (Gupta, 1986). The lectins (agglutinins) of several dipteran vectors have been isolated and their physico-chemical properties elucidated (Ingram and Molyneux, 1991).

Lectins have been implicated in insect vector-trypanosomatid parasite interactions *e.g.* in tsetse flies *(Glossina* spp.*),* the natural biological vectors of African trypanosomes (Ingram and Molyneux, 1991). It is thought that both haemolymph and midgut lectins may regulate *Trypanosoma* spp. infections in this species, potentially influencing vectoral capacity and transmission of trypanosomiasis (Maudlin, 1991). The stable fly, *Stomoxys calcitrans*, is a suggested mechanical vector of certain *Trypanosoma* spp. (*e.g T.b. gambiensis* and *T. evansi*) but despite being sympatric with tsetse is not a biological vector of trypanosomes. Lectins or lectin-like molecules may influence this refractory status (Ingram and Molyneux, 1991; Lehane, 1991).

In this chapter purification of haemolymph (HL) and reservoir homogenate (RH) was performed in order to more fully investigate their agglutination activities against RBC

(human [ABO], rabbit, horse and sheep) and against trypanosomatid haemoflagellate parasites (bloodstream forms of *T. brucei* and culture forms of *T. brucei*, *L. hertigi* and *C. fasciculata*) as described in chapter 1. The characteristic properties of purified HL and RH lectins and their relative molecular weights were determined.

Purification of the crude samples was performed by dialysis after adsorption with specific sugars. HL was adsorbed by immobilized synthetic oligosaccharides coupled with Synsorb (an inert silica matrix) via a spacer. RH was adsorbed by N-acetyl-D-glucosamine immobilized in 6% agarose. Dialysis is one of the oldest procedures used in the purification and characterisation of biomolecules. It separates dissolved molecules according to molecular size (Boyer, 1993).

The molecular weights of the protein subunits of the purified samples were estimated using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). This process is particularly useful for estimation of molecular weights (in the range 10,000 to 200,000 daltons) of different types of oligomeric protein subunits (Boyer, 1993).

The secondary, tertiary and quaternary structures of the protein molecules are disrupted in SDS treatment, resulting in production of linear polypeptide chains. Mercaptoethanol assists the protein denaturation by reducing all disulphide bonds. The detergent SDS then binds to hydrophobic regions of the denatured protein chain in a constant ratio of about 1.4g of SDS to 1.0g of protein. These bound detergent molecules carry a negative charge and therefore mask the protein's native charge. As

a result, polypeptide chains with a constant charge/mass ratio and uniform shape are produced (Boyer, 1993).

The electrophoretic mobility of the SDS-protein complex is influenced primarily by molecular size, larger molecules being retarded by the molecular sieving effect of the gel and smaller molecules having greater mobility. Empirical measurements have identified a linear relationship between the log of molecular weight and electrophoretic mobility (Boyer, 1993).

The Bio-Rad technique for determination of protein contents of crude and pure HL and RH samples is given in the Materials and Methods section. Determination of protein contents was carried out in order to investigate the relationship between protein content and agglutination activity. Lectin quantities in the samples were also determined in order to make comparisons of agglutination activity and trypanosome resistance between *Stomoxys* and *Glossina* spp.

MATERIALS AND METHODS

1(a). Maintenance of flies:

Flies were obtained from colonies maintained in the insect house at U.W., Bangor at 25-28^{0}C in a 12h light/dark cycle. Details of maintenance are given in chapter 1.

(b). Collection of haemolymph (HL) and reservoir homogenate (RH) samples:

Samples were collected as described in chapter 1.

2. Determination of protein contents in haemolymph (HL) and reservoir (RH) homogenates:

Samples used in this experiment were collected and prepared as described in chapter 1. Determination of the protein contents of samples was performed after dilution using the Bio-Rad Protein Assay method for detecting protein concentrations in the range 100 - 1500μg/ml (Bio-Rad Life Sciences Research Products Catalogue, 1995). This assay is based on the colour change (absorbance shift from 465 to 595 nm) of Coomassie brilliant blue G-250 dye in response to various protein concentrations. This colour change occurs as a result of the dye binding to the proteins in an acidic solution (Pierce, 1989). The dye binds to primarily basic (especially arginine) and aromatic amino acid

residues (Boyer, 1993). The assays were performed according to the protocol in the catalogue instructions.

3(a). Preparation of the standards:

The standard curve is prepared by plotting the average net absorbance at 595 nm for each known concentration of BSA (bovine serum albumin) which was used as a standard protein.

(i). Bio-Rad dye reagent was diluted to 1 in 5 (with distilled water).

(ii). Seven clean, dry 10 ml test-tubes were prepared and 5 ml of the dye was measured into each.

(iii). The tubes were then labelled 1 to 7 (standard 1 to standard 7).

(iv). BSA was added to the tubes according to the following:

Tube (standard) no.	**Amount of BSA added (µl)**
1	5
2	10
3	20
4	40
5	60
6	80
7	100

(b). **Preparation of Microtitre plates:**

A 96 well microtitre plate was prepared and then 100µl of standards and unknown test samples (HL and RH samples) were loaded (in duplicate) as shown in the plate plan below (7 standards and 16 test samples).

	1	2	3	4	5	6	7	8	9	10	11	12
A		B_1	B_2	T_1	T_1	T_9	T_9					
B		S_1	S_1	T_2	T_2	T_{10}	T_{10}					
C		S_2	S_2	T_3	T_3	T_{11}	T_{11}					
D		S_3	S_3	T_4	T_4	T_{12}	T_{12}					
E		S_4	S_4	T_5	T_5	T_{13}	T_{13}					
F		S_5	S_5	T_6	T_6	T_{14}	T_{14}					
G		S_6	S_6	T_7	T_7	T_{15}	T_{15}					
H		S_7	S_7	T_8	T_8	T_{16}	T_{16}					

B = Blank (dye only)
T = Test sample
S = Standard

Reading of the plate was carried out using the microtitre plate reader machine (DYNATECH MR5000).

(c). Analysis of results:

Results for test samples and standards were clearly shown on a paper output in the form of a plan (as above). From the results, the standard curve was plotted and from this the protein concentrations were calculated. The same method was used to determine the protein contents of HL and RH of *G. m. morsitans* and *G. palpalis*.

4. Purification of haemolymph (HL) and reservoir homogenate (RH) samples:

The purification was performed in the Tsetse Research Laboratory, University of Bristol, following the instructions of Drs. Maudlin and Welburn and according to the dialysis method mentioned by Boyer (1993).

(a). Purification of haemolymph (HL):

3 ml HL were obtained from approximately 1500-2000 flies for the purification process as described in Chapter 1. For more information see Appendix 3. These haemolymph samples were stored in the freezer. Prior to the purification process they were thawed at room temperature (25-26^{0}C) for 20-30 mins. and then centrifuged at 1000 rpm. 1 mg of Synsorb, an inert silica matrix (Chembiomed, Edmonton, Alberta, Canada), was added to 30µl samples of haemolymph (Piller *et al.*, 1990). These samples were then transferred into

a 5ml bottle, which was sealed and covered with a parafilm to avoid evaporation of the mixture. The bottle was put on the Spiramix for 4h at room temperature (25 - 26^{0}C).

1.71g of α-D(+)-melibiose was added to 5 ml of 10 mM calcium saline solution in order to obtain a molar solution. This solution was used to extract the Synsorb from the haemolymph by 'washing' the Synsorb/haemolymph mixture five times with it. The molar melibiose solution was added volume to volume and mixed with the Synsorb/haemolymph for 3 h at room temperature (25-26^{0}C) on each of the five 'washes'. This permitted the lectin to bind with melibiose. Protein contents were determined before and after melibiose addition.

The lectin was then dialysed. Dialysis tubing was moistened by placing it in freshly-made 10 mM calcium saline until supple. The Synsorb washings (*i.e.* a mixture of expected lectins and melibiose) were deposited in the tubing (each Synsorb washing of the 5 washes was dialysed separately). The dialysis tubing was then put in a bottle containing 500 ml fresh 10 mM calcium saline (see appendix 3 for preparation of this saline) and mixed with a magnetic stirrer in the fridge (4^{0}C) for 3-4h five times (the saline was changed at the 3-4-hourly intervals). The tubing was then carefully cut and the contents pipetted (using a 1.0 ml graduated pipette) into a labelled 1.0 ml aliquot. The protein contents of each aliquot were measured and it was then placed in the freezer (-20^{0}C).

(b). Purification of reservoir homogenate (RH):

Reservoirs were dissected as described in Chapter 1. The dissected reservoirs (approximately 8.0 x 10^3 reservoirs from flies fed two days previously, see Appendix 3) were obtained from the freezer and thawed at room temperature (25-26^{0}C). They were put into 1 ml of PBS, pH 7.4, containing 2µl 5mg/ml aprotinin, 2µl 5mg/ml leupeptin and 6µl 50 mM TLCK [1-chloro-3-tosylamido-7-amino-2-heptanone hydrochloride (from Aldrich)] and left for at least 4h in the fridge. The guts were then filtered into a 50 ml centrifuge tube through a 0.22µm filter at 1000 rpm for 2 mins. The liquid was then placed in a bijoux bottle.

An equal volume of agarose was added to the midgut homogenate. After mixing, it was left on the Spiramix overnight in the fridge (4^{0}C). After removal from the fridge it was allowed to settle. The supernatent was removed followed by addition of 2ml of cold saline. After mixing it was allowed to settle again. This process was repeated five times in all. An equal volume of 1M glucosamine was added with mixing and then it was put on the Spiramix for 3-4 hours.

Dialysis was performed as described above. After completion of this process, the dialysed samples were put in labelled 5ml tubes.

5. Investigation of the agglutination activities of the purified HL and RH lectins against RBC and parasites.

Haemagglutination assays were performed (as described in Chapter 1). In the case of purified HL and RH homogenates the doubling of dilutions was performed by adding only 2 µl of the purified samples (lectins) to the first well of the used row (neat). 1µ of this was then taken and added to 1 µl in the 2nd well, mixed thoroughly, then 1 µl of this mixture was added to the 3rd well and so on. The mean protein contents of pure HL and RH lectins were 3.6 mg/ml (SE 0.26) and 1.3 mg/ml (SE 0.10) respectively.

RBC's from man (A, B, AB, O), rabbit and sheep were used. Preparation of the RBC suspensions and haemagglutination assays was carried out as described in Chapter 1. Controls of unpurified (crude) HL and RH samples were used. For a clearer comparison of crude and pure sample agglutination activity, the crude samples were diluted to concentrations similar to those of the pure samples (see table 3.2a and b).

6. Investigation of the factors influencing the specificity of the agglutination activity of the purified RH and HL samples.

In the experiments samples of unpurified (crude) RH and HL were used as controls.

(a). Cross Adsorption Process:

In this experiment only rabbit RBC and *T. brucei*, *L. hertigi* and *C. fasciculata* parasites were used with purified samples. Unpurified samples were used only as controls of haemagglutination. Assays were performed as described in Chapter 2.

(b). Enzyme treatment of RBC:

Only rabbit RBC were treated with enzymes. This experiment was performed as discussed in chapter 2, using the same enzymes.

7. Sugar specificities of the purified RH and HL anti-parasite and anti-erythrocyte lectins:

Method (see chapter 2). Only carbohydrates with a degree of inhibition of 1+ or more for the activity of the samples (against rabbit RBC and *T. brucei* bloodstream and culture forms) were used.

8. Determination of the nature of the purified RH and HL samples.

(a). Enzyme treatment of the purified RH and HL samples:

This experiment was performed as discussed in chapter 2, using the same enzymes.

(b). Organic solvent and chemical treatment of the purified RH and HL samples:

The samples were treated according to the method described in chapter 2 and with the same solvents and chemicals.

(c). Sodium periodate ($NaIO_4$) oxidation:

The samples were treated according to the method described in chapter 2.

9. Physico-chemical treatment:

The following experiments were performed in order to investigate the influence of selected physico-chemical treatments of RH and HL (purified samples) on agglutination titres.

(a). Presence of metal ions:

This experiment was carried out in order to determine the importance of the presence of metal ions *e.g.* Ca^{2+} and/or Mg^{2+}. Treatment of the purified RH and HL samples with EDTA and EGTA were performed as described in Chapter 2.

(b). Dithiothreitol (DTT) reduction and exposure to urea:

The RH and HL samples were treated with DTT and urea as described in Chapter 2.

(c). Effect of variation in buffer pH.

PBS was adjusted to different pH values in order to determine the effect of buffer pH on haemagglutination activity of RH and HL samples (see chapter 2).

(d). Effect of temperature:

The samples were heated in water baths at 25, 35, 50, 65, 80 and 100^{0}C or cooled to 5^{0}C for 45 mins. They were then examined for haemagglutination activity.

10. Determination of the relative molecular weight of the purified MG and HL samples by SDS-PAGE using the Phast System and Phast Gel gradient (10-15).

(a). Dissociation of the sample proteins into their polypeptide subunits.

Proteins were first dissociated into their polypeptide subunits by using SDS and 2-mercaptoethanol. These subunits bind the same amount of SDS per unit

weight and take on a net negative charge. Therefore, all the polypeptides will have the same charge density and the separation will then be based on size alone.

(b). Separation media:

Phast Gel gradient medium for SDS-PAGE consists of 13 mm stacking gel zone with an acrylamide concentration of 4.5% and 3.0% cross-linkage, and a 32 mm gradient gel zone. The gels were used with Phast Gel SDS buffer strips (Pharmacia).

Prepared gels were purchased from Pharmacia as Phast Gel gradient ® 10 - 15 and had a continuous 10 - 15% gradient gel zone with 2% cross-linkage. The gel dimensions were 43 x 50 x 0.45 mm. The buffer system in the gels was 0.112 M acetate (leading ion), 0.20 M tris-HCl and 0.55% SDS, pH 7.4.

(c). Separation technique:

(i) Sample preparation:

Purified and unpurified (crude) samples of RH and HL were prepared in a sample buffer (10mM tris-HCl, 1mM EDTA, pH 8.0) which contained 2.5% SDS and 5.0% 2-mercaptoethanol. The samples were heated at 100^{0}C for 5 mins. before application.

A vial of low molecular weight (LMW) proteins was obtained from Pharmacia. It was dissolved in 200µl of sample buffer and treated in the same way as purified and unpurified samples. This was used to calibrate the results.

The following table shows the low molecular weight (LMW) protein standards which were used for protein molecular weight determination using SDS - PAGE.

Standard Protein	**Subunit Molecular weight (Daltons)**	**Source**
Phosphorylase b	94000	rabbit muscle
Albumin	67000	bovine serum
Ovalbumin	43000	egg white
Carbonic anhydrase	30000	bovine RBC
Trypsin inhibitor	20100	soybean
α–lactalbumin	14400	bovine milk

(ii) Sample application:

A sample well stamp (Pharmacia) was placed on a table, wells facing upwards. Parafilm was placed over the wells with the protective cover facing upwards. Depressions in the parafilm were made by running a rod along the lane of wells. The cover was then removed and the depressions filled with a volume of sample which was at least twice that of the volume applied to the gels. A drop

of water (approximately 100µl) was placed on the middle of the gel marked by the red line on the separation bed.

The gel was removed from its package with the aid of forceps. It was lowered on to one of the gel areas so that a film of liquid, free from air bubbles, formed between the gel support and the separation bed (excess water was removed with absorbent paper). The plastic film was removed from the gel surface. The Phast Gel buffer strip holder was placed over the gel by sliding it forwards so that the two black pins and the holes in the holder formed a hinge. The holder was then lowered onto the separation bed and two buffer strips were inserted, one in the anode and one in the cathode compartment. The buffer strips were gently pressed down to ensure good contact with the gel. The electrode assembly was then lowered so that the outer electrodes rested evenly on the buffer strips. The electrodes were gently pressed down and the sample applicator arm was lowered.

The sample applicators were lowered on to the surface of the samples allowing them to enter the capillaries (8 samples of 1µl). The applicators were then inserted into the slot nearest the cathode and the separation compartment lid closed. The system was then ready for electrophoresis.

The electrophoresis process (method 1: SDS-PAGE) was used. This method runs the electrophoresis at a gel bed temperature of 15^0C, at 60 volts.hour and with 1.3 sample separation. The process takes approximately 20-30 mins.

(d). Staining technique:

The fast Coomassie blue staining technique was used, derived from the method of Neuhoff *et al.* (1985). The average sensitivity limit of this technique is 20-30ng of protein per band (according to Phast System Development Technique, File No. 200, Pharmacia AB, Uppsala, Sweden).

The stain solution was made by dissolving 1 tablet of Phast Gel Blue R250 (obtained from Pharmacia) in 80ml distilled water. This required stirring for 5-10 mins. at room temperature. To this solution was added 120ml methanol with additional stirring for 2-3 mins. This was the stock solution, stored in the dark until required.

Immediately before use, the stain solution was filtered and mixed with an equal volume of 20% acetic acid. Meanwhile, the destaining and preserving buffer solutions were made. The destaining buffer solution consisted of acetic acid, methanol and distilled water (in the ratio 1:3:6). The preserving buffer solution consisted of glycerol, acetic acid and distilled water (in the ratio 1:2:8).

When the electrophoresis process was complete, the gels were immediately extracted from the machine and placed in the developing chamber (connected to the electrophoresis equipment). This chamber can be connected to a maximum of nine different buffer solutions via nine tubes. In this experiment,

only 3 tubes were required: staining solution (tube 1), destaining buffer solution (tube 2) and preserving buffer solution (tube 3).

After programming the equipment, the developing operation was performed. The gel was then examined and photographed. From the photograph, the relative molecular weights of the purified samples were determined (see below).

(e). Determination of molecular weights:

A calibration curve was constructed for the low molecular weight (LMW) standards (see Results). After staining with Coomassie blue, the gels were examined for individual band positions. They were then photographed. Photocopies of the photographs were obtained for the measuring process in which the positions of the individual bands of the LMW standards were established.

The relative migration distance (R_f) of each component was calculated from the formula:

R_f = distance of a band from origin/distance from origin to reference point

(where the origin was the position of sample application, and the reference point was the position to which bromophenol blue had migrated).

The standard curve was obtained by plotting R_f against $\log_{(10)}$ molecular weight for each standard. Regression analysis was used in order to obtain a line of best fit for the data.

The R_f values for the separated components of HL and MG purified samples (lectins) were calculated from the photocopies. These were then matched with those of the LMW standards on the calibration curve. Thus the relative molecular weights of the lectins in the HL and MG purified samples were calculated.

11. Statistics.

The means (x) and standard errors (6, SE) of treated and untreated samples were obtained. They were then analysed using the Student's t-test (Microsoft, Excel 5).

Determination of protein contents of unpurified (crude) samples of RH and HL.

The standard curve was obtained by plotting protein content of BSA (μg/ml) against optical density (OD) at 595nm. See Fig. 3.1. From this standard curve, the protein contents of the test samples were determined. The results are shown in Table 3.1(a), the results having been converted from μg/ml to mg/ml.

The protein content of RH samples started to increase from 6.4mg/ml at age < 12h, reached 7.2/ml at day 14, then decreased to 5.6 mg/ml at day 28. A similar trend was observed in the protein content of HL samples; the content started to increase from 25mg/ml at age < 12h, reached 28mg/ml at day 3, increased further to 28mg/ml at days 7-14, and then decreased to 22mg/ml at days 21-28 [see Table 3.1(a)]. The protein contents of samples used in purifications are given in Table 3.1(c). To make a comparison of *Stomoxys* and *Glossina spp.* agglutination activity the HL and RH protein contents of *G. m. morsitans* and *G. palpalis* were determined. Results are shown in Table 3.1(b).

Investigation of agglutination activities of purified HL and RH samples against the RBC's and parasites.

Results are given in Table 3.2(a). From these it can be seen that the agglutination activities of the purified HL and RH samples were lower than those of the unpurified (crude) samples before dilution. Purified samples of RH and HL showed a reduction

in activity titres of 4 - 64-fold against RBC (all types) compared with undiluted crude samples, the difference being significant ($p < 0.05$). Purified samples of RH and HL showed a reduction in activity titres of 8 - 16-fold against the parasites, again the difference beign significant ($p < 0.05$). This must be due to the fact that pure lectin samples have a lower protein content than that of the crude lectin samples. Crude lectin samples were therefore diluted to the same concentrations as those of the pure lectins in order to make a comparison of agglutination activity. Results are shown in Table 3.2(b). From these results it is clear that pure lectin agglutination activity is stronger than crude lectin agglutination activity (see Appendix 3).

Investigation of the factors influencing the specificity of the agglutination activity of the purified HL and RH samples.

1. Cross adsorption process.

The results are given in Table 3.3. From these it is clear that agglutination activity against agglutinogens was reduced in adsorbed samples. Agglutination activity against rabbit RBC was negated whilst it was reduced to 2^{-1} (RH) and 2^{-2} (HL) in the case of parasites. In the case of both HL and RH parasite-adsorbed samples, activity against the other parasites was reduced to the neat and to 2^{-1} against the RBC.

2. Enzyme-treated RBC.

Only rabbit RBC were treated with enzymes. Results are given in Table 3.4. From these it can be seen that treatment with neuraminidase significantly increased acitivity 8-32-fold ($p < 0.05$). Similar results were obtained with papain whilst trypsin only increased the activity 2-8-fold. Treating the samples with glycoside hydrolase enzymes reduced activity 2-16-fold ($p < 0.01$).

3. Sugar specificities of the purified HL and RH anti-parasitic and anti-erythrocytic agglutinins.

Results are given in Table 3.5. From these it can be seen that the degree of inhibition of agglutination increased with some sugars and not with others. For determination of the minimum concentration of specific inhibitor sugars to be used, see chapter 2.

4. Determination of the nature of the purified HL and RH samples.

(a). Enzyme treatment of the purified HL and RH samples:

Results are shown in Table 3.6. Neuraminidase has slight effect on the agglutination activity of either HL of RH agglutinins. However, trypsin reduced the activity 4-fold ($p < 0.01$) in the case of RBC and 1-2-fold in the case of parasites ($p < 0.001$). Chymotrypsin and pronase reduced the activity in all cases by 8-16-fold ($p < 0.05$). Papain and pepsin were not found to

reduce the agglutination activity significantly ($p > 0.01$) against RBC or parasites.

(b). Effect of organic solvents and chemical treatments on agglutination activity against rabbit RBC and parasites (*T. brucei, L. hertigi* and *C. fasciculata*).

Results are given in Table 3.7. From these it can be seen that ethanol, ether, ether/ethanol and xylene had no effect on the samples ($p > 0.05$). However, chloroform treatment reduced activity of both HL and RH against RBC 64-128-fold ($p < 0.01$) and against the parasites 32-64-fold ($p < 0.05$). The combination of chloroform/ethanol reduced the activity against RBC 128-256-fold and against parasites 16-32-fold in both HL and RH ($p < 0.01$). Acetone reduced activity against RBC 32-128-fold and against parasites 8-16-fold in both HL and RH samples ($p < 0.01$).

(c). Sodium periodate ($NaIO_4$) oxidation:

Sodium periodate caused a reduction in agglutination titres against rabbit RBC of 32-fold in the case of HL samples ($p < 0.05$) and 64-fold in the case of RH samples ($p < 0.05$). It caused a reduction in agglutination titres against all three parasites of 16-fold in both HL and RH samples ($p < 0.01$). The results are summarised in Table 3.8.

5. Physico-chemical treatments of the purified HL and RH samples:

(a). Presence of metal ions:

Results are given in Table 3.9. Compared to the untreated samples (controls), EDTA reduced titres 64-fold in both HL and RH samples against rabbit RBC ($p < 0.01$) and 16-fold in both HL and RH samples against parasites ($p < 0.01$). A similar reduction in activity was seen with EDTA + Mg^{2+} ions whilst EDTA + Ca^{2+} ions gave the same activity as untreated samples. Treatment with EDTA + Mg^{2+} ions + Ca^{2+} ions increased the titres 2-8-fold against RBC and parasites (HL and RH samples). Incubation of the HL and RH samples with PBS + Mg^{2+} ions + Ca^{2+} ions also caused an increase in titres, 8-fold against RBC ($p < 0.05$) and 16-fold against parasites ($p < 0.01$) for both samples. Treatment of the samples with EGTA reduced the titres against RBC 16-fold and against parasites 64-fold ($p < 0.001$). EGTA + Mg^{2+} ions reduced the titres against RBC 64-fold in the case of HL ($p < 0.05$) and 128-fold in the case of RH ($p < 0.05$).

(b). Exposure to DTT and urea:

Results are given in Table 3.10. DTT reduced the titres of both HL and RH against rabbit RBC 8-16-fold. Urea reduced the tires of both samples against rabbit RBC 16-32-fold and against parasites 4-8-fold ($p < 0.05$ in all cases).

(c). Variation in buffer pH:

Results are given in Table 3.11. From these the pH of PBS to optimise agglutination activity was found to be 7.4 The activity can be maintained at pH values of 6 to 8. A similar mode of activity was found in the case of unpurified samples of HL and RH (see Chapter 2).

(d). Effect of temperature:

Results are given in Table 3.12a. Heating the purified HL and RH samples had a similar effect to that seen in the heating of unpurified (crude) HL and RH samples. Boiling the samples (at 100^0C) reduced the activity titre to that of the neat in all cases whilst a temperature of 80^0C reduced the activity titre 10-24-fold in both HL and RH samples against all agglutinogens. The frequent freezing and thawing of both HL and RH samples was seen to reduce agglutination activity (see Table 3.12b).

Determination of the relative molecular weights of the purified HL and RH lectins:

1. Haemolymph (HL):

SDS-PAGE HL results are shown in Figs. 3.3 and 3.4. In Fig. 3.3 Lane 1 used for LMW, Lane 2 used for HMW, Lane 3 used for 1/8 crude HL, Lane 4 used for 1/4 pure HL, Lane 5 used for HMW, Lane 6 used for 1/2 crude HL, Lane 7 used for undiluted pure HL (3 bands can be seen clearly), and Lane 8 used for 1/16 crude HL. In Fig. 3.4, lanes 6 and 7 (½ non-adsorbed HL pure extracts), 3 bands are clearly visible. These bands have the relative molecular weights of 26,302, 16,218 and 14,028 daltons. After adsorption of the HL pure extracts by RBC 3-4 times, the bands disappeared. This also occurred when adsorption was done 4 times by *T. brucei* alone or 4 times by RBC alone.

2. Reservoir Homogenate (RH).

SDS-PAGE RH results are shown in Figs. 3.5 & 3.6. In Fig. 3.5 Lane 1 used for undiluted crude RH (from 200 reservoirs per ml), Lane 2 used for 1/2 crude RH, Lane 3 used for 1/4 crude RH, Lane 4 used for 1/4 HL and Lane 5 used for 1/4 crude RH. In Fig. 3.6 Lanes 6 and 7 were used for purified RH extracts (non-adsorbed and diluted to one in four). Three bands are clearly visible and have approximately the same relative molecular weights as those of

the HL (28,300, 16,218 and 14,600 Daltons). These bands disappeared when the sample was adsorped by RBC 4-5 times and *T. brucei* 4-5 times.

DISCUSSION

In 1981 Pereira and his co-workers discovered lectins in the crop, midgut and haemolymph of *Rhodinus prolixus*. They found that these lectins were capable of agglutinating *Trypanosoma cruzi* and consequently interest in insect lectins and their role in parasite-vector relationships was stimulated. Ibrahim *et al.* (1984) were the first to demonstrate the presence of glucosamine-binding lectin in the midgut of tsetse flies. However, it was Maudlin and Welburn (1987) who first showed that lectins prevent establishment of the trypanosomes in tsetse flies. High midgut infection followed addition of glucosamine to infective blood meals. It was concluded that glucosamine in the infective feed inhibited midgut lectin agglutination activity thereby facilitating the establishment of infection in the vector. However, lectins were found to be necessary for parasite differentiation (Maudlin, 1991). Maintenance of flies on a diet containing glucosamine was found to lead to the complete inhibition of lectin activity. A concomitant reduction in the number of parasites which established themselves within the flies was observed (Maudlin and Welburn, 1988 a and b).

My observations that lectins from *S. calcitrans* can agglutinate parasites supports the conclusion of Maudlin and Welburn (1987) that susceptibility to infection in tsetse flies is mediated, in part at least, by midgut lectins. Before dilution of the crude samples to the same concentrations as those of the pure lectins, the activities of the pure lectins were seen to be less than those of the crude samples. This might be due to the loss of lectins and other proteins during the purification process. It was therefore necessary

to adjust the crude sample concentrations to those of the pure HL and RH samples. Agglutination assays were then performed with all the samples against RBC and parasites. Results are shown in Table 2(a) and Table 2(b).

In general, pure *S. calcitrans* HL and RH lectin samples have higher agglutination activity against erythrocytes (RBC) and parasites than that of crude samples. Sugar and agglutinogen specificity was the same in both pure and crude samples. The results indicated that these samples were heterogeneous with regard to reactivity towards a diverse array of sugar moieties (except glucose and its deoxy-derivatives). This suggests that the lectins are not dependent for their activity on the presence of other 'helper' molecules in the crude homogenate. D(+)-glucosamine and melibiose showed the highest degree of inhibition in the case of both pure and crude *S. calcitrans* HL and RH samples followed by galactose and mannose and their derivatives or moieties with other combinations. These results are generally in agreement with the findings of Ingram and Molyneux (1988) who carried out similar sugar inhibition studies with tsetse flies.

Although my results showed that both pure and crude samples of *S. calcitrans* HL and RH agglutinated all RBCs and parasites used, only rabbit RBCs were enzyme-treated. Results of proteolytic and glycosidase enzyme treatment suggest the presence of glycoprotein or glycopeptide membrane receptors on the rabbit RBC cell wall. This is supported by evidence of elevated HL and RH pure and crude sample activity when RBCs were treated with neuraminidase and proteolytic enzymes. These enzymes may have partially digested the membrane-integrated proteins or peptides or terminal sialic

acid residues resulting in the exposure of potential sugar residues involved in lectin binding. Thus the removal of N-glycolneuraminyl and N-acetylneuraminyl linkages from the RBC membrane neuraminic acid by neuraminidase could unmask cryptic acid galactosyl and glucosyl residues present on the RBC surface. Conversely, use of glycosidase may have cleaved lectin binding sites from the RBC surface resulting in a decrease in activity titre. It can be concluded that *Stomoxys* HL and RH agglutinin specificities are directed mainly towards α-D- and/or β-D-galactose residues, and to a lesser degree towards α-D- and/or β-D-glucose moieties on the RBC surface. Ingram and Molyneux (1988; 1993) obtained similar agglutinin specificity results with tsetse fly HL samples (except in the case of *G. m. morsitans* and *G. p. gambiensis* anti-0 agglutinins). Their carbohydrate inhibition experiment results correlated well with those for glycoside hydrolase-treated rabbit RBCs.

The different stereochemical orientations of the cell membrane glycoconjugates could account for the multispecific binding nature of *Stomoxys* HL and RH samples. Ingram and Molyneux (1993) reported the same findings with tsetse HL lectins.

Nuclease treatment of *Stomoxys* pure HL and RH samples had a limited effect and treatments with phenol, TCA, chloroform, acetone and neuraminidase negated activity or markedly reduced it. This suggests that lectins are not nucleic acids or polysaccharides but are more likely to be glycoproteins, lipoproteins, lipids or glycolipids.

Treatment of *Stomoxys* pure HL and RH samples with lipase and organic solvents (xylene and a mixture of ether and phenol), which cause lipid hydrolysis and inactivation/denaturation respectively, had relatively little effect on agglutination activities. This finding tends to eliminate a potential glycolipid or lipoprotein involvement for the activities. Thus the lectins are most likely to be protein or glycoprotein in nature. This is supported by the evidence for glycosidic moieties in the samples obtained in the sugar inhibition studies and by results of treatment with $NaIO_4$ and neuraminidase (which hydrolyses 2-, 3-, 2,5- and 2,8-N-glycolneuraminyl and N-acetylneuraminyl linkages).

Agglutinin levels in HL and RH were found to be reduced in all neuraminidase-treated samples (especially in the case of rabbit RBC). A significant reduction in agglutination acitivity of pure HL and RH samples was seen after proteolytic enzyme treatment in all cases (RBCs and parasites).

Non-specific pronase and relatively specific pepsin break most peptide linkages. Treatment with pronase reduced the activity titres of *Stomoxys* pure HL and RH samples indicating that the lectins possess peptide bonds and are therefore proteinaceous in nature. Ingram and Molyneux (1990 and 1993) reported the same findings in their work with tsetse HL and human (ABO) RBCs.

Trypsin and chymotrypsin are more specific in their actions compared to pronase and pepsin. Treatment of *Stomoxys* pure HL and RH samples with chymotrypsin suggests that the haemagglutinin peptide bonds contain several aromatic amino acid residues.

Basic amino acid groups (lysine and argenine, preferentially split by trypsin) were found in tsetse HL haemagglutinins especially *G. m. morsitans* and *G. p. gambiensis* anti-AB RBC (Ingram and Molyneux, 1993).

It is not surprising to find that *Stomoxys* haemagglutinins exhibit trypsin sensitivity since this property has been reported for lepidopteran (Suzuki and Natori, 1983), dipteran (McKenzie and Preston, 1992; Ingram and Molyneux, 1990 and 1993) and orthopteran (Hapner and Jermyn, 1981; Stebbins and Hapner, 1985) agglutinins. There is a very high trypsin level in the *Stomoxys* midgut, 95% of which is found in the posterior midgut (Lehane, 1991). It is therefore not surprising to find that peak lectin activity occurs in the anterior reservoir region of the midgut.

Heat treatment of *Stomoxys* pure and crude HL and RH samples affected the titres in exactly the same way. Increasing the temperature reduced the titres. Boiling the samples at 100^{0}C almost negated activity. In contrast, samples from the tsetse flies *G. tachinoides* and *G. p. gambiensis* (active against human RBC group O and B respectively) were reported to be only slightly affected after 80^{0}C heat treatment (Ingram and Molyneux, 1993). However, most insect HL agglutinins are heat-labile (Komano *et al.*, 1980; Ingram and Molyneux, 1993; Ingram *et al.*, 1984). The thermolability of *Stomoxys* pure and crude HL and RH sample agglutinins against RBCs and trypanosomatid flagellate parasites and the influence of pH (outside the normal physiological range) on their activities indicate that these agglutinins are protein or glycoprotein in nature.

The pH for optimum activity of *Stomoxys* crude HL and RH sample agglutinins against RBCs and trypanosomatid flagellate parasites was typically in the range 7.0 - 7.4. The pH for optimum activity of the agglutinins of tsetse flies fell within the range 6.2 - 8.2 and, although *G. tachinoides, G. m. morsitans* and *G. p. gambiensis* HL was reported to be slightly acidic, the haemagglutinins required pH 5 - 6, 5 - 7 and 7 - 9 respectively for optimum agglutination.

DTT treatment of *Stomoxys* pure and crude HL and RH samples produced the same results; the agglutination activity titres against RBC and parasites were reduced in all cases. Urea treatment noticeably reduced the activity against the same agglutinogens (especially against the parasites). Similar results were obtained by Ingram and Molyneux (1993). They concluded that tsetse HL haemagglutinins against human (ABO) RBCs (especially groups B and AB in *G. m. morsitans*) might contain a higher number of sulphur-containing amino acids forming S - S bonds compared to a restricted number of H - H bonds which may be of greater significance in the structural configuration of HL haemagglutinins. This might be the case for HL and RH agglutinin samples of *Stomoxys* against RBCs and parasites.

Ca^{2+} ions were essential for optimum activity of *Stomoxys* crude HL and RH sample agglutinins against RBCs and parasites. Mg^{2+} ions were non-essential. This was the same in the case of *G. p. gambiensis* purified samples which were reported to require Mg^{2+} ions and both *G. tachinoides* and *G. m. morsitans* were found to require Ca^{2+} ions for their optimum HL haemagglutination activity (Ingram and Molyneux, 1993). However, haemolymph Mg^{2+} ion levels in adult dipterans tend to be higher than those

of Ca^{2+} ions (Sutcliffe, 1963). Since pure and crude *Stomoxys* HL and RH sample agglutinins require Ca^{2+} ions for optimum activity like HL sample agglutinins of *G. tachinoides* and *G. m. morsitans* (Ingram and Molyneux, 1993), they belong to the C-type class of animal lectins. C-type class lectins all require Ca^{2+} ions for functional activity (Drickamer, 1988).

In my studies of *Stomoxys* crude HL and RH samples I suggested that mixtures of enzymes, proteins and other organic materials present within them were capable of influencing results. The crude samples showed a reduction in activity titres against all the agglutinogens compared with the pure samples. However, the activities had the same mode and specificity of effect in both crude and pure samples. In conclusion, the reduced strength of agglutination activity in crude samples was due to the substances which were separated from them by purification. Detailed explanations are given in the General Discussion and Conclusions.

Stomoxys calcitrans pure HL sample protein contents were found to be approximately 10% of those of the crude HL sample. Pure RH sample protein contents were found to be approximately 4.30% of that of the crude RH sample (see Tables 3.1a and c). Whilst the same mode of specificity was shown towards the agglutinogens in both pure and crude samples, this explains why agglutination activity (against RBC and parasites) was stronger with crude samples. After dilution of the crude samples to concentrations equal to those of the pure samples, pure sample activity was found to be stronger than that of the crude samples (see Table 3.2a and b).

Stomoxys calcitrans crude HL and RH sample protein contents were shown to be less than those of *Glossina* spp. (see Table 3.1a and b). The protein contents in *Glossina* spp. are approximately 2 - 3 times higher than in *Stomoxys*. However, *Stomoxys* sample agglutination activity was seen to be stronger than that of *Glossina* against RBC and parasites (Ingram and Molyneux, 1988, 1990 and 1991). The total activity of *S. Calcitrans* RH (from 3 days old) against human RBC group B was 2^{-11} and inhibited by D(+) galactosamine (GalN) at 2.88mM/mg. RH protein, whilst that of *G. m. morsitans* was reported to be 2^{-6} against the same RBC (Ingram and Molyneux, 1988) and found to be inhibited by 1.139mM/mg. *G. m. morsitans* MG, hence *Stomoxys* lectin activity is approximately 1.5 - 2.5 times stronger than that of *Glossina.*

Pure and crude tsetse lectins were found to react with sugars in studies carried out by Ingram and Molyneux (1988). *Stomoxys* lectins were found to react with the same sugars in our study. In addition, lectin reaction with sugars on the trypanosome surface was also discovered (Jackson *et al.*, 1978; Mutharia and Pearson, 1987). With these discoveries and reports that tsetse lectins were involved in the regulation of parasite development within the midgut (Welburn and Maudlin, 1990), it was not surprising to find that the lectins reacted with *T. brucei* (bloodstream and culture forms), *L. hertigi* (culture forms) and *C. fasciculata* (culture forms).

Although the involvement of insect lectins or lectin-like molecules in both lysis and differentiation of the parasites has become widely accepted, their mechanisms of action are not clear (Osir *et al.*, 1995). In pilot experiments in our work whole gut extracts of *Stomoxys* showed haemagglutination activity against rabbit RBC with some

haemolysis (see Appendix 1). This haemolysis increased if the plates were incubated at 26^{0}C for more than 1.5 hours or if left at room temperature (approximately 20 C)^0 for 2 - 3 hours. Similar results were observed with hindgut extracts. The reservoir was the only part of the midgut to give stronger haemagglutination activity than other parts of the gut without haemolysis (wherever RH samples are mentioned, this refers to the reservoir). The haemolysis might be due to the accumulation of trypsin since 95% of this enzyme is located in the posterior midgut (Lehane, 1991). Trypsin or trypsin-like enzymes were reported to be involved in lectin activity (Imbuga *et al.*, 1992; Abubakar, 1995; Osir *et al.*, 1995). Osir *et al.* (1995) concluded that discovery of the bifunctional molecule, with both trypsin and lectin activity, might be a reconciliation of the two views regarding the role of lectins and trypsins in trypanosome differentiation and lysis (Maudlin, 1991; Imbuga *et al.*, 1992; Osir *et al.*, 1993 and 1995).

Osir *et al.* (1995) isolated a bloodmeal-induced lectin with proteolytic activity from midgut extracts of *Glossina longipennis* by a two-step procedure involving anion-exchange chromatography. They described it as a glycoprotein with a native molecular weight, M_r , of 61000 ± 3000 Da, composed of two noncovalently-linked subunits designated α (M_r ~27000 Da) and β (M_r ~ 33000 Da). The native protein was capable of agglutinating bloodstream form and procyclic trypanosomes as well as rabbit RBC. This activity was seen to be strongly inhibited by D-glucosamine and weakly inhibited by N-acetyl-D-glucosamine. Soya bean trypsin inhibitor abrogated agglutination of bloodstream forms whilst procyclics were unaffected. Agglutination acitivity was also found to be sensitive to temperatures above 40^{0}C but was unaffected

by chelators or metal ions. Osir and his co-workers concluded that, without doubt, the α subunit had trypsin activity as it bound tritiated (DFP) and its molecular weight compared favourably with that of trypsin from other tsetse species such as *G. p. palpalis* (M_r ~24000 and 26000 Da) and *G. m. morsitans* (M_r ~24000 Da).

In contrast, our purified *Stomoxys* HL and RH sample agglutinins agglutinated all the RBC types and parasites which had been agglutinated by crude samples. The same sugar specificities were observed with crude and pure samples. Results of SDS-PAGE of HL pure lectins revealed three bands with relative molecular weights of 26,302 Da, 16,218 Da and 14,028 Da. SDS-PAGE of MG pure lectins revealed three similar bands with relative molecular weights of 28,300 Da, 16,218 Da and 14,600 Da. *Stomoxys* RH and HL lectins are of approximately the same molecular weight, exhibit the same agglutination activity and possess the same sugar specificity. It can therefore be concluded that the lectin molecules in the haemolymph are identical to those found in the midgut; haemolymph lectins are secreted into the gut lumen to play a physiological and immunological role in the fly. Infection was not established in flies despite provision of high concentrations of inhibitor sugars with infective feed. The infection was probably prevented by lectins (see Chapter 4).

Antibodies raised against the protein were used against the isolated agglutinins from *Glossina* species in order to detect the cross reactivity of these agglutinins with those of some other blood-sucking insects (Osir *et al.*, 1995). Results revealed a single precipitation band in the case of *G. longipennis, G. m. morsitans* and *G. pallidipes* midgut extracts. However, midgut extracts from stable flies (*S. calcitrans*), sandflies

(*Phlebotomus duboscqui*) and mosquitoes (*Aedes aegypti*) showed no cross-reactivity (Osir *et al.*, 1995). The lack of cross-reactivity explains why lectins from *S. calcitrans* and other blood-sucking insects vary in their specificities to sugars and agglutinogens.

TABLE 3.1(a) **The mean ($\bar{x}$) protein contents (mg/ml) of unpurified (crude) samples of HL and RH from *S. calcitrans* collected at different ages.**

Fly Age	HL **	RH ***
≤ 12h	25.0	6.4
1d	24.0	6.4
3d	28.0	6.5
7d	31.0	6.8
14d	32.0	7.2
21d	22.0	5.8
28d	22.0	5.6

** 80µl of HL was collected from 100 - 120 flies on the same occasion.
*** RH was obtained from 40 flies.

TABLE 3.1(b) **The mean ($\bar{x}$) protein contents (mg/ml) of unpurified (crude) samples of HL and RH from *Glossina* spp. collected at two different ages.**

Fly Age	Species	HL **		RH ***	
		$\bar{x}$	SE	$\bar{x}$	SE
About 1 day (unfed)	*G. m. morsitans*	39.0	0.98	16.5	0.3
	G. palpalis	28.8	0.29	14.9	0.12
About 2 weeks	*G. m. morsitans*	49.8	0.95	20.0	0.6
	G. palpalis	45.0	0.6	18.1	0.21

** 80µl of HL was collected from 100 - 120 flies on the same occasion
*** RH was obtained from 40 flies.

TABLE 3.1(c) **The mean ($\bar{x}$) protein contents (mg/ml) of purified and unpurified samples of HL and RH from *S. calcitrans* collected at age 14 days.**

Samples before purification				Samples after purification			
HL		RH *		HL		RH	
$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
38.0	2.65	30.2	1.18	3.6	0.26	1.3	0.10

* RH samples in this case were produced by homogenization of approximately 160 - 170 reservoirs per ml PBS (pH 7.4).

TABLE 3.2 **The mean ($\bar{x}$) agglutination activity titres of purified and unpurified samples of HL and RH against RBC (human [A, B,AB, O], rabbit and sheep) and parasites *T. brucei* (culture forms, C.f; and bloodstream forms, B.f.), *L. hertigi* and *C. fasciculata*.**

(a) Before dilution of the unpurified samples:

Type of sample	human RBC				rabbit RBC	sheep RBC	*T. brucei* C.f. *	*T. brucei* B.f. *	*L. hertigi*	*C. fasciculata*
	A	B	AB	O						
Unpurified HL lectins	2^{-10}	2^{-15}	2^{-8}	2^{-12}	2^{-16}	2^{-8}	2^{-12}	2^{-11}	2^{-12}	2^{-11}
Purified HL lectins	2^{-7}	2^{-10}	2^{-7}	2^{-9}	2^{-10}	2^{-6}	2^{-8}	2^{-8}	2^{-8}	2^{-7}
Unpurified RH lectins	2^{-8}	2^{-14}	2^{-8}	2^{-12}	2^{-15}	2^{-7}	2^{-11}	2^{-10}	2^{-12}	2^{-10}
Purified RH lectins	2^{-6}	2^{-9}	2^{-7}	2^{-9}	2^{-10}	2^{-6}	2^{-8}	2^{-7}	2^{-8}	2^{-6}

(b) After dilution# of the unpurified samples:

Type of sample	human RBC				rabbit RBC	sheep RBC	*T. brucei* C.f. *	*T. brucei* B.f. *	*L. hertigi*	*C. fasciculata*
	A	B	AB	O						
Unpurified HL lectins	2^{-3}	2^{-5}	2^{-2}	2^{-5}	2^{-5}	2^{-2}	2^{-3}	2^{-2}	2^{-3}	2^{-3}
Purified HL lectins	2^{-8}	2^{-10}	2^{-7}	2^{-9}	2^{-10}	2^{-6}	2^{-8}	2^{-8}	2^{-8}	2^{-7}
Unpurified RH lectins	2^{-1}	2^{-3}	2^{-2}	2^{-3}	2^{-4}	2^{-1}	2^{-2}	2^{-3}	2^{-3}	2^{-1}
Purified RH lectins	2^{-6}	2^{-9}	2^{-7}	2^{-9}	2^{-10}	2^{-5}	2^{-8}	2^{-7}	2^{-7}	2^{-6}

NB * C.f. Culture forms *T. brucei*
B.f. Blood forms *T. brucei*

\# The unpurified samples were diluted to the following concentrations:
HL = 3.6 mg/ml, RH = 1.3 mg/ml.

TABLE 3.3 **The mean ($\bar{x}$) haemagglutination activity titres against rabbit RBC, *T. brucei*, *L. hertigi* and *C. fasciculata* in adsorbed and untreated pure samples of *S. calcitrans* HL and RH.**

(n = 3. SE 0.0)

Adsorbed Agglutinogen	HL				RH			
	rabbit RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*	rabbit RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*
RBC	0	2^{-2}	2^{-2}	2^{-2}	0	2^{-1}	2^{-1}	2^{-1}
T. brucei	2^{-1}	0	neat	neat	2^{-1}	0	neat	neat
L. hertigi	2^{-1}	neat	0	neat	2^{-1}	neat	0	neat
C. fasciculata	2^{-1}	neat	neat	0	2^{-1}	neat	neat	0
Untreated	2^{-10}	2^{-8}	2^{-7}	2^{-6}	2^{-9}	2^{-8}	2^{-7}	2^{-6}

TABLE 3.4. **Effect of neuraminidase, proteolytic enzyme and glycoside hydrolase enzyme treatment of rabbit RBC on the mean ($\bar{x}$) haemagglutination titres ($-\log_2 2^{-n}$) of *S. calcitrans* purified HL and RH samples.**

(n = 3).

Treatment	HL		RH	
	$\bar{x}$	SE	$\bar{x}$	SE
Untreated (non-enzyme treated)	11	0.6	9	0.6
Neuraminidase	14	0.6	14	0.6
Trypsin	12	0.6	12	0.6
Papain	16	0.6	15	0.6
α-galactosidase	7	0.6	6	0.6
β-galactosidase	7	0.6	7	0.6
α-glucosidase	9	0.6	8	0.6
β-glucosidase	10	0.6	9	0.6

TABLE 3.5.

Degree* of inhibition (in descending order) of *S. calcitrans* purified HL and RH agglutination activity against rabbit RBC, *T. brucei* procyclic ($T.b^1$) and bloodstream forms ($T.b^2$) with carbohydrates and glycoproteins.

Carbohydrate Inhibitor	HL			RH		
	RBC	$T.b^1$	$T.b^2$	RBC	$T.b^1$	$T.b^2$
D(+)-Glucosamine	6+	6+	6+	6+	6+	6+
D(+)-Galactosamine	6+	6+	6+	6+	6+	6+
D(-)-Mannosamine	6+	6+	6+	6+	6+	6+
N-Acetyl-D-Glucosamine	5+	5+	5+	5+	5+	5+
N-Acetyl-D-Galactosamine	5+	5+	5+	5+	5+	5+
D(+)-Galactose	6+	6+	6+	6+	6+	6+
D(+)-Mannose	6+	6+	6+	6+	6+	6+
D(+)-Melibiose	5+	5+	5+	5+	5+	5+
6.7-Dihydroxycoumarine-6-Glucoside	3+	2+	2+	3+	2+	2+
D-Galacturonic acid	3+	2+	2+	3+	2+	2+
2-Deoxy-D-Galactose	2+	2+	2+	2+	2+	2+
N-Acetyl-Neuraminic Acid	2+	2+	2+	2+	2+	2+
D-Manheptalose	2+	1+	1+	2+	1+	1+
2-Hydroxymethyl-phenyl-β-D-glucose	2+	1+	1+	2+	1+	1+
L(-)-Arabinose	2+	1+	1+	2+	1+	1+
L(-)-Sorbose	2+	1+	1+	1+/2+	1+	1+
D-Glucuronic acid	2+	1+	1+	2+	1+	1+
D-Glucurnamide	2+	1+	1+	1+	1+	1+
β-Methyl-Glucoside	2+	1+	1+	2+	1+	1+
Mannan	2+	1+	1+	1+	1+	1+
Bovine γ-globulins	2+	1+	1+	1+	1+	1+
D(+)-Trehalose	1+	1+	1+	2+	2+	2+
Rhamnose	2+	1+	1+	1+	1+	1+
D(-)-Ribose	1+	1+	1+	1+	1+	1+
6-Deoxy-L-Mannose	1+	1+	1+	1+	1+	1+
6-Deoxy-L-Galactose	1+	1+	1+	1+	1+	1+
α-Lactose	1+	1+	1+	1+	1+	1+
D(+)-Turanose	1+	1+	1+	1+	1+	1+

Table 3.5 continued

Carbohydrate Inhibitor	HL			RH		
	RBC	T.b[1]	T.b[2]	RBC	T.b[1]	T.b[2]
β-Gentiobiose	1+	1+	1+	1+	1+	1+
Fetuin	1+	1+	1+	1+	1+	1+
α-Amylase	1+	1+	1+	1+	1+	1+
Mucin	1+	1+	1+	1+	1+	1+
Albumin	1+	1+	1+	1+	1+	1+
β-Lactoglobulin	1+	1+	1+	1+	1+	1+
Invertase.	1+	1+	1+	1+	1+	1+
Glycogen	1+	No	No	No	No	No
D(+)-Glucose	No	No	No	No	No	No
D(+)-Xylose	No	No	No	No	No	No
β-D-Fructose	No	No	No	No	No	No
2-Deoxy-D-ribose	No	No	No	No	No	No
2-Deoxy-D-glucose	No	No	No	No	No	No
6-Deoxy-D-glucose	No	No	No	No	No	No
L(-)-Fucose	No	No	No	No	No	No
D(+)-Fucose	No	No	No	No	No	No
Maltose	No	No	No	No	No	No
Sucrose	No	No	No	No	No	No
D(+) Melezitose	No	No	No	No	No	No
Raffinose	No	No	No	No	No	No
Stachyose	No	No	No	No	No	No
Chitin	No	No	No	No	No	No
Xylan	No	No	No	No	No	No
Casein	No	No	No	No	No	No
β-Lactoglobulin	No	No	No	No	No	No
Human Chorionic Gonadotrophin	No	No	No	No	No	No
Glucose Oxidase	No	No	No	No	No	No

* Degrees of inhibition (D1) were scored according to the scale of Ingram and Molyneux (1988); thus a reduction in titre by 2 wells was scored as 1+, 3 wells as 2+, 4 wells as 3+, 5 wells as 4+ and 6 or more wells as 5+. Total inhibitions (100%) were scored as 6+. Inhibition of one well was denoted by ± and no inhibition was denoted by -.

TABLE 3.6. **Effect of enzyme treatment of *S. calcitrans* purified HL and RH samples on the mean ($\bar{x}$) agglutination activity titres ($-\log_2 2^{-n}$) against rabbit RBC and parasites (*T. brucei, L. hertigi* and *C. fasciculata*).** **(n = 3, SE 0.0).**

Treatment	HL				RH			
	RBC	*T.brucei*	*L. hertigi*	*C. fasciculata*	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*
Untreated	11	9	8	8	10	9	8	7
Neuraminidase	9	7	7	7	8	7	7	7
Trypsin	7	7	6	6	7	6	6	6
Chymotrypsin	6	5	5	5	7	6	5	5
Papain	10	9	8	7	10	9	8	7
Pronase	6	5	5	5	7	6	5	5
Pepsin	10	8	8	7	10	9	7	7

TABLE 3.7 **Effect of organic solvent treatment of purified *S. calcitrans* HL and RH samples on agglutination titres ($-\log_2 2^{-n}$) against rabbit RBC and parasites *T. brucei, L. hertigi* and *C. fasciculata.***
(n = 3, SE 0.0)

Treatment	HL				RH			
	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*
Untreated	11	8	8	7	12	8	8	7
Ethanol	9	7	7	7	10	7	7	7
Ether	10	8	8	7	12	8	8	7
Ether/Ethanol	9	7	7	6	10	7	7	7
Chloroform	5	4	4	4	6	4	3	3
Chloroform/ ethanol	4	4	3	3	4	3	3	3
Xylene	10	8	8	7	12	8	8	7
Acetone	6	5	5	4	5	4	4	4

HL and RH on agglutination activity titres ($-\log_2 2^{-n}$) against rabbit RBC and parasites *T. brucei, L. hertigi* and *C. fasciculata.* (n = 3, SE 0.0).

Treatment	HL				RH			
	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*
Untreated	11	8	7	7	12	8	8	8
With $NaIO_4$	6	4	3	3	6	4	4	4

TABLE 3.9. The effect of EDTA, EGTA and divalent cation treatments of purified samples of *S. calcitrans* HL and RH on agglutination activity titres ($-\log_2 2^{-n}$) against rabbit RBC and parasites *T. brucei, L. hertigi* and *C. fasciculata.* (n = 3, SE 0.0)

Treatment	HL				RH			
	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*
Untreated	12	9	8	8	12	9	9	8
PBS+Mg^{2+}+Ca^{2+}	15	11	11	11	15	12	12	12
EDTA	6	5	4	4	6	5	5	5
EGTA	8	6	6	6	8	6	6	5
EDTA + Mg^{2+}	7	6	6	6	7	5	6	6
EDTA+Ca^{2+}	11	10	9	9	10	10	9	9
EGTA+Mg^{2+}	6	6	6	6	5	5	6	6
EDTA+Mg^{2+}+ Ca^{2+}	13	12	12	11	13	12	11	11

TABLE 3.10 The effect of DTT and urea treatments of purified samples of *S. calcitrans* HL and RH on agglutination activity titres ($-\log_2 2^{-n}$) against rabbit RBC and parasites *T. brucei, L. hertigi* and *C. fasciculata.* (n = 3, SE 0.0)

Treatment	HL				RH			
	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*	RBC	*T. brucei*	*L. hertigi*	*C. fasciculata*
Untreated	12	9	9	8	11	9	9	8
DTT	8	7	6	6	8	6	6	6
Urea	7	6	6	6	7	5	5	5

TABLE 3.11. **The effect of varying PBS pH on *S. calcitrans* purified HL and RH titres ($-\log_2 2^{-n}$) against rabbit RBC, *T. brucei* (T.b.), *L. hertigi* (L.h.) and *C. fasciculata* (C.f.).**
(n = 3, SE 0.0)

pH value	HL				RH			
	RBC	T.b.	L.h.	C.f.	RBC	T.b.	L.h.	C.f.
2.2	neat	neat	neat	neat	neat	neat	neat	neat
3	1	1	1	1	1	1	1	1
3.6	4	2	2	2	2	2	2	2
4.0	4	3	3	3	4	3	3	3
5.0	4	4	4	4	4	4	4	4
6.0	8	6	6	6	8	6	6	6
7.0	12	8	8	8	12	9	8	8
7.4	13	9	9	8	12	9	8	8
8.0	9	5	5	5	8	5	5	5
9.0	5	4	4	4	5	4	4	4
10.0	2	2	2	2	2	2	2	2

TABLE 3.12(a) **Effect of temperature on the titres ($-\log_2 2^{-n}$) of agglutination activity of S. calcitrans HL and RH samples (purified) against rabbit RBC, *T. brucei* (T.b.), *L. hertigi* (L.h.) and *C. fasciculata* (C.f.).**

(n = 3, SE 0.0)

Temperature °C	HL				RH			
	RBC	T.b.	L.h.	C.f.	RBC	T.b.	L.h.	C.f.
Untreated	12	9	9	9	12	9	9	8
5	12	9	8	8	12	9	8	8
15	12	7	7	7	12	7	7	7
25	11	7	7	7	10	7	7	7
26	10	6	6	6	10	6	6	6
35	9	5	5	5	9	5	5	5
40	8	5	5	5	8	5	5	5
50	5	4	4	4	5	4	4	4
60	4	3	3	3	4	3	3	3
80	2	1	1	1	2	1	1	1
100	neat	neat	neat	neat	neat	neat	neat	neat

TABLE 3.12(b) **The effect of freezing and thawing *S. calcitrans* HL and RH purified samples on the titres ($-\log_2 2^{-n}$) of agglutination activity against rabbit RBC, *T. brucei* (T.b.), *L. hertigi* (L.h.) and *C. fasciculata* (C.f.).**

(n = 3, SE 0.0)

Treatment	HL				RH			
	RBC	T.b.	L.h.	C.f.	RBC	T.b.	L.h.	C.f.
Untreated	12	9	9	8	12	9	9	8
Frozen & thawed once	12	9	9	8	12	9	9	8
Frozen & thawed twice	11	8	8	8	11	8	8	8
Frozen & thawed 3 times	8	6	6	6	8	6	6	6
Frozen & thawed 5 times	6	5	5	5	6	5	5	5
Frozen & thawed 8 times	4	3	3	3	4	3	3	3

FIG. 3.1

Calibration curve established using bovine serum albumin (BSA) for the determination of the concentration of protein in HL and RH of *S. calcitrans*.

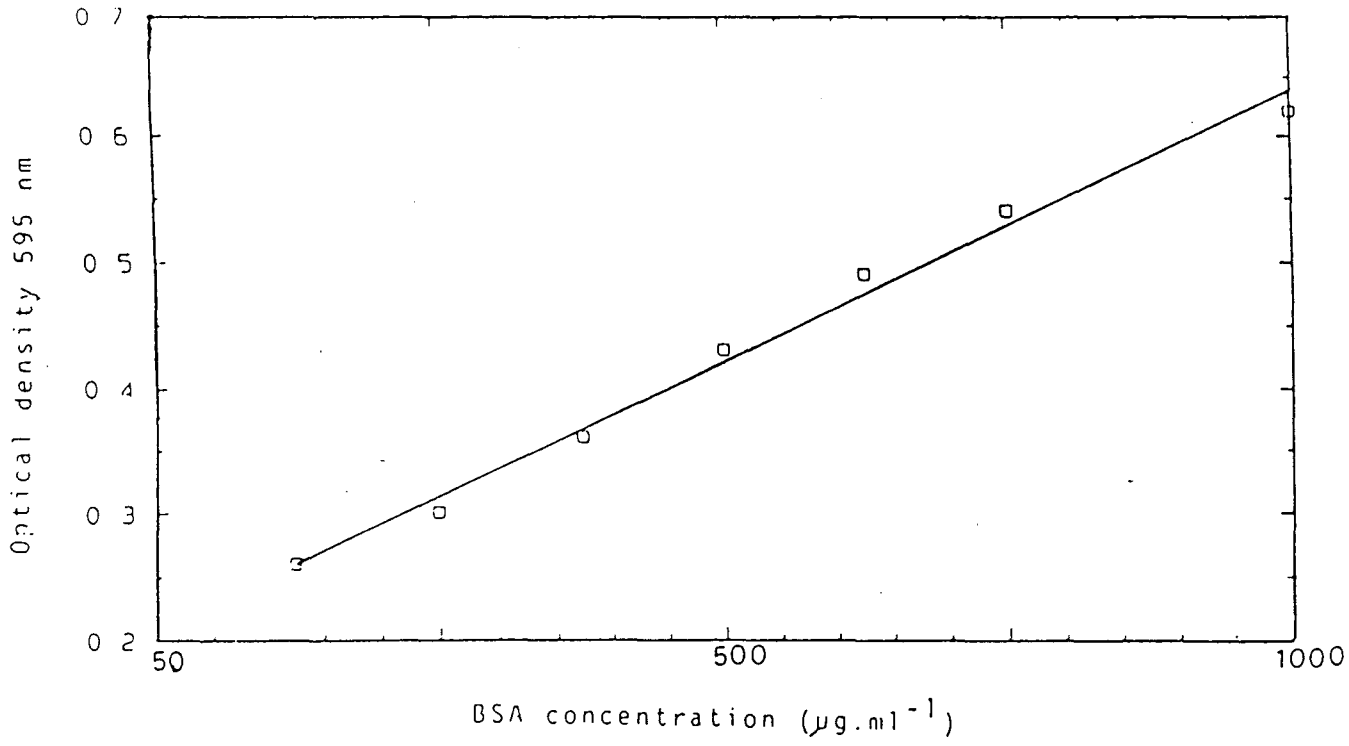


FIG. 3.2.

Calibration curve established with the low molecular weight (LMW) protein standards for the gels shown in Figures 1, 2, 3.

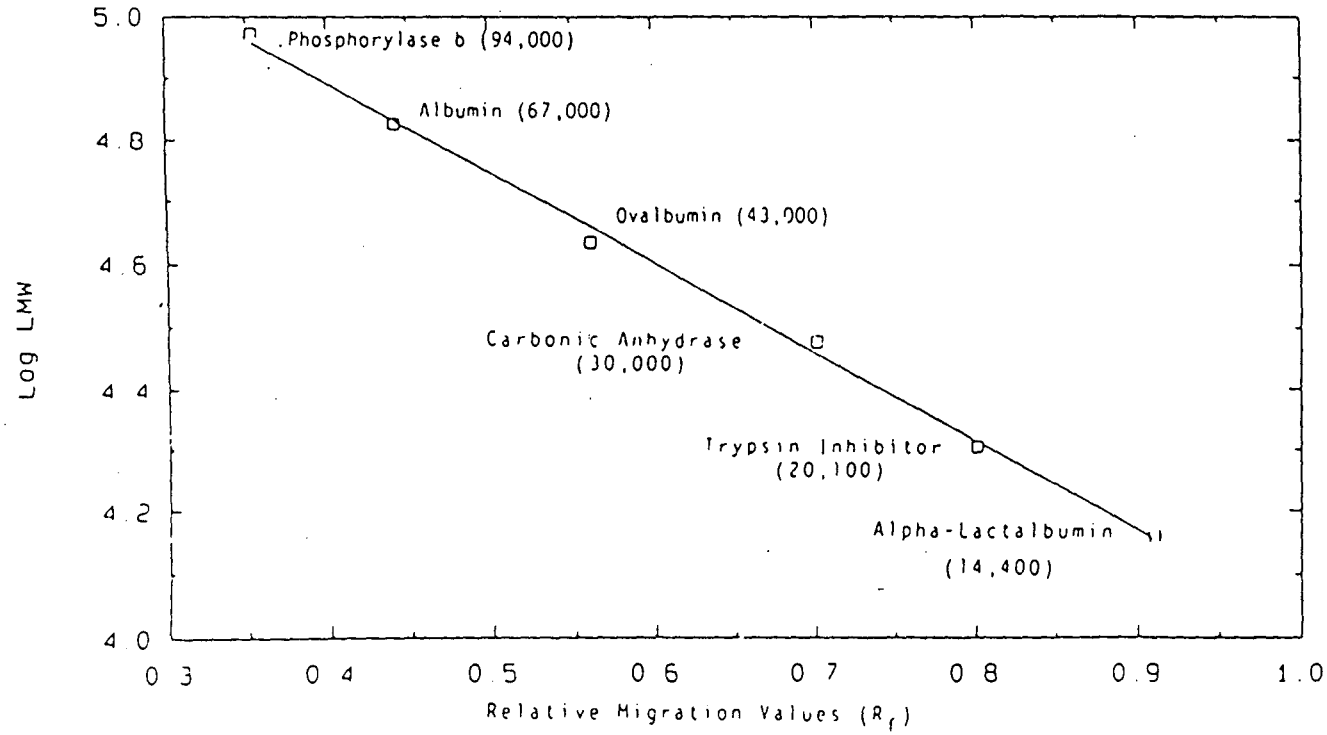
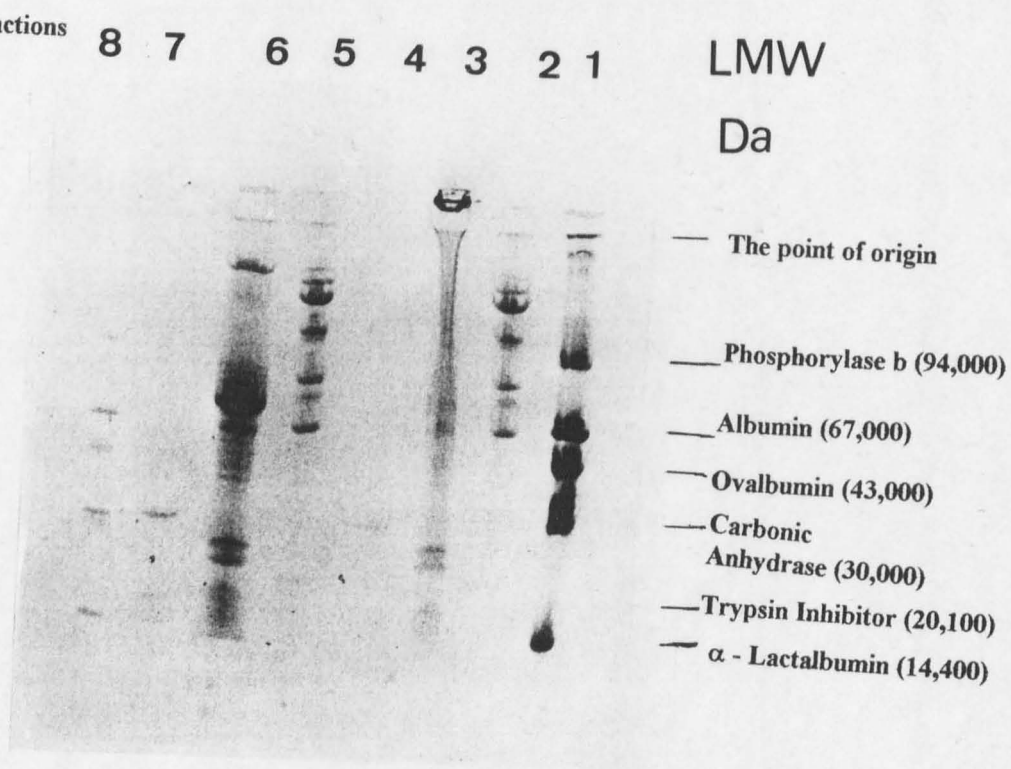


FIG.3.3

SDS-PAGE separation of HMW, LMW and *Stomoxys calcitrans* haemolymph (LH)



Lane 1 LMW protein standard
Lane 2 HMW protein standard
Lane 3 1/8 crude HL sample
Lane 4 1/4 pure HL sample
Lane 5 HMW protein standards
Lane 6 1/2 crude HL sample
Lane 7 Undiluted pure HL (note the 3 bands)
Lane 8 1/16 crude HL sample

a 28,300 Da

b 16,218 Da

c 14,600 Da

FIG.3.3

SDS-PAGE separation of HMW, LMW and *Stomoxys calcitrans* haemolymph (LH) (see facing page).

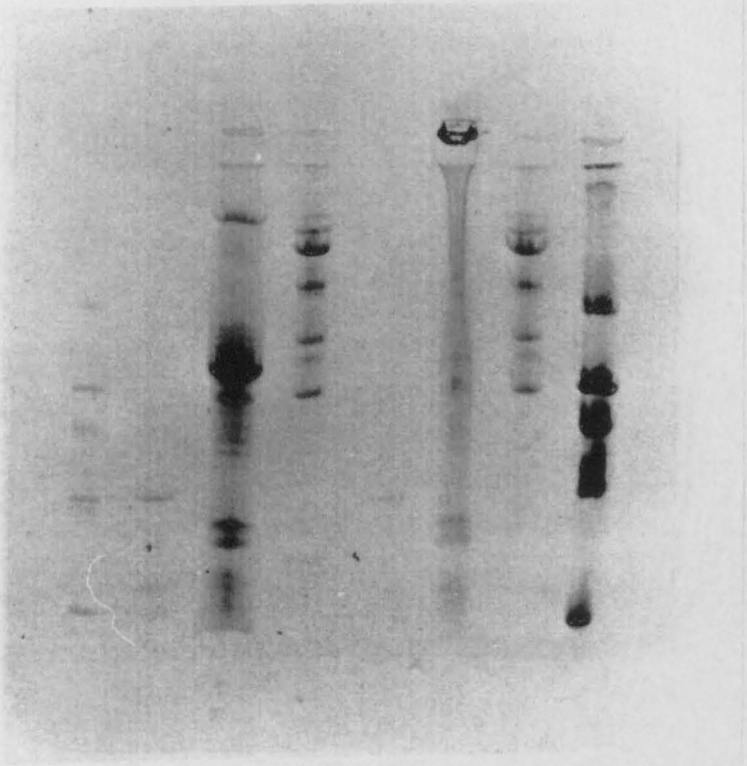
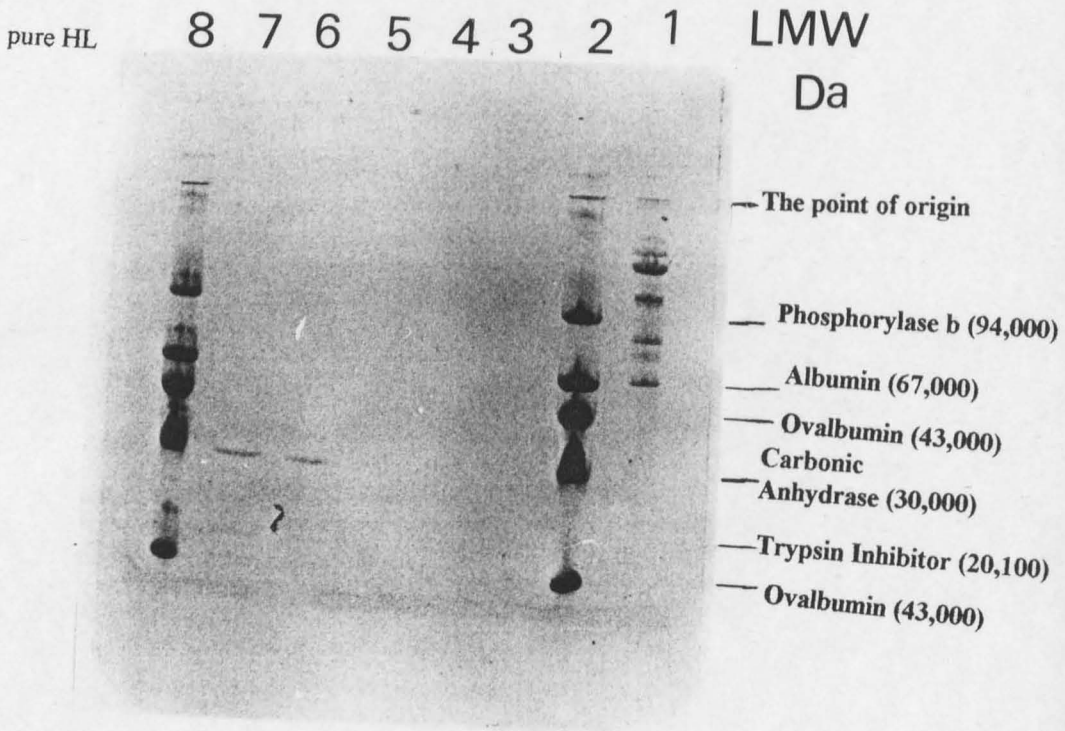


FIG. 3.4

SDS-PAGE separation of pure *Stomoxys* HL sample, LMW and HMW protein standards.



Lane 1 HMW protein standards

Lane 2 LMW protein standards

Lane 3 Adsorbed pure HL (4 times with RBC)

Lane 4 Adsorbed pure HL (4 times with *T. brucei*)

Lane 5 Adsorbed pure HL (5 times with RBC + *T. brucei*)

Lane 6 1/2 pure HL (non-adsorbed)

Lane 7 1/2 pure HL (non-adsorbed)

Lane 8 LMW

a 28,300 Da

b 16,218 Da

c 14,600 Da

FIG. 3.4

SDS-PAGE separation of pure *Stomoxys* HL sample, LMW and HMW protein standards (see facing page).

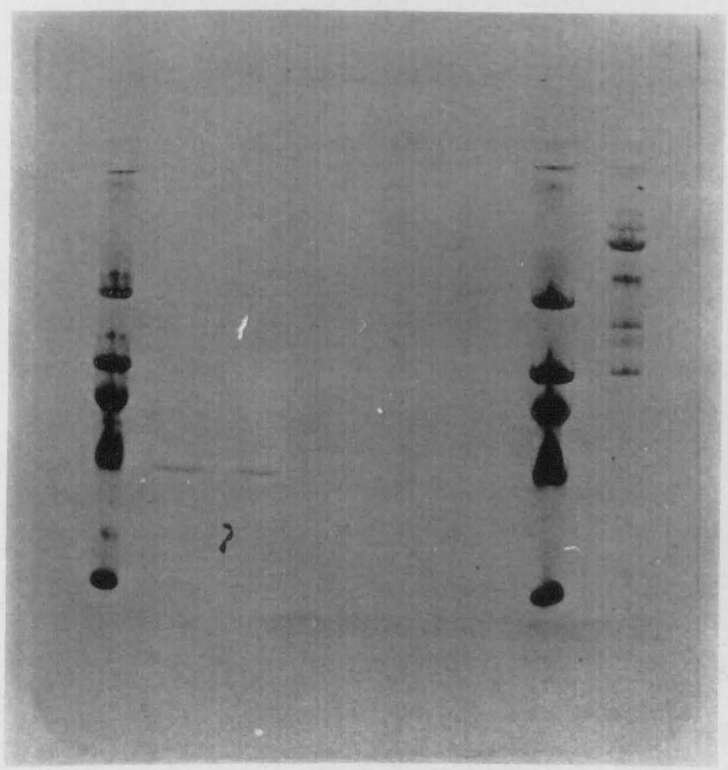
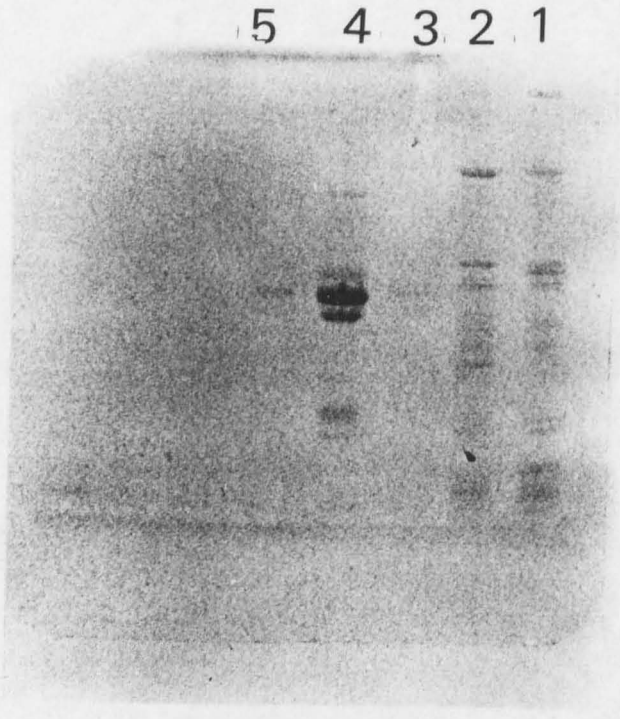


FIG. 3.5

HL SDS-PAGE separation of *Stomoxys calcitrans* haemolymph (HL) and reservoirs extracts RH (200 organs per ml).



Lane 1 undiluted crude RH

Lane 2 1/2 RH crude sample

Lane 3 1/4 RH crude sample

Lane 4 1/4 HL crude sample

Lane 5 1/4 RH crude sample

FIG. 3.5

HL SDS-PAGE separation of *Stomoxys calcitrans* haemolymph (HL) and reservoirs extracts RH (200 organs per ml) (see facing page).

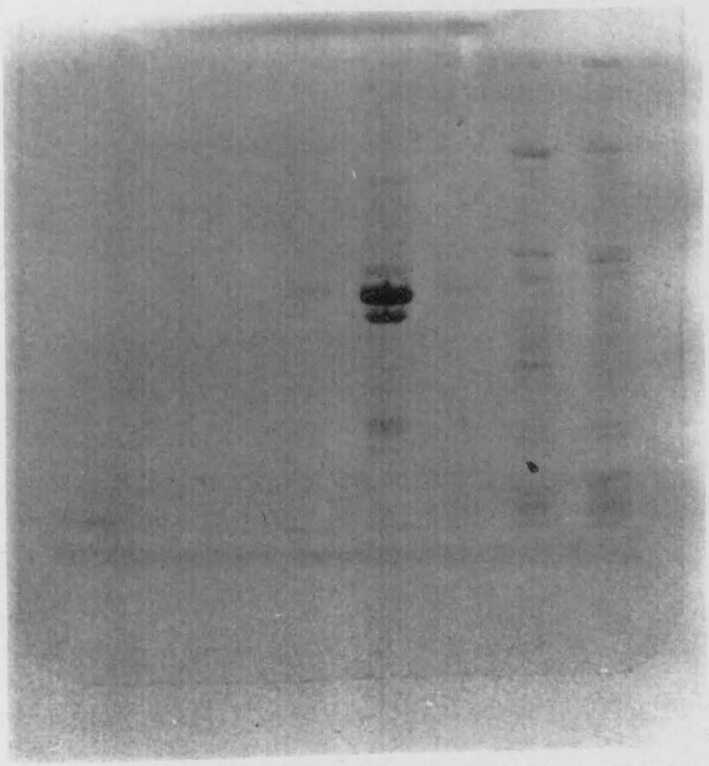
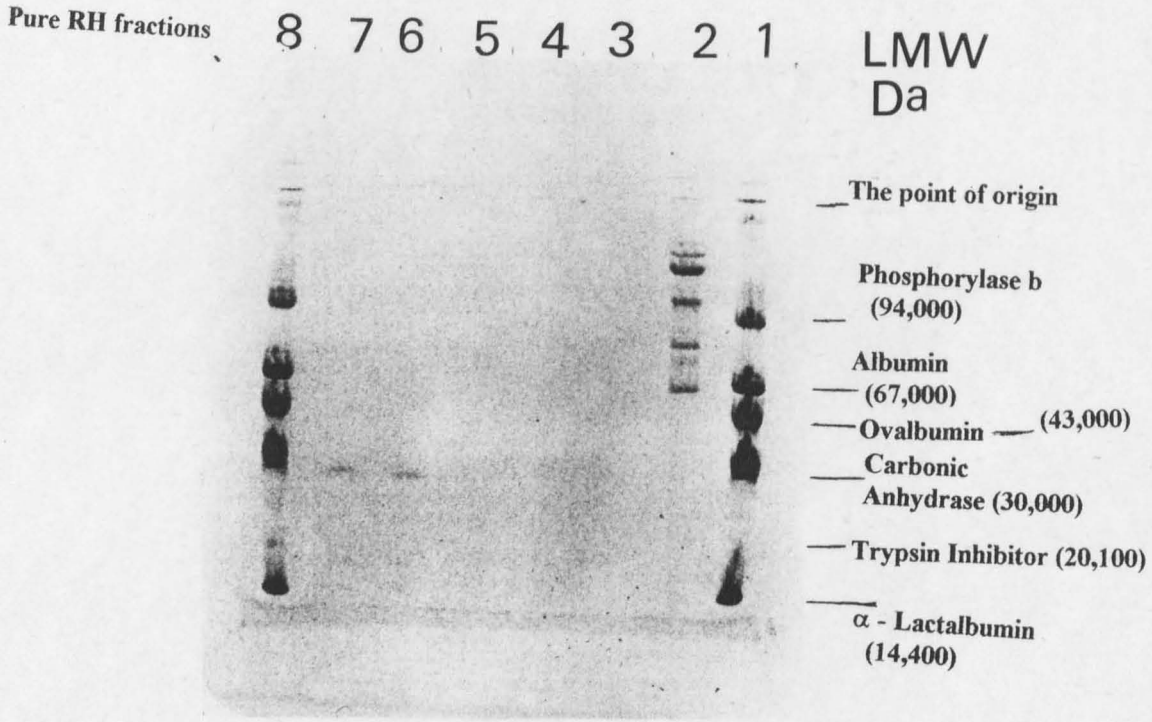


FIG. 3.6

SDS-PAGE separation of HMW and LMW protein standards and *Stomoxys calcitrans* pure RH sample.



Lane 1 LMW standards

Lane 2 HMW standards

Lane 3 Pure HL sample adsorbed by RBC 4 times

Lane 4 Pure HL sample adsorbed by *T. brucei* 4 times

Lane 5 Pure HL sample adsorbed by RBC and *T. brucei* 5 times

Lane 6 1/4 pure HL sample non-adsorbed

Lane 7 1/2 pure HL sample non-adsorbed

Lane 8 LMW standards

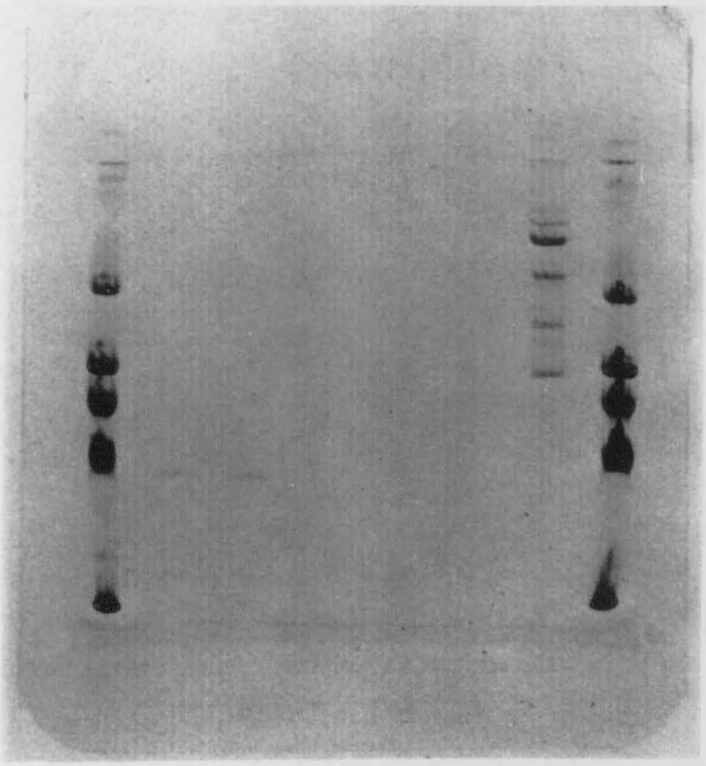
a 26,302 Da

b 16,218 Da

c 14,028 Da

FIG. 3.6

SDS-PAGE separation of HMW and LMW protein standards and *Stomoxys calcitrans* pure RH sample (see facing page).



Chapter 4

Infection of *Stomoxys calcitrans* with *Trypanosoma brucei brucei.*

ABSTRACT

Experiments were carried out to determine the survival rate of *Trypanosoma brucei brucei* trypomastigotes (bloodstream forms) in the midgut of *S. calcitrans* in the presence and absence of specific sugar inhibitors of lectin-mediated haemagglutination. The specific sugar inhibitors used were glucosamine, mannose, melibiose, galactose and N-acetyl-D-glucosamine. Glucose and fructose, non-specific sugars which did not prevent lectin-mediated haemagglutination, were also used. Each fly, irrespective of sex, was fed approximately 0.014 ml of rat blood either on its own, with inhibitor sugar or with sugar and trypanosomes.

Mortality of flies over a 4 day period (age 2 - 6 days) fed on blood with specific sugars was slightly higher than those fed on blood alone or on blood with non-specific sugars. The mortality of flies fed on uninfected blood with or without non-specific sugars was 18.75-20.00%, whilst those fed on uninfected blood with specific sugars was 26.25% (glucosamine), 28.75% (mannose and melibiose), 30.00% (galactose) and 32.50% (N-acetyl-D-glucosamine). The mortality of flies fed on infected blood with or without non-specific sugars was 37.50-38.75%, whilst those fed on infected blood with specific sugars was 82.50% (glucosamine), 78.75% (mannose), 83.75% (melibiose), 87.50% (galactose) and 88.75% (N-acetyl-D-glucosamine).

No parasites were found in the haemolymph or haemocoel. However, the parasites were seen agglutinated in the midgut (reservoir region) on the first day (1-2 hours and 5-7 hours post-infection) and in the morning of the second day in the case of flies fed

on infected blood (with or without non-specific sugars). The parasites were absent from the midgut by the end of the second day in these treatments. In the case of flies fed on infected blood with specific inhibitor sugars, most of the parasites were seen swimming freely in the midgut contents. A little agglutination had occurred by the first observation on the second day and most parasites were agglutinated by the end of the second day. A small number of agglutinated parasites were present on day 3 but were absent from the midgut on day 4.

Smears of gut contents, haemocoel and haemolymph (taken from flies at each observation time) were made. They were fixed and stained (with Giemsa) and examined in order to determine trypanosome occurrence. Parasites were only found in smears of midgut contents of flies 1-2 days post-infection. Parasites were not detected in the smears of haemolymph or haemocoel of any of the infected flies.

INTRODUCTION

Insects display a diverse array of defence mechanisms to counteract the wide spectrum of potential pathogens in the environment. These mechanisms include humoral and cellular immunity (Lackie, 1988). Dipteran haemolymph and midgut extracts both possess anti-parasite agglutinins and haemagglutinins which are in some cases inducible (Ingram and Molyneux, 1991). These agglutinins (lectins) are innate, naturally-occurring molecules which participate in the humoral immunity mechanisms of insects (Olafsen, 1986; Ingram and Molyneux, 1991) and they may play an antibody-like role (Rowley *et al.*, 1986) or may play an opsonic role in phagocytosis (Gupta, 1986).

Many haematophagous insects are vectors of parasites of man and other animals. These parasites cause diseases of medical and economic importance. Interest has therefore focused on insect haemolymph and midgut extract haemagglutinins (lectins) because it was thought that they could exert an influence on host-parasite interactions in the appropriate vectors (Molyneux and Killick-Kendrick, 1987; Ingram and Molyneux, 1991; Maudlin, 1991).

Lectins have been implicated in insect vector-trypanosomatid parasite interactions e.g. in tsetse flies, *Glossina* spp., the natural biological vectors of African trypanosomes. It is thought that both haemolymph and midgut lectins may regulate *Trypanosoma spp.* infections in these vectors thereby potentially influencing vectoral capacity and transmission of trypanosomiasis (Ibrahim *et al.*, 1984; Maudlin and Welburn, 1987

and 1988; Ingram and Molyneux, 1991). Maudlin and Welburn (1988b) reported that the midgut lectin of *Glossina m. morsitans* had a binding specificity for D(+) glucosamine, preventing colonization of the fly midgut by *T. brucei brucei* and *T. congolense.*

African trypanosomes undergo various developmental changes within the mammalian host and the tsetse fly vector (Hoare, 1972; Soulsby, 1986; Kreier and Baker, 1987). In the vector these changes start immediately after ingestion of an infected blood meal, the bloodstream forms being transformed into procyclic forms (Vickerman, 1965; Vickerman and Preston, 1976; Vickerman *et al.*, 1993). This process involves a series of complex morphological and physiological changes enabling the parasite to adapt to the radically-different environment of the fly midgut (see literature review). The transformation process is accompanied by loss of the surface coat, cessation of variable surface glycoprotein (VSG) synthesis, activation of mitochondrial enzymes and the cytochrome electron transport system *etc.* It ends in the formation of the procyclic forms which are characterized by the displacement of the nucleus and kinetoplast (Vickerman and Preston, 1976; Barry and Vickerman, 1979; Roditi and Preston, 1990; Vickerman *et al.*, 1993).

Transformation is the first important step in the establishment of infection in the vector and in the transmission of the trypanosomes from vector to mammalian host. Therefore recent studies have been concerned with investigation of the factors which trigger this transformation process (Bienen *et al.*, 1980; Overath *et al.*, 1983; Maudlin and Welburn, 1988; Durieux *et al.*, 1991; Imbuga *et al.*, 1992; Vickerman *et al.*,

1993). Epimastigote attachment (to the gut and then salivary gland in tsetse fly) was found to be necessary for metacyclic development (Vickerman *et al.*, 1988). Maudlin and Welburn (1987) reported that the establishment of midgut trypanosome infection was prevented by the secretion of tsetse midgut lectin. In another study (1988b), the same workers reported that this lectin was responsible for triggering maturation of procyclic *T. congolense* and *T. brucei*. They also reported that lectin stimulation of midgut trypanosome transformation varied between trypanosome species and between stocks of the same trypanosome species. These variations probably reflect differences in lectin binding site numbers which are determined by trypanosome genotypes

Welburn *et al.*, (1994) reported that midgut infection rates of *T. congolense* in *G. p. palpalis* and *T. brucei rhodesiense* in *G. pallidipes* were potentiated by the addition of D(+)-glucosamine to the infected blood meal (but not to the super-infection). They also reported that *G. m. morsitans*, *G. p. palpalis* and *G. pallidipes* possessed two trypanocidal molecules, a glucosyl-lectin (inhibited by D(+)-glucosamine) and a galactosyl-lectin (inhibited by D(+)-galactose). Addition of D(+)-glucosamine and D(+)-galactose to the teneral fly infective blood meal was therefore seen to promote super-infection of the midgut of *G. p. palpalis*. It was suggested that these glucosyl-lectins and galactosyl-lectins were responsible for the natural agglutination of trypanosomes in the fly midgut. This agglutination was thought to occur when the lectins bound to the procyclic surface coat prior to establishment in the ecto-peritrophic space.

A recent study was carried out by Abubakar *et al.* (1995) in order to determine the properties of blood meal-induced midgut lectin from *G. morsitans* by agglutinating *T. brucei in vitro*. They found that midgut homogenates from flies which had fed twice had the highest agglutination acitivity followed by those which had fed once, followed by the teneral (unfed) flies. A much lower concentration of the midgut homogenate was required for procyclic parasite agglutination compared to bloodstream form parasite agglutination, the process was heat-sensitive and inhibited by D(+)glucosamine. Agglutination of bloodstream form parasites was found to be abrogated by soybean trypsin-inhibitor and the agglutination activity co-eluted with trypsin activity at approximately 50% NaCl. Abubakar *et al.* therefore suggested that a very close relationship exists between the midgut trypsin-like enzyme and the agglutinin (lectin). Since successful agglutination of bloodstream form trypanosomes required protease activity, they concluded that the enzyme may cleave off some parasite surface molecules thereby exposing the lectin-binding sites.

From the above reviews, the role of lectins and the factors influencing tsetse fly infection with trypanosomes can be predicted. This chapter studies the factors influencing infection in stable flies, *S. calcitrans*. It also investigates the development of the parasite and the mortality rate of flies after *in vitro* feeding on the blood of infected white rats (with and without specific inhibitor sugars). It has been mentioned that *S. calcitrans* is a mechanical but not a biological vector of *T. b. gambiensis*, *T. congolense*, *T. b. brucei* and *T. evansi* (Soulsby, 1986; Lehane, 1991). Lectins or lectin-like molecules may influence this refractory status (see Chapter 1). As far as

I know, this is the first report of artificial infection of the stable fly (by using *in vitro* feeding) in order to study the defence mechanisms of this fly.

MATERIALS AND METHODS

Maintenance of the flies:

Flies were maintained in the insect house (as described in Chapter 1).

In vitro method of feeding *S. calcitrans:*

Flies were fed *in vitro* in glass food jars of approximately 250 ml capacity containing 1 - 2 ml pools of blood (see Appendix 4). 16 food jars were cleaned, dried and sterilized in order to minimise contamination of the infected blood. 80 dewinged flies 2-3 days old were put in each jar. The jars were then divided into two groups; A (for uninfected blood) and B (for infected blood).

Determination of blood intake by a fly:

This experiment was carried out in order to determine the amount of blood required for the *in vitro* feeding experiment. Firstly, 40 flies were sexed and divided into 2 groups (males and females). Each fly was put into a 20 ml tube (covered with a holed cap to permit entry of air). Tubes were weighed before and after feeding the flies in order to determine the blood intake for each fly.

Maintaining the parasite:

Bloodstream forms were obtained from an infected animal at the peak of parasitaemia because they are known to change their surface coats during prolonged infection and extended *in vitro* culture (Davidowicz *et al.*, 1975). The parasites were cryopreserved in 10% (v/v) glycerol plus RPMI 1640 (Roswell Park Memorial Institute, 1640 medium) plus HI-FCS (heat-inactivated foetal calf serum) under aseptic conditions (Alafiatayo, 1993 unpublished).

When cryopreserved parasites were required, the ampoules were removed from the liquid nitrogen, thawed in a water-bath (at 37^0C) and slowly diluted with 5 ml of cold culture medium (4^0C) and 10% HI-FCS. Rats were infected intraperitoneally (I.P.) with the recovered parasite suspension diluted with phosphate buffered saline glucose, (PBSG) to give a suspension containing approximately 10^5/ml of parasites as determined using a Neubaeur haemocytometer (American Optical Co., Buffalo, New York). Each rat received 0.1-1.2 ml of this suspension intraperitoneally (by injection to the left side of the flank, ensuring that no organ was inadvertently pierced). The infected animals were maintained in separate cages in an isolation room in the animal house. The animals were observed every day (after a period of 5-7 days) in order to determine the peak parasitaemia at which time exsanguination was performed.

Exsanguination of infected animals:

When infected blood was required, the rats were bled according to Home Office regulations. Each animal was anaesthetized using ether (Analar grade, B.D.H., U.K.) and killed by cervical dislocation. Submersion of the animal in water was carried out to moisten its fur thus preventing loose hairs from contaminating the incision area. The skin was incised in the flank region and deflected to expose the thoracic cavity thus facilitating access to the heart. The infected blood was then drawn aseptically (via a 23G or 25G needle) from the right ventricle into a 2 ml syringe (Beckton-Dickenson Co. Ltd., Ireland) until the flow stopped. The blood was deposited in heparinised collection vials and immediately placed on ice for transportation to the insect laboratory.

Infecting the flies:

Flies were put in jars (80 in each) and divided into 16 feeding groups as shown in Table 1 (see Appendix 4). Sugars were fed to the flies prior to their blood meals. This permitted combination of the sugars with midgut lectins before introduction of blood meals. Each fly was fed approximately 0.014 ml of rat blood according to the following protocol:

TABLE 4.1

Group	Jar No.	Type of Meal (Blood and sugar)	
A	I	Uninfected blood without sugar	
	II	" " with glucose	) non
	III	" " " fructose	) inhibitors
	IV	Uninfected blood with glucosamine	)
	V	" " " mannose	)
	VI	" " " melibiose	) inhibitors
	VII	" " " galactose	)
	VIII	" " " N-actyl-D-glucosamine	)
B	I	Infected blood without sugar	
	II	" " with glucose	) non
	III	" " " fructose	) inhibitors
	IV	Infected blood with glucosamine	)
	V	" " " mannose	)
	VI	" " " melibiose	) inhibitors
	VII	" " " galactose	)
	VIII	" " " N-acetyl-D-glucosamine	)

The flies were then kept in well-illuminated conditions and at a temperature of 26-28^{0}C for the duration of the sampling period (4 days). Each jar was observed about 5-7 times per day and a record of fly mortality kept. Fly midguts were dissected regularly and light microscopy used to detect the presence of parasites. The same procedure was carried out with haemolymph. Smears of gut contents and haemolymph were fixed and stained with Giemsa (see Appendix 4) and examined for trypanosome presence.

Statistics

The means (x) and standard errors (σ, SE) of treated and untreated samples were obtained. They were then analysed using the Student's t-test (Microsoft, Excel 5). See Appendix 1, Table 7 and Table 8.

RESULTS

Measurement of blood intake by weighing flies before and after feeding showed that there was no significant difference in intake between male and female flies (Chi squared test, X^2, $p > 0.05$). The average blood intake was found to be 0.0111g per fly. Allowing for the density of blood a fly therefore requires approximately 0.014 ml of blood. The results are show in Table 4.2.

Parasitaemia in mice and rats was found to be at an optimum 3-4 days post-infection when the number of parasites was 1.5×10^6/ml of blood. Even in the case of the control feeding group (uninfected group) there was fly mortality. In flies fed on normal (uninfected) blood only, mortality was 8.75% by the end of day 2 (increasing to 18.75% by day 4). In flies fed on uninfected blood with glucose or fructose, mortality was 8.75 - 10.0% by the end of day 2 (increasing to 18.75 - 20.0% by day 4). Mortality of uninfected flies increased when they were fed on specific inhibitor sugars (X^2, $p < 0.05$). In the infected groups there was a significant increase in the mortality of flies fed on infected blood alone, infected blood with glucose or fructose (X^2, $p < 0.01$), and infected blood with specific inhibitor sugars (X^2, $p < 0.001$). Statistical analysis of the above data is shown in Appendix 4.

In all flies fed on infected rat blood the wet smears of the midgut contents showed that at the beginning of the first day (1-2 hours post-infection) some parasites were free and others were agglutinated to each other. Motilities of free and agglutinated parasites were still very high. 5-7 hours post-infection, the smears revealed that all

the parasites were agglutinated in flies fed on infected blood alone and in flies fed on infected blood with glucose or fructose. In the morning of the second day a small number of parasites was present in the midgut but all had disappeared by the afternoon. In the case of flies fed on infected blood with specific inhibitor sugars, the parasites were found swimming free in the midgut contents during the first few hours (2-5 hours post-infection). The number of free parasites had decreased slightly at the second (afternoon) observation. The abdomens of the flies were seen to be swollen and their reservoirs were larger than those of flies fed on blood only or on blood with glucose or fructose. This was observed during the first day and the beginning of the second day (morning observation). At the first observation of the second day (morning observation) little parasite agglutination had occurred but most parasites were agglutinated by the end of the day and the swelling of the gut and abdomen decreased. On day 3 the parasites had disappeared in flies which had been fed on infected blood only and on infected blood with glucose or fructose. A small number of agglutinated parasites were found in flies which had been fed on infected blood with inhibitors. On day 4 the parasites had disappeared from all flies and the colour of midgut blood contents of all the flies appeared similar. Parasites were not found in the posterior gut of any flies and the blood contents of the posterior gut were 'chocolate' in colour. Stained slides of the midgut contents revealed bloodstream forms of the parasite (trypomastigotes). Parasites were not detected in the haemolymph or haemocoel of any of the infected flies.

DISCUSSION

Both male and female stable flies, *S. calcitrans*, are haematophagous (blood-sucking) insects. While a single fly can ingest about 25.8 mg blood (Kettle, 1984) under our laboratory conditions flies were found to take a mean weight of blood of 0.0221g (22.1 mg). No significant difference was found in average intake between the sexes (X^2, $p > 0.05$), see Appendix 4. Therefore flies were not sexed prior to the *in vitro* feeding experiment, the aim of which was to investigate the development of the *T. brucei* parasite in the midgut of the stable fly and the role of midgut lectins in this developmental process.

It is known that *T. brucei* is agglutinated by haemolymph (HL) and midgut (MG) haemagglutinins (see Chapter 1). Mshelbwala (1972) investigated *T. brucei* infection in the haemocoel of tsetse flies. He dissected 1,285 flies (consisting of *G. tachinoides, G. morsitans* and *G. palpalis*) in the period 6-80 days after the flies had been fed on *T. brucei*-infected rabbits. He found that 11.58% of flies had mature salivary gland infections and 8.65% of flies had immature infections. Parasites were found in the haemocoel of 40 (15.27%) flies. Despite this no trypanosomes were found in the haemocoel of *S. calcitrans* suggesting an effective barrier is in place. Although this barrier is not disrupted by inhibiting midgut lectins with specific sugar inhibitors the other evidence gathered clearly points to the lectins forming part of this barrier. Parasites ingested with the infected blood by *S. calcitrans* were found in the midgut reservoir region immediately after feeding. Parasites were not observed in any of the samples taken from haemolymph or haemocoel over the 4 day experiment.

In this experiment, parasites were found to be agglutinated in the reservoir region of the midgut of infected flies which had been fed on infected blood only or on infected blood with non-specific inhibitor sugars (glucose and fructose). This agglutination was seen during the first day of infection (in the first observation, 2-5 hours post-infection). In the second observation (afternoon) of the same day, the parasite population in this region had decreased. On the second day, the parasites had disappeared completely from the midgut. Given that parasite agglutination and elimination are postponed by feeding with specific inhibitors, it seems clear that lectins from at least part of an effective barrier system prevent infection of the fly with trypanosomes.

Whereas Mshelbwala (1972) had found well-developed parasites in the haemocoel (thoracic) and leg haemolymph samples (23-63 days post-infection in all three species) of tsetse flies, no parasites were found in the haemocoel and haemolymph samples taken from stable flies in my work. From these results and from knowledge of the trypanosome life cycle in *Glossina* spp. it can be concluded that *S. calcitrans* is not a suitable host for trypanosomes, e.g. *T. brucei*. This is not surprising as *T. brucei* and other related haemoflagellates were found to be agglutinated with samples of *S. calcitrans* haemolymph (HL) and midgut homogenate (MG) (see Chapter 1).

The agglutination activity was inhibited by glucosyl and galactosyl sugars and their related sugar groups (see Chapter 2: inhibition tests). Glucosamine, mannose, melibiose, galactose and N-acetyl-D-glucosamine were found to have the greatest agglutination inhibitory effect. These sugars were fed to the stable flies with *T.*

brucei infective rat blood and uninfective rat blood. A record of fly mortality was kept (see Table 4.2). Flies which fed on blood alone or blood with glucose or fructose showed lower mortality than those which fed on blood with the above-listed specific sugar inhibitors. Mortalities were seen to be greater in flies fed on infected blood with inhibitor sugars than in flies fed on infected blood alone.

From these results it can be concluded that *T. brucei* may be lethal to the stable fly, the parasite's virulence increasing when the flies are fed on inhibitor sugars. Interference between these sugars and the midgut haemagglutinins (lectins) may play a role in the fly's resistance to trypanosomes and other pathogenic organisms.

Insect midgut lectins probably have an additional physiological function (diuresis and digestion), facilitating digestion of the meal and/or excretion of the excessive amount of water from the blood or food. This may explain why inhibition of the specific sugars in the absence of trypanosomes still led to an increase in the mortality of the flies over that in control insects (see Table 4.2). To understand the role of lectins in insect immunity (resistance to infection), it is necessary to look at the work of Maudlin and Welburn (1987).

They found that D(+) glucosamine was a specific inhibitor of midgut lectin agglutination activity against trypanosomes and that, when fed to *G. m. morsitans* with infective meals (*T. congolense* and *T. b. rhodesiense*), it significantly increased midgut infection rates. All flies infected with *T. b. rhodesiense* and maintained on a blood with D(+)-glucosamine diet throughout their lives developed midgut infections.

Maudlin and Welburn (1987) therefore concluded that the susceptibility to trypanosome infection in tsetse flies was mediated through midgut lectins. Perhaps this is also the case for stable flies.

In another study (Welburn *et al.*, 1994), D(+) glucosamine was found to increase or potentiate the infection rates of *T. congolense* in *G. palpalis* and *T. b. rhodesiense* in *G. pallidipes* (but not to the level of super-infection). D(+)-galactose fed to teneral flies (*G. p. palpalis*) with infected blood promoted super-infection of the midgut. From these findings they suggested that the tsetse midgut lectin was responsible for the agglutination of trypanosomes by binding to the procyclic surface coat prior to establishment in the ecto-peritrophic space. The same can be assumed in stable flies as agglutination was inhibited by the glucosamine and galactose sugars mentioned above and the mortality increased when infected flies were fed with these sugars. The increased mortality might be due to interference of these sugars with the function of midgut lectins which may have other physiological roles. The lectins may bind to the sugars instead of to other blood contents and trypanosomes as implicated in the work of Abubakar *et al.*, (1995). They found that agglutination of bloodstream and procyclic forms with midgut homogenates of *G. m. morsitans* was inhibited by D(+) glucosamine. Only bloodsteam form agglutination was abrogated with soybean trypsin inhibitor. In addition they found that agglutination of separated (pure) midgut homogenate (separated by anion-exchange chromatography) co-eluted with trypsin activity at approximately 50% NaCl. They therefore suggested that a very close relationship exists between midgut trypsin-like enzymes and the agglutinins. Successful agglutination of bloodstream form trypanosomes was found to require

protease activity and it was suggested that the enzyme might cleave off some trypanosome surface molecules thereby exposing the lectin-binding sites. A similar increase in lectin efficiency was found with haemolymph (HL) and midgut (MG) lectins in the stable fly (see Chapters 2 and 3). Thus when the flies were fed on blood with specific inhibitor sugars the agglutination of the blood contents was inhibited. The inhibition of agglutination may affect the other physiological (e.g. digestion) and immunological functions of the lectins in the flies, the effects being reflected by an increase in mortality of flies fed with infected blood and specific sugar inhibitors.

TABLE 4.2

The individual intakes and the mean ($\bar{x}$) blood intake (g) ingested by the stable fly *S. calcitrans* in a single meal.

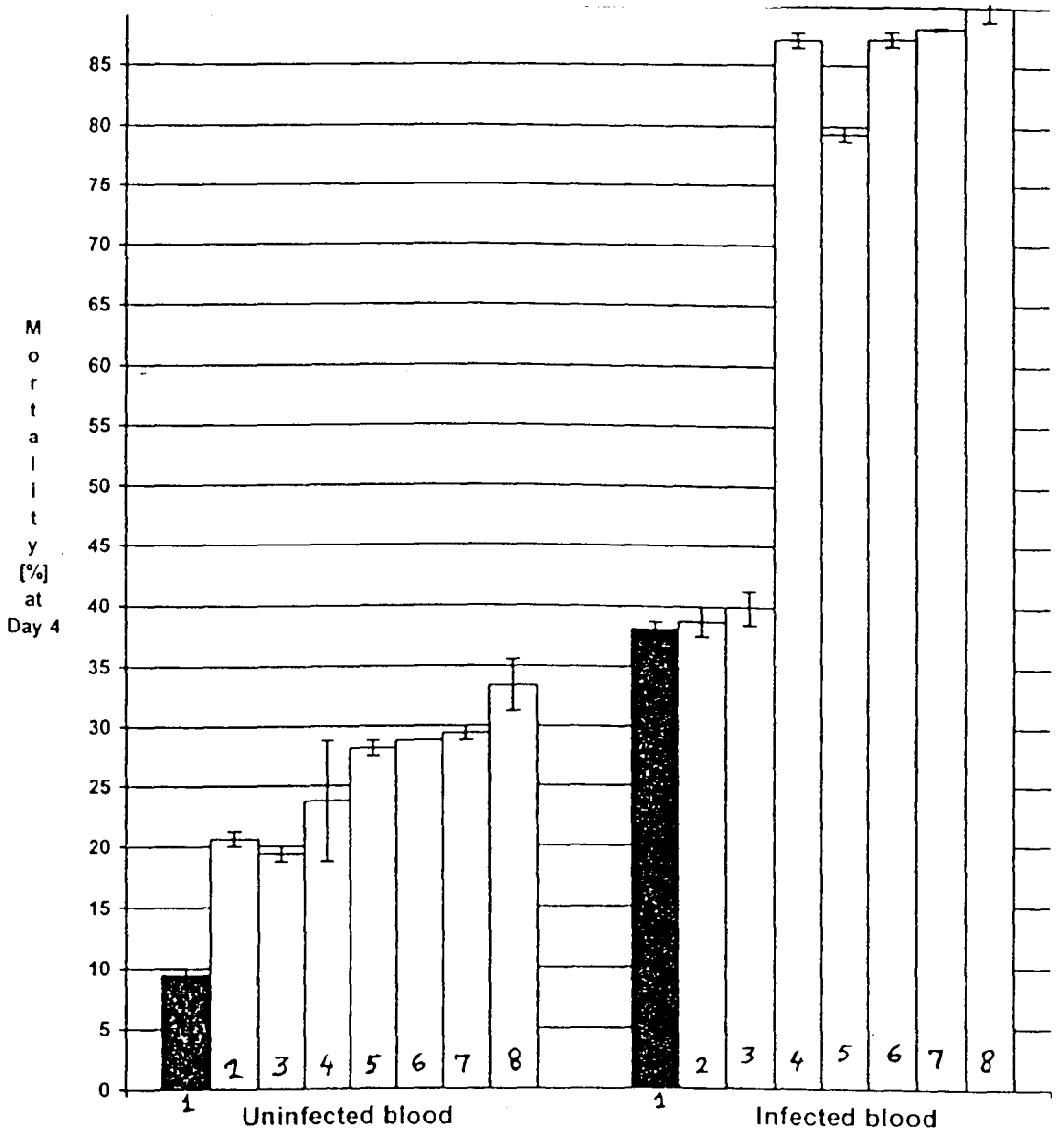
No.	Blood intake in grams		
	Male flies	Female flies	Unsexed flies
1	0.002	0.002	0.003
2	0.002	0.004	0.001
3	0.010	0.001	0.006
4	0.004	0.001	0.005
5	0.003	0.003	0.001
6	0.014	0 022	0.010
7	0.021	0.012	0.001
8	0.014	0.014	0.014
9	0.024	0.025	0.023
10	0.012	0.001	0.020
11	0.016	0.011	0.001
12	0.013	0.018	0.016
13	0.020	0.006	0.009
14	0.002	0.014	0.020
15	0.001	0.007	0.010
16	0.023	0.021	0.011
17	0.010	0.013	0.018
18	0.001	0.004	0.016
19	0.018	0.020	0.001
20	0.002	0.015	0.008
Mean	0.011	0.011	0.010
SD	0.008	0 008	0.007

TABLE 4.3 *Means (x) and standard errors (SE) of Experiments 1 and 2 to show the effect of inclusion and omission of specific or non-specific sugar inhibitors on the % mortalities of stable flies fed uninfected and T. brucei brucei - infected rat blood. Glucose (a), fructose (b), glucosamine (S1), mannose (S2), melibiose (S3), galactose (S4) and N-acetyl-D-glucosamine (S5). (n = 2)* *

	FEED TYPE	DAY 1		DAY 2		DAY 3		DAY 4	
		$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
16	Uninfected Blood only	-	-	8.750	0.000	12.500	0.000	19.375	0.625
15	" Blood + a	-	-	9.375	0.625	12.500	0.000	20.625	0.625
14	" Blood + b	-	-	10.000	0.000	14.375	0.625	19.375	0.625
13	" Blood + S_1	-	-	10.625	0.625	23.125	0.625	23.750	2.500
12	" Blood + S_2	-	-	11.875	0.625	19.375	0.625	28.125	0.625
11	" Blood + S_3	-	-	11.875	0.625	18.750	0.000	28.750	0.000
10	" Blood + S_4	-	-	10.625	0.625	20.000	0.000	29.375	0.625
9	" Blood + S_5	-	-	14.375	0.625	24.375	0.625	33.375	2.125
8	Infected Blood only	2.500	0.000	21.250	1.250	34.375	0.625	38.125	0.625
7	" Blood + a	1.875	0.625	21.250	0.000	34.375	0.625	38.750	1.250
6	" Blood + b	2.500	0.000	23.125	0.625	32.500	1.250	39.875	1.375
5	" Blood + S_1	21.875	0.625	48.125	0.625	48.750	0.000	83.125	0.625
4	" Blood + S_2	22.500	2.500	50.610	0.610	62.500	0.000	79.375	0.625
3	" Blood + S_3	2.500	1.250	53.125	0.625	64.375	0.625	83.120	0.625
2	" Blood + S_4	26.250	0.000	51.875	0.625	68.750	1.250	88.125	0.125
1	" Blood + S_5	28 750	0.625	61.875	0.625	81.875	0.625	90.000	1.250

* This is a result of 2 experiments (See Appendix 4)

FIG.4.1. Effect of inclusion and omission of specific or non-specific sugar inhibitors on the % mortalities of stable flies (*S. calcitrans*) fed uninfected and *Trypanosoma brucei brucei* - infected rat blood.



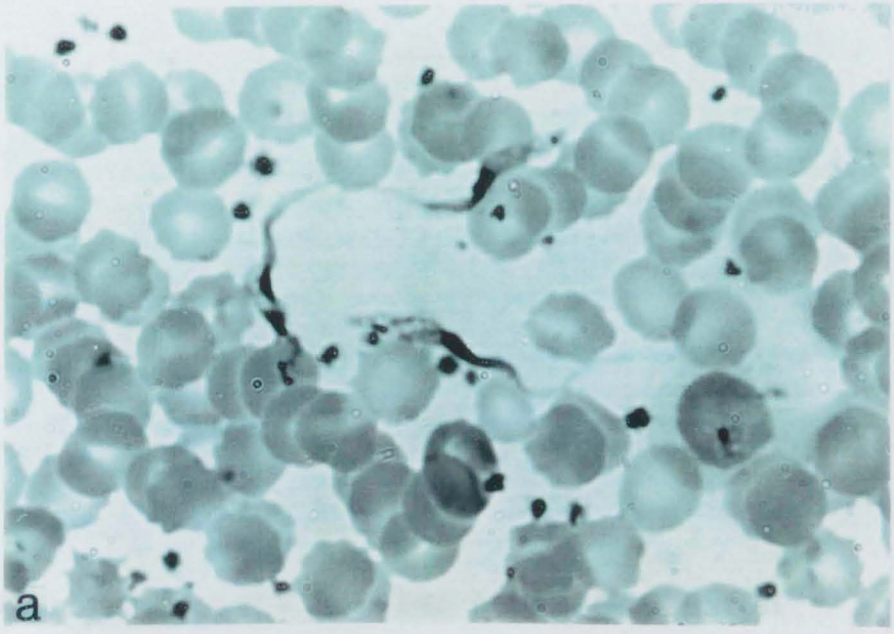
Experimental Groups

1	I Blood alone	I Blood alone
2	II " with glucose	II " with glucose
3	III " " fructose	III " " fructose
4	IV " " glucosamine	IV " " glucosamine
5	V " " mannose	V " " mannose
6	VI " " melibiose	VI " " melibiose
7	VII " " galactose	VII " " galactose
8	VIII " " N-acetyl-D-glucosamine	VIII " " N-acetyl-D-glucosamine

PLATE 2 a and b Giemer stained smears of rat blood infected with *T. brucei*.

a) Smear of the infected blood after being taken from the animal directly.

b) The smear infected blood taken from the reservoirs of some infected blood fed flies with inhibitor (Glucosamine) by the end of the first day of infecting the flies.



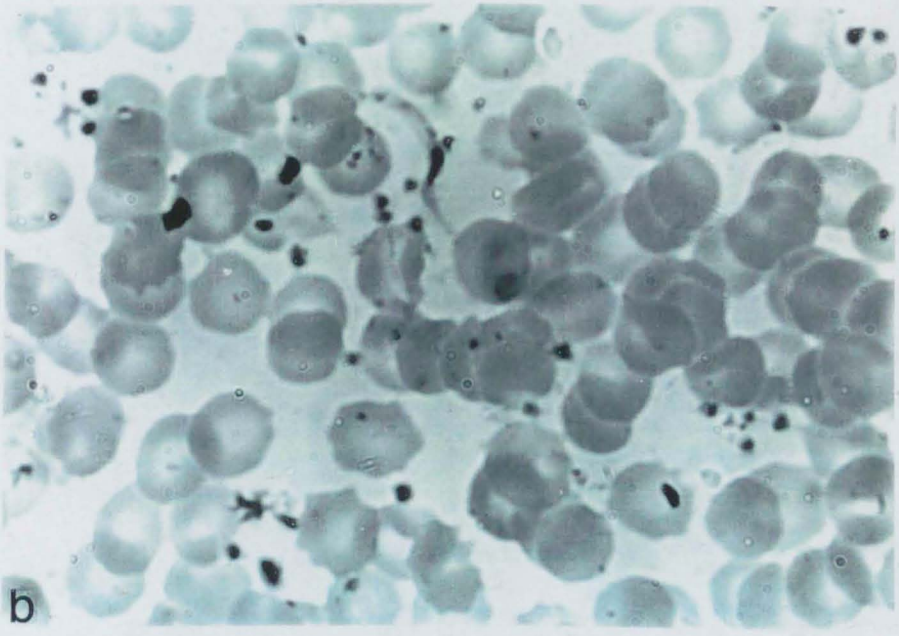


Plate 2

General Discussion and Conclusion

In 1981 Pereira and his co-workers discovered lectins or lectin-like molecules in the crop, midgut and haemolymph of *Rhodinus prolixus*. They found that these lectins were capable of agglutinating *Trypanosoma cruzi* and consequently interest in insect lectins and their role in parasite-vector relationships was stimulated. Croft *et al.* (1982) found good evidence for the production of tsetse lectins or lectin-like substances which were observed to be active *in vitro* against African trypanosomes. However, at this time it was only speculation that the presence of these substances had an effect on the life cycle of the typanosome invading the fly. In 1983 East *et al.* suggested that the haemolymph lectins (agglutinins) prevent invasion of the tsetse haemocoel by *Trypanosoma brucei* infections. However, the precise role of lectins in the fly could not be determined by these *in vitro* studies, (Maudlin and Welburn, 1987).

Ibrahim *et al.* (1984) were the first to report the presence of a glucosamine-binding lectin in the midgut of tsetse flies. Evidence for the possible involvement of this lectin in the establishment of trypanosomes within the vector came later from observation of high midgut infections following an infective blood meal which contained the lectin inhibiting sugar glucosamine (Maudlin and Welburn, 1987). This observation led to the conclusion that the role of lectins was to prevent establishment of parasites within the fly (Maudlin and Welburn, 1987; Osir *et al.*, 1995). The presence of glucosamine in the infective feed relieved the inhibition and thus facilitated the establishment of infection. However, the complicating observation soon arose that lectins were also needed in order for the parasites to differentiate (Maudlin. 1991). Consequently. maintaining flies on a diet incorporating glucosamine led to complete inhibition of

lectin activity and a concomitant reduction in the number of parasites which established themselves within the fly (Maudlin and Welburn, 1988 a and b).

In my work the potential role of lectins in the refractoriness of stable flies, *S. calcitrans*, to trypanosomes was investigated. Although this fly has worldwide distribution and inhabits the same areas as tsetse flies, *Glossina* spp. (Kettle, 1984; Service, 1986; Soulsby, 1986; Lehane, 1991), it only transmits trypanosomes mechanically unlike *Glossina* spp. However, *S. calcitrans* is the biological vector of the nematode *Habronema majus* (a horse stomach worm) and a parasite of cattle, *Setaria cervi* (Kettle, 1984; Soulsby, 1986; Lehane, 1991). It has also been implicated in the transmission of other pathogenic organisms, such as the polio virus, anthrax and fowlpox (Lehane, 1991). What makes *S. calcitrans* a biological vector of the latter organisms but not of the trypanosomes? What are the differences between *Glossina* spp. and *Stomoxys* which make the former susceptible to *Trypanosoma spp.* infection and the latter not susceptible?

Both *Stomoxys* and *Glossina* spp. are haematophagous dipterans and have type II peritrophic membranes (Lehane, 1991). The peritrophic membrane (p.m.) functions as a physical barrier (a primary barrier) separating blood contents and parasites from the midgut epithelial cells (Dunn, 1986; Lehane, 1991). However, Ellis and Evans (1977) reported that trypanosomes could penetrate the peritrophic membrane 9 - 11 days after ingestion of the infective blood. This indicates that the membrane is not a physical barrier to the penetration of the trypanosomes to the haemocoel of infected insects (Lehane and Msangi. 1991). The ability of the trypanosomatids to penetrate

the peritrophic membrane of their vectors may be enhanced by their ability to produce chitinase (Schlein and Jacobson, 1992). Although this may be true in some trypanosomatids, production of such enzymes was found in *T. Lewisi* or *T. brucei* (Arnold *et al.*, 1992).

Lectins are now known to play a very important role as humoral defence factors in the immune mechanisms of insects (Dunn, 1986; Lackie, 1988; Lehane, 1991). They were found in the haemolymph of *G. m. morsitans* and showed anti-parasite agglutination activity against *T. brucei, T. vivax* and *T. congolense in vitro* (East *et al.*, 1983). Agglutination activity was also observed in the haemolymph, mid and hindguts of *G. m. morsitans* and *G. austeni* against *T. brucei* and RBC of several animal species. Lehane and Msangi (1991) found no activity against human RBC group AB Rhesus negative in whole gut extracts of newly-emerged *G. m. morsitans* flies. Only a trace of activity was observed in flies aged 3 - 8 days post-emergence (p.e.) with titres neat -2^{-3}. Flies aged 14 days p.e. showed activity of 50% (2+) and titres ranged neat -2^{-8}. Ingram and Molyneux (1988) found that the same RBC type was agglutinated by MG extracts of *G. m. morsitans* (titre 2^{-7}), *G. p. gambiensis* (titres 2^{-3} - 2^{-5}) and *G. tachinoides* (titre 2^{-3}).

In my work RH extracts from stable flies (*S. calcitrans*) aged 6 hours p.e. showed very weak agglutination activity (trace to neat). This activity increased with age to reach 1+ at 12 hours and 2+ at 1 day p.e. with titres of 2^{-3} - 2^{-4}. It then continued to increase until a titre of 2^{-8} - 2^{-9} was reached at days 6 - 7 p.e. for flies of both sexes.

Gray (1990, unpublished) found that very young *S. calcitrans* flies (0 days) showed activity against rabbit RBC, the highest titres ranging from 2^{-5} - 2^{-7}. The strongest activity was observed in flies aged 4 - 8 days p.e. with titres 2^{-7} - 2^{-8}. Activity decreased slightly when flies reached 12 - 14 days p.e. (titre 2^{-7}). In our work rabbit RBC were found to be agglutinated by RH extracts from unfed flies of both sexes, the activity increasing with age to 2+ to 3+ (neat to 2^{-7}). Comparison of these results with mine revealed some variations despite experimentation with extracts from the same insect species against the same RBC type. The variations might be due to differences in the way the insects were maintained and prepared for dissection. Gray collected flies every 4 days whilst flies [6h, 12h, 1d and 2d p.e. (unfed)] were dissected every day in my work. In addition, Gray fed the insects on 5% glucose solution for 2 days in order to flush out any residual blood from the gut. This procedure could have washed out some of the lectins or diluted them thereby affecting the titres. In my work this flushing out procedure was not performed. However, RH extracts of flies fed on 5% glucose solution in my work showed similar activity against rabbit RBC to that in Gray's study. Unfed flies were dissected immediately after collection whilst fed flies were kept in the laboratory at room temperature for 24 hours after their last feed.

My *S. calcitrans* RH and HL sample results were compared with samples from other insects against various agglutinogens. Ingram and Molyneux (1988) found that MG extracts from *G. tachinoides* and *G. p. gambiensis* [against human RBC(ABO)] caused haemolysis of all the RBC types tested with titres ranging from 2^{-3} to 2^{-5} in the former and from 2^{-5} to 2^{-9} in the latter. No haemagglutination was seen. *G. m. morsitans* lysin activity was reported with titres ranging from 2^{-4} to 2^{-8} and

haemagglutination occurred with RBC group AB. The neat hindgut extracts of the same insect agglutinated RBC group B rhesus positive to a degree of 1+ (1) - 20%) whilst trace (tr) agglutination was observed with group B rhesus negative. Weak agglutination (tr to 1+) was observed with group O rh+ and the remainder of blood types used were agglutinated to a degree of 3+/2+ (75 - 100%). The workers reported that only the hindgut extracts of *G. tachinoides* agglutinated both A rh+ and A rh- [to a degree of 3+ (100%) in the former and trace in the latter].

In my work, only midgut extracts (reservoir region) were generally used as these had been found to exhibit maximum agglutination activity against all blood types and parasites without haemolysis. Rabbit RBC were seen to be the most sensitive cells to agglutination by *Stomoxys* HL and RH samples followed by human RBC group B, human RBC group O and horse RBC. Human RBC groups A and AB and sheep RBC were the least sensitive. Almost all the trypanosomatid flagellate parasites *(T. brucei* bloodstream form and *T. brucei, L. hertigi* and *C. fasciculata* procyclic forms) were seen to be sensitive to agglutination by *Stomoxys* HL and RH samples. The activity started at 6 - 12h p.e. and increased with age until the maximum titre was reached at approximately 2 weeks p.e. It then decreased, returning to the initial level of activity by the end of the 4th week p.e. (in flies fed on whole blood). Flies fed on 5% glucose solution died at the end of the third week p.e.

The range of *S. calcitrans* HL and RH haemagglutination activity titres against human (ABO) RBC and animal RBC is generally much higher than that recorded for other species of adult insects (Scott, 1971; Hapner and Jermyn, 1981; Hapner, 1983). See

Chapter 3, Table 3.1 (a, b and c). Furthermore, similar haemagglutination activity to that observed in *S. calcitrans* in my work was seen in *Glossina* against human (ABO) RBC (Ingram and Molyneux, 1988), *Periplaneta americana* haemolymph against human RBC group O (Lackie, 1981), and *Sarcophaga bullata* haemolymph against human RBC group B (Stynen *et al.*, 1985) and against animal RBC (Lackie, 1981).

It is possible that the human (ABO) RBC (especially group B) and animal RBC (especially rabbit) used in my work may have more exposed cell membranes with larger numbers of reactive surface receptors for the *Stomoxys* haemagglutinins compared with those for haemagglutinins of other insect species. Alternatively, *Stomoxys* HL and RH extracts may contain a large number of haemagglutinin molecules with a high affinity or high degree of combining specificity. The latter might be due to structural variations in the molecules towards the RBC membrane.

In general, no significant difference in agglutination activity titres between the sexes was observed in *Stomoxys* HL and RH samples. The same was true of *Blaberus carnifer* (Donlon and Wemyss, 1976), *Teleogryllus commodus* (Hapner and Jermyn, 1981) and *Glossina* spp. (Ingram and Molyneux, 1988). In contrast, adult female *Melanoplus bivittatus* (Jurenka *et al.*, 1982) and *Sarcophaga bullata* (Stynen *et al.*, 1985) exhibited higher anti-O haemagglutination activity titres than males at certain stages during metamorphosis. In my work, the use of adult *S. calcitrans* eliminated the possibility of results being affected by such potential variations. Adult *Glossina* were used by Ingram and Molyneux (1988) who also found no difference in lectin activity between males and females. This is not surprising as both sexes of the two

dipteran species are haematophagous. However, *Stomoxys* is a facultative blood-sucking insect and *Glossina* is an obligative blood-sucking insect. It is therefore not surprising to find differences in lectin activity between the two species. Agglutination of the parasites (procyclic and bloodstream forms of *T. brucei* and procyclic forms of *C. fasciculata* and *L. hertigi*) by HL and RH samples from both sexes of *Stomoxys* revealed no difference in activity between the two sexes.

The occurrence of non-specific haemolymph agglutinins active against the human (ABO) RBC Rhesus system appears to be common amongst insects (Scott, 1971; Molyneux *et al.*, 1986; Ingram and Molyneux, 1988). However, two exceptions are the gypsy moth, *Lymantria dispar* (Umetsu *et al.*, 1979), and *S. bullata* (Stynen *et al.*, 1985), in which lectins of anti-B specificity and with reactivity towards D-galactose were reported.

The highest haemagglutination activity titres of *S. calcitrans* HL and RH samples were observed with rabbit RBC and human group B RBC (almost identical), followed by horse RBC, human group O, and human group A. The lowest titres were observed with human group AB and sheep RBC. Similar titres were seen with the procyclic forms of *T. brucei, L. hertigi* and *C. fasciculata* and the bloodstream form of *T. brucei*. In view of the diverse array of sugar inhibitors, which prevent agglutination of the various agglutinogens, it is possible that more than one type of agglutinatory molecule (hetero-agglutinins or lectins) may exist in *S. calcitrans* HL and RH samples. These hetero-agglutinins may possess broad specificities and similar agglutination capacities and therefore be capable of combining with a wide range of carbohydrates.

Similar findings were reported in tsetse HL (Ingram and Molyneux, 1988). The high degree of heterogeneity and wide sugar reactivities of *S. calcitrans* lectins seem to depend on the agglutinogen studied. This is comparable with the observations of Ingram and Molyneux (1988) who concluded that the degree of heterogeneity in tsetse might depend on the tsetse species and the RBC types used. This is not surprising since various structural carbohydrate configurations (constituting terminal sequences of oligosaccharide chains) on the RBC surfaces are presented to the lectins (Ingram and Molyneux, 1988).

The ABO(H) blood group antigenic determinants are partly glycosphingolipids (containing sugar moieties of L-fucose, galactose, glucose, N-acetylglucosamine or N-acetylgalactosamine in the case of group A RBC) and glycoproteins (Watkins, 1980). Galactose, N-acetylglucosamine and N-acetylgalactosamine were amongst the sugars which gave the highest degree of RBC and parasite agglutination inhibition in my work with *Stomoxys* HL and RH samples. Fucose caused moderate to weak inhibition. In my work the immunodominant terminal sugar residues N-acetylgalactosamine, D-galactose and L-fucose were found to be amongst the strongest inhibitors of agglutination of many of the blood types and parasites used. The deoxy-form of D-galactose only weakly inhibited agglutination activity in most cases. Ingram and Molyneux (1988), working with *G. p. gambiensis* and *G. tachinoides*, reported that the first three residues were not effective inhibitors of blood group A, B and O agglutination whilst the deoxy-form of D-galactose was a strong inhibitor of group B and AB agglutination. They found that susceptible tsetse species contained anti-erythrocyte midgut lectins which were inhibited by mannose derivatives,

glucose (1,2-) and (1,3-) - linked fructose and suggested that these substances were able to mediate salivarian trypanosomatid flagellate reproduction, stimulating rather than limiting parasite development and subsequent transmission.

Pereira and his co-workers (1981) demonstrated the presence of lectins having distinct reactivity for the surface receptors of *T. cruzi* epimastigotes and selective specificity towards N-acetylmannosamine, N-acetylgalactosamine and β-D-galactose in *R.. prolixus* crop, midgut and haemolymph respectively. Similar results were obtained in our work with *S. calcitrans* pure and crude HL and RH samples in which the parasites (procyclic *T. brucei, L. hertigi* and *C. fasciculata* and bloodstream form *T. brucei)* were agglutinated.

In view of the above findings it seems likely that insect lectins could affect host-parasite interactions by influencing the development of trypanosomatid flagellates within the gut of the insect vector (Molyneux *et al.*, 1981; Ibrahim *et al.*, 1984; Maudlin and Welburn, 1987 and 1988 a and b; Ingram and Molyneux, 1988).

In my work, the only part of the *S. calcitrans* gut to show strong haemagglutination activity against animal RBC and human (ABO) RBC without haemolysis was the reservoir region of the midgut. The thoracic and posterior midgut regions possessed only weak activity (tr at the neat or 2^{-1} occasionally). Posterior midgut extracts caused complete haemolysis of all the RBC tested. Furthermore, dissection of the *S. calcitrans* flies fed on *T. brucei* infected rat blood revealed that the parasites had been digested (the blood changing to a chocolate-like substance). This is perhaps due to

the fact that most of the proteolytic digestive enzymes such as trypsin are found in that part of the midgut. This is not surprising as 95% of midgut trypsin was reported to be located in the posterior midgut (Lehane, 1991).

Ingram and Molyneux (1988) concluded from their tsetse haemagglutinin inhibition studies that the haemagglutinins were heterogeneous with regard to reactivity towards a diverse array of sugar moieties. A similar conclusion can be drawn from our work with *S. calcitrans* pure and crude HL and RH samples with the exception of glucose and its deoxy derivatives. D+ glucosamine, galactose and melibiose were shown to be the most effective inhibitors. This might be the reason for the specificity of *S. calcitrans* HL and RH agglutinins towards galactose and mannose simple sugars and their derivatives or moieties with other combinations. Glucose, fructose and sucrose (found to be non-specific inhibitors of *S. calcitrans* HL and RH agglutination activity against all the RBC types and parasites used) can be obtained naturally by the fly.

Only rabbit RBC were treated with enzymes in my work. Treatment with proteolytic enzymes and glucosidase suggested the presence of potential glycoprotein or glycopeptide membrane receptors on the rabbit RBC cell wall. This is supported by evidence for elevation of pure and crude HL and RH sample activity titres when RBC were treated with neuraminidase and proteolytic enzymes. These enzymes partially digested the membrane integrated proteins or peptides resulting in exposure of potential sugar residues involved in the lectin binding. The removal of N-glycolneuraminyl and N-acetylneuraminyl linkages from the RBC membrane neuraminic acid by neuraminidase would unmask cryptic acid galactosyl and glucosyl

residues present on the RBC surface. Conversely, the use of glycosidase decreased the activity titres. These findings indicate that *S. calcitrans* RH and HL agglutinin specificities are mainly directed towards α-D and/or β-D-galactose residues, but to a lesser degree towards α-D and/or β-D-glucose moieties on the RBC surface. These findings are similar to those of Ingram and Molyneux (1988, 1993) who studied haemagglutination of human (ABO) RBC by tsetse HL. Tsetse HL agglutinins had the same specificities as those found by us in *Stomoxys* except in the case of *G. m. morsitans* and *G. p. gambiensis* anti-O agglutinins. Furthermore, the results of the previous carbohydrate inhibition experiment in our work correlated well with those for the use of glycoside hydrolase-treated rabbit RBC. However, different membrane structural glycoconjugate orientations (caused by varied oligosaccharide chains, glycoproteins or glycolipids of RBCs and the trypanosomatid flagellates used in this work) may be presented in various stereochemical configurations. This could account for the multispecific binding nature of the *S. calcitrans* HL and RH lectins. This finding was also reported by Ingram and Molyneux (1993) in the case of tsetse lectins.

In my work, the limited effects of the nucleases, together with negation or marked reduction in the activity titres after treatment of *S. calcitrans* crude and pure HL and RH samples with phenol, TCA, chloroform, acetone or neuraminidase, suggest that the agglutinins (lectins) are not nucleic acids or polysaccharides. Instead they may be glycoproteins, lipoproteins, lipids or glycolipids. However, treatment of the same HL and RH samples with lipase and organic solvents (xylene, ether and phenol) which cause lipid hydrolysis and inactivation/denaturation respectively, had relatively little effect on agglutination activity. This finding would tend to eliminate a potential

glycolipid or lipoprotein involvement for the activities. Thus the *Stomoxys* HL and RH agglutinins (lectins) are most likely to be protein or glycoprotein in nature. This is supported by evidence for glycosidic moieties in the samples in the results of the sugar inhibition studies coupled with exposure of the samples (pure and crude) to $NaIO_4$ or neuraminidase (which hydrolyses 2-, 3-, 2,5- and 2,8- N-glycolneuraminyl and N-acetylneuraminyl linkages). *Stomoxys* HL and RH (crude and pure) sample agglutination titres were seen to decrease when treated with neuraminidase in all cases (especially anti-rabbit RBC).

Treatment of HL and RH (crude and pure) samples with proteolytic enzymes caused a significant titre reduction in all cases (RBCs and parasites). Both non-specific pronase and relatively-specific pepsin break most peptide linkages, whilst trypsin and chymotrypsin are more specific in their actions. The use of pronase and pepsin reduced the activity titres of both samples but the latter enzyme to a lesser extent in the case of RBCs and parasites. This implies that HL and RH anti-RBC agglutinins and anti-parasite agglutinins possess peptide bonds and are therefore protein in nature. Ingram and Molyneux (1990 and 1993) reported similar findings in the case of tsetse HL haemagglutinins against human (ABO) RBC.

Work with chymotrypsin suggests that *Stomoxys* HL and RH haemagglutinin peptide bonds contain several aromatic amino acid residues. Tsetse HL haemagglutinins (especially *G. m. morsitans* and *G. p. gambiensis* anti-AB RBC) were found to contain basic amino acid groups (lysine and arginine), preferentially split by trypsin. It is not surprising that *Stomoxys* haemagglutinins possess trypsin sensitivity as this property

has been reported for lepidopteran (Suzuki and Natori, 1983), dipteran (McKenzie and Preston, 1992; Ingram and Molyneux, 1990 and 1993) and orthopteran (Hapner and Jermyn, 1981; Stebbins and Hapner, 1985) agglutinins.

The thermolability of *Stomoxys* HL and RH (pure and crude) sample agglutinins (aginst RBC and the trypanosomatid flagellate parasites) with rise in temperature and influence of pH (outside the normal physiological range) on their activities are also indicative of proteins or glycoproteins. Heating *Stomoxys* HL and RH (pure and crude) samples affects the agglutination activity. Increasing the temperature reduces the titres. Boiling the samples at 100^0C almost negates activity. In contrast the tsetse *G. tachinoides* and *G. p. gambiensis* (active against human RBC group O and B respectively) were reported to be affected only slightly after heating the samples at 80^0C (Ingram and Molyneux, 1993) However, most insect HL agglutinins (lectins) are heat-labile (Ingram *et al.*, 1984; Komano *et al.*, 1990; Ingram and Molyneux, 1993).

The pH for maximum activity of *Stomoxys* HL and RH agglutinins aginst RBCs and the trypanosomatid flagellate parasites normally occurred within the range 7.0 - 7.4. Ingram and Molyneux (1993) reported that the HL agglutinin activity of tsetse flies occurred within the range 6.2 - 8.2 and although their HL was slightly acidic the *G. tachinoides, G. m. morsitans* and *G. p. gambiensis* haemagglutinins required pH 5 - 6, 5 - 7 and 7 - 9 respectively for optimum agglutination activity. Perhaps the difference in these pH ranges between tsetse and *Stomoxys* is due to their physiological and feeding habit differences (see the Literature Review). *Stomoxys* can be

maintained on sugar syrup *i.e.* glucose solution (5%) or wet dirty animal excreta as well as on blood sucked from animals. In contrast, the tsetse fly is an obligate haematophage (Soulsby, 1986; Lehane, 1991).

Treatment of *S. calcitrans* crude and pure HL and RH samples with DTT reduced agglutination activity titres (against RBCs and parasites). Urea treatment reduced the activity noticeably. These findings might be due to the fact that DTT splits disulphide linkages and urea splits hydrogen bonds in protein and glycoprotein molecules (Ingram and Molyneux, 1993). Similar observations in tsetse were reported by Ingram and Molyneux (1993). This led them to conclude that tsetse HL haemagglutinins [active against human (ABO) RBC especially groups B and AB in the case of *G. m. morsitans*] might contain larger numbers of sulphur-containing amino acids (forming S - S bonds) relative to the number of H - H bonds. This may be of great significance in the HL haemagglutinin structural configuration. The same conclusion could be drawn for HL and RH agglutinins [active against human (ABO) RBC, animal RBC and parasites] examined in our work.

Treatment of *S. calcitrans* crude and pure HL and RH samples with EDTA (which chelates all divalent cations) and EGTA (which only chelates Ca^{2+} ions) was carried out with or without the addition of excess $MgCl_2$ and/or $CaCl_2$. Results showed that, for optimum activity of HL and RH agglutinins against the RBCs and parasites, Ca^{2+} ions were more of a requirement than Mg^{2+} ions and both *G. tachinoides* and *G. m. morsitans* required Ca^{2+} ions for optimum haemagglutination activity (Ingram and Molyneux, 1993). However, haemolymph Mg^{2+} ion levels in adult dipterans tend to

be higher than those of Ca^{2+} ions (Sutcliffe, 1963). In some insects it was reported that Ca^{2+} ions and/or other divalent metal ions were essential for normal HL haemagglutinin function (Kubo and Natori, 1987; Stebbins and Hapner, 1985) whereas in other insects they are not essential (Ingram *et al.*, 1984; Umetsu *et al.*, 1984). As the *S. calcitrans* HL and RH agglutinins required Ca^{2+} ions for optimum activity, like those of *G. tachinoides* and *G. m. morsitans* HL samples (Ingram and Molyneux, 1993), they may belong to the C-type class of animal lectins, so-termed because of their Ca^{2+} requirement for functional activity (Drickamer, 1988). It is clear that cations (especially Ca^{2+} ions) are essential for the maintenance of structural integrity and activity of the *Stomoxys* HL and RH agglutinin molecules.

The use of purified *S. calcitrans* HL and RH samples means that conclusions regarding the physico-chemical properties of the agglutinins can be drawn with confidence. From my findings, it is highly plausible that these molecules are proteins or glycoproteins in agreement with the findings in other insect studies (Hapner and Jermyn, 1981; Pendland and Boucias, 1986; Richards *et al.*, 1988).

Determination of protein contents of pure and crude HL and RH samples indicated that pure samples had stronger activities than those of crude samples. Although *Stomoxys* crude samples (HL and RH) were found to have lower protein contents than those of *Glossina* spp. (see Chapter 3), it was concluded that *Stomoxys* had stronger agglutination activities than *Glossina* spp. (about 1.5 - 2 times stronger) against RBC and parasites (see Chapter 3, Discussion, p. 170.

Lectins in all HL and RH samples reacted with sugars in my work (see inhibition tests in Chapters 2 and 3) and therefore might react with sugars on the trypanosome surface membrane (Jackson *et al.*, 1983; Mutharia and Pearson, 1987). The lectins might have reacted with procyclic forms of *T. brucei, L. hertigi* and *C. fasciculata* and the bloodstream form of *T. brucei*. This is not surprising as lectins were reported to be involved in the regulation of parasite development within the midgut of the tsetse fly (Welburn and Maudlin, 1987, 1988 a and b, 1990; Maudlin, 1991).

Abubakar *et al.* (1985), studied tetse midgut extract activity against trypanosomes. They reported that when midgut homogenate of *G. m. morsitans* was separated by anion-exchange chromatography the agglutination activity co-eluted with trypsin activity at approximately 50% NaCl. They therefore suggested that there exists a very close relationship between the midgut trypsin-like enzyme and the agglutinins (lectins). They also suggested that the lectin-binding sites might be exposed by the enzyme cleaving off some of the parasite surface molecules as successful agglutination of bloodstream form trypanosomes required protease activity.

In another study, it was found that trypsin or trypsin-like enzymes might be involved with lectin or lectin-like molecules in lysis and differentiation of the parasites (Imbuga *et al.*, 1992). A bifunctional molecule with both trypsin and lectin activities was suggested to be a reconciliation of the two views regarding the role of lectins or trypsins in trypanosome differentiation and lysis (Maudlin, 1991; Imbuga *et al.*, 1992; Osir *et al.*, 1993; Abubakar *et al.*, 1995).

In a later study, the blood-meal-induced lectin isolated from midgut extracts of *G. longipennis*, by a two-step procedure involving anion-exchange chromatography, was found to be a glycoprotein. It had native molecular weight, M_r , 61000 ± 3000 da and was composed of two noncovalently-linked subunits designated α (M_r , 27000 da) and β (M_r , 33000 da). The trypsin and the glycosyl residues were found on the α and β subunits respectively. The native protein was capable of agglutinating both bloodstream form and procyclic trypanosomes as well as rabbit RBC. This activity was seen to be strongly-inhibited by D-glucosamine and weakly-inhibited by N-acetyl-D-glycosamine. It was also found that soya bean trypsin inhibitor abrogated agglutination of bloodstream form trypanosomes but not procyclic trypanosomes. Agglutination activity was seen to be sensitive to temperatures above 40^0C but was not affected by chelators of metal ions (Osir *et al.*, 1995).

These workers used antibodies raised against the protein (the above native molecule) in immunoblotting experiments. They found a similar protein in several *Glossina* species. However, no cross-reactivity was detected with midgut extracts prepared from sandflies, mosquitoes or stable flies. They therefore proposed that this protein might play an important role in differentiation of bloodstream form trypanosomes into procyclic (midgut) forms. This differentiation is accompanied by complex morphological and physiological changes which enable the parasites to adapt to a harsh environment within the fly midgut (Ghiotto *et al.*, 1979; Roditi and Pearson, 1990). The efficiency of the differentiation process appears to be crucial in determining the success of infection establishment within the vector (Osir *et al.*, 1995). Consequently, most studies on tsetse-trypanosome relationships have concentrated on trying to

elucidate the fly midgut factors influencing differentiation of the parasites from bloodstream into procyclic forms (Maudlin, 1991).

It is now generally accepted that lectins mediate both lysis and differentiation of the trypanosomes (Maudlin and Welburn 1987, 1988 a and b; Osir *et al.*, 1995). According to a model, which aims to explain the lectin-mediated establishment of trypanosomes, the action of an endochitinase enzyme produced by relatively large numbers of Rickettsia-like organisms (RLOs) degrades chitin and leads to an accumulation of glucosamine within the midgut of suceptible flies (Maudlin, 1991; Maudlin and Welburn, 1988a and b; Osir *et al.*, 1995). The glucosamine therefore blocks the lectin-mediated trypanocidal activity. On the other hand, refractory flies with few RLOs produce relatively less glucosamine and the parasites entering such flies are affected by the lectins.

Investigation of RLOs in *S. calcitrans* in my work was carried out in order to determine their influence on HL and RH activity. A control sample was obtained from *G. m. morsitans*. The organisms were detected in HL and MG homogenates obtained from unfed flies. No RLOs were detected in *S. calcitrans* samples. This together with evidence for lectin presence in the reservoir of *S. calcitrans* may help to explain their refractoriness to trypanosomes.

In order to draw conclusions regarding the refractoriness of *S. calcitrans* to trypanosomes *in vitro* feeding of the flies with *T. b. brucei*-infected rat blood was carried out (see Chapter 4).

From inhibition test results it was shown that agglutination activity was inhibited by glucosyl and galactosyl sugars and their related sugar groups (see Chapter 2). Glucosamine, mannose, melibiose, galactose and N-acetyl-D-glucosamine were found to be the strongest inhibitors. They were therefore fed to *S. calcitrans* flies with *T. brucei* infective and uninfective rat blood. A control experiment was performed in which flies were fed on blood alone or blood with glucose or fructose. A record of fly mortality was kept. Results showed that flies fed on blood alone or blood with glucose or fructose had lower mortality than those fed on blood with the above-listed specific sugar inhibitors. Mortalities were seen to be greater in flies fed on infected blood with inhibitor sugars than in flies fed on infected blood alone.

From these results it can be concluded that *T. brucei* may be lethal to the stable fly, *S. calcitrans*, the parasite's virulence increasing when the flies are fed on inhibitor sugars. Interference between these sugars and the reservoir haemagglutinins (lectins) may play a role in the fly's resistance to trypanosomes and other pathogenic organisms.

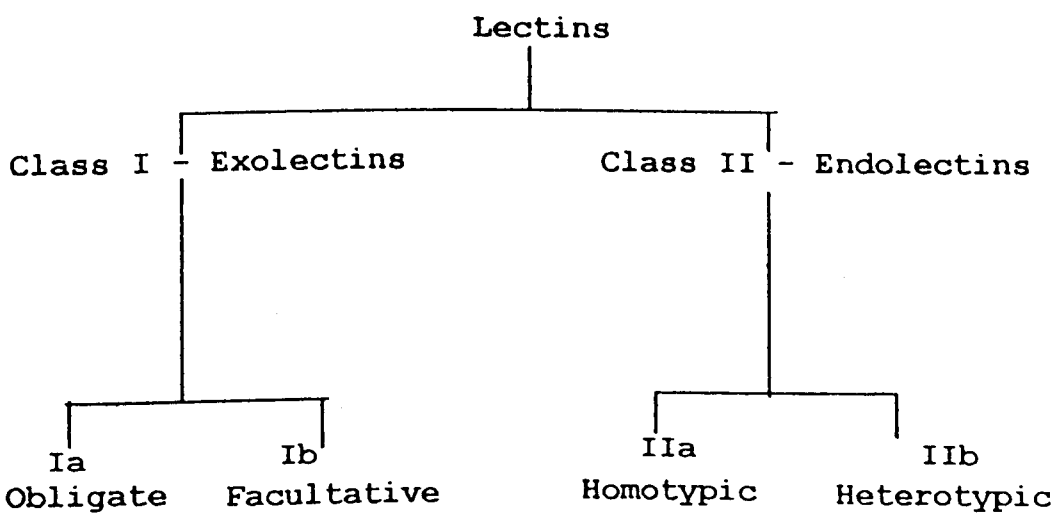
In order to understand the role of lectins in insect refractoriness (resistance) to pathogenic organisms it is necessary to look at the work of Maudlin and Welburn (1987). They found that D(+)glucosamine was a specific inhibitor of midgut lectin agglutination activity against trypanosomes and, when fed to *G. m. morsitans* with infective meals *(T. congolense* and *T. b. rhodesiense*), it significantly increased midgut infection rates. All flies infected with *T. b. rhodesiense* and maintained on a diet of blood with D(+)-glucosamine developed midgut infections. Maudlin and Welburn

therefore concluded that the susceptibility to trypanosome infection in tsetse flies was mediated by midgut lectins. Perhaps this is also the case in stable flies.

In another study (Welburn *et al.*, 1994), D(+)-glucosamine was found to increase or potentiate the infection rates of *T. congolense* in *G. palpalis* and *T. b. rhodesiense* in *G. p. pallidipes* (but not to the level of super-infection). However, D(+)-galactose fed to *G. p. palpalis* teneral flies with infected blood promoted super-infection of the midgut. From these findings it was suggested that the tsetse midgut lectin was responsible for the agglutination of trypanosomes by binding the procyclic surface coat prior to establishment in the ecto-peritrophic space. The same can be assumed in stable flies as agglutination activity with RH extracts was inhibited by glucosamine and galactose and the mortality increased when infected flies were fed with these sugars. The increased mortality might be due to interference of these sugars with reservoir lectin function. As well as causing agglutination, these lectins may have other physiological roles such as elimination of excess water (Gray, 1991) and digestion (Abubakar *et al.*, 1995). The lectins may bind to the sugars intead of other blood contents and parasites as implicated in the work of Abubakar *et al.* (1995). They found that agglutination of bloodstream and procyclic forms with midgut extracts of *G. m. morsitans* was inhibited by D(+)-glucosamine. They also found that only bloodstream form agglutination was abrogated with soya bean trypsin inhibitor. It was suggested that a trypsin-like enzyme with a very close relationship to the midgut agglutinins existed and they concluded that successful agglutination of the bloodstream form trypanosomes required protease activity. It was suggested that the protease might cleave off some trypanosome surface molecules thereby exposing the

lectin-binding sites. This phenomenon was observed with stable fly haemolymph (HL) and reservoir (RH) lectins (see Chapters 2 and 3). Thus, when flies were fed on blood with specific inhibitor sugars, the agglutination of the blood contents was inhibited. This agglutination inhibition may affect other physiological (*e.g.* digestion) and immunological lectin functions in the flies. It is therefore not surprising to find an increase in mortality of flies fed with infected blood and specific sugar inhibitors.

To draw conclusions with confidence regarding the specificities of stable fly lectins one needs to have knowledge of lectin classification. Gallagher (1989) divided the lectins into two classes as shown below:



The class I lectins show primary specificity for a single sugar in a complex oligosaccharide and will always recognize that sugar when it is found at the non-reducing end of a carbohydrate chain, hence the name exolectin. Some exolectins have a mandatory requirement for end-chain sugars and are called the "obligate exolectins" (Class Ia). Others recognize both peripheral and internal sugars and are described as "facultative exolectins" (Class Ib).

The endolectins are an interesting group of proteins which display a more complex mode of carbohydrate binding than the exolectins. They bind to specific carbohydrate sequences but no individual sugar in these sequences plays a predominant role in the binding process. It follows that an interaction with one or more internal sugars is essential for carbohydrate recognition (end-recognition). This class is also divided into two subclasses; the homotypic endolectins (class IIa) which recognize sequences of identical sugar units and the heterotypic endolectins (class IIb) which bind most strongly to sequences composed of two or more different monosaccharides.

From my results and the results of other workers discussed here, the characteristic properties of *S. calcitrans* HL and RH agglutinins can be summarised as follows: They exhibit anti-human (ABO) RBC, anti-animal RBC, and anti-trypanosomatid flagellate activity. They do not appear to be nucleic acids, lipids or glycolipids but are probably glycoprotein or lipoprotein in nature (this conclusion is based on results of treatment of both HL and MG extracts with organic reagents and various enzymes). The agglutinins may be lectins or lectin-like molecules, specific mainly towards galactose and the amino/acetylated derivatives of glucose. However, they are only

specific towards mannosyl moieties on the rabbit RBC surface membrane and are probably only specific towards mannosyl moieties on the human (ABO) RBC and other animal RBC surface membranes. Therefore we conclude that *Stomoxys* is likely to belong to class IIb (heterotypic endolectins).

S. calcitrans HL and RH lectins are glycoprotein in nature (this conclusion is based on the results of phenol, chloroform/ethanol, neuraminidase and $NaIO_4$ treatment of samples). A lipoprotein haemagglutinin active against RBC especially those of the rabbit is implicated (this conclusion is based on lipase, xylene and ether/ethanol treatment of samples). The lectins are thermolabile, freeze/thaw sensitive and possess peptide bonds containing either chymotrypsin-specific amino acid residues or basic amino acids (preferentially split by trypsin). This only occurs in the case of anti-rabbit RBC haemagglutinins in both HL and RH.

Both disulphide and hydrogen bonds occur in the haemagglutinin molecules but not in the anti-parasite agglutinin molecules. The lectins are neutral to slightly alkaline and require divalent cations (mainly Ca^{2+} ions and additionally Mg^{2+} ions in the case of the haemagglutinin molecule).

Reciprocal adsorptions of the samples revealed the presence of more than one type of agglutinin molecule in HL and RH. SDS-PAGE of the purified HL and RH samples showed 3 bands having subunit relative molecular masses of M_r : ~26,300, 16,218 and 14,028 daltons.

Lower haemagglutinin levels were detected in unfed compared to fed stable flies. There were no differences in haemagglutination titres between the sexes although values varied according to whether the insects were fed on glucose or whole blood and which red blood cell agglutinogen was employed. Optimum haemagglutinin activity occurred in blood-fed flies with the use of rabbit red blood cells as indictors of agglutination. Haemagglutinin levels were increased following either glucose or blood meal uptake in both haemolymph and reservoir samples with maxima reached slightly earlier in glucose-fed insects. Levels had declined to pre-fed values about 7 days earlier in glucose-fed compared to the blood-fed flies.

Inhibition of RH haemagglutinin activity with specific sugars resulted in two- to three-fold increases in *S. calcitrans* mortalities post-trypanosome infection compared to the other experimental groups with concurrent decreased detection of midgut parasites. Lower mortalities in the control groups coupled with absence of midgut trypanosomes suggest an agglutinin (lectin) parasite-killing action that would render *Stomoxys* an ineffective vector of trypanosomes.

Suggestions for Further Work

Purification of *Stomoxys calcitrans* HL and RH extracts may be done by high-performance liquid chromatography (HPLC) methods [in particular the lectin affinity chromatography (Gallagher, 1989)]. This, together with electron microscopy, will give clearer information about the immunity of *Stomoxys calcitrans* to trypanosomes.

I was not able to infect animals with *T. brucei* at Bangor because of Home Office regulations. I therefore carried out this experiment at Salford University in which only rats were infected and the flies fed on their infected blood.

If flies had been fed directly on the infected animals this would probably have given more accurate results regarding the natural infection mechanism of the flies and their resistance to it.

A research survey of people infected with trypanosomiasis in Saudi Arabia could be carried out with particular reference to those who come into the Country from infected areas of Africa.

More attention should be paid to investigation of *Glossina* species in Saudi Arabia especially in places inhabited by game animals brought from different parts of the world (especially Africa).

I hope for cooperation between King Faisal University, other Universities and Ministeries in Saudi Arabia and those in the U.K. (especially Schools of Biological Sciences, Medicine and Tropical Medicine and Hygiene) in order to carry out such projects because the government of Saudi Arabia funds and supports workers of such research generously.

BIBLIOGRAPHY

Abubakar L., Osir E.O., Imbuga M.O. (1995). "Properties of blood-meal-induced midgut lectin from the tsetse fly *Glossina morsitans*." Parasitol. Res. 81: pp 271-275.

Aikawa M. and Sterling C.R. (1974) "Intracellular Parasitic Protozoa." New York Academic Press.

Alafiatayo R.A. (1993) "Studies on *Trypanosoma brucei brucei* and Effector/Modulator Substances of The Nerve and Immune Systems." Ph.D. thesis at University of Salford.

Araugo Fausto G., Handman Emanuela and Remington Jack S. (1980) "Binding of lectins to the cell surface of *Trypanosoma cruzi*". J. Protozool. 27(4), pp.397-400.

Arnold K., Gooday G. and Chappell L. (1992) "Chitinases in Trypanosomatidae: a Cautionary Note." Parasitology Today, Vol. 8, No. 8, p. 273.

Ashcroft M.T. (1959) "The Tinde experiment: a further study of the long-term cyclical transmission of *Trypanosoma rhodesiense*". Ann. Trop. Med. Parasitol., 53, pp. 137 - 146.

Azambuja Patriciade, Freitas C. and Garcia S. (1986) "Evidence and partial characterization of an inducible antibacterial factor in the haemolymph of *Rhodinus prolixus*". J. Insect Physiol. Vol.32, No.9, pp.807-12.

Barondes S.H. (1981) "Lectins: their multiple endogenous cellular functions". Ann. Rev. Biochem. 198, 50: pp.207-231.

Barry J.D., Vickerman K. (1979) "Trypanosoma brucei: loss of variable antigenens during transformation from bloodstream to procyclic forms *in vitro*." Exp. Parasitol. 48, pp.313-324.

Bienen E.J., Hammadi E., Hill G.C. (1980) "Initiation of trypanosome transformation from bloodstream trypomastigotes to procyclic trypomastigotes." J. Parasitol.. 66, pp.680-682.

Billingsley P.F. (1994) "Approaches to Vector Control - New And Trusted. 2. Molecular Targets In The Insect Midgut." Transactions Of The Royal Society Of Tropical Medicine And Hygiene, Vol. 88, No. 2, pp. 136-140.

Blakemore D. (1994) "Control of trypsin secretion in *Stomoxys calcitrans*." Ph.D. thesis submitted to the University of Wales Bangor.

Blakemore D., Lehane M.J. and Williams S. (1993) "Cyclic AMP can Promote the Secretion of Digestive Enzymes in *Stomoxys calcitrans*." Insect Biochem. Molec. Biol. Vol.23, No.3, pp.331-335.

Boman H.G. and Hultmark D. (1987) "Cell-free immunity in insects." A. Rev. Microbiol. 41, pp.103-126.

Boyed W.C. (1962) "Introduction to Immunochemical Specificity". Wiley-interscience, New York, pp. 1 - 158.

Boyer R.F. (1993) "Modern Experimental Biochemistry." 2nd Ed. The Benjamin/Cummings Publishing Company Inc., 390 Bridge Parkway, Redwood City, California, U.S.

Bursell E. (1970) "Feeding, digestion and excretion." In: The African Trypanosomiases, ed. H.W. Mulligan, pp. 305-315, New York: Wiley - Interscience.

Buxton P.A. (1955) "The Natural History of Tsetse Flies." Mem. No.10, London School Hyg. Trop. Med, London.

Cheesbrough M. (1991) "Medical Laboratory Manual for Tropical Countries." Vol.1, 2nd.Ed. ed. Monica Cheesbrough, Tropical Health Technology, 14 Bevills Close, Doddington, Cambridgeshire, U.K.

Christensen B.M. and Nappi A.J. (1988) "Immune responses of arthropods." Anim. Plant Sci. 1, pp.15-19.

Croft S.L., East J.S. and Molyneux D.H. (1982) "Anti-trypanosomal factor in the haemolymph of *Glossina*." Acta Trop. 39, pp293-302.

Cunningham I. (1977). "New Culture Medium for Maintenance of Tsetse Tissues and Growth of Trypanosomatids." Protozoology. 24(2), pp.325-329.

Cunningham M.P. and Vickerman K. (1962) "Antigenic analysis in the *Trypanosoma brucei* group using the agglutination reaction." Trans. Roy. Soc. Trop. Med. Hyg., 56, pp. 48 - 59.

Davidowicz K., Hernandez A.G. and Infante R.B. (1975) "The surface membrane of Leishmania. 1. The effects of Lectins on different stages of Leishmania brazilien-sis." J. Parasitology, 61, pp. 950 - 953.

Desowitz R.S. and Watson H.J.C. (1953) "Studies on *Trypanosoma vivax*, IV. The maintenance of a strain in white rats without sheep-serum supplement." Ann. Trop. Med. Parasitol., 47, pp. 62 - 67.

Donlon W.C and Wemyss C.T. (1976) "Analysis of the hemagglutinin and general protein element of the hemolymph of West Indian cockroach, *Blaberus craniifer*." J. Invert. Path. 28. pp. 191 - 194.

Drickamer K. (1988) "Two distinct classes of carbohydrate-recognition domains in animal lectins." J. Biol. Chem. 263. pp. 9557 - 9560.

Dunn P.E. (1986) "Biochemical aspects of insect immunology". Ann. Rev. Ent. 32, pp.321-339.

Dunn P.E. (1990) "Humoral immunity in insects". Bioscience 40, pp.738-744.

Durieux P.O., Schütz P., Brun R., and Köhler P. (1991) "Alterations in Krebs cycle enzyme activities and carbohydrate catabolism in two strains of *Trypanosoma brucei* during *in vitro* differentiation of their bloodstream to procyclic stages." Mol. Biochem. Parasitol. 45, pp. 19 - 28.

East J., Molyneux D.H., Maudlin I. and Dukes P. (1983) "Effect of *Glossina* haemolymph on salivarian trypanosomes *in vitro*." Ann. Trop. Med. Parasitol. 77, pp.97-99.

El-Bihari S. and Kawasmeh, Z.A. (1980) "Occurrence and seasonal variation of some gastrointestinal helminths of the dromedary, *Camelus dromedarius* in Saudi Arabia." Proceedings of the Saudi Biological Society, 4, pp. 297 - 303.

El-Bihari S., Kawasmeh Z.A., Ashour N.A. and El-Naiem A.H. (1984) "Experimental infection of sheep by the camel stomach worm, *Haemonchus longistips.*" Vet. Parasitol. 15, pp. 257 - 261.

Ellis D.S. and Evans D.A. (1977a) "The passage of *Trypanosoma brucei rhodesiense* through the pretrophic membrane of *Glossina palpalis.*" Annals of Tropical Medicine and Parasitology, pp.52, 18.

Ellis D.S. and Evans D.A. (1977b) "The passage of *Trypanosoma brucei rhodesiense* through the peritrophic membrane of *Glossina morsitans morsitans".* Journal of Protozoology, 26: pp.557-563.

Englund P.T., Jadjuk S.L., Marini J.C. (1982) "The molecular biology of trypanosomes." Ann. Rev. Biochem. 51, pp. 695 - 726.

Fo:d (1970) "The geographic distribution of *Glossina.*" In: African Trypanosomiases, ed. H.W. Mulligan, pp.274-297. New York: Wiley - Interscience.

Gallagher J.T. (1989) "Affinity chromatography of complex carbohydrates using lectins." In H.P.L.C. of macromolecules, a practical approach. ed. R.W.A. Oliver, 1st Ed. Oxford University Press, Walton St., Oxford, U.K.

Ghiotto V., Brun R., Jenni L. and Hecker H. (1979) "*Trypanosoma brucei*: morphometric changes and loss of infectivity during transformation of blood stream forms to procyclic forms *in vitro*." Exp. Parasitol., 48, pp. 447 - 456.

Gilboa-Garber Nechama and Sharabi Yedida (1980) "Increase of growth rate and phagocytic activity of tetrahymenal induced by *Pseudomonas* lectins". J. Parasitology 27(2), pp.209-211.

Glasgow J.P. (1970) "The *Glossina* community. In: The African Trypanosomiases ed. H.W. Mulligan, pp. 304-381. New York: Wiley - Interscience.

Goldstein I. and Etzler M.E. (1983) "Chemical taxonomy, molecular biology and function of plant lectins". Pubs: Alan Liss INC, New York.

Gray A.S. (1990) "Early events in blood meal management by *Stomoxys calcitrans* < (Muscidae, Diptera). MSc thesis,U.C.N.W., Bangor.

Grubhoffer Libor and Dusbabek Frantisek (1991) "Lectin binding analysis of *Argas polonicus* tissue glycoproteins". Veterinary Parasitology 38, pp.235-247.

Gupta A.P. (1986). "Haemocytic and Humoral Immunity in Arthropods." J. Wiley, New York.

Hapner K.D. and Jermyn N.A. (1981) "Haemagglutinin activity in the haemolymph of *Teleogryllus commodus* (Walker)." Insect Biochem. 11, pp.287-295.

Hapner K. D. (1983) "Haemagglutinin activity in the haemolymph of individual Acrididae (grasshopper) specimens". Journal of Insect Physiology, 29, pp.101-106.

Hawkey C.M. and Dennett T.B. (1989) "Normal and abnormal blood cells in mammals, birds and reptiles" in "A Colour Atlas of Comparative Veterinary Haematology". Pubs: Wolfe Publishing Limited.

Hoare C.A. (1964) "Morphological and taxonomic studies on mammalian trypanosomes, X. Revision of the systematics." J. Protzool. 11, pp. 200 - 207.

Hoare C.A. (1972) "The trypanosomes of mammals." Blackwell Scientific, Oxford Edinburgh.

Honigberg B.M., Balamuth W., Bovee E.C., Corliss J.O., Gojdics M., Hall R.P., Kudo R.R., Levine N.D., Loeblich A.R.Jr., Weiser J. and Wenrich D.H. (1964) "A revised classification of the phylum *Protozoa*." J. Protozol. 11, pp. 7-20.

Honigberg B.M., Cunningham I., Stanley H.A., Su-Lin K.E. and Luckins A.G. (1976) "*Trypanosoma brucei*: antigenic analysis of blood-stream, vector and culture stages by the quantitive fluorescent antibody methods." Exp. Parasit. 39, pp.496-522.

Hornby H.E. (1952) "African Trypanosomiasis in Eastern Africa." pp. 39. London: H.M.S.O.

Ibrahim E.A.R., Ingram G.A. and Molyneux D.H. (1984) "Haemagglutinins and parasite agglutinins in haemolymph and gut sections of *Glossina*". Trop. Med. Parasit. 35, pp.151-156.

Imbuga M.O., Osir E.O., Labongo V.L., Darji N. and Otieno L.H. (1992). "Studies on tsetse midgut factors that induce differentiation of the bloodstream *Trypanosoma brucei brucei in vitro*. Parasitol. Res. 78, pp.10-15.

Ingram G.A., East J. and Molyneux D.H. (1984) "Naturally occurring agglutinins against trypanosomatid flagellates in the haemolymph of insects." Parsitol. 89, pp. 435 - 451.

Ingram G.A. and Molyneux D.H. (1988) "Sugar specificities of anti-human ABO(H) blood group erythrocyte agglutinins (lectins) and haemolytic activity in Haemolymph and gut extracts of three *Glossina* species." Insect Biochemistry, 18, pp.269-279.

Ingram G.A. and Molyneux D.H. (1990) "Lectins (haemagglutinins) in the haemolymph of *Glossina fuscipes fuscipes*: isolation, partial characterization, selected physico-chemical properties and carbohydrate-binding specificities." Insect Biochem.. 20, pp.13-27.

Ingram G.A. and Molyneux D.H. (1991) "Insect lectins: Role in Parasite-Vector Interactions, pp.103-127." In: Lectin Reviews Vol.1. ed. D.C. Kilpatrick, E. Van Driessche and T.C. Bg-Hansen. Sigma Chemical Comp.

Ingram G.A. and Molyneux D.H. (1993) "Comparative Study of Haemagglutination Activity in the Haemolymph of Three Tsetse Fly *Glossina* Species." Com. Biochem. Physiol. Vol. 106B, No.3, pp.563 -573.

Jackson P.R., Honigberg B.M. and Holt S.C. (1978) "Lectin analysis of *Trypanosoma congolense* bloodstream trypomastigote and culture procyclic surface saccharides by agglutination and electron microscopic techniques." J. Protozool., 25, pp. 471 - 481.

Jennings F.W., Whitelaw D.D., Holmes P.H., Chizyuka H.G.B., Urquhart G.M. (1979). "The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy." Int. J. Parasitol. 9, 381-384.

Jordan A.M. (1978) "Recent developments in techniques for tsetse control." In: Medical Entomology Centenary Symposium Proceedings, ed. S. Willmott, pp. 76-84. London: Royal Society of Tropical Medicine and Hygiene.

Jordan A.M. (1986) "Trypanosomiasis control and African rural development." London: Longman.

Jordan A.M. (1995a) "Tsetse-flies (Glossinidae)." In Medical Insects and Arachnids. Edited by Richard P. Lane and Roger W. Crosskey. 2nd Ed. pp. 333-388.

Jordan A.M. (1995b) "Control of Tsetse-flies (Diptera, Glossinidae) With Aid of Attractants." Journal of the American Mosquito Control Association. Vol. 11, No. 2, Pt 2, pp. 249-255.

Jurenka R., Manfredi Kirk and Hapner K. D. (1982) "Haemagglutinin activity in Acrididae (grasshopper) haemolymph". J. Insect. Physiol. 28, pp.177-181.

Kaaya G.P. (1989) "A review of the progress made in recent years on research and understanding of immunity in insect vectors of human and animal diseases." Insect, Sci. Applic. 10, pp.751-769.

Kaaya G.P. and Darji N. (1988) "The humoral defence system in tsetse: differences in response to age, sex and antigen types." Dev. Comp. Immunol. 12, pp.255-268.

Kaaya G.P., Flyg C. and Boman H.G. (1987). "Insect Immunity: Induction of cecropin and attacin-like antibacterial factors in the haemolymph of *Glossina morsitans morsitans.*" Insect Biochem. 17, pp. 309-315.

Kaaya G.P., Ratcliffe N.A. and Alemu P. (1986) "Cellular and humoral defences of *Glossina* (Diptera; Glossinidae): reactions against bacteria trypanosomes and experimental implants". J. Med. Ent. 23, pp. 30-43.

Kabat E.A. (1971) "Experimental Immunochemistry". 2nd edition Thomas, Springfield, Illinois.

Karp R.D. (1990). "Cell-mediated immunity in invertebrates." Bioscience 40, pp.732-737.

Kettle D.S. (1984) "Medical and Veterinary Entomology". Croom Helm Ltd, Provident House, Burrel Row, Beckenham, Kent BR3 1AT, U.K.

Komano H., Mizuno D., and Natori S. (1980) "Purification of lectin induced in the haemolymph of *Sarcophaga peregrina* larvae on injury. J. Biol. Chem. 255, pp. 2919-2926.

Kreier J.P. and Baker J.R. (1987) "Parasitic Protozoa." Allen & Unwin, Inc., 8 Winchester Place, Winchester, Mass. 01890, U.S.A.

Kubo T. and Natori S. (1987) "Purification and some properties of a lectin from the haemolymph of *Periplaneta americana* (American cockroach)." Eur. J. Biochem. 168, pp.75-82.

Lackie A.M. (1980) "Invertebrate Immunity". Parasitology 80, pp.393-412.

Lackie A.M. (1981). "The specificity of the serum agglutinins of *Periplaneta americana* and *Schistocerca gregaria* and its relationship to the insect's immune response." J.Insect Physiology. 27, pp.139-143.

Lackie A.M. (1982) "Immune mechanisms in insects". Parasitology Today Vol. 4, No.4, pp.98-105.

Lackie A.M. (1988). "Immune Mechanisms in Insects."Parasitology Today Vol.4, No.4 pp. 98-105.

Langley P.A. (1983) "Tsetse flies: vectors of the African trypanosomiases." Biologist 30, pp.281-287.

Lehane M.J. (1976) "Digestive Enzyme Secretion in *Stomoxys calcitrans* (Diptera: Muscidae)." Cell Tiss. Res. 170, pp.275-287.

Lehane M.J. (1991) "Biology of blood-sucking insects". 1st Ed. Berne Convention, Harper Collins Academic, London, U.K.

Lehane M.J. and Msangi A. (1991) "Lectins and peritrophic membrane development in the gut of *Glossina morsitans morsitans* and a discussion of their role in protecting the fly against trypanosome infection". Medical and Vet. Entomology 5, pp. 495-501.

Liener Irvin E., Sharon N. and Goldstein I. J. (1986) "The lectins' properties, functions and applications in Biology and Medicine". Pubs: Academic Press INC.

Maudlin I. (1982) "Inheritance of susceptibility to *Trypanosoma congolense* infection in *Glossina morsitans*". Annals of Tropical Medicine and Parasitology Vol.76, No.2, pp.225-227.

Maudlin I. (1991) "Transmision of African trypanosomiasis: interactions among tsetse immune system, symbionts and parasites." Advances in Disease Vector Research, Vol.7, pp.117-149. Springer, New York.

Maudlin I. and Welburn S.C. (1987) "Lectin mediated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina morsitans*. Tropical Medicine and Parasitology 38: 3, pp.167-170.

Maudlin I. and Welburn S.C. (1988a) "Tsetse immunity and the transmission of trypanosomiasis". Parasitology Today Vol.4, No.4, pp.109-111.

Maudlin I. and Welburn S.C. (1988b). "The role of lectins and trypanosome genotype in the maturation of midgut infections in *Glossina morsitans*. Tropical Medicine and Parasitology 39:1, pp.56-58.

McKenzie A.N.J. and Preston T.M. (1992) "Purification and characterization of a galactose - specific agglutinin from the haemolymph of the larval stages of insect *Calliphora vomitoria*". Dev. Comp. Immunol. 16, 31-39

McKenzie A.N.J., Preston T.M. (1992) "Purification and characterization of a galactose-specific agglutinin from haemolymph of the larval stages of the insect *Calliphora vomitoria.*" Dev. Comp. Immunol. 16, pp.31-39.

Mendez Y. and Honigberg B.M. (1972) "Infectivity of *Trypanosoma brucei* - subgroup flagellates maintained in culture." J. Parasitol. 58, pp. 1122-1136.

Mercado T.I. and Katusha K. (1979) "Isolation of *Trypanosoma cruzi* from The Blood of Infected Mice by Column Chromatography." Preparative Biochemistry, 9(1), pp. 79 - 106.

Mohamed H.A., Ingram G.A. and Molyneux D.H. (1992) "Carbohydrate-binding specificities of anti-erythrocyte lectins (haemagglutinins) in *Anopheles gambiae* gut extracts and haemolymph." 6, pp.217-224.

Molyneux D.H. (1983) "Host-parasite relationships of trypanosomatidae in vectors." In: Current Topics in Vector Research (ed. by Harris K.F.), Vol.1, pp.117-148. Praeger, New York.

Molyneux D.H. and Ashford R.W. (1983) "The Biology of *Trypanosoma* and *Leishmania*." Parasites of Man and Domestic Animals. Tylor and Francis, London.

Molyneux D.H. and Killick-Kendrick R. (1987) "Morphology, Ultrastructure and life cycles of *Leishmania*." The Leishmaniases (ed. by W. Peters and R. Killick-Kendrick), Vol. 1, pp. 121-176.

Molyneux D.H., Takle G., Ibrahim E.A.R. and Ingram G.A. (1986) "Insect immunity to *Trypanosomatidae*". Sym. Zool. Soc., London, 56: pp.117-144.

Molyneux D.H., Stiles J.K. (1991) "Trypanosomatid-vector interactions." Ann. Soc. Belg. Med. Trop. 71, pp.151-166.

Msangi A. R. (1988) "Lectin activity in the gut of *Glossina morsitans morsitans* Wiedemann." Msc Thesis, U.C.N.W., Bangor.

Mshelbwala A.S. (1972) *"Trypanosoma brucei* infection in the haemocoel of tsetse flies." Transactions The Royal Society of Tropical Medicine and Hygiene, 4, pp.637-643.

Murray M., Trail J.C.M., D'leteren G.D.M. (1990) "Trypanotolerance in cattle and prospects for control of trypanosomiasis by selective breeding." Rev. Sci. Tech. of Int. Epiz. 9, pp.369-386.

Muthuria L.M. and Pearson T.W. (1987) "Surface carbohydrates of procyclic forms of African trypanosomes studies using fluorescence-activated cell sorter analysis and agglutination with lectins." Molec. Biochem. Parasitol., 23, pp. 165 - 172.

Natori S. (1990) "Dual functions of insect immunity properties in defence and development." Res. Immunol. 141, pp.938-939.

Neuhoff V., Stamm R. and Eibl H. (1985) "Clear background and highly sensitive protein staining with Coomassie Blue dyes polyacrylmide gels: a systematic analysis." Electrophoresis., 6, pp. 427 - 448.

Olafsen, J.A. (1986) "Invertebrate lectins; biochemical heterogeneity as possible key to their biological function." Immunity in Invertebrates (ed. by M. Brehelin) pp. 94-111, Springer, Berlin.

Osir E.O., Abubakar L., Imbuga M.O. (1995). "Purification and characterization of a midgut lectin - trypsin complex from the tsetse fly *Glossina longipennis*." Parasitol. Res. 81: pp.276-281.

Osir E.O., Imbuga M.O., Onyango P. (1993). "Inhibition of *Glossina morsitans* midgut trypsin activity by D-glucosamine. Parasit. Res. pp.79:93-97.

Overath P., Czichos J., Stock U. and Nonnengaesser C. (1983) "Repression of glycoprotein synthesis and release of surface coat during transformation of *Trypanosoma brucei*." E.M.B.O. J. 2, pp. 1721 - 1728.

Pearlmann E., Slutzky G.M. and Grenblatt C.F. (1982) "Difference in sensitivity to Con A mediated agglutination between strains of *Leishmania*". J. Protozool., Vol.29, No.2, p.316.

Pendland J.C. and Boucias D.G. (1986) "Characteristics of a galactose-binding hemagglutinin (lectin) from hemolymph of *Spodoptera exigua* larvae." Dev. Comp. Immunol. 10, pp. 477 - 487.

Pereira M.E.A., Andrade A.F.B. and Ribeiro J.M.C. (1981) "Lectins of distinct specificity in *Rhodinus prolixus* interact selectively with *Trypanosoma cruzi*". Science, N.Y. 211, pp.597-600.

Pharmacia (1987) "Calibration Kits for Molecular Weight Determination using Electrophoresis." Uppsala, Sweden.

Pierce (1989) "Coomassie Protein Assay Reagent. Instructions." Pierce Chemical Company, P.O. Box 117, Rockford 1L, U.S.A. 61105.

Piller V., Piller F. and Carton J.P. (1990) "Comparison of the carbohydrate-binding specificities of seven N-acetyl-D-galactosamine-recognizing lectins." Eur. J. Biochem. 191, pp. 461 - 466.

Poltera A.A. (1985) "Pathology of human African trypanosomiasis with reference to experimental African trypanosomiasis and infections of central nervous system."

Potts W.H. (1973) "*Glossinidae* (Tsetse-flies)." In: Insects and other Arthropods of Medical Importance, ed. K.G.V. Smith, pp. 209-249. London: British Museum of Natural History.

Richards E.H., Ratcliffe N.A. and Renwrantz L. (1988) "Isolation and characterization of a serum lectin from the giant stick insect *Extatosoma tiaratum*." Insect Biochem. 18, pp.691-700.

Roberts C.J., Gray M.A., Gray A.R. (1969) "Local skin reactions in cattle at the site of infection with *Trypanosoma congolense* by *Glossina morsitans* and *G. tachinoides*." Trans. R. Soc. Trop. Med. Hyg. 63, pp.620-624.

Roditi I., Pearson T.W. (1990) "The procyclin coat of African trypanosomes." Parasitol. Today 6(3), pp.79-82.

Roitt Ivan, Brostoff Jonathan and Male David (1989) "Immunology". Pubs: Gowan Medical Publishing, London, New York.

Rowley A.F., Ratcliffe N.A., Leonard C.M., Richards E.H. and Renwrantz L. (1986) "Humoral recognition factors in insects with particular reference to agglutinins and prophenoloxidase system." Hemocytic and Humoral Immunity in Arthropods (ed. by A.P. Gupta), pp. 381-406, Wiley and Sons, New York.

Rudin W., Schwarzenbach M. and Hecker H. (1989) "Binding of lectins to culture vector forms of *Trypanosoma rangeli* Tejera, 1920 (Protozoa: Kinetoplastida) and to structures of the vector gut". J. Protozool. 36(6) pp.532-538.

Schlein Y. and Jacobson R.L. (1992) "Chitinases in Trypanosomatidae and in *Trypanosoma brucei brucei*. Parasitology Today, Vol. 8, No. 11, p. 367.

Schlein Y. And Jacobson R.L. (1994) "Hemoglobin Inhibits The Development Of Infective Promastigotes And Chitinase Secretion In *Leishmania major* Cultures." Parasitology, Vol. 109, No. 1, pp. 23-28.

Service M.W. (1986) "Lecture note on Medical Entomology". Pubs: Blackwell Scientific Publications, First Edition.

Scott M.T. (1971) "A naturally occurring haemagglutinin in the haemolymph of the American cockroach." Archs. Zool. exp. gen. 112, pp.73-80.

Sharon N. and Liss H. (1972) "Lectins: Cell-Agglutinating and Sugar-Specific Proteins." Science, Vol. 17, No. 4053, pp. 949 - 959.

Sigma (1991) "Concanavalin A from *Canavalia ensiformis* (Jack Bean) (Product No. C2010: Agglutination test)." Sigma Chemical Co., Ltd., Fancy Road., Poole, Dorset BH17 7TG, U.K.

Siraj A. (1985) "Climatological features of Saudi Arabia." Fauna of Saudi Arabia, 6, pp. 32 - 52.

Smyth J.D. (1976) "Introduction to Animal Parasitology." Chapter 5: Haemoflagellates.

Soulsby E.J.L. (1986) "Helminths, Arthropods and Protozoa of Domesticated Animals". 7th edition pubs: Baillier Tindall, London.

Spates G.E. (1981) "Proteolytic and Haemolytic Activity in the Midgut of the Stable Fly *Stomoxys calcitrans* (L.): Partial Purification of the Haemolysin." Insect Biochem. Vol.11, 2, pp.143-147.

Spates G.E. and Deloach J.R. (1980) "Hemolysin of the Stable Fly, *Stomoxys calcitrans*." Insect Biochem. Vol. 67B, pp.121-125.

Stebbins M.R. and Hapner K.D. (1985) "Preparation and properties of haemagglutinin from haemolymph of *Acrididae* (grasshoppers)." Insect Biochem. 15, pp.451-462.

Stynen D., Vansteenwegen K. and De.Loof A. (1985) "Anti-galactose lectins in the haemolymph of Sarcophaga bullata and three other caliphorid flies." Comp. Biochem. Physiol. 81, pp171-175.

Sutcliffe D.W. (1963) "The chemical composition of haemolymph in insects and some other arthropods, in relation to their phylogeny." Comp. Biochem. Physiol. 9, pp.121-135.

Suzuki T. and Natori S. (1983) "Identification of a protein having haemagglutinating activity in the haemolymph of the silkworm, *Bombyx mori*." J. Biochem. 93, pp.583-590.

Trigg P.I. and Guttride W.E. (1977) "Morphological, biochemical and Physiological changes occurring during the life cycles of parasitic protozoa." In - Parasite Invasion, Eds. A.E.R. Taylor & R. Miller, pp. 57 - 81. Oxford: Blackwell Scientific Publications.

Umetsu K., Koasaka S. and Suzuki (1984) "Purification and characterization of a lectin from the beetle, *Allomyrina dichotoma*." J. Biochem. 39, pp.583-590.

Umetsu K., Koasaka S., Kashimura S. and Suzuki T. (1979) "Studies on anti-B specificity and heterogeneity of some invertebrate hemagglutinins." Tohoku J. exp. Med. 129, pp. 161 - 167.

Urquhart G.M., Armour J., Duncan J.L., Dunn A.M. and Jennings F.W. (1987) "Veterinary Parasitology." Longman Scientific & Technical, Longman Group, U.K. Ltd.,

Vickerman K. (1965) "Polymorphism and mitochandrial activity in sleeping sickness trypanosomes." Nature 208, pp.762-766.

Vickerman K. (1966) "Genetic systems in unicellular animals." Sci. Progr., Oxford, 54, pp. 13 - 26.

Vickerman K. (1971) "Morphological and physiological considerations of extracellular blood protozoa." In - Ecology and Physiology of Parasites, Ed. by A. M. Fallis, pp. 58 - 91, Toronto University Press.

Vickerman K. (1972) "The host-parasite interface of parasitic protozoa. Some problems posed by ultrastructural studies." In - Functional Aspects of Parasitic Surfaces, Ed. A.E.R. Taylor & R. Muller, Vol. 10, pp. 71 - 91. Oxford: Blackwell Scientific Publications.

Vickerman K. (1985) "Developmental cycles and biology of pathogenic trypanosomes." Br. Med. Bull. 41, pp.105-114.

Vickerman K., Myler P.J. and Stuart K.D. (1993) "African trypanosomiasis." In - Immunology and Molecular Biology of Parasitic Infections. Ed. by Kenneth S. Warren, Blackwell Scientific Publications, Oxford, London and Edinburgh, U.K.

Vickerman K., Preston T.M. (1976) "Comparative Cell biology of the Kinetoplastid flagellates." In: Lumsden W.H.R., Evans D.A. (eds) Biology of the kinetoplastida, Vol.1. Academic Press, New York, pp.35-130.

Vickerman K., Tetly L., Hendry K.A.K. and Turner C.M.R. (1988) "Biology of African trypanosomes in the tsetse fly." Biol. Cell, 64, pp. 109 - 119.

Wallbanks K.R., Ingram G.A. and Molyneux D.H. (1986) "The agglutination of erythrocytes and *Leishmania* parasites by sandfly gut extracts: evidence for lectin activity." Trop. Med. Parasitol. 37, pp.409-413.

Watkins W. (1980) "Biochemistry and genetics of the ABO, Lewis and P Blood group systems." Adv. Hum. Genet. 10,pp.1-137.

Weitz B.G.F. (1970) "Hosts of *Glossina*." In: The African Trypanosomiases, ed. H.W. Mulligan pp. 317-326. New York: Wiley - Interscience.

Welburn S.C. (1991) "Rickettsia-like organisms (RLOs) in Tsetse Flies; *Glossina* species." Ph.D. thesis submitted at Bristol University.

Welburn S.C. and Maudlin I. (1990) "Haemolymph lectin and the maturation of trypanosome infections in tsetse." Med. Vet. Entomol. 4, pp. 43 - 48.

Welburn S.C., Maudlin I. and Ellis D.S. (1989). "Rate of trypanosome killing by lectins in midguts of different species and strains of *Glossina*. Medical and Veterinary Entomology 1989, 3. pp.77-82.

Welburn S.C., Maudlin I. and Molyneux D.H. (1994) "Midgut lectin Activity And Sugar Specificity In Teneral And Fed Tsetse." Med. Vet. Entomology, Vol.8, No.1, pp. 81 - 87

Whitcomb R.F., Shapiro M. and Grandos R.R. (1974) "Insect defence mechanisms against microorganisms and parasitoids." In: the Physiology of Insecta (Ed. by Rockstein M. Vol.5, pp. 448-537.Academic Press. New York.

Whitelaw D.D., Gardiner P.R., Murray M. (1988) "Extravascular foci of *Trypanosoma vivax* in goats: the central nervous system and aquous humour of the eye as potential sources of relapse infections." Parasitol. 97, pp.51-61.

Yeaton R.W. (1981a) "Invertebrate lectins: I. Occurrence". Develop. Comp. Immunol. 5, pp.391-404.

Yeaton R.W. (1981b) "Invertebrate lectins II: Diversity of specificity, biological synthesis and function in recognition". Develop. Comp. Immunol. 5, pp.535-545.

Yeaton R.W. (1982) "Are invertebrate lectins primordial receptors?" in E.L. Cooper, M.A. Brazier (eds) "Developmental Immunology: Clinical problems and ageing" pp. 73-83. Pubs: Academic Press, New York.

APPENDIX

Determination of the region of the gut which gives the maximum haemagglutination activity.

Tissues from different parts of the *S. calcitrans* gut were homogenized and their haemagglutination activities determined.

Results are shown in Table 1.

TABLE 1 (a).

Mean ($\bar{x}$) agglutination activity titres ($-\log_2 2^{-n}$) of different parts of the *S. calcitrans* gut against rabbit RBC. (Experiments were repeated 6 times and flies reared as described in Chapter 1).

Tissue	Age of the flies					
	≤ 12 h		1 Day		3 Days	
	$\bar{x}$	SE	$\bar{x}$	SE	$\bar{x}$	SE
Fore gut	0.0	0.0	0.0	0.0	1.0	0.0
Whole mid-gut	4 *	0.58	4 *	0.82	8 *	1.30
Reservoir zone only	5.00	1.15	6.00	0.58	10.00	1.16
Hind gut	3 **	0.0	3 **	0.0	6 **	0.77

* **haemolysis occurs after the first 1-2 hours**

** **haemolysis and change of colour**

TABLE 1(b). **Haemagglutination titres in HL and RH samples of *S. calcitrans* against human (ABO) and horse fresh and stabilized RBC (from Sigma)**

RBC	HL		RH	
	Fresh	Stabilized	Fresh	Stabilized
Man A	2^{-8}	2^{-8}	2^{-10}	2^{-9}
Man B	2^{-15}	2^{-14}	2^{-14}	2^{-14}
Man AB	2^{-7}	2^{-8}	2^{-8}	2^{-8}
Man 0	2^{-13}	2^{-12}	2^{-12}	2^{-12}
Horse	2^{-8}	2^{-7}	2^{-12}	2^{-12}
Sheep	2^{-6}	2^{-6}	2^{-7}	2^{-7}

(Each ml of RH contains 40 guts and each 80 l HL was obtained approximately from 40 flies)

TABLE 1(c). **Haemagglutination titres in HL and RH of *S. calcitrans* and fresh normal serum from rabbit, sheep, pig and bovine foetus.** (These experiments were performed with the assumption that the agglutinations were not due to the antibodies present in the blood meal ingested by the flies.)

RBC	Flies HL & RH *		Animal Fresh Normal Sera **			
	HL	RH	Rabbit	Sheep	Pig	Bovine foetus
Man A	2^{-8}	2^{-8}	N.A.	N.A.	N.A.	N.A.
Man B	2^{-14}	2^{-4}	N.A.	N.A.	N.A.	N.A.
Man AB	2^{-8}	2^{-8}	N.A.	N.A.	N.A.	N.A.
Man 0	2^{-12}	2^{-12}	N.A.	N.A.	N.A.	N.A.
Rabbit	2^{-15}	2^{-15}	N.A.	N.A.	N.A.	N.A.
Horse	2^{-9}	2^{-11}	N.A.	N.A.	N.A.	N.A.

* RH = 7.2 mg/ml (obtained from 40 guts)
HL = 32.0 mg/ml (each 80 1 HL obtained from 40 flies)

** Each animal fresh serum was used as 7.2 mg/ml (diluted in PBS)

N.A. No agglutination was seen

Estimation of the emergence (eclosion) percentage.

In order to ensure that all flies used for haemolymph collection or reservoir dissection were of precisely the same age results of Experiment 1 (see Table 2) were used as base information for determination of the factors especially age and sex influencing the agglutination activities.
The instrument used was constructed by myself (see Fig. 2).

TABLE 2. Results of Experiment 1.

Stomoxys pupae collected on 23.7.91 (eggs laid on 5.7.91)
Emergence record of 100 pupae.

Day	Date	No. Emerged
1.00	23.7.91	-
2.00	24.7.91	-
3.00	25.7.91	-
4.00	26.7.91	-
5.00	27.7.91	-
6.00	28.7.91	9.00
7.00	29.7.91	-
8.00	30.7.91	17.00
9.00	31.7.91	6.00
10.00	01.8.91	0
	02.8.91	0
	03.8.91	0

In this experiment we put pupae of various ages in the container to detect the emergence percentage with time. It showed that about 9% of pupae emerged by Day 5 and 17% by days 6 to 7. 44 flies emerged by 6.8.91.

* Eclosion of 100 pupae by 6.8.91 (eggs were collected between 19.7.91 and 22.7.91).
* Fully ecloded flies - 82
  ecloded flies - 3
  "dead" pupae - 15

Results were obtained from the calibrated standard curve (see Materials and Methods, Chapter 3) and were multiplied by the dilution factor of the wells from which the samples had been collected.

TABLE 3. Results of Experiment 2.

Flies emerging from eggs all of which had been laid on the same day.

Eggs laid: 1.8.91

Pupae collected: 23.8.91 (300)

	Date	No. of Flies	%
AUGUST	24th Sat.	4.00	1.33
	25th Sun.	14.00	4.66
	26th Mon.	9.00	5.00
	27th Tues.	67.00	22.33
	28th Wed.	69.00	23.00
	29th Thurs.	59.00	19.66
	30th Fri.	43.00	14.33
SEPTEMBER	31st Sat. 1st Sun.	18.00	6.00
	2nd Mon.	3.00	1.00
	3rd Tues.	0	0
	Total	286.00	
	+dead *	14.00	
	Total	291.00	

* Intact pupae (they were observed for a few days from Sept. 4th until Sept. 14th but no flies emerged).

TABLE 4. Pilot experiment to investigate the time needed for remains of the blood meal to be cleared from the reservoir region (10 flies were dissected in each investigation time)

Investigation time after feeding (hours)	Observations			
	Number of flies dissected	Presence of blood remains	Clearance of remains	Notes
Immediately	10	blood observed in all flies	-	The reservoirs were engorged with whole blood
1	"	"	-	"
4	"	"	-	"
8	"	"	Some of the blood found in all the flies	About 50-60% of ingested blood seen in each fly
10	"	"	"	"
12	"	"	"	"
14	"	5 Flies were seen to have some blood	5 Flies were clear from blood	Flies seen with blood show about 20% of the ingested blood
16	"	2 Flies seen to have blood	8 Flies were seen to be clear from blood	Flies seen with blood show about 10-20% of ingested blood
18	"	2 Flies seen to have some blood	8 Flies were seen to be clear from blood	Flies seen with blood show about 5% of ingested blood
24	"	"	9 Flies were seen to be clear from blood	Flies seen with blood show about 1% of ingested blood
36	"	-	10 Flies clear	No blood found in the reservoirs

TABLE 5. Summary of carbohydrate-binding specificities and nature of insect haemolymph lectins (agglutinins).*

Species	Agglutinogen*	Sugar Specificity	Nature
Diptera			
Sarcophaga peregrina (larvae)	sheep	lactose & D-galactose	protein
S. bullata (all stages)	human A, B, O	α-D-(+)-Melibiose	ND
Calliphora vicina	human A, B, O	D-galactose	ND
Lucilia caesar	human A, B, O	D-galactose	ND
Phormia terraenovae (all larvae)	human A, B, O	D-galactose	ND
Orthoptera			
Teleogryllus commodus (adults)	human A, B, O & 8 vertebrate species	NeuNac, D-GalNAc, D-GlcNaC	protein
Locusta migratoria (larvae)	rabbit & 4 vertebrate species	D-galactosides	ND
Melanoplus sanguinipes (adults)	human O & 4 vertebrate species	D-galactosides, galactosidic & glucosidic oligosaccharides	protein
4 *Melanoplus* spp. (adults)	human A, B, O & 5 vertebrate species	α-linked glucosides & galactosides	ND
M. sanguinipes (larvae & adults)	human A, B, O & 8 vertebrate species	several glycoproteins, mono- and oligosaccharides	ND
16 Acridid species (adults)	human O only	ND	ND
Schistocerca gregaria (adults)	human O & 4 mammalian species	sucrose & stachyose	ND
Extatosoma tiaratum (adults)	human A, B, O & 6 vertebrate species	D-(+)-galactose & lactose	glycoptn

TABLE 5 (continued)

Species	Agglutinogen[a]	Sugar Specificity	Nature
Coleoptera			
Allomyrina dichotoma (all stages)	human A, B, O	lactose & β-linked D-galactose	protein (Allo A-1)
Allomyrina dichotoma (all stages)	human A, B, O	lactose & β-linked D-galactose	protein (Allo A-1)
Leptinotarsa decemlineata (larvae & pupae)	human A, B, O & 4 mammalian species	sulfated polysaccharides	protein
Dictyoptera			
Blaberus craniifer (adults)	rabbit	ND	glycoptn
B. craniifer (larvae & adults)	rabbit, horse & sheep	ND	ND
Leucophaea maderae (adults)	rabbit	ND	protein
Periplaneta americana (adults)	human O & 4 mammalian species	L-rhamnose & D-fucose	ND
P. americana (adults)	sheep & 5 vertebrate species	ND	protein
P. americana (adults)	*Aerobacter aerogenes*	ND	ND
P. americana (adults)	human A, B, O & sheep	D-GalNAc & D-fucose	protein
P. americana (adults)	*Trypanosoma brucei* *Leishmania hertigi* & *Crithidia fasciculata*	ND	glycoptn

TABLE 5 (continued)

Species	Agglutinogen*	Sugar Specificity	Nature
Lepidoptera			
Bombyx mori (larvae)	sheep	glucuronic acid & heparin	glycoptn
Spodoptera exigua (larvae)	human O & rabbit	galactose & its derivatives	glycoptn
Pieris brassicae (larvae)	rabbit	GlcNAc & its derivatives	ND (ecml)
Manduca sexta (larvae)	sheep	glucose	protein
Antheraea pernyi (pupae)	rabbit	α-methyl D-glucoside α galactose derivatives	protein
Hyalophora cecropia (larvae & pupae)	human A, B, O & 7 mammalian species	D-galactose & its derivatives	protein
A. pernyi (larvae & pupae)	human A, B, O & 7 mammalian species	D-galactose	ND
Lymantria dispar (larvae)	human B, AB & rabbit	D-galactose, melibiose, raffinose mainly, plus several other sugars	ND
H. cecropia (larvae & pupae)	human & mammalian	ND	ND
Larval lepidopterans	human A, B, O	ND	ND
Protoparce sexta *Ceratomia undulosa* *Samia cecropia* *Actias luna* *Halisidota tessellaris* *H. caryae* *Datana integgerima* *Sibine stimulae* unidentified caterpillars			

ND, not determined; NeuNAc, N-acetylneuraminic acid; D-GalNAc, N-acetyl-D-galactosamine; D-GlcNAc, N-acetyl-D-glucosamine; glycoptn, glycoprotein; ecml, epidermal cell membrane lectin.
* Agglutinogens are erythrocytes unless otherwise stated.

* Ingram and Molyneux (1991).

TABLE 6. Carbohydrate-binding specificities and agglutination titres of antiparasite and anti-red blood cell lectins (agglutinins) in fresh haemolymph and gut extracts* of *Glossina* spp. (tsetse flies). *

Species	Tissue	Agglutinogen	Titre	Major Sugar Specificities
Glossina morsitans morsitans	hemolymph	human O	2^{-10}-2^{-12}	trehalose, lactose, 2-dGal
		A	2^{-10}-2^{-13}	sorbose, 2-dGal trehalose, turanose, melezitose
		B	2^{-9}-2^{-11}	trehalose, GalNAc, melibiose
		AB	2^{-10}-2^{-12}	glucose, sorbose, 6-dGlc, mannose, fructose, trehalose
	midgut	human O	2^{-4}-2^{-8}(2^{-5}-2^{-8})[b]	ND
		A	2^{-4}-2^{-7}(2^{-6})	ND
		B	2^{-5}-2^{-8}(2^{-5}-2^{-6})	ND
		AB	2^{-5}-2^{-10}(-)	galactose, ManMe ManNAc, ManN, mannoheptulose
		Trypanosoma brucei	2^{-6}-2^{-9}(-)	mannose, lactose, melezitose
	hindgut	human O	2^{-3}-2^{-5}	galactose, GalN, GalNAc, GlcN, GlcMe
		A	2^{-4}-2^{-7}	galactose, mannose, GalNAc, ManNAc, ManN
		B	2^{-1}-2^{-2}	ND
		AB	2^{-4}-2^{-8}	GlcN, GlcA, GlcMe
G. austeni	hemolymph	human O	2^{-7}-2^{-9}	ND
		A	2^{-6}-2^{-9}	ND
		B	2^{-6}-2^{-10}	ND
		calf	2^{-6}	GlcNAc, GalNAc, ManNAc
		guinea pig	2^{-6}	GlcNAc, GalNAc, ManNAc
		chicken	2^{-4}	ND
	midgut	human O	2^{-3}-2^{-4}	ND
		calf & guinea pig	2^{-6}	GlcN
		chicken	2^{-4}	ND
		T. brucei	2^{-7}	GlcN
	hindgut	human O	2^{-5}	ND
		A	2^{-6}	ND
		calf, guinea pig & chicken	2^{-6}	GlcN
		T. brucei	2^{-8}	GlcN

TABLE 6 (continued)

Species	Tissue	Agglutinogen	Titre	Major Sugar Specificities
G. palpalis gambiensis	hemolymph	human O	2^{-10}-2^{-12}	2-dGlc,6-dGlc, 2-dGal,GlcMe, gentiobiose, stachyose
		A	2^{-10}-2^{-12}	2-dGlc, 2-dGal, gentiobiose, stachyose
		human B	2^{-10}-2^{-12}	glucose, 2-dGal, mannose, manno-heltulose, sorbose, 2-dGal, maltose, trehalose, sucrose, turanose
		AB	2^{-10}-2^{-13}	glucose, sorbose 2-dGal, maltose
	midgut	O	-(2^{-9})	ND
		A	-(2^{-7}-2^{-9})	ND
		B	-(2^{-7}-2^{-9})	ND
		AB	-(2^{-5}-2^{-7})	ND
	hindgut	O	-(2^{-3}-2^{-6})	ND
		A	-(2^{-2}-2^{-6})	ND
		B	2^{-1}-2^{-5}(-)	glucose, 6-L-dGal, ManN, GalNAc
		AB	-(2^{-3}-2^{-6})	
G. tachinoides	hemolymph	human O	2^{-11}-2^{-16}	glucose, sorbose, GlcNAc, stachyose
		A	2^{-11}-2^{-16}	sorbose, 2-dGal, maltose, glycogen
		B	2^{-10}-2^{-15}	2-dGal
		AB	2^{-10}-2^{-14}	2-dGal
	midgut	O	-(2^{-2}-2^{-5})	
		A	-(2^{-3}-2^{-5})	
		B	-(2^{-5})	
		AB	(2^{-3}-2^{-5})	
	hindgut	O	-(2^{-3}-2^{-5})	
		A	2^{-5}-2^{-7} (2^{-1}-2^{-2})	6-L-dGal, GalN GalNAc
		B	2^{-4}-2^{-5} (2^{-2}-2^{-3})	2-dGlc, GlcN, GalN, GalNAc, chitin
		AB	-(2^{-2}-2^{-5})	

TABLE 6 (continued)

Species	Tissue	Agglutinogen	Titre	Major Sugar Specificities
G. fuscipes fuscipes	hemolymph	human O	2^{-11}-2^{-16}	ribose, 2-dGal, 2-dGlc, 6-dGal, GlcN, GalN, sucrose, melibiose, melezitose
		A	2^{-12}-2^{-15}	ribose, 2-dGal, GalNAc, sucrose, xylan, melibiose
		B	2^{-11}-2^{-16}	L-xylose, 2-dGal, GalN, sucrose, xylan, melibiose
		AB	2^{-14}-2^{-18}	L-xylose, ribose, 2- and 6-dGlc, GalNAc, ManNAc, melibiose, raffinose
	midgut	O	-(2^{-2}-2^{-5})	
		A	-(2^{-2}-2^{-5})	
	midgut	B	-(2^{-2}-2^{-4})	
		AB	-(2^{-8}-2^{-9})	
	hindgut	O	2^{-4}	ND
		A	2^{-4}	ND
		B	2^{-4}	ND
		AB	2^{-8}	2-dGal, ManN, ManNAc, GalNAc

Abbreviations and symbols: 2-dGal, 2-deoxygalactose; GalNAc, N-acetyl-D-galactosamine; 6-dGlc, 6-deoxyglucose; ManNAc, N-acetyl-D-mannosamine; ManMe, methyl mannoside; ManN, mannosamine; GalN, galactosamine; GlcN, glucosamine; GlcMe, methyl glucoside; GlcA, gluconic acid; GlcNAc, N-acetyl-D-glucosamine; 2-dGlc, 2-deoxyglucose; 6-L-dGal, 6-deoxy-L-galactose; ND, not determined; -, negative.

[a] Titers based on 100 hind- or midguts per ml diluent buffer.

[b] Hemolysin levels given in parentheses.

* Ingram and Molyneux (1991).

TABLE 7. Effect of inclusion and omission of specific or non-specific sugar inhibitors on the % mortalities of stable flies fed uninfected and *T. brucei* Experiment 1. *brucei* infected rat blood. Glucose (a), fructose (b), glucosamine (S1), mannose (S2), melibiose (S3), galactose (S4) and N-acetyl-D-glucosamine (S5).

Food Type	DAY 1				DAY 2				DAY 3				DAY 4			
	Reading			Mort. % R_3/80	Reading			Mort % R_3/80	Reading			Mort. % R_3/80	Reading			Mort.% R_3/80
	R_1	R_2	R_3		R_1	R_2	R_3		R_1	R_2	R_3		R_1	R_2	R_3	
Uninfected Blood only	-	-	-	-	6	6	7	8.75	9	9	10	12.50	15	15	16	20
" Blood + a	-	-	-	-	5	6	8	10	9	9	10	12.50	15	15	17	21.25
" Blood + b	-	-	-	-	6	6	8	10	8	8	12	15	16	16	16	20
" Blood + S_1	-	-	-	-	6	6	8	10	10	13	19	23.75	13	14	17	21.25
" Blood + S_2	-	-	-	-	7	8	9	11.25	12	14	15	18.75	19	19	22	27.50
" Blood + S_3	-	-	-	-	5	6	10	12.50	12	13	15	18.75	17	18	23	28.75
" Blood + S_4	-	-	-	-	6	7	9	11.25	11	14	16	20	18	20	23	28.75
" Blood + S_5	-	-	-	-	5	8	12	15	14	14	20	25	22	25	25	31.25
Infected Blood only	1	1	2	2.50	6	9	18	22.50	21	22	28	35	29	30	31	38.75
" Blood + a	-	1	2	2.50	5	12	17	21.25	23	25	27	33.75	31	32	32	40
" Blood + b	1	1	2	2.50	3	10	19	23.75	20	23	25	31.25	29	33	33	41.25
" Blood + S_1	-	2	17	21.25	26	29	39	48.75	35	37	39	48.75	55	59	67	83.75
" Blood + S_2	1	3	16	20	36	41	41	51.22	42	48	50	62.50	60	60	64	80
" Blood + S_3	1	1	21	26.25	40	41	43	53.75	50	50	52	65	60	66	66	82.50
" Blood + S_4	1	2	21	26.25	30	38	41	51.25	51	54	54	67.50	59	69	71	88.75
" Blood + S_5	1	3	23	28.75	29	38	50	62.50	59	61	66	82.50	70	72	73	91.25

R1 is the reading taken at ~ 9.00 a.m.
R2 is the reading taken at ~ midday
R3 is the reading taken at ~ 5.00 p.m.
Where R = total number of dead flies at a reading time + that of the previous time
% mort. for any day = $\frac{R3 \text{ (for that day)}}{80} \times 100$

TABLE 8. Effect of inclusion and omission of specific or non-specific sugar inhibitors on the % mortalities of stable flies fed uninfected and *T. brucei brucei* infected rat blood. Glucose (a), fructose (b), glucosamine (S1), mannose (S2), melibiose (S3), galactose (S4) and N-acetyl-D-glucosamine (S5).

Experiment 2.

Food Type	DAY 1				DAY 2				DAY 3				DAY 4			
	Reading			Mort. % R_3/80	Reading			Mort. % R_3/80	Reading			Mort. % R_3/80	Reading			Mort. % R_3/80
	R_1	R_2	R_3		R_1	R_2	R_3		R_1	R_2	R_3		R_1	R_2	R_3	
Uninfected Blood only	-	-	-	-	-	5	7	8.75	8	9	10	12.50	14	14	15	18.75
" Blood + a	-	-	-	-	6	6	7	8.75	7	8	10	12.50	13	15	16	20
" Blood + b	-	-	-	-	5	5	8	10	8	8	11	13.75	12	14	15	18.75
" Blood + S_1	-	-	-	-	5	6	9	11.25	10	15	18	22.50	18	18	21	26.25
" Blood + S_2	-	-	-	-	4	7	10	12.50	11	14	16	20	16	17	23	28.75
" Blood + S_3	-	-	-	-	5	6	9	11.25	12	14	15	18.75	18	20	23	28.75
" Blood + S_4	-	-	-	-	5	7	8	10	10	16	16	20	19	19	24	30
" Blood + S_5	-	-	-	-	5	8	11	13.75	14	14	19	23.75	20	24	26	32.50
Infected Blood only	-	1	1	2.50	5	9	16	20	24	25	27	33.75	29	30	30	37.50
" Blood + a	-	-	1	1.25	5	12	17	21.25	26	26	28	35	29	29	30	37.50
" Blood + b	-	-	2	2.50	3	10	18	22.50	19	24	37	33.75	28	28	31	38.75
" Blood + S_1	-	-	18	22.50	28	30	38	47.50	36	36	39	48.75	50	58	66	82.50
" Blood + S_2	-	-	25	31.25	36	40	40	50	42	48	50	62.5	55	64	63	78.75
" Blood + S_3	-	-	19	23.75	39	40	41	51.25	49	50	51	63.75	59	62	67	83.75
" Blood + S_4	-	2	21	26.25	30	39	42	52.50	54	55	56	70	58	65	70	87.50
" Blood + S_5	-	-	22	27.50	29	39	49	61.25	56	59	65	81.25	70	70	71	88.75

R1 is the reading taken at ~ 9.00 a.m.
R2 is the reading taken at ~ midday
R3 is the reading taken at ~ 5.00 p.m.
Where R = total number of dead flies at a reading time + that of the previous time
% mort. for any day = $\frac{R_3 \text{ (for that day)}}{} \times 100$