ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF TRANSFERRIN FROM TSETSE FLY, *Glossina morsitan centralis.*

BY

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A thesis submitted in partial fulfilment of the requirement for the degree of Master of Science in Biochemistry at Egerton University.
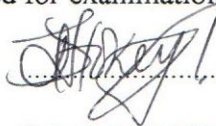
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DECLARATION

This thesis is my original work and has not been presented to any other University.

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This work is dedicated to my beloved parents, brothers and sisters.

ACKNOWLEDGEMENT

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ABBREVIATIONS

2-D	Two dimensional
BPB	Bromophenol blue
BSA	Bovine serum albumin
CASMAC	cascade mode multi affinity chromatography
CD	Circular Dichroism
Con A	concanavalin A
DPP	dipicolylamine
EDTA	ethylene diamine tetra-acetic acid
IEF	isoelectric focussing
IMAC	immobilised metal ion affinity chromatography
IRE	Iron responsive element
IRE-BP	Iron responsive element binding protein
KBr	Potassium bromide
PAGE	polyacrylamide gel electrophoresis
PAS	periodic acid schiff reagent
PAS	periodic acid solution
PBS	phosphate buffered saline
PI	isoelectric point
PMSF	Phenyl methyl sulfonyl fluoride
RHBP	*Rhodnius* heme binding protein
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
STD	standard
TRIS	Tris-hydroxy methyl amino methane
V/V	volume by volume
W/V	weight by volume
W/W	weight by weight

UNIT ABBREVIATIONS

μg   microgram

g   gram

h   hour

kDa   kilodaltons

km   kilometre

M   molar concentration

mA   milliamperes

mg   milligram

Min   minute

ml   millilitre

μl   microlitre

mM   millimolar

M_r molecular weight

nm   nanometre

pH   -log [hydrogen ion concentration]

pI   isoelectric point

rpm   revolutions per minute

sec   second

LIST OF FIGURES

ABSTRACT

Tsetse flies are biting insects that transmit trypanosomiasis to man and domesticated animals. Trypanosomiasis is commonly known as Nagana in cattle, Surra in camels and Sleeping sickness in humans. No sustainable control methods for this disease are currently available and yet in tropical Africa it is one of the most important vector-borne disease of medical and veterinary importance.

Tsetse flies ingest large amounts of blood from their hosts and therefore face an oxidative challenge due to iron release. Iron binding proteins are known to play a major role in controlling this toxicity by regulating iron uptake, transport and storage. Understanding how this detoxification mechanisms work can be very important in designing novel control strategies.

This study focused on purification and biochemical characterization of transferrin from *Glossina morsitan centralis*. To achieve these goal, a number of experiments were carried out and this included screening the hemolymph and midgut samples for iron binding proteins using Ferene S (0.75mM 3-[2-Pyridyl]-5,6-bis (2-[-Furyl sulfonic acid])-1,2,4-Triazine, 2% acetic acid, 0.1% thioglycollic acid). Ferene S staining of hemolymph samples on polyacrylamide gels indicated presence of two distinct iron binding proteins, one of high molecular weight (490 kDa) and the other of low molecular weight (140 kDa). However, midgut samples did not stain significantly for iron binding proteins. The first step of transferrin purification from the tsetse hemolymph involved KBr density gradient ultracentrifugation. This was followed by preparatory gel electrophoresis and electroelution of the lower molecular weight Ferene S stained iron binding protein. The electroeluted protein was applied on Concanavalin A affinity column and the bound fraction eluted with α-methyl-D-mannopyranoside. This fraction was finally subjected to 95% ammonium sulphate precipitation. The homogeneity of the purified protein was

confirmed by silver stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional (2-D) gel electrophoresis. Separation on SDS-PAGE indicated that the protein had an approximate molecular weight of 80 kDa. This indicates conservation in terms of size as it had been shown by earlier studies that 121 amino acid residues are conserved in vertebrate, cockroach and *Manduca sexta* transferrin. However native gels indicated a higher molecular weight of approximately 140 kDa and this difference was attributed to the structure and charge properties of the protein in the native state. The isoelectric point of transferrin was estimated at 6.5 and this is consistent with previous studies that have shown that insect hemolymph proteins have a pI of 4 - 8. Staining with Periodic acid Schiff reagent and binding to con A affinity column revealed that transferrin was glycosylated and of high mannose type, respectively. Absorption spectroscopy spectrum of transferrin showed two maxima (565,370 nm) and a weak absorption at 280 nm.

Key words: *Transferrin, Iron binding, Glossina morsitan centralis.*

1.0 INTRODUCTION

Tsetse flies are the disease vectors of trypanosomes the protozoan parasite that causes trypanosomiasis to man and his domestic animals. The disease is caused by different species of the protozoan trypanosomes whose distribution coincides with the distribution of tsetse fly of *Glossina* species. In the tsetse host, parasites undergo part of the development cycle, which culminates in the generation of metacyclic trypomastigotes that are infective to the mammalian host. This means that infected flies are reservoirs of the trypanosome parasite (KETRI, 1994). Such a fly has the potential to infect any animal from which it subsequently takes a blood meal. The circulation of trypanosomes between wild animals and tsetse flies is of no practical significance until man or his domestic animals, which are usually susceptible to the pathogenic effects of the trypanosomes, intrude into the life cycle and become hosts for the parasite.

Tsetse flies cause enormous economic problems in African continent with its rapidly expanding human population (Mehlhorn, 1998). In Kenya they occupy about 60% of Kenyas range lands, which is equivalent to one quarter of the country. They are responsible for limiting the pace and extent of rural development in much of tropical Africa.

The threat posed to man and his domestic livestock by trypanosomiasis in Africa is related entirely to the distribution of infected tsetse flies (Maudlin, 1991). An estimated 45 million people are exposed to the risk of infection resulting in untold misery and death. Trypanosomiasis of domestic animals is however, a more serious problem than the human disease, thus making tsetse flies economically more important vectors. The socio-economic impact of the tsetse flies is therefore enormous and this

makes their control desirable in the interest of health and economy. The control of African trypanosomiasis, to date, depends on two main activities namely: parasite control, which targets the parasite mainly in the vertebrate host, and vector management, which targets the tsetse vector mostly in its habitat.

Chemotherapy is the most widely employed approach towards parasite control- and in many countries of Africa the only approach towards the control of trypanosomiasis (Jordan, 1986). Other strategies towards parasite control include use of trypanotolerant cattle as well as efforts to develop anti-trypanosome vaccines (ILRI, 1998). Almost all the anti-trypanosomal drugs currently in use have been shown to have toxic side effects, do not act on all stages of the disease and require repetitive administrations. Another in use of these drugs is the exorbitant cost associated with them especially in the developing world where poverty is very rampant. Furthermore, the parasites tend to develop resistance to most of the drugs (Mehlhorn, 1998). The commonly used anti-trypanosomal drugs include; tryparsamide, pentamidine, suramin, pyrithidium, melarsoprol, nitrofurazone and quinapyramine. (Jordan, 1986).

It has not been easy to develop vaccines due to the ability of the parasite to change the antigenic composition of their surface coat. This serves for the trypanosome as a simple but effective strategy of survival in the immune system of the host. Trypanotolerance, as a means of parasite control is only applicable in animal trypanosomiasis. The West African Ndama cattle (ILRI, 1998) display this tolerance. Trypanotolerance is a more appropriate control method where effective tsetse control by other methods is not feasible (Mehlhorn, 1998).

Vector management in trypanosomiasis control basically aims at reducing the population of tsetse flies sufficiently to break the disease transmission cycle. Most common methods of tsetse control today involve use of insecticides. Ecological considerations and non target-effects against the aquatic fauna and flora restrict the wide spread repetitive use of insecticides (Hutson and Roberts, 1985). Furthermore, the insects develop resistance when chemical insecticides are used repeatedly because of selective mortality of the more susceptible genotypes following the application of insecticides (Brown, 1977). It is evident that most of the trypanosomiasis control methods currently in use have proved to be unsuitable and ineffective. Hence there is need for extensive research into approaches or technologies that take into account environmental and land use issues in the different agro-ecological zones infested with tsetse flies. Knowledge about intra and extra - cellular antioxidant defenses of blood-sucking insects; iron transport and utilization in invertebrates particularly insects (Locke and Nichol, 1992), could have application in the development of biological pesticides and in the control of blood-sucking insects that are disease vectors as well as consumers of iron -rich diet (Law *et al.*, 1992).

1.1   Importance of the study

The socio-economic impact of trypanosomiasis is enormous. It is estimated that over 50 million cattle and 100 million goats die from the disease annually (KETRI 1994). The disease can destroy rural communities and fear of the disease can lead to desertion by people of otherwise productive land, causing serious losses to food production. Current control methods of trypanosomiasis mainly involve chemotherapy against the parasites and use of insecticides against the tsetse flies. These control methods have not been effective due to resistance and the negative environmental impact

of insecticides. Thus there is need for extensive research into environmentally friendly approaches or techniques to control or reduce tsetse flies which are the disease vectors. Exploiting specific biochemical processes like iron transport and utilization in tsetse flies can provide a way out.

Iron transport in vertebrates has been investigated intensively over recent-decades, but then the subject has received little attention in invertebrates. Understanding the metabolism of iron can help in solving many problems associated with them (Nichol and Locke,1992). For example increased dietary iron was shown to cause oxidative stress in *Musca domestica* (Sohal *et al.*,1985). Bipyridillium herbicides such as paraquat act on ferritin to free iron that catalyzes the production of damaging free radicals and enhances lipid peroxidation in membranes (Saito *et al.*, 1985). Paraquat is toxic to aphids (Nichol and Locke, 1989) possibly because aphid cells contain cytosolic ferritin. Iron-catalyzed free radical production may also cause paraquat toxicity in other insects. Therefore the aim of the study was to use tsetse fly *G morsitan centralis* which is a vector for trypanosomiasis as a model to understand the nature and functioning of iron binding protein, transferrin, in controlling iron transport and toxicity.

In addition, the role that these proteins play in the development of parasites is of paramount importance. It has long been established that both malarial parasites and trypanosomes do sequester iron from mammalian hosts. *Leishmania chagasi* promastigotes can acquire iron from hemin, ferrilactoferrin and ferritransferrin (Wilson *et al.*, 1994). This capacity to utilize several iron sources may contribute to the organism's ability to survive in the diverse environments it encounters in the insect and mammalian hosts. While there is no evidence of the same process in the invertebrate hosts it is most likely that parasites obtain their iron requirements from the inveterbrate

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hosts as well.

While iron-binding proteins in hematophagous insects would be vital for their survival, the proteins of these insects are yet to be characterized biochemically. Results from this study will go along way in contributing to the possible use of iron binding proteins in the control of hematophagous insects. Possibility towards this end exists in altering the iron binding capacity of the proteins thereby exposing the insect to toxic levels or in limiting the availability of iron to parasites for which they are vectors hence limiting their development. This may provide a possible strategy for control of these insects as well as other blood feeding insect vectors of medical and veterinary importance.

1.2   Aims and objectives

The overall aim was to study the nature and function of iron binding proteins in *G. Morsitan centralis*.

The specific objectives were:

(i)   Screening for iron binding proteins in *G. morsitan centralis* midgut and hemolymph.

(ii)   Isolation and purification of transferrin.

(iii)   Biochemical characterization of transferrin.

2.0   LITERATURE REVIEW

2.1   Tsetse flies as disease vectors

Trypanosomes are transmitted from one vertebrate host to another by blood sucking tsetse flies of the genus *Glossina* (Mehlhorn,1998).   The trypanosomes, which are protozoan in nature, invade the blood stream and nervous system of the host in the advanced stages of the disease. Infection by one or other of the trypanosomes is known as trypanosomiasis. The transmission of trypanosomes from one host to another by tsetse flies (*Glossina*) is cyclical and obligatory, for they undergo part of their lifecycle within the insects (Ford, 1971).   The trypanosomal stage occurs in the blood of mammalian host (Fig.1).

Inspite of some recent advances in the field of epidemiology and control, human and animal *Glossina* - borne African trypanosomiasis continues to present a formidable challenge to health and economy in many countries of tropical Africa by crippling animal husbandry and causing sleeping sickness (Laird, 1977). Infectious disease constraints livestock productivity in developing countries. Vector control measures, drugs, vaccinations, movement control and slaughter of infected animals are unavailable, inappropriate or inadequate for control of trypanosomiasis. Vector control is difficult to sustain and raises environmental concerns. Drug efficacy is threatened by drug resistance in target organisms. Vaccines are not available and movement control and slaughter are not options for this disease (ILRI, 1998).

The World Health Organization estimated that some 45 million people are at risk of contracting sleeping sickness, but only ten thousand cases are reported each year (Jordan, 1986). It has been estimated that the total area infested with tsetse flies and

Figure 1: The life cycle of trypanosomes (Mehlhorn, 1998)

Tsetse fly

Mammal

Metacyclic forms are injected into the skin of a mammalian host

Epimastigote forms multiply in the salivary glands

Procyclic forms multiply in the gut

Long slender forms multiply asexually by binary fision

Short stumpy forms do no divide

Bloodstream forms
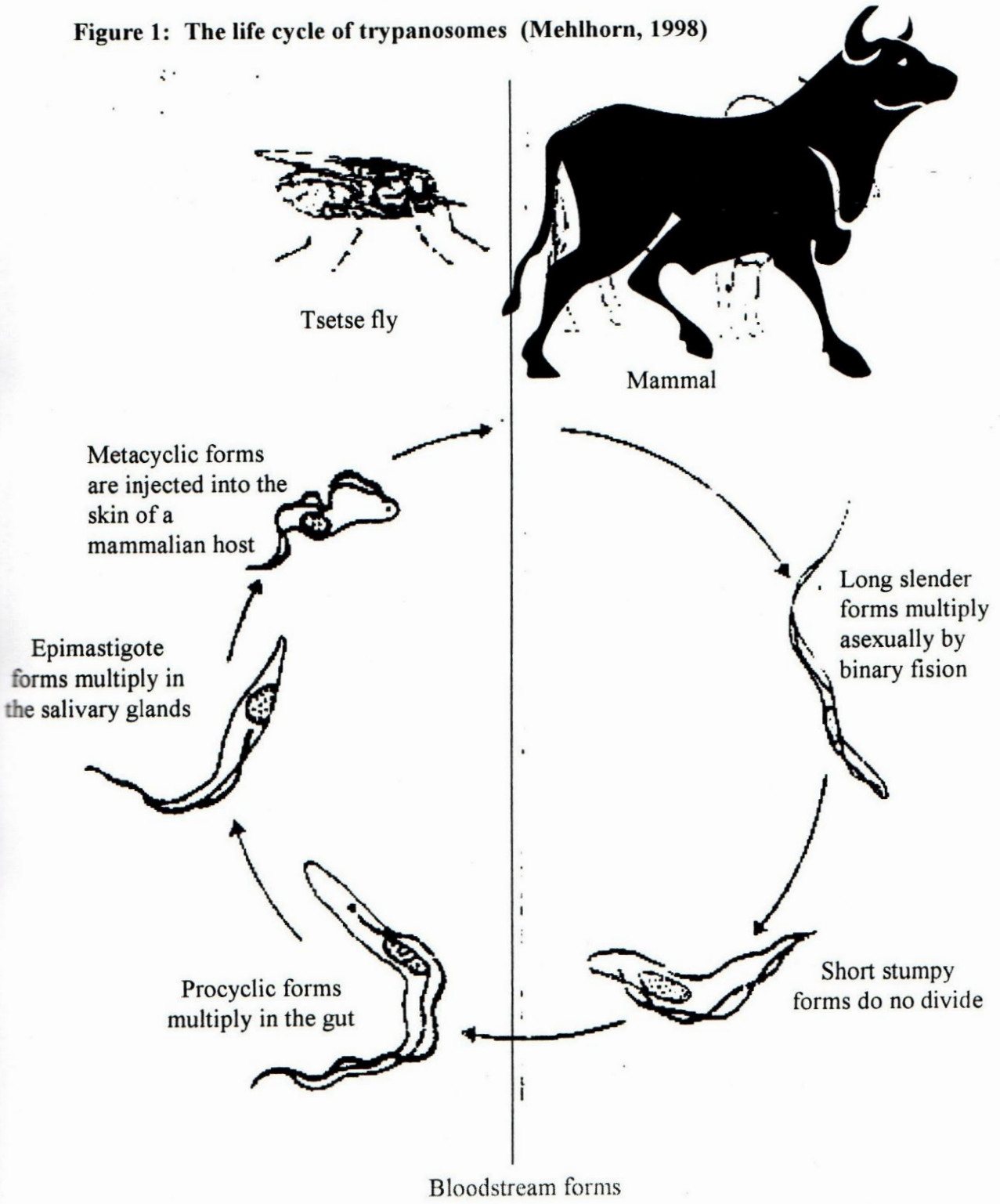
precluded from productive cattle husbandry has the potential of supporting about 125 millions heads of cattle.

There are three groups of *Glossina*:-

(i)   The *fusca* group

(ii)   The *palpalis* group

(iii)   The *morsitans* group

Rates of infection with *Trypanosoma* in *Glossina* vary from species to species and from one locality to another. In general, overall infection rates are lower in the *fusca* and *palpalis* groups than in the *morsitans* group - in which typically some ten to fifteen percent of individuals in a population might be infected with trypanosomes (Harris, 1991).

Jordan (1986) suggested six major factors that influence fly infection rates; temperature, age of fly at the time of infection, age structure of fly population, hosts of flies, species of fly, and variation between individual flies.

2.2   Distribution of tsetse fly in Africa

The genus *Glossina* occurs in over 11 million Km^2 of Africa. Its Northern limits extend across the continent from Senegal in West to Southern Somali in the East. This limit is about 14^0N, but in Somalia it is only about 4^0 North. These limits are determined by climate, often through its effects on vegetation. Figure 2 shows that the *fusca* group is found in lowland rain forest and along the edge of the rain forest, along water courses in the Savanna and in forest islands far from the main blocks of forest. In general these species are of negligible economic importance. Figure 2 shows that like the *fusca* group, the distribution of *palpalis* is centered on the two blocks of lowland rain forest, but some species extend far out through the humid savanna and into drier savannas

Figure 2: Distribution of tsetse fly (*Glossina*) in lowland rain forests in Africa (Jordan, 1986).
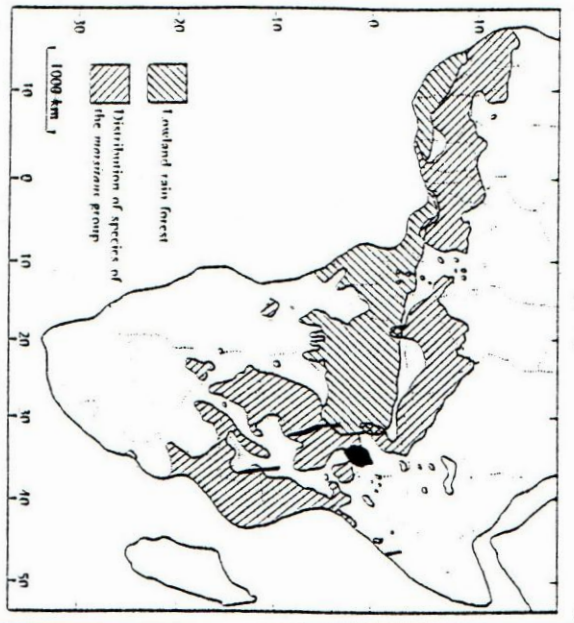
Distribution of the *Glossina morsitans* group of species, in relation to the two zones of lowland rain forest.
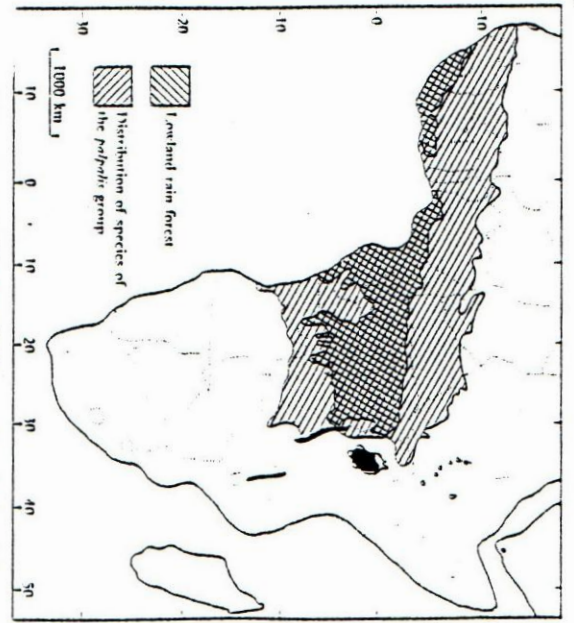
Distribution of the *Glossina palpalis* group of species, in relation to the two zones of lowland rain forest.
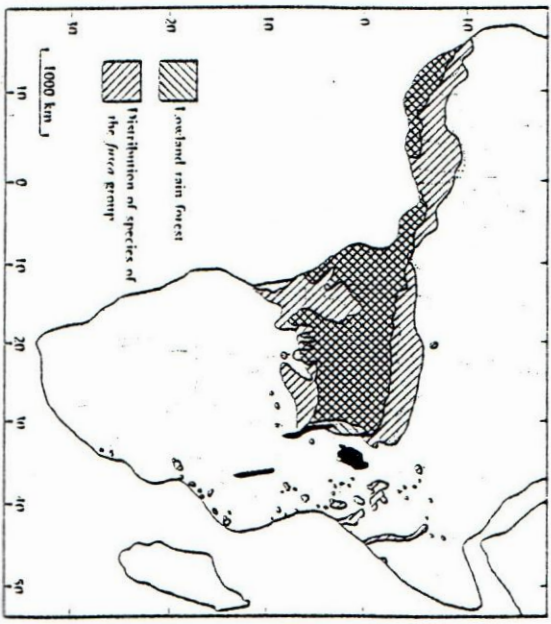
Distribution of the *Glossina fusca* group of species, in relation to the two zones of lowland rain forest.

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along rivers and streams e.g. Nile and the inland drainage systems of some of the great African lakes. As indicated in figure 2 the *Morsitans* group is restricted to the savanna woodlands around lowland rain forests. In wetter areas the flies roam widely over the woodland and also in the drier parts of this range, such as Sudan, Savanna of West Africa and Southern Africa (Mozambique, Zimbabwe) to Tanzania in the north. The Miombo woodland of East Africa is a typical habitat and the Mopane woodland in Zambia valley. *G. morsitans* in pan-African terms is the most important species of *Glossina* as it infests an enormous area (Jordan, 1986). The distribution of *G.m. centralis* concides with the distribution of the *morsitans* group.

2.3   Acquisition and digestion of a bloodmeal by the flies

Tsetse flies discharge saliva whilst probing the host for a blood meal. As saliva may contain trypanosomes, parasites can be exuded each time the fly probes before successfully obtaining a blood meal. Vector saliva plays an important role in arthropod disease transmission by creating a suitable environment for the disease agent in both the host and the vector (Titus and Ribeiro, 1990).

These flies ingest large amounts of vertebrate blood which has about 10 mM heme bound to hemoglobin. The large increase in weight of tsetse flies after feeding causes difficulty in flying, and increase their vulnerability to predators. It is therefore important for them to loose some of the weight of the blood meal rapidly. This is done by rapid excretion of excess water from the blood meal whilst maintaining the water balance and without risking desiccation (Bursell *et al.*, 1974). The water content of a tsetse bloodmeal is reduced from 79% to about 55% within three hours after feeding. This process leads to very high heme concentrations in the tsetse fly. The large amount

of nitrogen from amino acids that the tsetse fly's food contains must be excreted in order to avoid water loss and desiccation. Uric acid is the main excretory product, representing the final product of nitrogen metabolism in most insects, and makes up more than 60% of the dry weight of tsetse faeces. These insects have evolved solutions to the common problem of circumventing host hemostatic mechanisms that prevent blood loss. Because hemostasis is a complex and redundant physiological phenomenon that involves blood clotting, platelet aggregation, and vasoconstriction, blood-sucking arthropods, in their struggle for survival, have become natural pharmacologists with the means to neutralize or manipulate host hemostasis to their advantage (Law *et al.*, 1992; Limo *et al.*, 1991).

Tsetse saliva like that of many other blood-sucking insects contains a powerful anticoagulant enzyme identified as antithrombin (Lester and Lloyd, 1928). Saliva has vasodilatory and anticoagulant properties that make it easier for the fly to find and obtain its blood meal (Mant and Parker, 1981). Saliva may also have immunosuppressive, anti-inflammatory properties that would prevent an adverse reaction in the host. Tsetse saliva does not breakdown blood meal erythrocytes, but saliva of *G. morsitans* exhibits cholinesterase activity that is similar to enzyme activity of saliva in most insects in its reaction to inhibitors and ability to hydrolyze a wide range of substrates (Golder and Patel, 1982).

A number of studies have been carried out to investigate the enzymes involved in the digestion of a bloodmeal. It has however been shown that proteins in the tsetse blood meal stimulate enzyme activity in the midgut. For example trypsin is stimulated by the feeding process (Gooding, 1977a). Other proteolytic enzymes include carboxypeptidases, chymotrypsin-like enzyme and aminopeptidases which have been

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isolated from *G. morsitans* and subspecies of *G. palpalis*. A heamolytic agent, heamolysin, which lyses erythrocytes had also been detected in the digestive section of *G. morsitans* midgut (Gooding, 1977a). Two fibrinolysin proteases from the midgut of adult female *G. morsitan centralis* have been purified and characterized (Endege *et al.*, 1989). A protease inhibitor in the anterior midgut of *G.m. morsitans* is produced cyclically in relation to the host feeding cycle.

Digestion of bloodmeal proteins appears to take place only in the posterior section of the midgut and involves the six enzymes identified above which convert proteins to peptides and free amino acids. Interactions between digestive enzymes and trypanosome infection are very complex. It has been shown that *Trypanosoma brucei brucei* causes a concentration dependent decrease in trypsin or trypsin-like enzymes, in crude midgut homogenates of G. *m. morsitans*, suggesting that trypanosomes might overcome the hostile tsetse fly midgut barrier by inhibition of this enzyme activity (Imbuga *et al.*, 1992). Experimentally, D-glucosamine inhibits the activity of midgut trypsin in *G. morsitans* and could play a role in the susceptibility of tsetse to trypanosome infections (Osir *et al.*, 1993). Products of a blood meal digestion are stored as lipids, which constitutes the main food reserve of tsetse flies.

Tsetse flies use a bloodmeal for growth, reproduction, lipid synthesis, energy release, proline synthesis, nitrogenous excretion and as a source of carbohydrates (Bursell *et al.*, 1974). Early studies indicated that proline plays an important part in the flight metabolism of the tsetse (Bursell, 1963), and subsequent investigations have served to emphasize the central importance of this amino acid as a substrate for oxidative metabolism and as a source of energy for muscle during rest as well as during flight. According to Konji *et al.*, (1988), isocitrate synthesis stimulates the synthesis of

proline. A hormone stored and released by the corpora cardiaca, controls proline oxidation and appears to be dependent upon the presence of calcium ions (Pimley, 1985).

2.4.1 Constraints in hematophagy

Tsetse flies face an oxidative challenge due to heme and iron release following digestion of hemoglobin by midgut proteinases. Nearly all cells require iron, which is essential for synthesis of iron containing proteins such as hemoglobin and cytochromes (Locke and Nichol, 1992). On the other hand, excess iron is destructive to many cell processes and may occur at dangerous levels in reducing environment. Free iron catalyzes the production of hydroxyl radicals in the Haber-Weiss reactions (Crichton and Charloteaux-Waters, 1987) as shown below:

$$Fe^{2+}+O_2 \rightarrow Fe^{3+}+O_2^-$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow {}^\bullet OH + OH^-$$
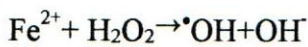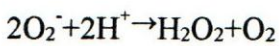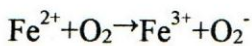
Free radical reactions injure cells and tissues by causing oxidative damage to several classes of biomolecules. Iron and iron containing organic molecules such as heme are well known catalysts in the formation of activated oxygen species (Halliwell and Gutteridge 1989). In order to limit iron toxicity several defensive mechanisms have arisen during evolution to protect cells from iron - induced oxidative injury. These include synthesis of proteins such as ferritin and transferrin, which bind iron and form complexes that do not promote formation of free radicals (Halliwell and Gutteridge, 1990).

2.4   Structure and function of Iron binding proteins

Hematophagy has evolved independently in several insect orders and a great diversity of ways to digest and use blood have arisen during the course of evolution. Research on genetic mechanisms controlling the synthesis of transferrin, ferritin, and their receptors has intensified in recent years especially in mammalian systems (Locke and Nichol, 1992).

2.5.1   Structure and function of ferritin

The presence of ferritin in insect tissues was suspected earlier by the observation of ferritin like particles in electron micrographs. In a survey by Nichol and Locke (1989), ferritin particles were observed in tissues of insects representing the following orders; *Orthoptera, Diptera, and Hemiptera*. These particles were localized exclusively in the vacuolar system.

Ferritin is a protein which has its principle function as the intracellular storage of iron in a non-toxic and bio-available form (Theil, 1987). Ferritin isolated from the midgut and hemolymph of insects has been characterized biochemically as well as by electron microscopy. Nichol and Locke, (1989) isolated ferritin from the larvae of *Calpodes ethlius (Lepidoptera, Hesperiidae)*. It is glycosylated like mammalian serum ferritin. It has 2 principal subunits of 24 and 31 kDa and minor ones of 26 and 28 kDa. All except the 24 kDa subunits are glycosylated by N-linked high mannose oligosaccharides. The native ferritin has a molecular size above 600 kDa consisting of three isoforms that have PIs of 6.5-7.0. Ferritin from *Manduca sexta* larvae hemolymph has a native molecular weight of 490 kDa with a principle subunit of 26 kDa (Heubers *et al.*, 1988). In a recent study, Winzerling *et al.*, (1995) isolated *Manduca sexta* ferritin through KBr

density gradient ultracentrifugation and its molecular weight estimated at 660 kDa consisting of two major subunits (24 and 30 kDa). The protein exists in dimeric form, and showed similar properties as previously described by Nichol and Locke (1989).

Mammalian ferritin is composed of two subunits, termed H and L (Rucker, *et al.*, 1996). The H subunit has a key role in rapid iron oxidation. Amino acid residues Glu-62 and His-65 in this subunit are involved in this process. The L subunit is involved in protein stability and iron mineralization. In vertebrates iron overload leads to increased synthesis of L-rich ferritins.

Ferritin in contrast to other iron transport proteins appears to have a more constant structure with an outer protein shell and an inner iron core, identifiable by electron microscopy (Huebers *et al.*, 1988).

2.5.2. Structure and function of transferrin

Studies on *Manduca sexta* by Huebers *et al.*, (1988) showed that transferrin is a transport protein of 80 kDa. It absorbs iron at the gut, shuttles it between peripheral sites of storage and use, and maintains the metal at circulating concentrations sufficient to support cells having special demand for iron (Jamroz, *et al.*, 1993). Transferrin and ferritin serves to sequester iron in a form that is unavailable to parasites or pathogens and this forms part of the defense of the vertebrate against these agents. This has also been reported in Mosquito and *Drosophila melanogaster* transferrin (Yoshiga *et al.*, 1997; Yoshiga *et al.*, 1999) where on infection with pathogens, the synthesis of transferrin is increased for the purpose of protecting these insects.

Insects have transferrin (Bartfeld and Law 1990; Huebers and Finch 1987), showing that its evolution anteceded iron transport for hemoglobin metabolism. *Manduca sexta* transferrin is similar to that of vertebrates in size (77 kDa) in tertiary and

secondary structure. It is also similar near the iron-binding site in primary structure (Bartfeld & Law 1990). It differs in having only one iron binding site .The greatest area of similarity was seen to be around the two iron binding sites, although the insect protein seems to contain only one functional domain. Earlier, Heubers, *et al.*, (1988) isolated a similar protein from the same insect but did not consider it to be transferrin. Transferrin (78 kDa) from the cockroach *Blaberus discoidalis* hemolymph while showing high homology to that of *M. sexta*, binds two iron atoms per protein molecule as do the vertebrates in contrast to previously described insect transferrin which bind one atom of iron per protein molecule (*Jamroz et al.*, 1993). The Circular Dichroism (CD) spectrum of transferrin indicated a structure low in α-helix (13%) and high β-pleated sheets (55%) (Bartfeld and law 1990). The CD spectrum of human serum transferrin and human lactoferrin also indicate low α-helix and high β-sheet respectively implying similar secondary structures as compared with insect protein.

To explore possible functional and evolutionary relationships between the cockroach iron -binding protein and the transferrin super family, Jamroz *et al.*, (1993) compared the deduced primary structure of *Blaberus discoidalis* sequence with those of nine vertebrate transferrins and *M. sexta* transferrin. An alignment of cockroach sequence with those of *M.* sexta transferrin and human serum transferrin was done. One hundred and twenty one amino acid residues were found to be conserved in the three proteins, with the most striking global feature being identity of cysteine residues at 19 positions (conserved among all transferrins) (Bartfeld and Law, 1990). The high level of cysteinyl identity suggests similarity of tertiary structure, although vertebrates' transferrins can accommodate more disulfide bonds than can the insects' proteins.

In a recent study, Kurama *et al.,* (1995) demonstrated in *Sarcophaga* that maternally supplied transferrin plays a crucial role in insect embryogenesis. Iron carried by 65kDa protein with characteristic features of transferrin family were transferred to another soluble protein in the eggs, presumably, ferritin.

2.5.3   Structure and function of other iron binding proteins

Recently, a heme binding protein (RHBP) has been isolated from hemolymph and oocytes of the blood-sucking bug *Rhodnius* (Oliveira *et al.,* 1995). A special problem generated by having vertebrate blood as the sole food source in this insects is the large amounts of free hemin that is produced upon digestion of hemoglobin. However, evidence has been obtained to show that RHPB acts as an anti-oxidant capable of blocking hemin induced lipid peroxidation (Dansa-petreski *et al.,* 1995). This is important because hemin is a powerful generator of free radical reduction products of dioxygen that are capable of causing biological injury through peroxidation of lipids, protein and DNA. The RHBP is composed of a single 15 kDa polypeptide chain coiled in a highly α-helical secondary structure which binds non-covalently one heme/polypeptide chain. RHBP from hemolymph is not saturated with heme and promptly binds heme added to the solution. The oocyte protein is however fully saturated and is not capable of binding additional heme. According to Oliveira *et al,* (1995), the spectrum of the dithionite-reduced protein has peaks at 426,530 and 559 nm and resembles that of a β-type cytochrome.

Vertebrates plasma also has a heme- binding protein hemopexin which has been shown to diminish the effectiveness of heme as a pro-oxidant (Gutteridge, 1988). Hemopexin is also involved in heme transport in vertebrate plasma.

2.6 Regulation of Iron binding proteins

Excess free iron is toxic and the iron level to which individual cells are exposed to can be reduced by preventing over accumulation and by increasing excretion of iron from the whole organism. Therefore organisms prevent over accumulation by converting ferrous irons to ferric ions and by using siderophraxes to bind ferrous ions, coupled with the repression of genes for iron transport and storage molecules (Locke and Nichol, 1992). In vertebrate cells, both transcriptional and translational control of ferritin synthesis is sensitive to iron levels (Theil, 1990a,b;). This is also true for insects because in a recent study, Pham *et al.*, (1999) and Georgieva *et al.*, (1999) found transcriptional and translational regulation of ferritin synthesis in a mosquito and *Drosophila* respectively. Iron regulates the synthesis of two proteins critical for iron metabolism, ferritin and transferrin receptor, through novel mRNA/ protein interactions (Theil, 1990a; Klausner and Hartford, 1989). The presence of an iron responsive element (IRE) in both ferritin and transferrin receptor mRNAs allows iron levels in the cell to influence both mRNAs in concert. The mRNA regulatory sequence, (IRE) occurs in the 5'-0untranslated region of all ferritin mRNAs and is repeated as five variations in the 3'-untranslated region of transferrin receptor mRNA. When iron is in excess, cells use stored ferritin mRNA to synthesise more ferritin for iron storage (Theil, 1987). At the same time the stability of the transferrin receptor mRNA decreases, which diminishes receptor synthesis and iron uptake. Conversely when iron levels are low, transferrin receptor mRNA is stabilised, more receptors is synthesised and iron uptake increases, while ferritin mRNA is masked, ferritin synthesis declines and iron storage decreases. Therefore iron delivered to a cell by the binding of transferrin to its receptor is partitioned between ferritin and biosynthesis requiring iron (Theil,1990). The iron

responsive factor is conserved in evolution and occurs in *Drosophilla melanogaster* (Rothernberger *et al.*, 1990). Iron-responsive elements- binding protein [IRE-BP] has been defined and identified as an RNA-binding protein found in iron-deprived eukaryotic cells (Basilion, *et al.*, 1994). IRE-BP binds to stem-loop structures, iron-responsive elements (IREs), which are located in the untranslated regions of the mRNAs for several genes including ferritin, and the transferrin receptor. When cells are iron replete, iron-sulfur cluster is ligated to the IRE-BP, the protein loses RNA binding properties.

Further studies have shown that juvenile hormone suppressed transferrin mRNA levels drastically in the adult female cockroach (Jamroz, *et al.*, 1993). Kurama *et al.*,(1995) demonstrated that *Sarcophagha* transferrin transports iron ions into eggs during oogenesis and delivers them to ferritin and no significant activation of the transferrin gene was detected during embryogenesis.

Thus, it may be expected that insects have the same molecular mechanisms for the control of ferritin and transferrin receptor as the vertebrates. This is true because most recently, translational and transcriptional regulation of an iron binding protein (ferritin) has been reported in the mosquito (Pham *et al.*, 1999). This confirms the fact that in both vertebrates and insects, ferritin and transferrin are sensitive to iron levels. Studies on the mosquito have also shown that transferrin is bloodmeal induced. Further studies have also indicated that just like in vertebrates, ferritin and transferin forms an important defense mechanism of the insect against pathogens (Yoshiga *et al.*, 1997; Yoshiga *et al.*, 1999). On the basis of these central importance of transferrin in protecting both vertebrates and insects, this study embarked on the purification and further study of this protein with the view of using knowledge gained there of to control insect disease vectors.

CHAPTER THREE

3.0   MATERIALS AND METHODS

3.1   Chemicals

The laboratory chemicals used in all experiments were of analytical grade and were obtained from Sigma Chemical Company, St. Louis, USA; Pierce Chemical Company, Rockford, USA; Bio-Rad, Richmond, USA; Promega, Madison, USA; Pharmacia and Diagnostic Chemicals, Uppsala, Sweden.

3.2   Experimental insects

Tsetse flies, *Glossina morsitan centralis* were obtained from a rearing unit at the International Livestock Research Institute (ILRI). The flies were maintained on a 12:12 hour (light-dark) photoperiod at 75-80% relative humidity at 25°C. They were starved for 72 hours after which they were fed on a bloodmeal prior to bleeding them.

3.3   Hemolymph and midgut collection

For hemolymph collection, chilled insects were used. Hemolymph samples were obtained from cut legs of the insects using microcapillary tubes (Osir *et al.*, 1991) and collected in ice-chilled tubes containing 200 μl of a bleeding solution (130 mM *NaCl*, 5 mM *KCl*, 19 mM *NaH$_2$P0$_4$*, 1.7 *K$_2$HPO$_4$*, 10 mM EDTA, 0.02% *NaN$_3$*, pH 7.5) containing antiprotease, PMSF (4mM), and a cocktail of protease inhibitors consisting of 0.5μg/ml each of leupeptin, pepstatin and antipain. To avoid any interference from these inhibitors, they were used in extremely low concentrations. A few crystals of Phenylthiourea (PTU) were used to prevent melanization, and an antioxidant, glutathione (25mg per volume of bleeding buffer) was used. These samples were then centrifuged (1000g, 10 min, 4° C) to remove

hemocytes and clotted materials (Winzerling *et al.*, 1995). Samples were then kept at −20°C until use.

Midguts with attached malpighian tubules were dissected out and the gut contents removed. The midgut contents were then washed in phosphate buffered saline (PBS) (100 mM sodium phosphate pH 7.2/0.15 M NaCl). After homogenization of the midgut, it was centrifuged (15,000 g, 5 min, 4°C) to remove clotted materials. The pellet was discarded and the supernatant kept at −20° C.

3.4   Separation of total protein from the midgut sample on Sephadex G-25 column

Before any analysis was done for the midgut sample, clotted materials, pigments and any other contamination that could interfere with protein determination and subsequent analysis were separated from the total protein using Sephadex G-25 column chromatography.

A column of sephadex G-25 was packed (1cm wide, 80cm long) and equilibrated with PBS before loading 3 mls of the midgut sample (supernatant) onto the column. 3ml fractions were collected and absorbences read at 280 nm. Measurements of absorbance at 280 nm for the fractions collected indicated a clean separation (see appendix I). The total protein (peak on the chromatogram) was pooled, concentrated using polyethyleneglycol and kept at −20⁰ C until use. The midgut sample was now ready for protein determination (see appendix 2) and subsequent analyses.

3.5   Protein estimation

Protein estimation was carried out using the Pierce Bicinchoninic acid (BCA) protein assay method as described by Smith *et al.*, (1985). Samples were incubated at 37°C for 30 minutes

and then absorbences measured at 562 nm using Pharmacia Biotech Novaspec (II) Spectrophotometer. Bovine Serum albumin (BSA) (Bio-Rad) was used as a standard. The standard curve (see appendix II) was used to estimate the amount of protein in unknown samples.

3.6   Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Gradient gels and linear gels were used. The gradient gels (5-15%) were cast using BIO-RAD gradient former model 385. The resolving gel (acrylamide: bisacrylamide, 30:0.8) was used. The gels were then layered with water. After polymerization, water was poured out and a stacking gel (3%) was cast on top of the resolving gels.

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), samples that had previously been dialyzed against PBS were dissolved in an equal volume of sample buffer (0.13M Tris-HCl, 20% glycerol, 0.002% BPB, 4% SDS, 1% ß-Mercaptoethanol, pH 6.8) and boiled for 5 minutes in a water bath, before application on to the gel. Electrophoresis was carried out at a constant current of 30 mA at room temperature until the tracking dye, bromophenol blue was no longer in the gel matrix. Non-denaturing PAGE was carried out on gradient gels (5-20%) and linear gels of 7.5% at room temperature with a constant current of 30 mA. Samples for the electrophoresis were dissolved in an equal volume of non-denaturing sample buffer (0.13 M Tris-HCl, 20% glycerol, 0.002% BPB) before application on the gel.

3.6.1   Staining of PAGE gels

3.6.1.1 Staining of gels for proteins

After electrophoresis the gels were stained for proteins with 0.6% Coomassie Brilliant Blue R.250 (Sigma Co., St. Louis, USA), in acetic acid, methanol and distilled water in the ratios of 9.2:50:40.8, respectively overnight. The gels were then treated with several changes of destaining solution (acetic acid, methanol, and distilled water in ratios of 9.2:50:40.8) at room temperature. Separated proteins were also detected through the silver staining (Merril *et al.*, 1986). This staining method is based on the ability of proteins to bind silver and is 100 times more sensitive than coomassie blue. Distilled water was used with freshly prepared reagents. The first step involved fixation of the proteins onto the gel using 10% acetic acid, 50% methanol and 2.5% glutaraldehyde (30 min incubation). Before adding the silver nitrate reagent to the gels, they were washed once with distilled water for 5 minutes and twice with 50% methanol. The gels were left in the silver solution for 15 minutes. They were then rinsed 3 times in a lot of distilled water. Protein bands were developed in the reducing solution (2.5% of 1 % citric acid, 125 µl of 38% formaldehyde made up to 250 mls with distilled water). Development was done by shaking slowly until a clear background was observed. This reaction was stopped with 5% acetic acid.

3.6.1.2 Staining of gels for iron binding proteins

Polyacrylamide gels were stained for iron containing proteins with Ferene S stain (Diagnostic chemicals, CT) (0.75mM 3-[2-Pyridyl]-5,6-bis (2-[-FurylSulfonicacid])-1,2,4- Triazine, 2% acetic acid, 0.1% thioglycollic acid) according to the method of Chung, (1986). This stain is specific for iron binding proteins and was prepared just before use. For colour development, each gel was immersed in 50 mls of staining solution. Destaining in 2 % acetic acid cleared

the gel background. This stain is based on the interaction between the bound iron atoms of the protein and a sensitive chromogenic ligand (Ferene S) to give a blue coloured complex in PAGE

gels.

For increased sensitivity of the iron stain, the gels were incubated in a freshly prepared solution of 0.05 M $FeSO_4$ for 30 minutes at room temperature, washed thoroughly four times in distilled water before immersing them in 50 mls of Ferene S stain. Thioglycollic acid reduces ferric iron in iron-binding proteins forming a dark blue reaction product with Ferene S. Its sensitivity depends on iron content and rate of iron release. After 5-10 minutes, the gels were removed from Ferene S and destained for 30 minutes in 2% acetic acid at room temperature.

The destained gels for Coosmassie Brilliant Blue – R 250, silver stain and Ferene S were stored in 7.5% acetic acid in sealed polythene bags until they were photographed using Panatomic - X films (Kodak).

3.7   Purification of transferrin from the hemolymph

3.7.1. Potassium bromide (KBr) density gradient ultracentrifugation

Hemolymph samples were subjected to KBr density gradient ultracentrifugation (50,000 rpm, 10°C, 4 hours) according to Ryan et al., (1986). After ultracentrifugation 1 ml aliquots were taken and their absorbances determined at 280 nm. The refractive indices of the same fractions were also determined. Density (D) values were calculated from the formula D= 6.4787 RI – 7.6430 (where RI is the refractive index at 25^0 C) (Ogoyi et al., 1995). A graph of density and absorbance at 280 nm, against fraction number was plotted. The fractions from the peak containing lipophorin were pooled. The subphase containing the

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rest of the protein was pooled and subjected to further purification.

3.7.2 Electroelution

The proteins from the ultracentrifugation subphase were separated on a preparative 7.5% native-PAGE using constant current of 30 mA until the tracking dye, Bromo phenol blue was no longer in the gel matrix. At the end of electrophoresis, two side pieces of the gel were cut, and stained with Ferene S. Destaining was done with several changes of the destain solution (2% acetic acid). The middle piece of the gel flanked by the two sides was meanwhile kept moist with distilled water. The destained side pieces were then aligned with the middle piece. The portion of the middle piece of the gel coinciding with the positively iron stained protein at approximately 140 kDa was carefully cut out and chopped into small pieces. The pieces were then placed into model 422 electroeluter glass tubes (Bio-Rad laboratories, Richmond, USA) using forceps. The assembly of the electroelution apparatus was carried out according to instructions supplied by the manufacturer (Bio-Rad). A current of 8 mA per glass tube was used and electroelution done overnight. Electroeluted samples were analyzed on native and SDS-PAGE to confirm presence of the protein.

3.7.3 Affinity chromatography on Con A Sepharose column

The electroeluted samples were dialyzed overnight at 4°C against Concanavalin A buffer (0.01M Tris, 1Mm $MgCl_2$, 0.5M NaCl, 1mM $Ca^{2+,}$ 0.02% NaN_3, PH 7.5). A column of concanavalin A sepharose (Sigma) was packed (10 cm length, 1 cm width). This column was equilibrated with con A buffer before loading the protein sample (400 µg). The column was left to stand for 15 minutes to allow for maximum binding before the unbound fraction was washed and collected in a batch of 10 mls in con A buffer. Elution of the bound fraction

was done using 500 mM α-methly-D mannopyranoside. The two fractions, bound and unbound were subjected to 95% (NH$_4$) SO$_4$ precipitation at 4°C and centrifuged in microfuge tubes at 13,000 rpm for 15 minutes.

The supernatant was discarded and the pellet pooled together, redissolved in PBS and dialyzed against distilled water for one hour and then PBS for 12 hours to remove (NH$_4$)$_2$ SO$_4$. The purity and homogeneity of this preparation was checked by SDS-PAGE and two-dimensional gel electrophoresis. The transferrin sample was then kept at -20°C for biochemical characterization.

3.8 Carbohydrate analysis

The presence of a carbohydrate moiety on transferrin was determined according to the method of Kapitany Zebrowski (1973) and Sergio et al., (1998). Transferrin was separated by a linear (12%) SDS-PAGE at a constant current of 30 MA. The gel was fixed in 10% acetic acid and 30% ethanol overnight.

Glycoproteins in the gel were oxidized with 1% periodic acid before detecting them with Schiff- silver staining. Incubation in the Schiff reagent lasted for 15 minutes. This reagent was prepared as follows; 1 gram of basic fuchsin was dissolved in 200 mls of boiling distilled water, stirred for 5 minutes and then cooled to 50^0 C. This solution was filtered and 20 mls of 1 N HCl added. This solution was cooled to 25^0 C and then 1 gram of sodium metabisulphite added. This solution was left to stand in the dark for 12 hours. 2 grams of activated charcoal were added and then filtered. The filtrate was stored at room temperature. After incubation in the Schiff reagent, the gel was put in the silver nitrate reagent (0.2% AgNO$_3$ (w/v), 0.03% formaldehyde (v/v)) for 30 minutes at room temperature. After this, colour development was done. Destaining of the gel was carried

out in several changes of 7% acetic acid. The destained gel was then stored in sealed polythene bags containing 7.5% acetic acid until photographed.

3.9 Isoelectric focussing (IEF)

Isoelectric focussing was done on mini protein II (Bio-Rad) system according to the method of O'Farrel (1975). Casting of IEF gel was done using a gel solution containing 5% acrylamide, 3% ampholytes and 15% (w/v) glycerol. Samples were dissolved in an equal volume of first dimension sample buffer (9.5 M Urea, 2.0% Triton x - 100, 5% ß mercaptoethanol, 1.2% (pH 3-10) ampholyte.

The carrier ampholytes in the IEF gel were prefocused for 10 minutes at 200 volts, 15 minutes for 300 and 500 volts respectively to generate a stable pH gradient. 4 µg of transferrin in 25 µl PBS was then applied in each tube, overlayed with 25 µl of sample overlay buffer (9 M Urea, 0.8% 3/10 ampholyte (Sigma), 0.0025% BPB) and focussed at 500V for 10 minutes and 750 V for 3.5 hours. The tube gels were extruded from the glass tubes and stained for protein using Coomassie Brilliant-Blue. Isoelectric points were determined by pH measurements of gel slices from concurrently run blanks equilibrated in distilled water (Saleem and Atkinson, 1976). The pI value for transferrin was obtained from a plot of pH versus distance moved by the ampholytes from the cathode.

3.9.1 Two-dimensional gel electrophoresis (IEF-SDS-PAGE)

The isoelectric focussing gel was extruded from the glass tube onto a petri dish containing distilled water and left there for about 30 seconds to dissolve any precipitated urea. The water was drained off and the gel transferred onto a second petri dish containing equilibration buffer (0.0625 M Tris HCl, 2.3% SDS, 5.0% ß-mercaptoethanol, 10% glycerol,

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0.05% BPB, pH 6.8). The gel was left to incubate at room temperature for about 30 minutes with occasional gentle shaking to replace as much of the protein associated urea with SDS as possible.

After equilibration, the gel was slid onto a piece of parafilm and loaded onto the second dimensional gel. As soon as the isoelectric focussing gel was in position, molten agarose sealing solution (0.1% agarose in 0.125 M Tris- Hcl pH 6.8) was injected below the rod gel until the space between the stacking gel and the rod gel was filled. The agarose was allowed to polymerize for about 30 min and then the running buffer (0.1% SDS, 0.25 mM Tris, 192 mM Glycine, pH 8.3) was added and the second dimension run started at 30 mA at room temperature. The gel was stained with silver stain and destained in 50% methanol to remove the background.

3.10 Absorption spectroscopic analysis of transferrin

Visible spectroscopy studies were carried out for both reduced and oxidized forms of 250 μg of transferrin using Perkin Elmer (550 S) Spectrophotometer, according to Oliveira *et al.*, (1995). Scanning of the native, reduced and oxidized forms of the protein was carried out at a scan speed of 60 nm/min and paper speed of 30 mm/min between 200 - 600 nm. The reduced form of the protein was obtained by addition of small amounts of Sodium dithionite. Reoxidation was achieved by progressive addition of potassium ferricyanide.

CHAPTER FOUR

4.0   RESULTS

4.1   SDS and native PAGE analysis of midgut and hemolymph samples

This was done essentially to show the protein profile in the two samples.

Analysis of hemolymph samples on SDS-PAGE (Fig.3) showed 6 major protein bands (400kDa, 270 kDa, 200 kDa, 80 kDa, 50 kDa, and 45 kDa) with several minor ones. Midgut samples on the same gel showed 3 major low molecular weight protein bands (60 kDa, 45 kDa, and 40 kDa) and 3 minor bands of 212 kDa, 80 kDa, and 90 kDa respectively.

Analysis of hemolymph samples on native PAGE (12%)(Fig.4) indicated several major proteins bands at 490 kDa, 212 kDa, 272 kDa, 140 kDa, 29 kDa, 22 kDa, and 18 kDa with a few minor bands. The midgut sample showed four major protein bands at about 600 kDa, 66 kDa, 29 kDa and 14 kDa.

To determine presence of iron binding proteins in any of these bands, these samples had to be subjected to a more specific analysis (Ferene S staining).

Figure 3: 5-15% SDS-PAGE analysis of tsetse hemolymph and midgut samples (silver stained).

Lane 1: 100 µg of tsetse midgut protein

2: 75 µg of tsetse midgut protein

3: Molecular weight markers

All the samples were dissolved in the same sample buffer before electrophoresis. Electrophoresis was done until the tracking dye reached the bottom of the gel. The Promega high molecular weight standards were used.
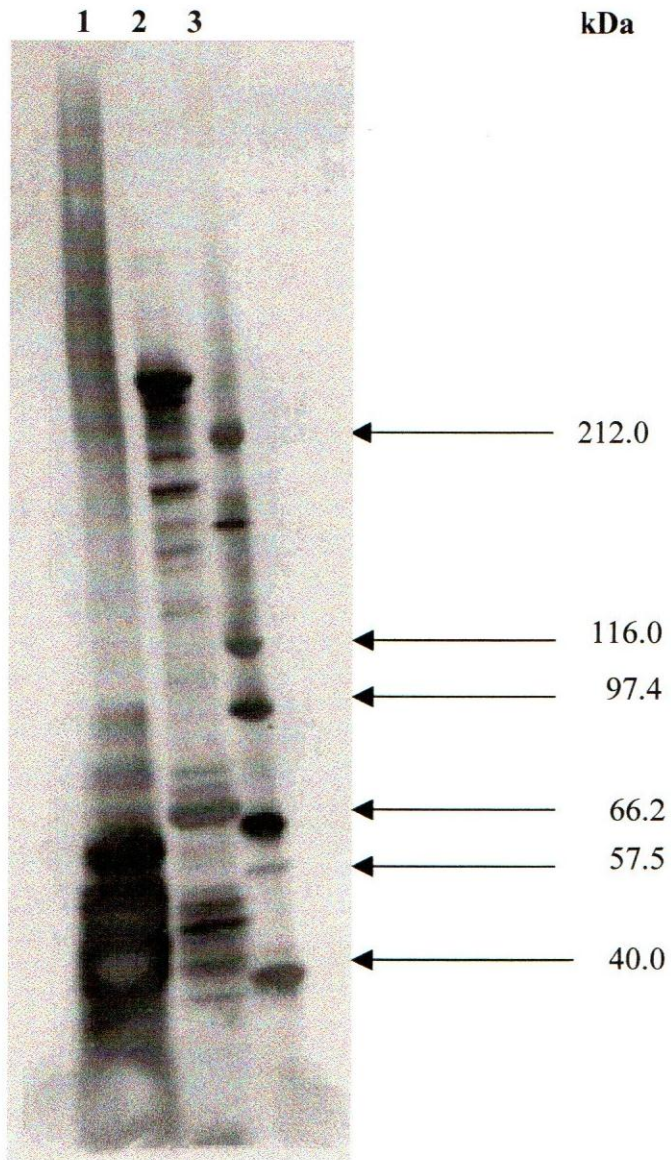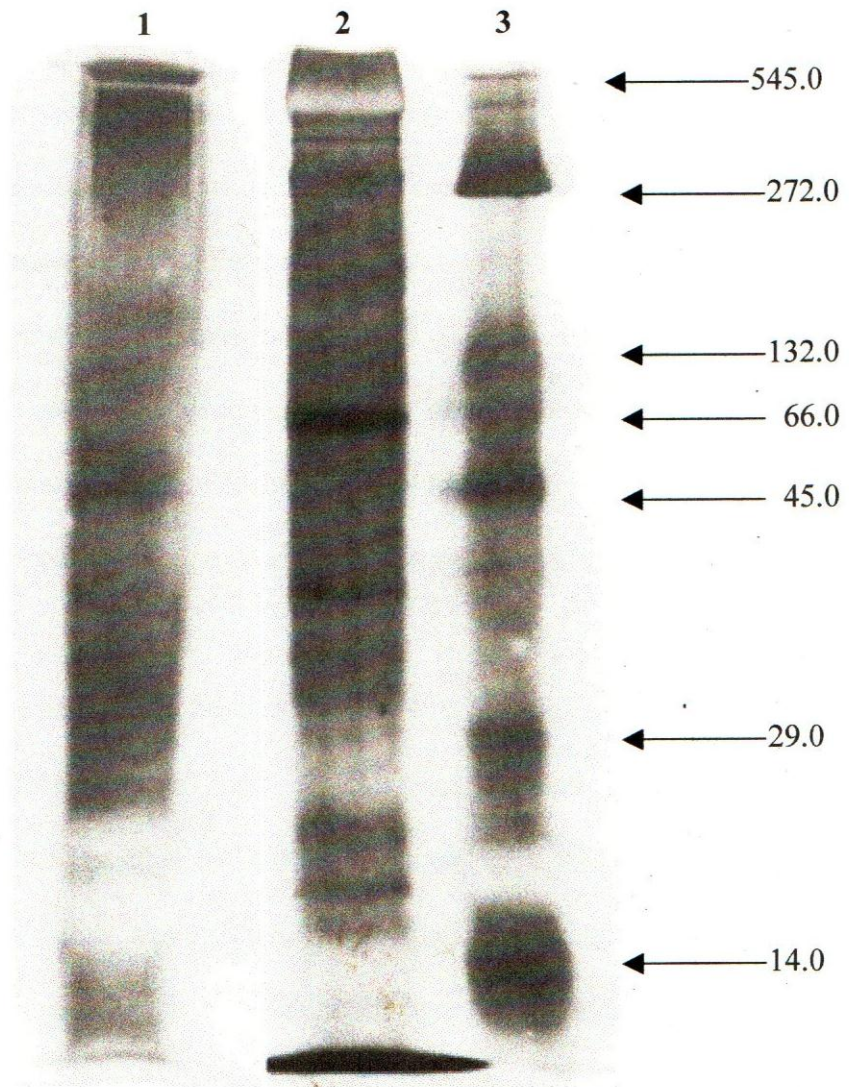
Figure 4: 12% Native PAGE analysis of midgut and hemolymph samples.

Lane   1:   50 μg of Midgut samples

        2:   50 μg of hemolymph samples

        3.   molecular weight markers

All samples were treated similarly before electrophoresis was done. This gel was silver stained.

4.2   Iron binding proteins in hemolymph and midgut samples

The presence of iron binding proteins in hemolymph and midgut samples was evaluated by staining proteins separated on native and SDS-PAGE with Ferene S (Specific for iron binding proteins). Initial experiments indicated that staining directly with Ferene S was not sensitive enough to detect the iron binding proteins in the midgut and the hemolymph. However when the improved methodology using 0.05 M $FeSO_4$, was used the sensitivity improved drastically. This method involved incubation of gels in 0.05 M $FeSO_4$ for 30 minutes before staining them with Ferene S.

SDS-PAGE analysis of hemolymph samples (Fig. 5) indicated presence of 3 iron binding proteins. One of this had a molecular weight of approximately 80 kDa. The other two were of low molecular weight of about 30 and 24 kDa respectively. Analysis of midgut samples on this gel did not stain for any iron binding protein even after loading 100 μg of the protein.

Analysis of the hemolymph samples in native PAGE indicated presence of two iron binding proteins (Fig.6). One of these proteins was of high molecular weight of approximately 490 kDa. The other one showed a low molecular weight of about 140 kDa. The higher molecular weight iron binding protein (putative ferritin) took the iron stain strongly and faster and is evidently more abundant than the lower molecular weight one (putative transferrin). The hemolymph also showed a very insignificant band at around 60 kDa. The midgut too faintly showed two low molecular weight proteins (50 and 20 kDa respectively). The two proteins, ferritin and transferrin were distinguished due to differences in molecular weight and subunit composition. Only hemolymph samples stained significantly for iron binding proteins and therefore 9 mg of total hemolymph protein was used for subsequent purification of transferrin. It is important to note that iron binding proteins

stained with intense blue bands while other proteins that seemed to take the stain had
yellowish colour. This aspect was used to distinguish the two categories of proteins.

Figure 5: Ferene S staining of tsetse midgut and hemolymph samples on 12% SDS-PAGE

Lane 1:   Molecular weight markers

2:   75 µg of tsetse hemolymph protein.

3:   100 µg of tsetse midgut protein.

This gel was incubated in 0.05 M $FeSO_4$ before it was stained with Ferene S for 15 minutes.

It was destained in 2% acetic acid at room temperature.

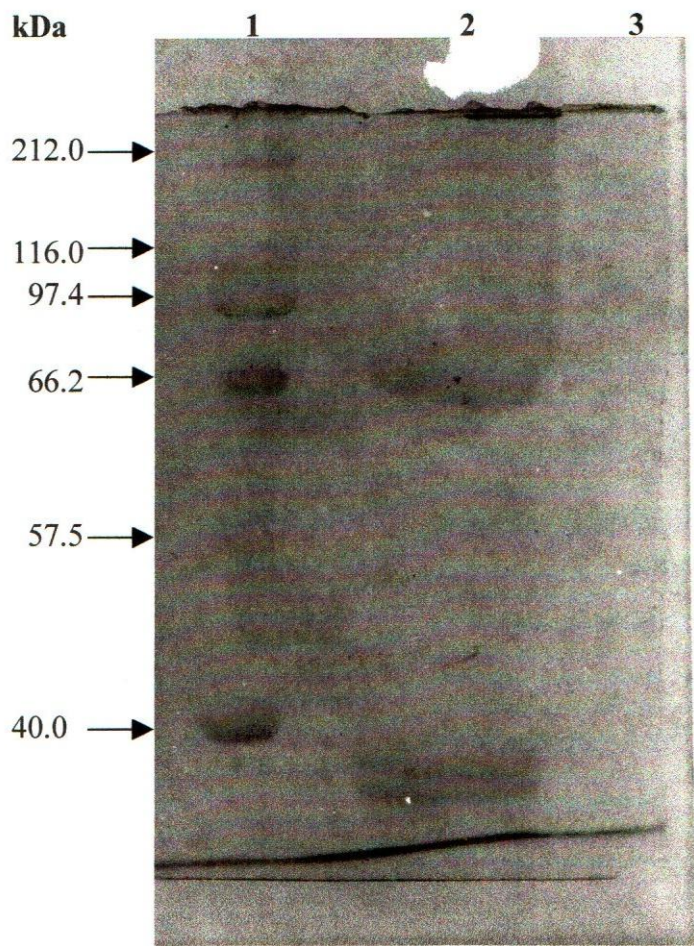**Figure 6: Analysis of tsetse hemolymph and midgut samples for iron binding proteins using Ferene S on 7.5% Native gel**
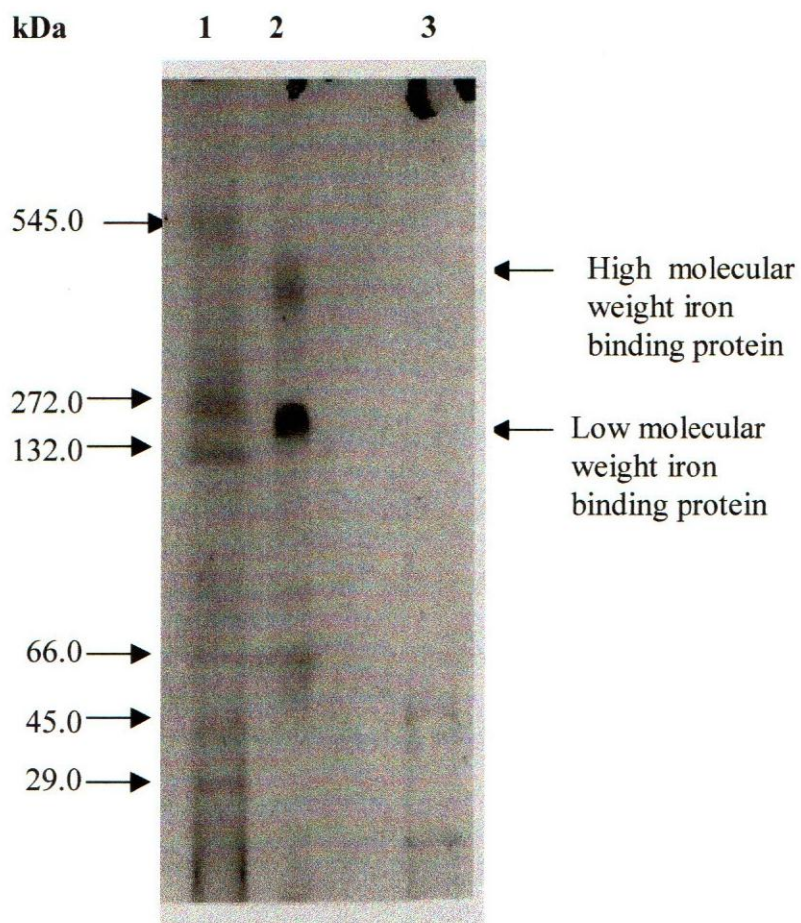
Lane 1:   Molecular weight markers

    2:   75 µg of tsetse hemolymph protein

    3:   100 µg of tsetse midgut protein

The two samples were treated similarly in terms of sample buffers and staining process

This gel was incubated in 0.05 M $FeSO_4$ before staining it with the Ferene S for 15 minutes.

4.3   Isolation and purification of transferrin from the hemolymph

An 80 kDa (80 ± 2) protein was purified from the tsetse hemolymph using KBr density gradient ultracentrifugation, electroelution from a preparatory gel electrophoresis, affinity chromatography on con A affinity column and 95% ammonium sulfate precipitation as described in materials and methods. Subsequent biochemical characterization and comparison with previous studies indicated that the purified protein was tsetse transferrin.

4.3.1   KBr density gradient ultracentrifugation of tsetse hemolymph proteins

KBr density gradient ultracentrifugation of hemolymph removed lipophorin and other major hemolymph proteins. The density of the fractions was obtained using the expression: D = 6.4787 RI − 7.6430 (where RI is the refractive index at 25^0 C). Peak between fraction 3-15, represent lipophorin as shown in figure 7(i). Lipophorin is a low density protein and was therefore easily separated from the rest (subphase).   Analysis of the ultracentrifugation subphase from which transferrin was purified indicated complete removal of lipophorin because their was no evidence of contaminating apolipophorin I (250kDa) and apolipophorin II (80 kDa) Fig.7(i). The subphase (fraction 21-41) was then used for the next purification strategy (electroelution).

Figure 7(i): KBr Density gradient ultracentrifugation profile of tsetse hemolymph

Samples in 44% KBr were ultracentrifuged (50,000 rpm, 10oC, 4 hours). Proteins were separated according to their density along the increasing density gradient.

A graph of density and absorbence of each fraction versus fraction number was constructed.

Density Gradient Profile

Density g/ml vs Fraction Number
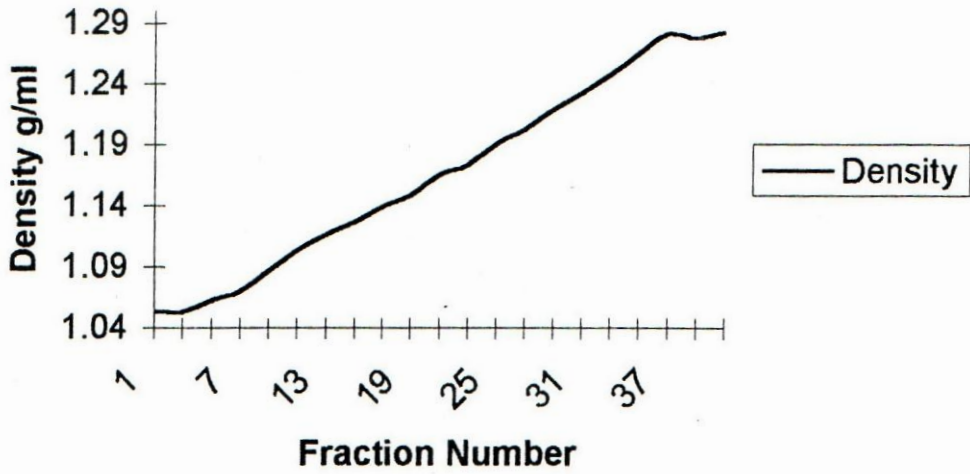
Absorbance Against Fraction Number

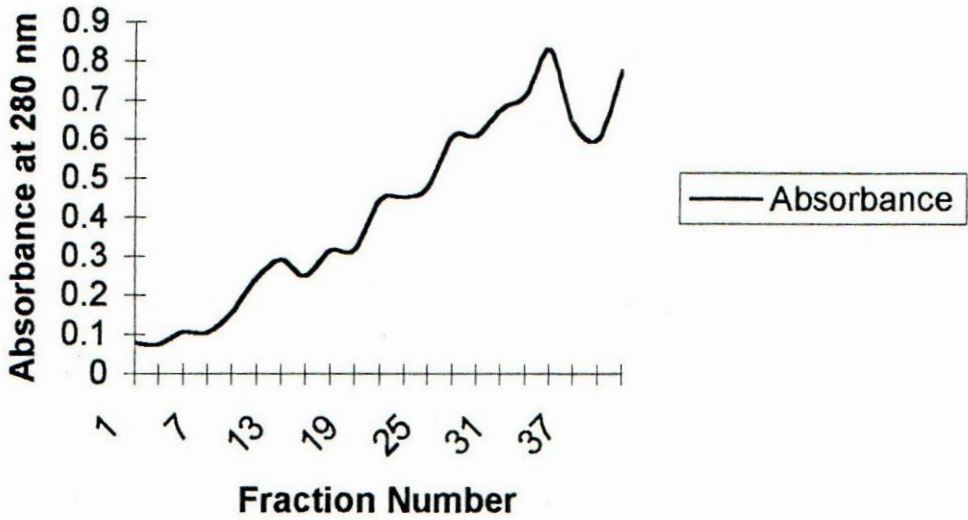Absorbance at 280 nm vs Fraction Number

Figure7(ii): 7.5% SDS-PAGE analysis of hemolymph samples after

ultracentrifugation (fraction 21-41).

Lane 1: Molecular weight markers

2: 50 µg of hemolymph protein

3: 40 µg of ultracentrifugation protein (fraction 21-41)

After electrophoresis the gel was stained with silver. It was then used to evaluate the removal of lipophorin from the rest of the proteins.
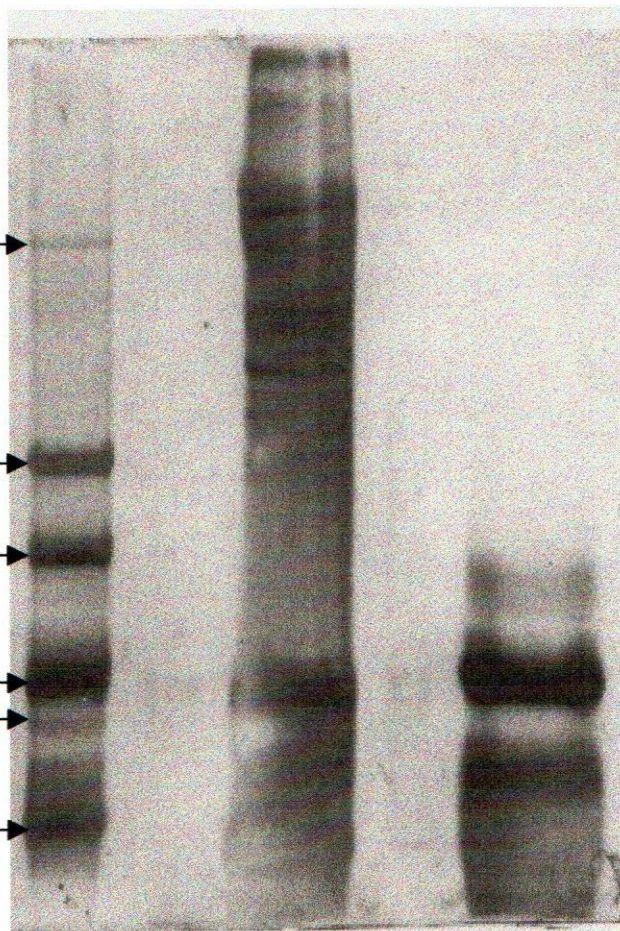
4.3.2   Electroelution from a preparatory gel electrophoresis

The 80 kDa protein in Fig. 5, was the putative transferrin as shown in native PAGE (Fig.6).   Therefore to obtain transferrin, a 7.5% preparative gel electrophoresis for hemolymph samples was done and after successful staining with Ferene S, a positively iron stained band (~ 140 kDa) was cut out from the gel and chopped into small pieces and then the protein electroeluted overnight. The electroeluted protein was then analysed by native and SDS PAGE as shown in figure 8, and 9 and was found to stain positively with Ferene S (Fig. 8). However as shown in figure 9, the process of elecroelution did not result into a pure preparation and therefore the sample had to undergo further purification to remove the contaminating proteins.

Figure 8: Ferene S staining of the electroeluted protein on 7.5% native gel.

Lane 1: High range molecular weight standards

2: 50 μg of crude hemolymph protein.

3: 50 μg of the electroeluted protein

Protein separation on this gel was done until the tracking dye was no longer at the end of the gel. It was then incubated in 0.05 M $FeSO_4$ before it was stained with Ferene S stain.

				kDa
3	**2**	**1**		
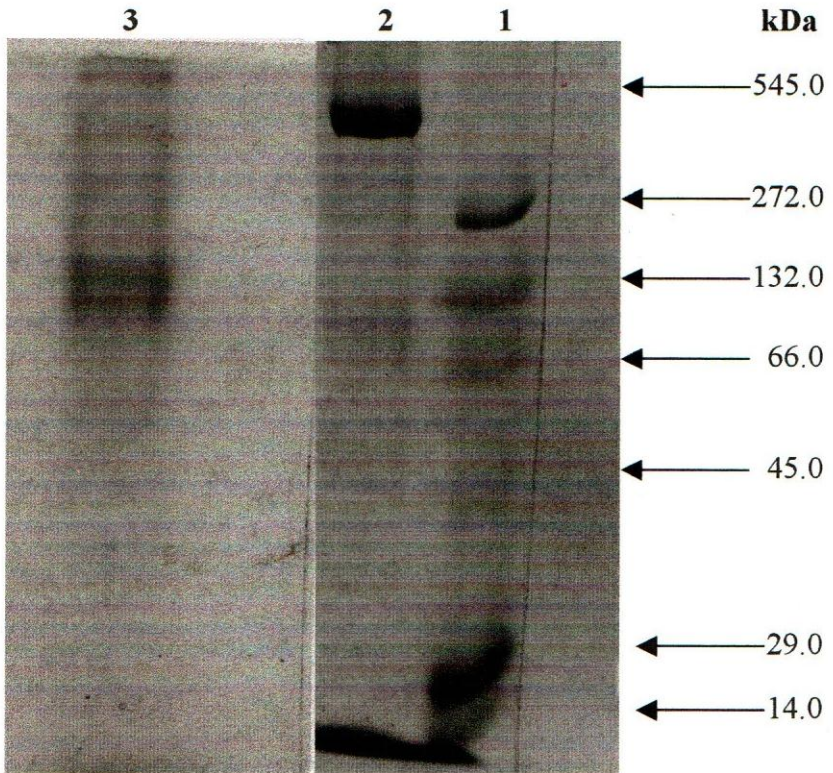
4.3.3 Purification of transferrin through Con A affinity chromatography

An electrophoretically pure preparation of transferrin was obtained after running the electroeluted protein sample through Concanavalin A Sepharose affinity column, followed by 95% $(NH_4)_2SO_4$ precipitation of the bound fraction from Con A column (figure 9). 132 µg of transferrin' was purified from the original 9mg total hemolymph protein. Analysis of the purified protein with Ferene S indicated that it binds iron (figure 10). Native PAGE analysis stained with silver further confirmed the purity of this preparation (Fig. 11). However compared to SDS PAGE analysis, native PAGE showed a higher molecular weight similar to that of the protein that had been electroeluted early. This observation confirmed that it is the positively iron stained protein that was bound to the con A affinity column.

Figure 9: 12%SDS-PAGE analysis of tsetse hemolymph samples stained with silver.

Lane 1: Molecular weight markers

2: 50 μg of tsetse hemolymph protein

3: 3 μg of electroeluted protein

4: 3 μg of the unbound fraction

5: 6 μg Con A affinity purified protein followed by 95% $(NH_4)_2SO_4$ precipitation.

The unbound and the bound fractions (lane 5) were dialyzed extensively with PBS to remove excess $(NH_4)_2SO_4$ before they were used for this electrophoresis.
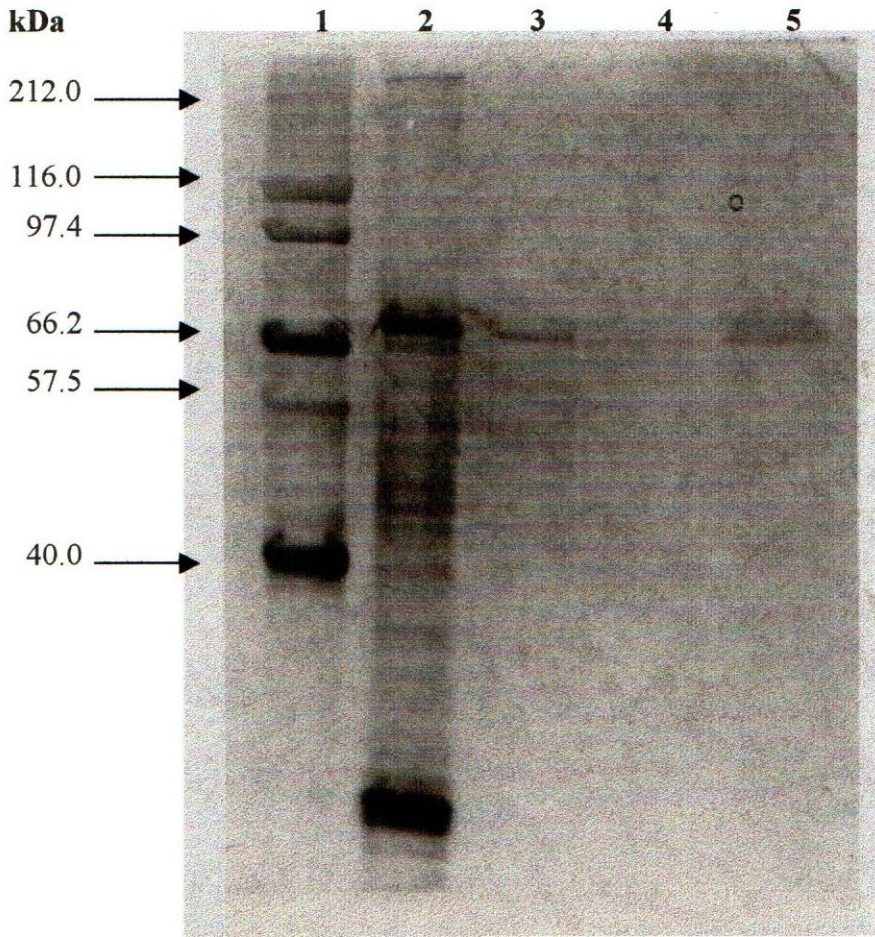
Figure 10: 7.5% Native PAGE analysis of the purified protein stained with Ferene S

50 μg of the purified protein was used for this analysis. The gel was processed as in figure 8.
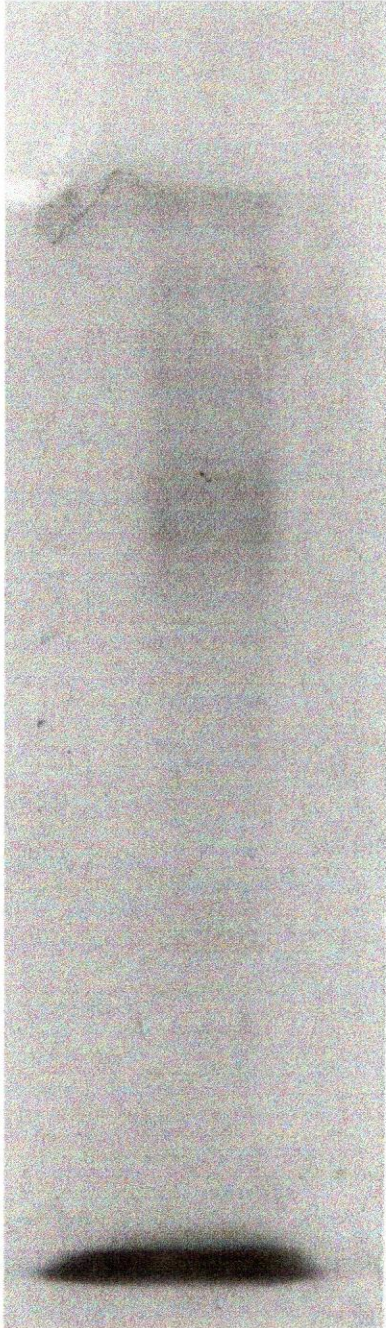
Purified protein

Figure 11: 7.5% Native PAGE of 3.5 μg of transferrin

Lane 1:   Molecular weight markers

2:   3.5 μg of transferrin

3:   Molecular weight markers.

After electrophoresis, this gel was stained with Coomassie Brilliant Blue. It was then washed several times with distilled water and then subjected to silver staining. Tf represents the transferrin band.
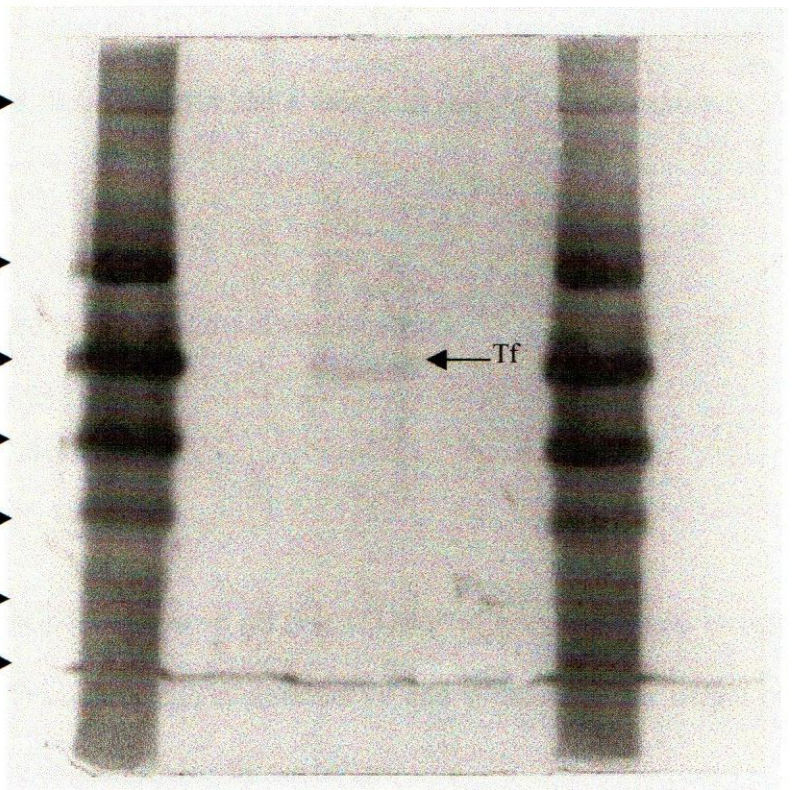
4.4   Biochemical characterization of transferrin

The tsetse fly transferrin was purified to homogeneity as shown by silver stained SDS-PAGE and two-dimensional gel electrophoresis.   It was characterized as a single polypeptide of 80 kDa with PI 6.5 possessing a carbohydrate moiety.   Absorption spectroscopic analysis of transferrin suggest presence of a heme group. With these alternative techniques, it was possible to purify transferrin that is found in small quantities.

4.4.1   Molecular weight estimation of transferrin

A standard curve (see appendix III) of log molecular weight against relative mobility on 12% SDS-PAGE showed that transferrin had a molecular weight of approximately 80 kDa (Fig.9).

The expression below was used to work out the molecular weight.

Log. mol.weight = 2.2504 –1.042 x Rf

$\qquad$ = 2.2504 – 1.042x 0.3295

$\qquad$ = 1.9070

Mol. wt   = 80.73 kDa.

A standard curve for this protein from a native gel (Fig.11) showed a high molecular weight of approximately 140 kDa.   From the graph in appendix IV and V the molecular weight of the protein in the native state was estimated as shown below:

Log. Molecular weight = 2.145

Therefore the actual molecular weight = 139.636

$$\approx 140 \text{kDa.}$$

4.4.2 Characterization of carbohydrate moiety

Periodic acid and Schiff-silver staining of transferrin on 12% SDS-PAGE showed that the protein had a carbohydrate moiety. The gel in figure 12 shows intense staining for transferrin. This is indicative of heavy glycosylation of this protein. Other glycosylated proteins of high molecular weight were also evident in the hemolymph samples.

Binding to Concanavalin A Sepharose column as shown during the purification process, indicated that the carbohydrates is of a high mannose type.

Figure 12: PAS-Schiff silver staining of transferrin in 12% SDS-PAGE.

Lane 1:   15 µg of purified transferrin.

    2:    50 µg of crude tsetse hemolymph sample.

After fixation, the gel was incubated in periodic acid and then stained with Schiff reagent. This was followed by incubation in silver nitrate reagent. Development was done as described in methods.
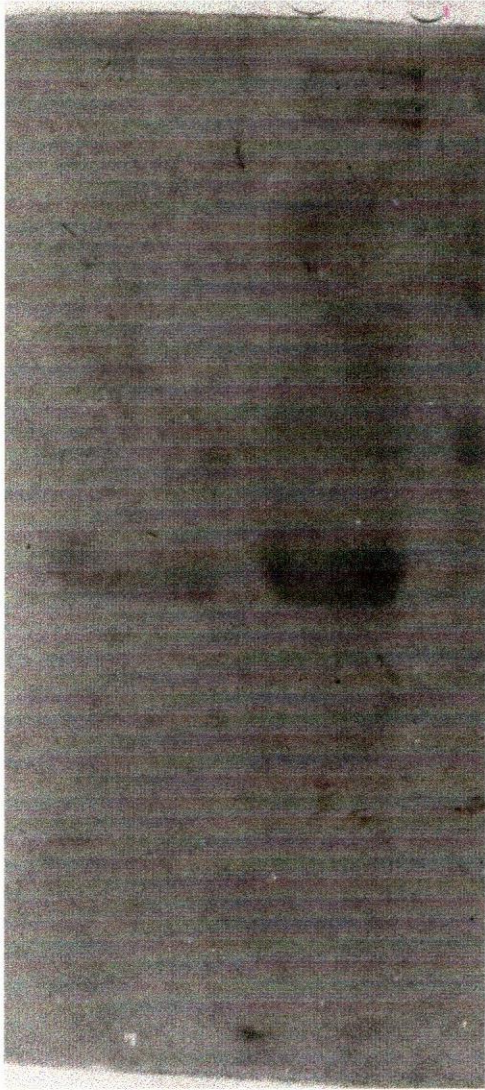
4.4.3   Determination of the isoelectric point (pI) of transferrin

The isoelectric point of transferrin was determined by pH measurements of gel slices of concurrently run blanks equilibrated in distilled water versus distance moved by the ampholytes from the cathode. The pI of transferrin was estimated at 6.5 from the standard curve (see appendix IV).

Figure 13: Isoelectric focussing of transferrin.

4 µg of transferrin was loaded in the tube gel (4%) and separated on isoelectric focussing (Ampholyte range pH 3-10). Focussing was done for 3 hours. The gel was the stained with Coomassie Blue.

pH 10.0
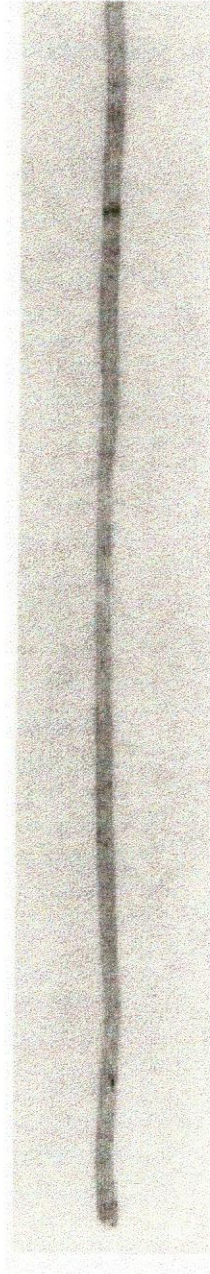
Purified protein band

pH 3.0

4.4.4 Two-dimensional gel electrophoresis

2-Dimensional gel electrophoresis confirmed the homogeneity (purity) of the purified protein by producing only one spot. It also confirmed earlier estimations of pI (6.5) and molecular weight of transferrin (80 kDa) (Fig.14). This experiment further confirmed that transferrin is a single polypeptide.

Figure 14: Two-dimensional gel electrophoresis of transferrin on a 12% gel.

4 μg of the protein was run through the isoelectric focussing tube gel (4%), and then on 12% SDS-PAGE. The gel was stained with silver as described in methods.

SDS PAGE
Electrophoresis

I.E.F

kDa

212.0

116.0
97.4

66.2

57.5

40.0
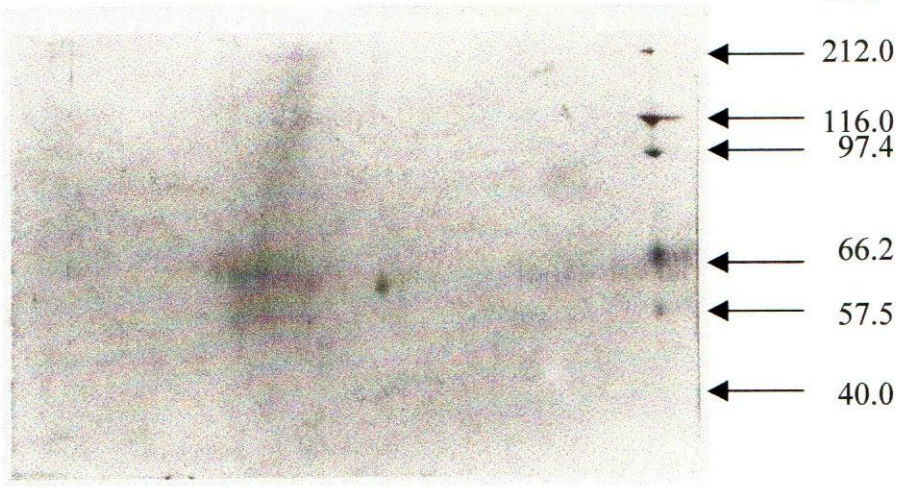
4.4.5 Absorption spectroscopic analysis of transferrin

250 μg of transferrin was used to determine the absorption spectrum of this protein over a ultraviolet - visible range of 200-600 nm. The spectroscopic analysis showed that the native form of transferrin had peaks at 565 nm, 370 nm and 298 nm. The reduced form of transferrin showed a slight shift of these peaks at 370 nm to 360 nm and 295 nm to 290 nm. The oxidized form had exactly its peaks as those of native transferrin (Fig.15).

Figure 15: Absorption spectroscopic analysis of transferrin.

This analysis was done at a scan speed of 60 nm/min and a paper speed of 30nm/min.

(a) & (b) =Native protein

(c)   = Dithionite reduced protein.

(d)   = Reoxidized by potassium ferricyanide

200nm   400nm   600nm

5.0   DISCUSSION

5.1   General discussion

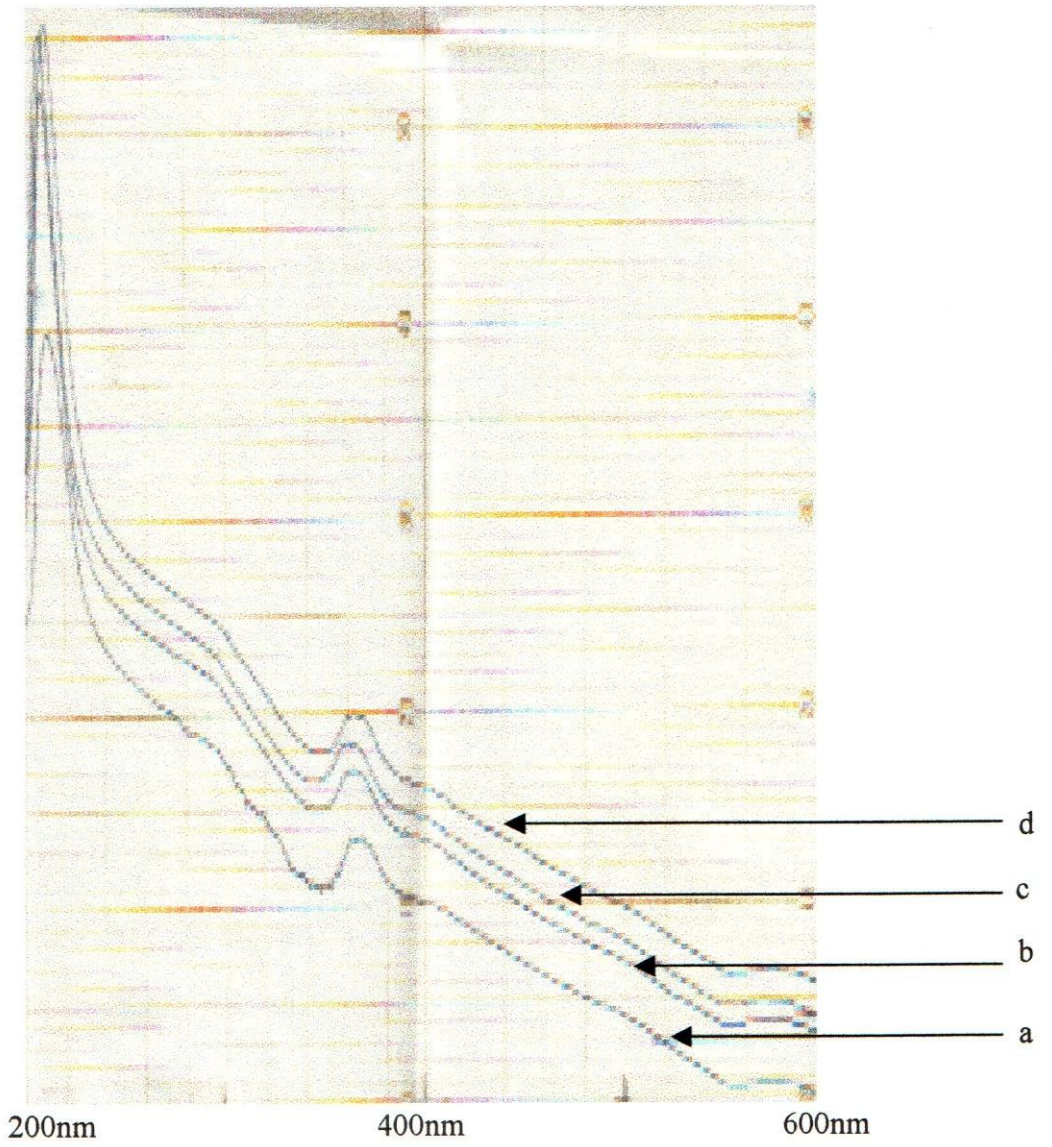Using conventional methods, it has been possible to isolate and characterize major hemolymph proteins in insects like *Manduca sexta* (Kanost *et al.*, 1990). Bartfeld and law (1990) isolated transferrin from the hemolymph of this insect using five chromatographic steps and this resulted into heavy losses of the protein. Earlier Huebers *et al.*, (1988) had isolated two iron binding proteins from the *Manduca sexta* using *in vivo* radio iron labelling and subsequent elution of radioactive protein from the gel using 0.01 M NaHCO$_3$ in 0.15 M saline and anion exchange chromatography on DEAE-Sephacryl S 300. Jamròz *et al.*, (1993) also used Sephacryl S 300 with nickel-dipicolyamine (DPA) metal ion affinity chromatography to purify transferrin from the cockroach *Blaberus discoidalis*.

Among the new technologies that have been useful for separating iron binding proteins include immobilized metal ion affinity chromatography (IMAC) and a combination of IMAC with other affinity methods for example Cascade-mode multi affinity chromatography (CASMAC) (Porath *et al.*, 1975). Most recently Winzerling *et al.*, (1995) described a rapid and efficient isolation of transferrin from *Manduca sexta* hemolymph. They isolated insect transferrin from the density gradient ultracentrifugation subphase by IMAC using a new gel Novarose SE 1000/40. This gel was coupled to dipicolylamine (DPA) (Porath and Hansen, 1991). Using this method, two iron binding proteins from the larval hemolymph of *M. sexta* were purified, one of high molecular mass of 490 kDa and a second of 80 kDa. The lower molecular weight protein was characterized as an insect transferrin, which made up 1.3% of the total hemolymph protein hence a minor constituent. Staining of total hemolymph protein from the tsetse fly also indicated presence of two iron binding

proteins, one of high molecular weight (490 kDa) and the other of a low molecular weight (140 kDa) (Fig.6) on native gels. The high molecular weight protein was presumably tsetse ferritin because on SDS-PAGE gels it showed two low molecular weight (24 &30 kDa) iron binding proteins. Similar ferritin polypeptides of the same size were characterized in *M. sexta* hemolymph (Winzerling *et al.*, 1995). Each of the two samples faintly stained positively with Ferene S (between 50 and 14 kDa) and this was attributed to non specific binding because other analyses did not detect them. In this study, an 80 kDa iron binding protein (putative transferrin) was isolated from the tsetse hemolymph using KBr density gradient ultracentrifugation, electroelution, and affinity chromatography on Con A Sepharose.

In contrast to *M. sexta* where hemolymph samples of up to 250 mls were obtained for transferrin purification, the amount of hemolymph that was available from each tsetse fly for this purpose was about 2 μl. A total of ~1000 flies were used. This is a very small amount of sample as compared to *M. sexta*. This made it very difficult to handle the sample without severe losses either due to degradation or adsorption to the walls of apparatus. At the same time, the protein being isolated makes up a very small proportion of the starting material. In order to purify transferrin, which is a minor protein in the hemolymph of *G. morsitans*, alternative techniques had to be resorted to. Alternative techniques for obtaining pure samples of proteins present in μg amounts are mainly electrophoretic. This led to the choice of direct elution of the protein band from the gel after ascertaining its identity by using iron staining. Iron staining was very critical in this purification strategy and was done according to Chung (1986). It is however important to note that incubation of the gels in 0.05 M $FeSO_4$ for 30 minutes before immersing them in Ferene S stain significantly improved the sensitivity, speed and simplicity of this staining. Apart from producing sharp bands, the time required for the gel to remain in Ferene S solution was reduced to about 5-10 minutes

with optimum staining instead of the normal 15 minutes or overnight incubation; and also the blue stain seemed to be very stable, almost permanent. This is evident from gels that have been stored in 2 % acetic acid for the last one year and still have retained the intense dark blue bands without loosing the original intensity. Tsetse Midgut and hemolymph samples were first screened for the presence of iron binding proteins before subjecting them to the purification of transferrin. These experiments indicated that only hemolymph samples had significant amounts of iron binding proteins and was therefore used in the purification. According to Huebers *et al.*, (1988) insects undoubtedly require a large supply of iron to be used in the cytochrome heme structure of their highly aerobic muscle system. This implies that at any one given time, much of transferrin will not be found in the midgut, but rather in the circulation in the hemolymph.

The purification steps for transferrin are summarised in a flow diagram as shown below:

Hemolymph Sample

Ultracentrifugation (50,000 r.p.m)

Lipophorin fraction

Subphase

Preparatory gel electrophoresis

Putative transferrin

Electroelution

Affinity chromatography on con A Sepharose

bound fraction

Unbound fraction

95% $(NH_4)_2SO_4$

Homogeneous transferrin
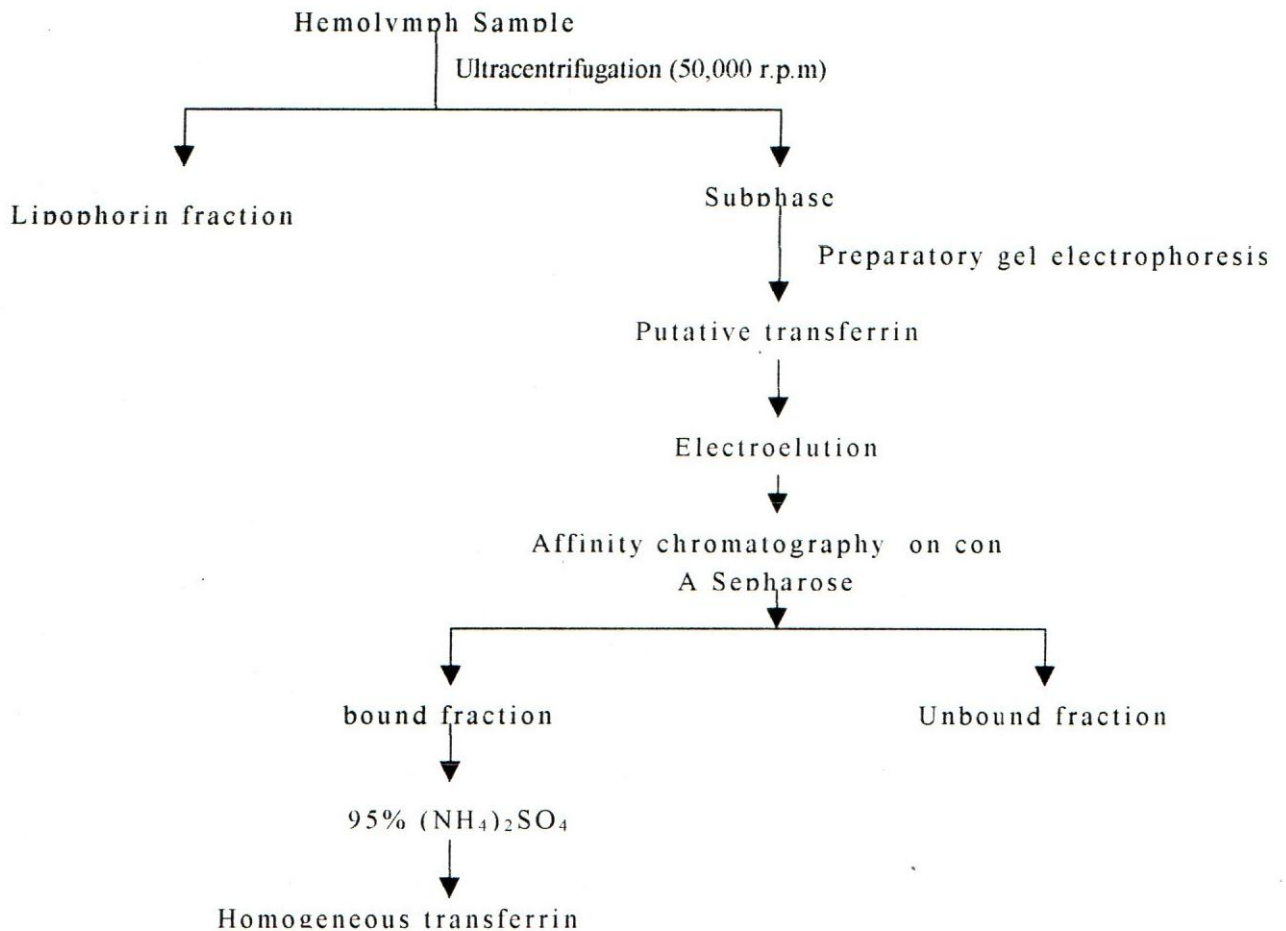
The first step in the isolation process of transferrin from the hemolymph was KBr density gradient ultracentrifugation. This step removed lipophorin, which is an abundant protein in the insect hemolymph that often interfere with purification of other minor proteins (Ochanda et al., 1991). Since transferrin is a minor protein, this was a very important step. The lipophorin devoid sample was ran on a preparatory gel electrophoresis and the Ferene S stained band was electroeluted. This process of electroelution did not result into pure transferrin as shown by SDS-PAGE in figure 9.

The electroeluted protein was further purified through Concanavalin A Sepharose affinity chromatography. The bound fraction from the column was then subjected to 95% ammonium sulfate precipitation. This strategy yielded a homogenous preparation of transferrin as shown by SDS-PAGE and IEF-SDS-PAGE and was in its native rather than denatured state. From a total hemolymph protein of 9 mg, 132 μg of transferrin was purified representing about 1.45 % of the total protein. This is consistent with *M. sexta* transferrin (Winzerling *et al.*, (1995) which is a minor hemolymph protein (1.3 % of the total hemolymph protein). In contrast vertebrate sera transferrins approximate 2-3mg/ml (3-5% of total protein) (Vieira and Schneider, 1993).

Carbohydrate analysis of the purified protein showed that it is indeed glycosylated. This is in agreement with the carbohydrates analysis results of *Manduca sexta* transferrin (77 kDa) (Winzerling *et al.*, 1995; Huebers *et al.*,1988) *Sarcophaga peregrina* flies (65kDa) (Jamroz *et al.*, 1993) and Cockroach *Blaberus discoidalis* transferrin (78) kDa). Studies on insects and vertebrate transferrin show that it constitutes a super family of single chain 80 kDa glycoproteins (Jamroz *et al.*, 1993). This is consistent with tsetse transferrin, which had a molecular weight of 80 kDa and has a carbohydrate moiety high in mannose. This shows conservation in terms of size. In a study to explore functional and evolutionary relationships

between vertebrate and insect transferrin, Jamroz *et al.*, (1993) compared the amino acid sequence of 9 vertebrate transferrins and that of *M. sexta* and *B. discoidalis*. 121 amino acid residues were found to be conserved in the three proteins, with cysteine residues at 19 positions, conserved among all. However in native gels, transferrin showed a high molecular weight (approximately 140 kDa). This observation was made early in *Manduca sexta* transferrin by Winzerling *et al.*, (1995) who noted that probably the protein structure was responsible for this electrophoretic mobility difference between native gels and reduced ones. It is also important to note that under non-denaturing conditions, the charge properties of the protein may also lead to differences in electrophoretic mobility (Laemmli, 1970).

Early studies on insect hemolymph proteins have shown that they have isoelectric points ranging from 5 to 8 (Palli and Locke 1987 a,c). Tsetse fly transferrin which was purified from the hemolymph had a pI of 6.5 and therefore in total agreement with the early findings. This property allows transferrin to be taken up by the fat body like other hemolymph proteins. This is in agreement with Huebers *et al.*, (1988) who found that during the rapid growth stage in the 5th instar of *Manduca sexta*, transferrin concentration was very high and that it functioned to carry absorbed iron to the fat body where it was incorporated into ferritin and released to the hemolymph. Also the importance of charge has been reported by Locke and Leung (1984) whereby the binding of cationic (pI 8.5 - 9.5) and anionic (pI 4.0 - 4.4) horse ferritin to insect surface suggests that charge could play an important role in the movement of hemolymph proteins by controlling passage across basal laminae. High charge may also relate to iron content since within the range of normal glycosylated serum ferritin isoforms, only the most basic contain stainable iron (Cragg *et al.*, 1981). This perhaps explains why it was difficult to stain transferrin (acidic PI) significantly without first incubating it in iron sulfate. Charge may also be important for retention within

the vacuolar system or processing for secretion.

The transferrin absorption spectrum was found to correlate closely and significantly with the spectrum of *Manduca Sexta* transferrin (Huebers *et al.*, 1988) and *Rhodnius prolixus* heme binding protein (Oliveira *et al.*,1995). The spectrum showed 2 maxima (565,370nm) and a weak absorption of between 280-300 nm is in agreement with *Manduca sexta* transferrin. These spectral characteristics suggest the presence of a heme group. The *Manduca sexta* transferrin bound only one atom of iron instead of 2 bound by mammalian transferrins (Huebers and Finch 1987). The spectral profile of the dithionite reduced tsetse transferrin looked strikingly similar to those of a single heme binding protein from the hemolymph and oocytes of the blood-sucking insects *Rhodnius prolixus* (Oliveira *et al.*, 1995). These similarities suggest that tsetse transferrin may have a single iron binding site just like other insects transferrins.

It is true that the study of the physiological role that iron plays in insect biology has been neglected. In contrast iron has a major place in prokaryote, plant and vertebrate studies, with many papers relating to human biology. Much of this work is relevant while not specific to insects. The role of iron in insects is no doubt very important owing to the amount ingested especially by hematophagous ones. Given the fact that ferritin and transferrin provide a highly effective storage and transport mechanism for iron respectively to meet the demands of insects, knowledge gained from their study can be exploited to develop insecticides that enables ferritin to release its iron and transferrin not to bind iron leading to its accumulation in the gut. This can be a novel control strategy because the insect gut is highly sensitive to iron levels implying a day- to-day reliance on dietary iron by insects (Dallman, 1986). If this is true, pesticides working through the gut might be more effective when delivered with a chelator that reduces the availability of iron. It is also important to

note that ferritin and transferrin serve to sequester ferric ions in a form that is unavailable as a nutrient for parasites or pathogens, and thus constitute a part of the defense of the vertebrate against these agents. This phenomenon has also recently been observed in mosquito and *Drosophila melanogaster* transferrin (Yoshiga *et al.*, 1997; Yoshiga *et al.*,1999), where on infection with bacteria or some parasites, the synthesis of insect transferrin is upregulated significantly. This is believed to be an iron-withholding defense strategy. This implies that if ferritin and transferrins' ability to restrict the availability of iron to pathogens is altered, the tsetse vector will be exposed to the pathogenic effects of these parasites and hence serve as a control strategy.

5.2 Recommendations and suggestions

♦ One of the essentially untapped resources of insect biochemistry, is the tremendous variety of insect species, which have been evolving and diversifying for hundreds of million years. One area in which this rich diversity can be put into good use is in studies of protein evolution. For example future studies on the comparative sequence analysis of transferin from insect species and /or between insects and vertebrates may identify highly conserved regions that are essential for the biological activity of this protein. Likewise such knowledge holds promise of a treasury of new materials with unique properties that will have practical importance.

♦ From these results, further studies need to be done on transferrin and other iron binding proteins to determine ways in which knowledge gained from this can be exploited in developing better control methods for tsetse flies and other disease causing blood-sucking insects.

♦ Another area that needs further study in blood sucking insects is the midgut because it is the site of bloodmeal digestion and is usually the initiating organ for major post feeding development events like pathogen transmission, oogenesis and vitellogenesis (Billingsley et al., 1990).

♦ These insects are also good candidates for the study of cellular induction and in this respect the impact of extra iron load on the titres and expression of transferrin as a result of a bloodmeal needs further study.

♦ Future studies need to be done on immunological cross-reactions of tsetse transferrin with the existing relatives and subsequent sequencing of the protein for amino acids to enable comparison with the existing transferrins.

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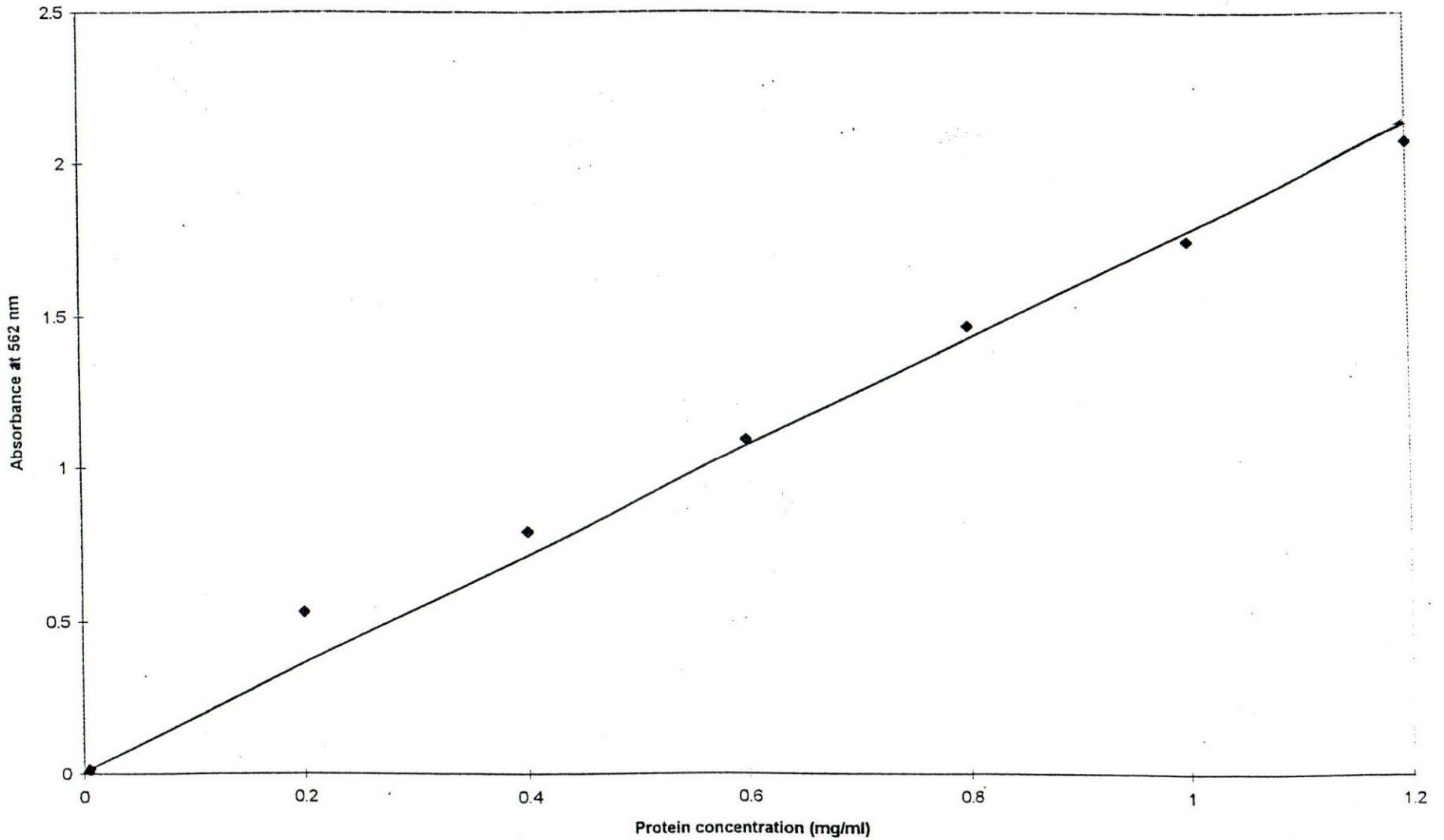
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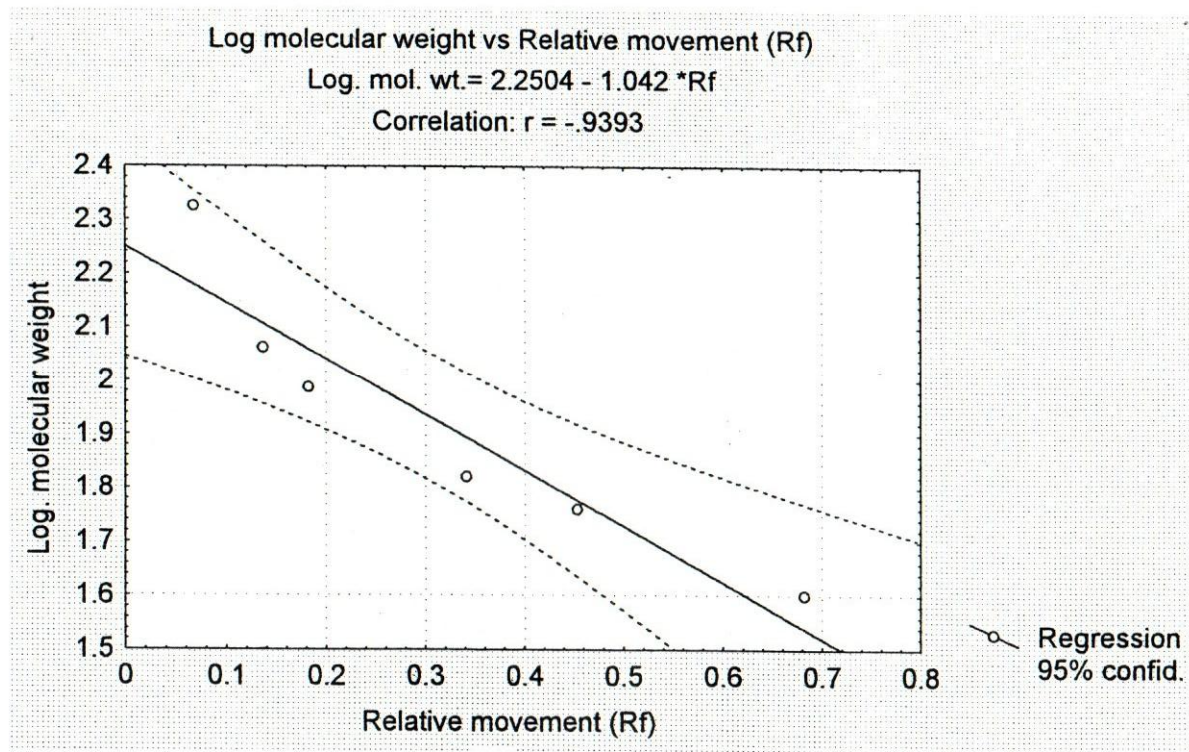**Titus, R.G. and Ribeiro, J.M.C.** (1990) The role of saliva in transmission of arthropod-borne disease. Parasitology Today, 6, 157-160.

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Appendix I : Separation of total protein from the midgut sample on Sephadex G-25 column
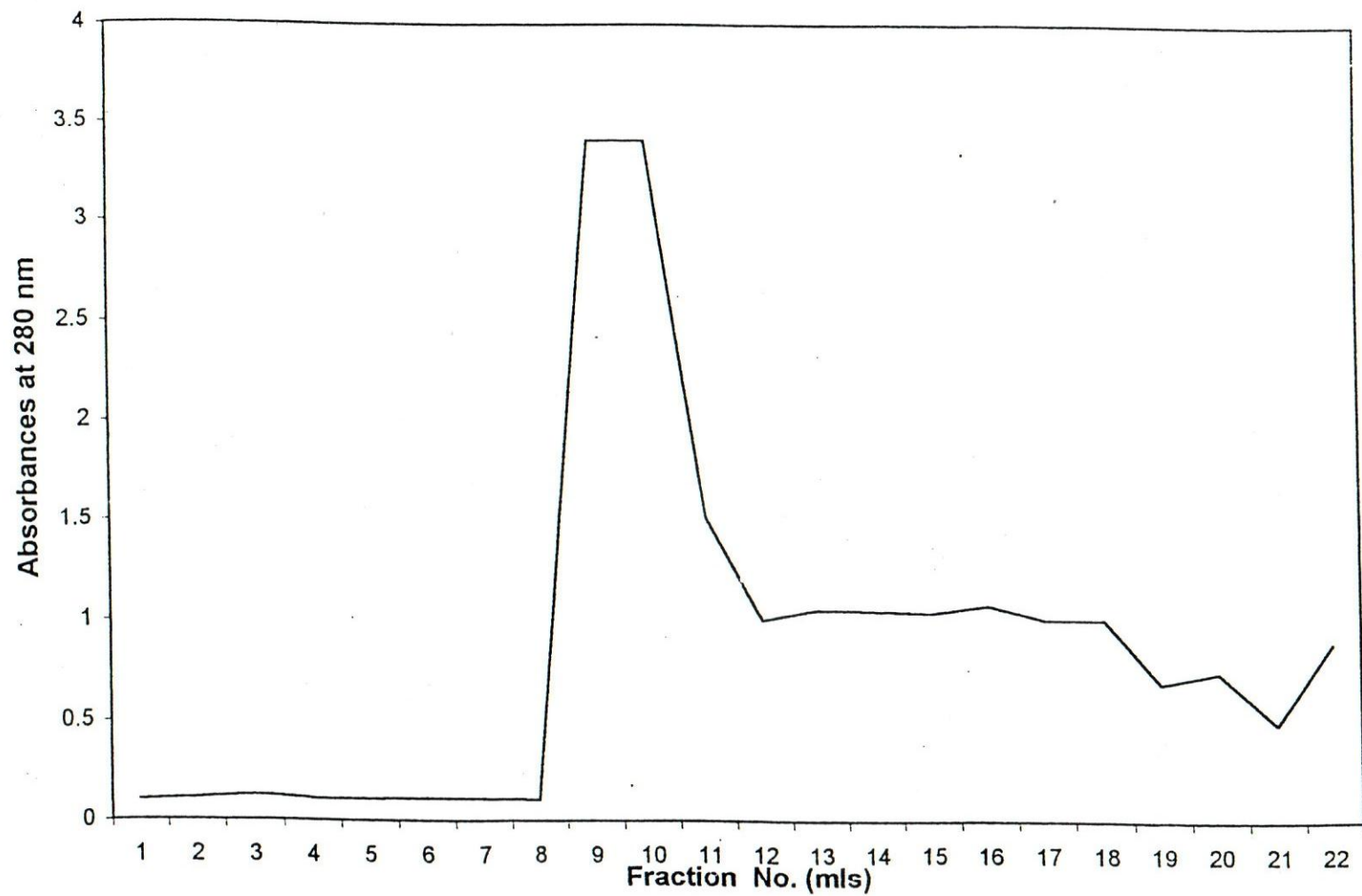
Log molecular weight vs Relative movement (Rf)
Log. mol. wt.= 2.2504 - 1.042 *Rf
Correlation: r = -.9393

APPENDIX IV: Molecular weight determination of transferrin on SDS PAGE

Dye-front distance = 4.04 cm

Mr. standards	Mr. (kDa)	Log. Mr.	Distance(cm)	Rf
1	212 kDa	2.326	0.3	0.068
2	116 kDa	2.064	0.6	0.136
3	97.4 kDa	1.988	20.8	0.182
4	66.2 kDa	1.821	1.5	0.341
5	57.5 kDa	1.760	2.0	0.454
6	40.0 kDa	1.602	3.0	0.682
Sample (transferrin)			1.45	0.3295

Appendix V: Molecular weight estimation of transferrin on 7.5% Native PAGE
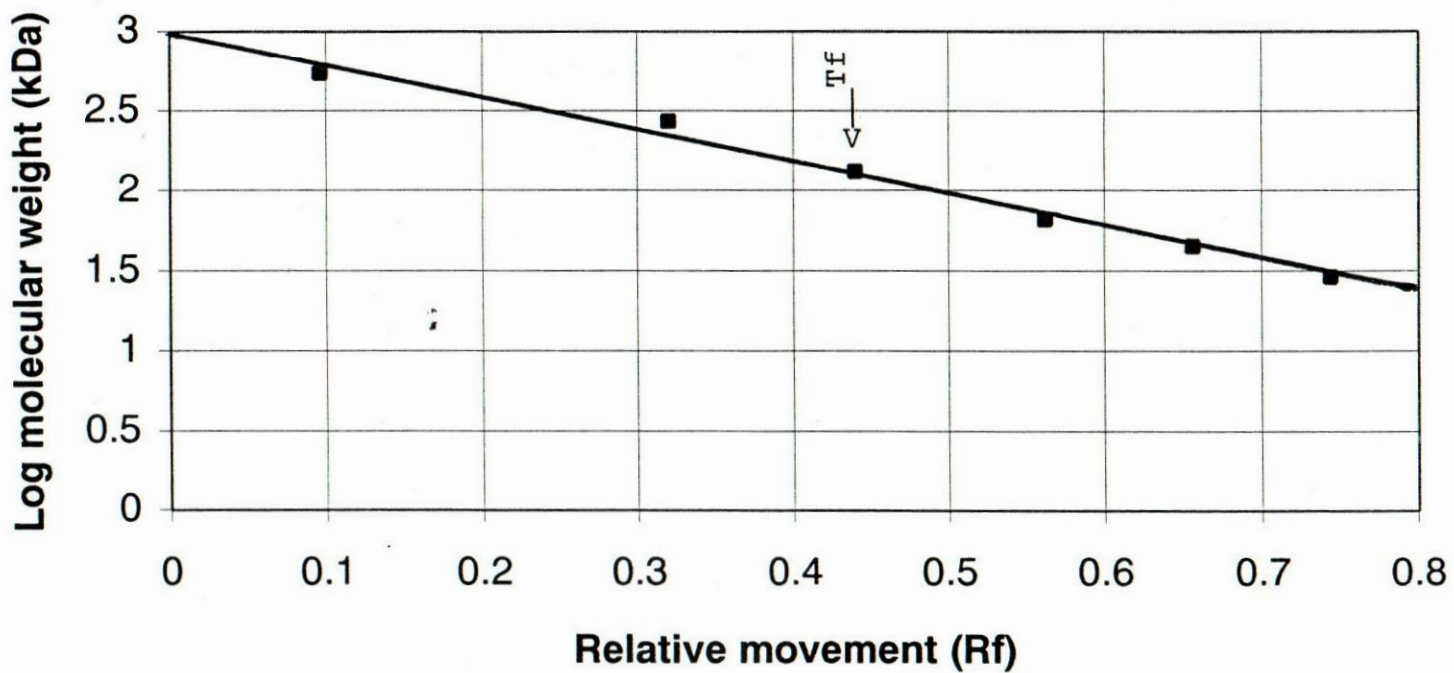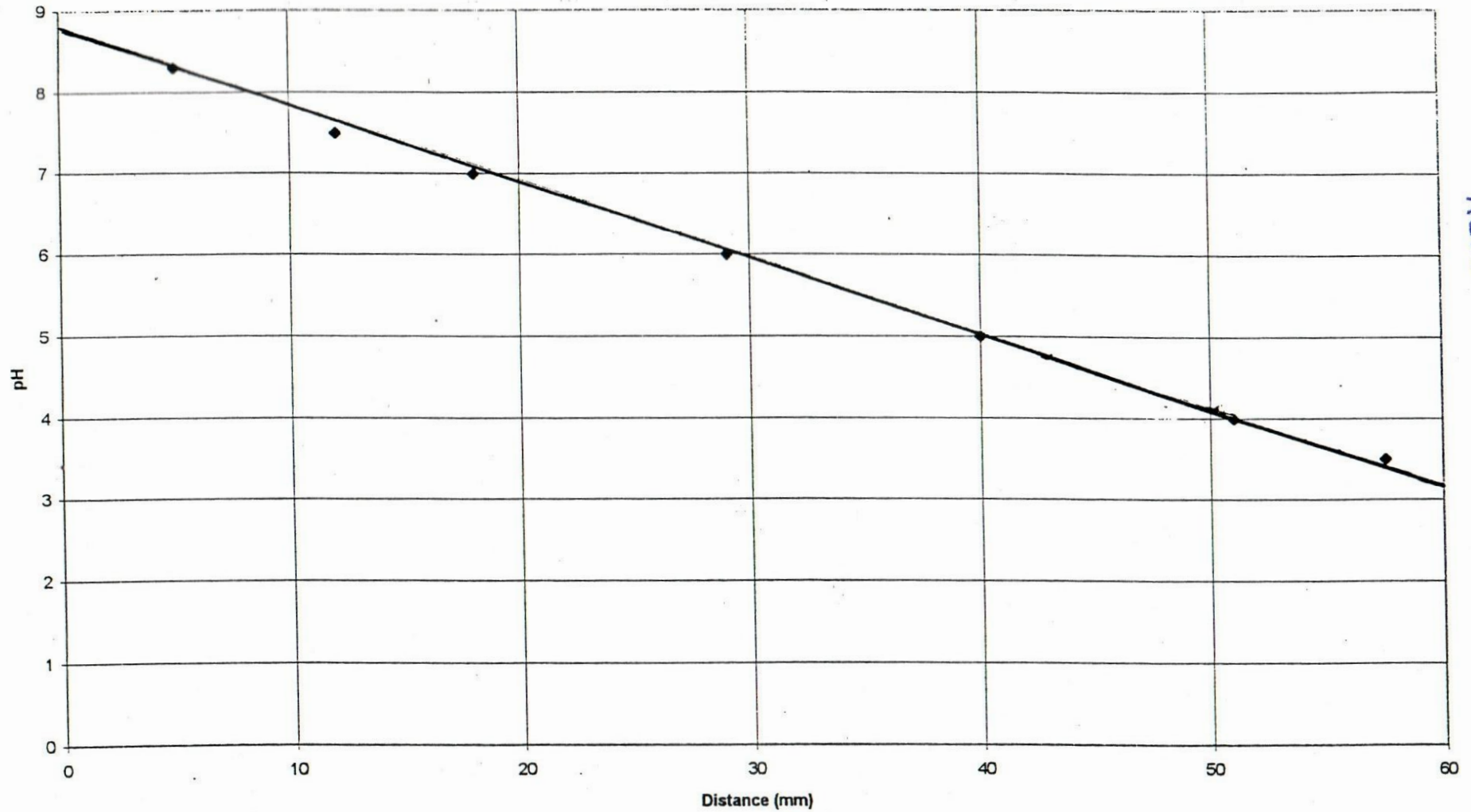
Appendix V: Molecular weight estimation of transferrin on native PAGE

Dye-front distance = 6.25 cm

Mr. standards	Mr.	Log. Mr.	Distance(cm)	Rf
1	545.0	2.7363	0.6	0.096
2	272.0	2.4346	2.0	0.32
3	132.0	2.1206	2.75	0.44
4	66.0	1.8195	3.51	0.562
5	45.0	1.6532	4.10	0.656
6	29.0	1.4624	4.65	0.744
Sample (transferrin)			2.70	0.4320

Appendix VI: Standard curve for the determination of the isoelectric point of transferrin.