STUDY ON TRYPANOSOMA EVANSI-DERIVED HAEMOLYTIC ACTIVITY

BY

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A

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D E C L A R A T I O N

This thesis is my original work and has not been presented for a degree in any other University.

Signed.....

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This thesis has been submitted with our approval as University supervisors:-

Signed.....

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Chairman, Department of Biochemistry.

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DEDICATED

TO

MY WIFE ROSEMARY

AND

OUR CHILDREN

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

L I S T OF A B R E V I A T I O N S

Abreviation	Full name/meaning
ml	millilitre
µl	microlitre
M	Molar
mM	millimolar
µM	Micromolar
nMole	nanomole
nm	namometers
pMole	picomole
xg	Number of times the gravitation force
g	gram
mg	milligram
µg	microgram
PCV	Packed cell volume
HA	haemolytic activity
KCN	Potassium cyanide
E.D.T.A.	Ethylenediamine-tetra acetic acid
K	Kilo
RaTe	Rabbit anti-T.evansi serum
Penstrep	Penicillin and streptomycin
PSG	Phosphate saline glucose buffer
Hb	Haemoglobin
h	hour or hours
min	minutes

SUMMARY

Anaemia and tissue damage are characteristic of African trypanosomiases. The underlying pathophysiologic mechanisms are still obscure. It is, however, known that lysed African trypanosomes generate haemolytic activity (HA). During the course of this investigation it was shown by other workers that pathogenic trypanosomes possess phospholipase A_1 activity. This investigation attempts to define the basis of T.evansi HA.

It has been shown, in this investigation, that purified and bloodstream T.evansi parasites possess haemolytic, lymphocytolytic and phospholipases A_2 and B activities. The HA was present only in the particulate fraction of T.evansi lysate (i.e. the fraction with HA was completely sedimented at 100,000xg) while the lymphocytolytic activity was present in both the crude lysate and soluble 100,000xg supernatant. All the three fractions: the crude lysate, the 100,000xg pellet and the 100,000xg supernatant possessed both phospholipases A_2 and B activities.

The HA and phospholipases A_2 and B were completely inactivated by heating a freshly prepared parasite lysate at 89°C for fifteen minutes. However, the HA of the parasite lysate that was first incubated at 37°C for sixteen hours and then heated at 89°C/15 min was not inactivated by the heat. This HA was regenerated when commercial phospholipase A_2 or 100,000xg T.evansi lysate supernatant was added to the heat inactivated fresh parasite lysate. The HA was present

only in the chloroform extract of the parasite lysate that had been incubated at 37°C for sixteen hours. However, the chloroform extracts of non-incubated and heat-inactivated parasite lysate or fresh unheated parasite lysate had no HA. The HA and phospholipase B (but not phospholipase A_2) were inhibited by 5mM KCN or E.D.T.A.

HA and phospholipase B (but not phospholipase A_2) were detected in heavily parasitaemic plasma from rats infected with T.evansi. Anaemia could be induced in mice by a single intravenous dose of T.evansi lysate extracts, commercial phospholipase A_2, lysolecithin or palmitic acid. However, a similar injection of heat-treated (89°C, 15 min) T.evansi lysate failed to cause anaemia.

An antiserum (RaTe) raised in rabbits against crude T.evansi lysate blocked HA. Neither phospholipase A_2 nor B activity was blocked by this antiserum.

It is concluded that T.evansi contains phospholipases A_2 and B activities. The T.evansi derived HA is generated by phospholipase B enzymatic reaction on the parasite endogenous substrates. The haemolysis itself is caused by heat-resistant, chloroform soluble products (probably free fatty acids) of phospholipase action on parasite phospholipids. Phospholipases released in vivo by lysed trypanosomes may be responsible for the anaemia and tissue damage characteristic of African trypanosomiases.

Part of this study: "Haemolytic Activity of Trypanosomes" has been published in the East African Medical Journal, 58, (12), 907-911, (1981).

CHAPTER ONE

INTRODUCTION

1.1 What is currently known about anaemia in trypanosomiasis and trypanosome derived haemolytic activity.

Although anaemia has long been established as a hallmark of animal trypanosomiasis (Murray, 1974; Jennings *et al*, 1974; Ikede *et al*, 1977; Woo and Kobayashi, 1975; Assoku, 1975), its aetiology remains unknown. It is, however, thought by many investigators to be one of the most significant factors in the pathophysiologic process of bovine trypanosomiasis (Murray, 1974). It has been postulated that the anaemia is haemolytic in origin (Jennings *et al*, 1974, Mamo and Holmes, 1975; Kobayashi *et al*, 1976; Ikede *et al*, 1977), but the underlying mechanisms have not been conclusively established. Assoku (1975), working with *Trypanosoma evansi*, observed severe anaemia with increased bone-marrow erythropoietic activity in rats. He was also able to induce moderately severe anaemia by repeated intravenous injections into rats of soluble *T.evansi* crude antigens and attributed the anaemia to an antibody-mediated mechanism: soluble antigens coat the erythrocytes; antibodies produced by the host react with these antigens to form immune complexes on the erythrocytes and in the presence of complement the erythrocytes are lysed. Direct and indirect Coomb's tests

used to detect these antigens and antibodies gave only sporadic positive reactions thus giving doubt to the strength of the mechanism he proposed. His postulations also suggested that erythrophagocytosis, direct toxins and haemolytic or enzymatic effect of T.evansi or its products on red cells may act multifactorially to contribute to the anaemia. Similar postulations about the multifactorial mechanisms of anaemia have been expressed by Murray et al (1979) and Murray (1979).

T.evansi stabilate used in this study always resulted in very high parasitaemia in rats and, depending upon the infecting dose, the infection almost always resulted in death of the rodents between the third and tenth day of infection. At such high parasitaemia there is likely to be massive circulating immune complexes and toxins released from parasites killed by immune response, the combined effect of which would undoubtely be fatal. The question is, what factor is the most lethal? Anaemia is merely a sign of erythrocyte damage and cannot be the sole cause of the sudden onset of death observed in rats infected with highly virulent T.evansi. There must be damage to other vital cells, tissues and organs. the combined effect of which would explain the sudden onset of death. Cossio et al (1974) have shown that in T.cruzi infections (American trypanosomiasis called Chaga's disease) immune response generates antibodies which cross-react with vital organs and tissues of the host. Although such a phenomenon is currently not accepted, it is likely that anti-host cell antibodies can be induced by host

cell surface alterations (most probably caused by trypanosome enzymes) resulting from hydrolysis of surface molecules by parasite products. Thus, it seems likely that this latter mechanism may be operative in T.evansi infections. It is also likely that toxins released by killed and ruptured parasites will not only cause lysis of erythrocytes but also lysis of other host cells and damage to vital tissues and organs such as the central nervous system.

Anaemia, in most parasitic infections, is probably an early and sensitive indicator of potential damage that will soon be inflicted upon other areas of the host before death. The cause of anaemia may also be due to factors that also cause the most serious or fatal damage during the progression of disease. It therefore seems worthwhile to study the mechanisms of anaemia in parasitic diseases such as trypanosomiasis so that the causes, which may also be responsible for more serious injury elsewhere in the host, can be elucidated for preventive or therapeutic purposes. In African trypanosomiasis the search for the causes of anaemia and attempts to elucidate its mechanism have attracted the interest of many investigators (Murray, M., 1974; Mamo and Holmes, 1975; Jennings et al, 1974; Kobayashi et al, 1976; Tizard and Holmes, 1976; Mackenzie and Gruickshank, 1973; Mackenzie et al, 1978; Davis et al 1974; Tizard et al, 1977; Ikede et al, 1977; Woo and Kobayashi, 1975; Huan et al, 1975; Tizard et al, 1978; Assoku, 1975; Masake, 1980; Hambrey et al, 1981).

Landsteiner and Raubischek (1907) showed that an ethanol-extractable haemolysin was released by autolysing Trypanosoma equiperdum. The solubility of this principle in absolute alcohol suggested its lipid nature. Huan et al (1975), however, demonstrated a soluble protein -like haemolysin in T.brucei which was capable of causing slow lysis of unsensitized sheep erythrocytes. Huan et al (1975) partially purified the haemolysin using Sephadex G-100 and G-25 and associated its haemolytic activity with a soluble protein peak of 10,000 daltons. This appeared to be a different factor from that demonstrated by Landsteiner and Raubitschek (1907). Tizard and Holmes (1976) demonstrated a haemolysin generated by T.congolense upon autolysis and this haemolysin was postulated by Tizard, et al (1977) to be free fatty acids generated by phospholipase A_1 action on endogenous phosphatidylcholine substrates.

The discrepancy between Huan's and Tizard's work has not been experimentally resolved. Phospholipases of pathogenic and non-pathogenic Trypanosoma species were investigated and published (Hambrey, et al 1981) during the course of this thesis research. Hambrey, et al (1981) demonstrated high levels of phospholipase A_1 in pathogenic Trypanosoma species and low levels in non-pathogenic Trypanosoma species and consequently suggested a hypothesis that phospholipases are associated with pathogenesis of trypanosomes. Whether this pathogenesis includes haemolytic anaemia, and damage to other cellular elements caused by trypanosomes remains to be shown.

Mammalian cell membranes contain phospholipids in their structural organisation (Parsons, 1975). Specific phospholipids that are likely to be hydrolysed by phospholipases are phosphatidyl choline, sphingomyelin, phosphatidyl inositol and lysophosphatidyl choline. All these phospholipids are structural components of not only plasma membranes but also other membranes of the subcellular organelles. In rat erythrocyte membranes it has been shown by Renooij et al (1976) that lysophosphatidylcholine, sphingomyelin and phosphatidylcholine are bound, in large proportions, on the outside fracture face of the membranes. These phospholipids are therefore accessible to the action of phospholipases A and B as was shown by Renooij et al (1976). Studies by these investigators revealed that the outer surface phospholipids are hydrolysed by phospholipases but no haemolysis occurs. These workers observed, however, that the erythrocyte shape was distorted and action by sphingomyelinase caused even greater distortion. Such a perturbation in the erythrocyte shape was accompanied by decreased cell survival. Similar observations were made by Van Deenen (1981), who reported morphological changes and great disorganisation of phospholipids on the outer surface of the erythrocytes after treatment with phospholipase A_2. He also noted that there was increased osmotic fragility of the cells previously exposed to the action of phospholipases.

Zwaal et al (1975), using phospholipases A_2 from bee venom and Naja naja showed that these enzymes were able to cause a non-haemolytic breakdown of 50% of the phosphatidylcholine in the human erythrocytes, while sphingomyelinase C from Staphlococcus aureas was able to produce a non-haemolytic degradation of more than 80% of the sphingomyelin. Phospholipase C from Clostridium welchii, on the other hand, produces direct haemolysis of the human cells (Zwaal et al, 1975). Some phospholipases, depending on their source, are neither haemolytic nor hydrolytic to phospholipids in intact cells (Zwaal et al, 1975). Zwaal et al observed that phospholipase A_2 from pancreas and Crotalus adamanterus, phospholipase C from Bacillus cereus and phospholipase D from cabbage produced neither haemolysis nor hydrolysis of phospholipids in intact human erythrocytes.

In this investigation it was considered necessary to establish the generation of a similar haemolytic factor in vitro by purified Trypanosoma evansi. Assoku (1975) working with the same organism did not show the presence of such a factor. Nevertheless, it was anticipated that perhaps all the pathogenic African trypanosomes generate this factor in vitro. The haemolysins reported by Landsteiner and Raubistchek (1907), Huan et al (1975), and Tizard and Holmes (1976) and Murray et al (1979) were generated by allowing the purified trypanosomes to autolyse at room temperature or at 37°C before the presence of this activity was sought. The haemolytic factor studied in this investigation was generated

by artificially lysing freshly isolated parasites by rapid freezing and thawing using liquid Nitrogen (-196^{o}C) and 37^{o}C waterbath respectively. This approach, which was also adopted by Murray (1979), minimized the possibility of the haemolytic factor being generated by metabolism of trypanosomes when left to incubate at room temperature or at 37^{o}C as was the case in Huan's and Tizard's work. Thus the haemolytic factor detected in parasite lysates prepared in this manner, for this investigation, was thought to originate from the parasite's normal body constituents.

1.2 Objectives of this Investigation.

(a) Why T.evansi has been studied.

T.evansi is the causative organism of the classical "surra", a disease known to affect several mammalian species and is characterized by fever, anaemia, followed by oedema and cachexia. Surra is widely distributed in countries with warm and hot climate such as the Middle East and the southern part of Soviet Union, Iran, India, Indo-China, Central and South Africa, Morocco, Algeria, Tunisia, Sudan, Egypt, Senegal, Mali, Chad and parts of West Africa lying at latitude 15-16^{o} North. The domestic animals most seriously affected by surra are camels, horses and dogs. T.evansi is also pathogenic to most laboratory mammals.

Unlike T.brucei, its close associate, T.evansi is typically monomorphic although some strains exhibit some

form of polymorphism but only sporadically whereas in T.brucei polymorphism is a constant characteristic feature of normal strains.

The principle intermediate vector is the tabanid fly which transmits the parasite mechanically. T.evansi is, so far, not known to undergo any cycle of development in the intermediate host, for instance it is incapable of developing in Glossina genus (tsetse) for the trypanosomes ingested by the fly are killed in the gut within six hours and digested together with the blood.

T.evansi has many features in common with T.brucei but unlike the latter it is, as mentioned earlier, typically monomorphic and occurs only outside the area of distribution of Glossina. On the other hand laboratory forms of T.brucei also become monomorphic and lose their capacity to develop in the tsetse fly. The parallelism between the two organisms support the hypothesis that they are phylogenetically closely related. The parasite is, therefore, economically important for study in this investigation. Furthermore, current literature indicates that T.evansi is not as extensively studied as T.brucei.

(b) Why haemolytic activity (HA) of T.evansi has been studied in this investigation.

African trypanosomes are extra cellular parasites but the injury they cause in susceptible mammals attracts interest in the understanding of the mechanism of the cellular injury.

The main question is the nature of cell injury; is it caused by physical interaction of the parasite with the cell, by toxins released during parasite metabolism and/or parasite death, or by parasite exhaustion of nutrients vital to host survival? This question was the main guideline in this investigation. Anaemia is the hallmark of African trypanosomiasis and since it can be caused by increased lysis or removal of red blood cells it was decided to investigate haemolytic property of T.evansi. It was speculated that factors that brought about red cell lysis may also cause lysis of other cells of the host. Once these factors are characterized they may help in the understanding of the pathophysiological mechanisms of African trypanosomiases. It was also anticipated that purified forms of these cytopathogenic factors might be used to immunize animals against the deleterious effects of the factors and thus provide some or substantial protection against the pathogenesis of the parasites. In this way the animal might be in a better condition to eliminate the parasite by its own immune mechanisms.

This investigation also defines the chemistry of haemolytic factors, their general localisation in the parasite, their in vivo significance and pathogenic relevance to other host cells of the susceptible mammals and also attempts to purify enzymes involved in the generation of the HA.

CHAPTER TWO

MATERIALS AND METHODS

2.1.1 Source of Experimental Parasites:

(a) T. evansi:-

A strain of Trypanosoma evansi obtained from a stock initially isolated from a camel in Sudan (Mahmood and Malik, 1978) had been extensively syringe passaged in rats. This stock was cryopreserved in liquid Nitrogen at Chiromo Department of Biochemistry, University of Nairobi, Kenya.

(b) T.musculi:-

Was obtained as a stock stabilate of CBA mouse blood from Chester Beatty Research Institute (Institute of Cancer Research), London in the United Kingdom by courtsey of Professor A.J.S. Davis. The parasite had been initially adapted in CBA mice. The stock was kept cryopreserved in liquid Nitrogen at ILRAD. For use in this investigation the parasite was first cultured in either AJ mice or C3H/He mice. It was allowed to grow to about 1×10^8 parasites per ml of blood in these mice before the mice were sacrificed for purification of the parasite. After the first peak of parasitaemia the parasites were always undetectable from the circulation.

(c) T. brucei 221 (MITat 1.2, cloned from stock 427):
This was a cloned parasite stabilate maintained at ILRAD. The form used in this investigation had been extensively syringe passaged in rats. It was an extremely virulent and pathogenic parasite. Rats infected with this parasite were almost always killed within four days of infection.

2.1.2 Source of experimental animals:

(a) Rats:- strain, random bred Sprague Dawley rats. All the rats used in this investigation were reared at ILRAD.

(b) Mice:-
AJ, C3H/He and CBA strains of mice used in this investigation were all reared and maintained at ILRAD.

(c) Rabbits:- strain, N.Z.W. New Zealand White rabbits. The rabbits used in the production of anti-sera were also bred and maintained at ILRAD.

2.1.3 Reagents:
All reagents used in this investigation were of the ANALAR grade as supplied by Sigma Chemical Company, Pharmacia Fine Chemicals, Merck, Amersham or BDH, unless otherwise stated.

2.2. Infection of experimental animals.
All laboratory rodents used in this investigation were inoculated intraperitoneally with the appropriate

parasite stabilate previously thawed at 37°C and kept on ice. Rats and mice were inoculated with $2x10^6$ and $1x10^6$ live parasites from the stabilate respectively. Parasitaemia was monitored daily until it was heavy enough for harvest.

2.3 Purification of T.evansi from infected rat or mouse blood.

A strain of T.evansi initially isolated from a camel in the Sudan (Mahmood and Malik, 1978) was propagated in albino rats by syringe passage. At peak parasitaemia, usually between three and seven days after infection, the rats were bled through the heart and blood was collected into heparin containers (50 commercial heparin units per 50 ml of blood, plate 1). Parasites were recovered from infected blood using DEAE cellulose (Whatman De 52) ion exchange chromatography (as described by Lanham and Godfrey, 1970). The purified parasites were washed three times in 0.0607M phosphate buffer, 0.0436M sodium chloride, 1% D-Glucose (PSG) pH 8.0 by spinning at 600xg for five minutes. The washed parasites were resuspended in PSG to a concentration of 1×10^9 parasites per ml (plate 2).

2.4 Preparation of Trypanosome lysates.

Before lysis the above washed parasites were pelleted down by spinning at 600xg for ten minutes and then resuspended in the same volume of PSG pH 8.0 containing the antibiotic, penstrep or gentamycin, at a concentration of lug/ml. The parasite lysate was prepared by alternately freezing the washed parasite at -196°C in liquid Nitrogen and thawing at

37°C in water-bath incubator. The disrupted parasites were homogenized to obtain a uniform suspension using a loose-fitting Dounce Homogenizer.

Parasite lysates were either used while fresh or stored at -80°C until needed.

2.5.1 Determination of HA in trypanosome lysates and extracts, infected rat plasma and chromatographic fractions.

Initially HA in the samples prepared from T.evansi lysate was determined by incubating 500µl of each sample with 50µl of washed and pelleted rat erythrocytes at 37°C in a waterbath. At 1h, 4h, 6h, 8h, 10h and 19h intervals 50µl of test mixture (i.e. 500µl sample and 50µl erythrocytes) were withdrawn and placed into a 10-ml centrifuge containing 5ml of PSG. The tube and contents were spun at 1000xg for five minutes. The clear supernatant was carefully withdrawn, placed in a separate tube and its absorbance read at 415nm on a Gilford 260 spectrophotometer against a PSG blank. A PSG negative control comprising 500µl of PSG buffer and 50µl of rat erythrocytes was processed identically as the test. In other cases HA was assayed on samples by a single incubation at 37°C for only sixteen hours. HA expressed as mgHb/ml was read off from the standard curve shown (Fig.1).

Plate 1: A photomicrograph of Giemsa stained blood film prepared from rat blood heavily infected with T.evansi (Magnification .....960x).

A stock stabilate of T.evansi was cryopreserved at -196°C in a liquid Nitrogen tank in one millilitre vials. The stabilate was rapidly thawed in a water-bath at 37°C and then placed on ice. Rats were given intraperitoneal injections of 0.2ml and undiluted stabilate and then monitored for parasitaemia three days after infection until they attained peak parasitaemia (about 1×10^9 parasites/ml). At the peak parasitaemia the rats were anaesthetized in a beaker containing cotton wool soaked in either chloroform or diethyl ether. The chest cavity was cut open and 0.5 to 1 ml of PSG containing 5 units of commercial heparin was added. The aorta was cut open allowing the blood to flow into the cavity. The anticoagulated blood containing live parasites was transferred to a suitable receptacle for purification of parasites.

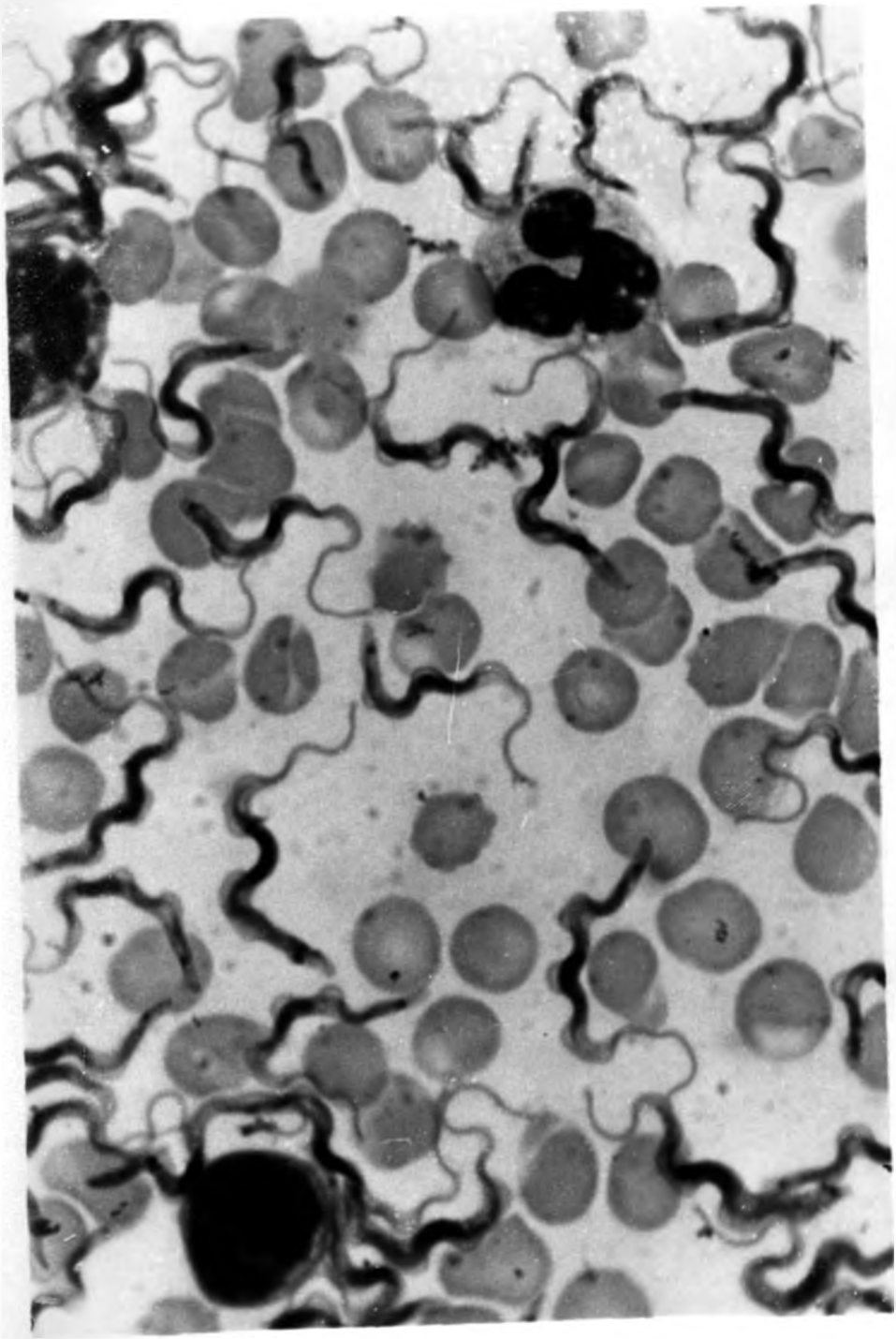
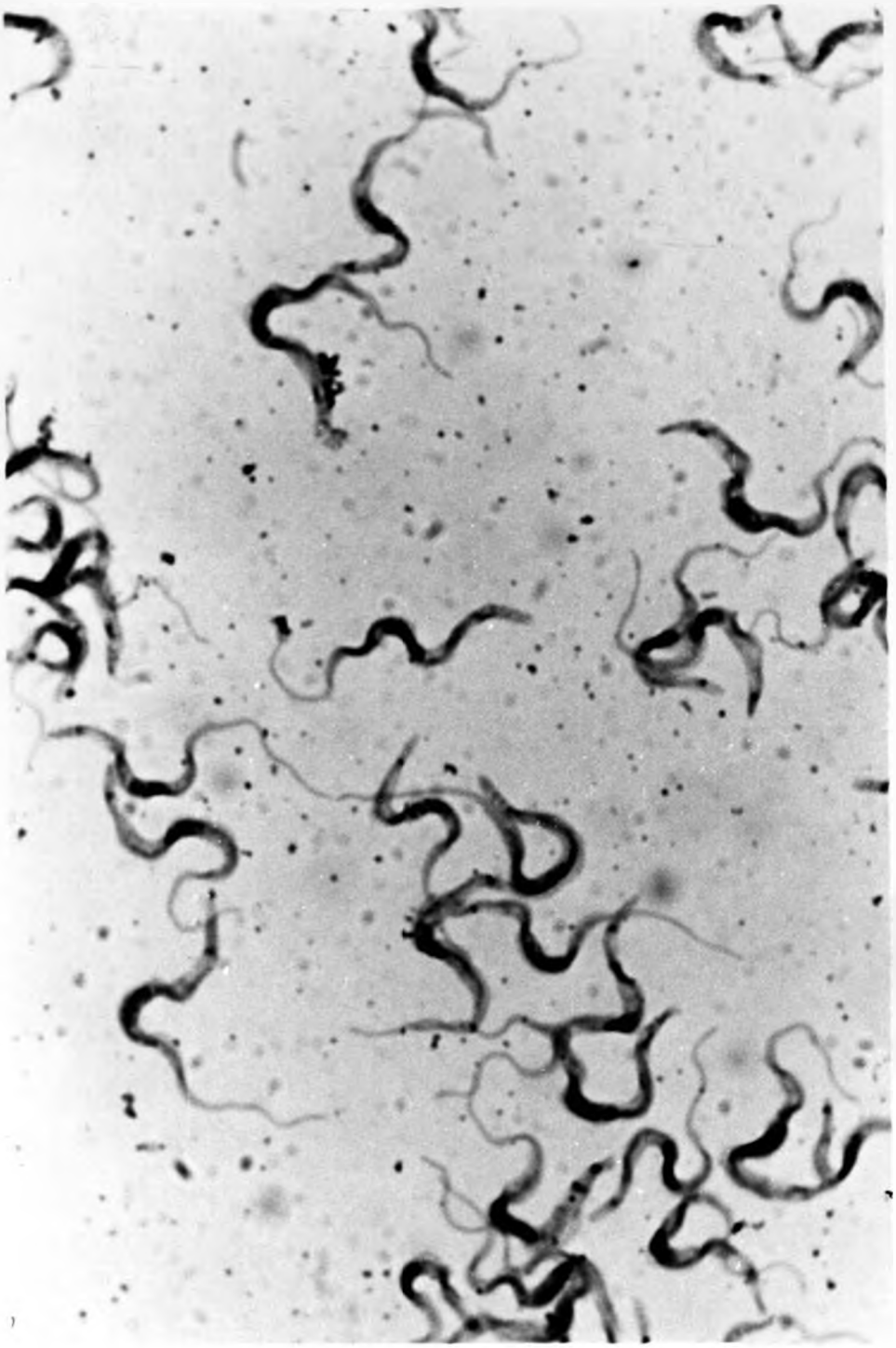
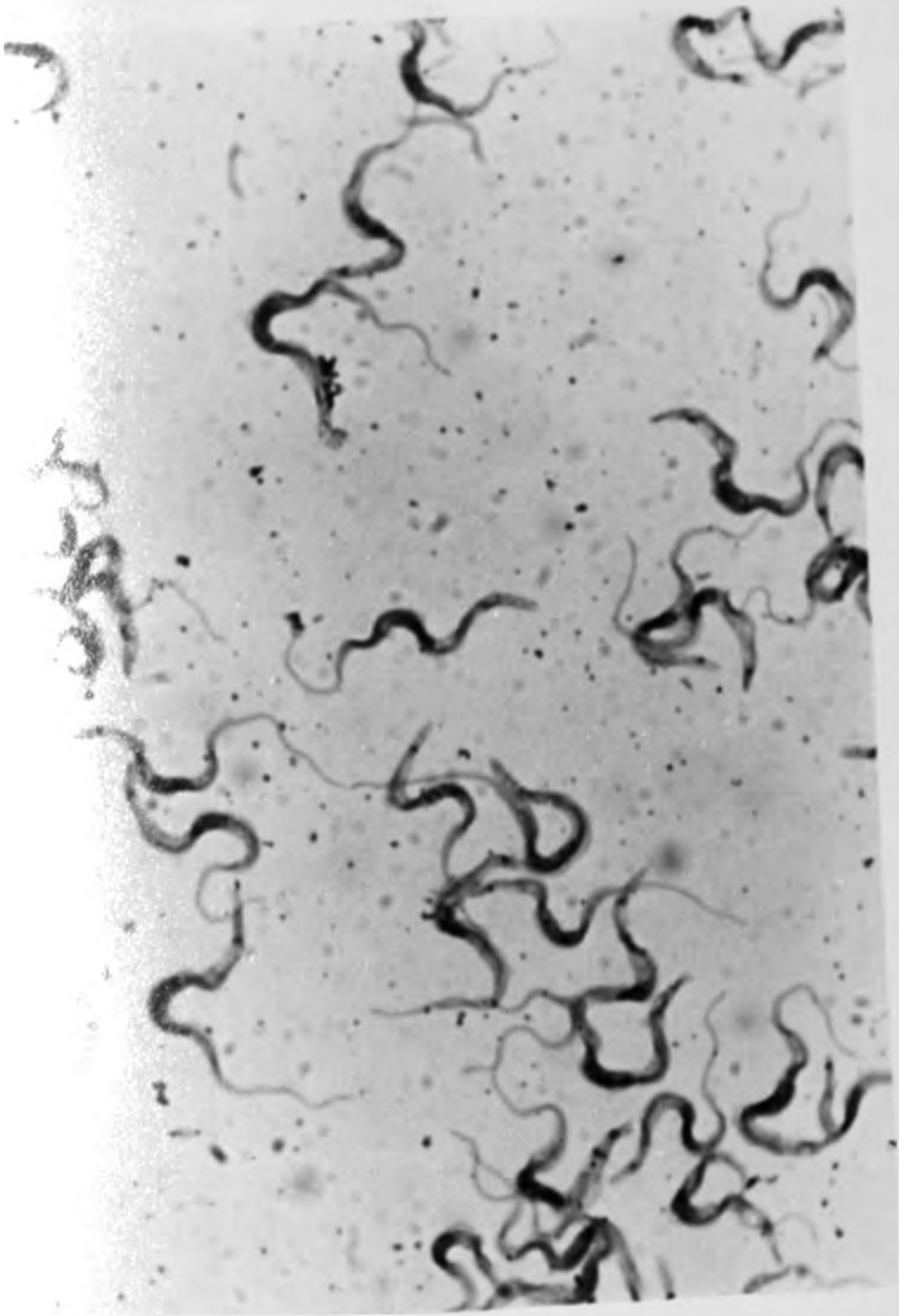


Plate 2: A photomicrograph of Giemsa stained film prepared from a sample of T.evansi purified by DE-52 ion exchange Chromatography as described below. (Magnification .....960x).

The infected blood was first centrifuged at 2000xg for ten minutes in 10ml conical centrifuge tubes. The interface between the packed red blood cells and the plasma (or the buffy coat) contained most of the parasites in concentrated form. After removing the excess plasma the buffy coat was recovered and placed into a separate receptacle. Some red blood cells were removed together with the buffy coat to facilitate maximum harvest. The recovered buffy coat (containing a mixture of parasites, white blood cells, platelets and red blood cells) was diluted three times with PSG. It was then loaded onto a DE 52 column equilbriated in PSG so that the packed slury volume was twice the volume of diluted buffy coat. The parasites were isolated from the rest of blood cellular elements by ion exchange chromatography as described by Lanham and Godfrey (1970). The isolated parasite suspension was centrifuged at 600xg for five minutes to a pellet form and the supernatant decanted off. After dispersing the pellet by gentle tapping fresh PSG equal in volume to the supernatant was added. The parasite suspension was centrifuged again at 600xg for five minutes, and the supernatant decanted off. The pellet was finally resuspended in PSG containing lug/ml gentamycin to a concentration of $1x\ 10^9$ parasites/ml.





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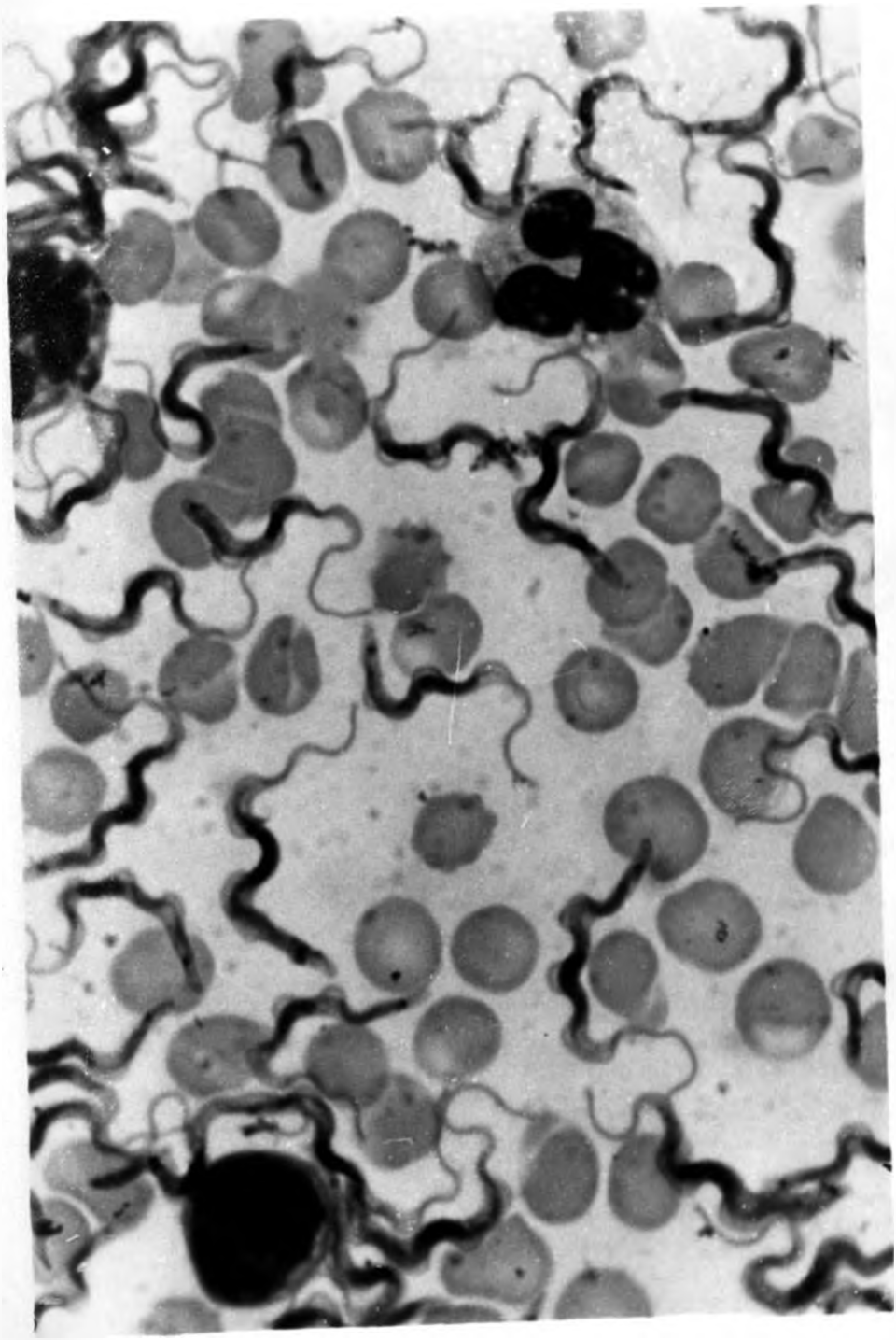
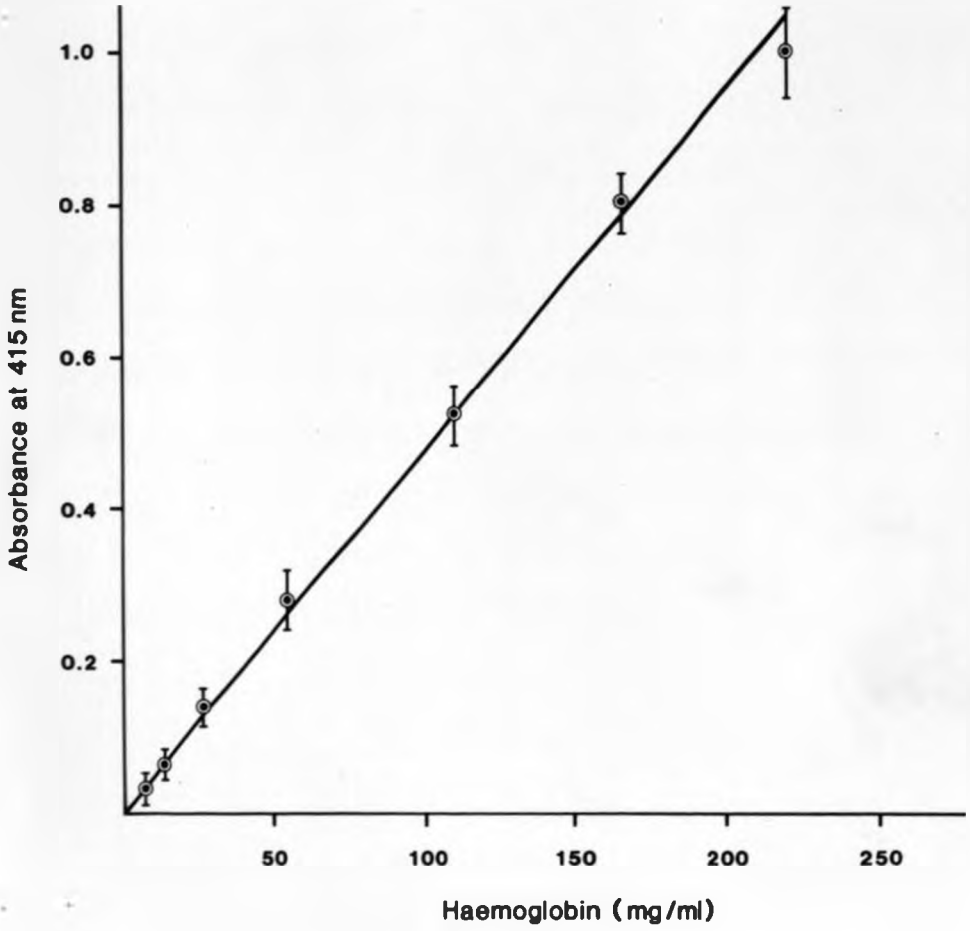


To determine the HA of T.evansi lysates prepared as described previously it was important to ensure that the reaction mixture was isotonic to the cell constituents of the erythrocytes used. This was achieved by initially preparing the parasite lysate in a physiological buffer and the PSG used in this investigation provided this condition. The procedures used to purify the parasites and prepare parasite lysates were not aseptic. Thus bacterial contamination of the samples was unavoidable. As a result bacterial growth in these samples was a serious problem since these bacteria generated haemolytic activity. It was therefore necessary to arrest bacterial growth in all assays for trypanosome derived haemolytic activity by addition of antibiotic to the PSG buffer used in sample preparation. A mixture of pencillin and streptomycin (penstrep) or gentamycin was found to be very effective in arresting bacterial growth. Before reading haemolysis, however, the reaction mixtures were checked for absence of bacterial growth using darkground-phase microscopy.

The incubation period for haemolytic activity assay was decided by time-response curve. Maximum haemolysis occurred after twelve to twenty hours of incubation. Negative controls usually gave 0-5 mg/ml of Haemoglobin. Haemolytic activity was considered significant when haemolysis was equivalent to 10mg Hb/ml and above. Haemoglobin release by some negative controls within the 0-10mg/ml was regarded as due to autohaemolysis of the batch of erythrocytes used.

Fig 1 A standard curve for haemolytic activity determination.

Whole blood was aseptically obtained from a normal rat by bleeding through the heart into a sterile syringe containing heparin anticoagulant. The blood was transfered to a sterile plastic conical centrifuge tube and ten times its volume of sterile PSG was added. The red blood cells were washed by spinning at 600xg for five minutes. The washing was repeated twice. The supernatant was removed using a Pasteur pipette, leaving washed red blood cells in a pellet form. Haemoglobin concentration in the pelleted red blood cells was determined by Coulter Counter. An aliquot from the pellet (50 µl) was artificially lysed by adding it to 500 µl of distilled water. This was regarded as a stock standard lysate. This was then diluted 100-fold with PSG to give 100% haemolysis equivalent to 220 mg/ml haemoglobin released. Other standards of lower haemoglobin values were prepared by diluting the 220mg/ml haemoglobin standard. The absorbances of different standard dilutions were obtained by reading at 415 nm on a Gilford 260 spectrophotometer using PSG buffer as a blank reference. The standard curve given was obtained from four assays for each point on the curve.


1.0
0.8
0.6
0.4
0.2
Absorbance at 415 nm
50
100
150
200
250
Haemoglobin (mg/ml)

A longer period of incubation (24-48 hours) was allowed for samples that contained low haemolytic activity, for instance, infected plasma and chromatographic elution fractions.

2.5.2 Determination of packed cell volume (PCV) and HA before and during the progression of parasitaemia in rats infected with T.evansi.

Twenty one adult rats (same age and breed) were divided into seven groups of three's. PCV was then determined for each rat in each group. PCV values were averaged in each group. On day zero rats in groups 2,3,4,5 and 6 were uniformly infected with T.evansi parasites recovered from a stabilate preserved in liquid Nitrogen as described previously. On day 'one' all rats were checked for parasitaemia. Three rats from any of the groups which were positive for parasitaemia had their PCV determined. Blood was collected from all the three rats into heparinized containers as described elsewhere in chapter two. The blood was immediately centrifuged at 3000xg for ten minutes and the plasma was recovered and stored at $-80^{o}C$ till required. On days 2, 3, 4, 5 and 6 a similar procedure of parasitaemia count, PCV determination and plasma sample recovery and storage was used. PCV and parasitaemia values were averaged for each group and sera were also pooled before storage. On day 'six' group 1 (uninfected control group) had PCV determined and plasma collected as described. The haemolytic activity in the plasma samples was determined as previously described.

2.6 Determination of phospholipases A_2 and B activities in trypanosome lysates, rat plasma and chromatographic fractions.

Phospholipase activity was determined using methods used by Nashijima et al (1974). For a single assay 15 nano moles of radio labelled substrate (L-α- phosphatidylcholine, β-[1-^{14}C] palmitoyl - Palmitoyl (for phospholipase A_2 assay) or L-Lysophatidylcholine, 1-[1-^{14}C] palmitoyl (for phospholipase B assay) contained in Toluene was evaporated to dryness under Nitrogen gas atmosphere. The radio labelled substrate was resuspended in 500µl of 0.1M tris-HCl buffer pH8.0 and thoroughly mixed by vortexing. Triton X-100 was added to a final concentration of 0.4% (this eventually diluted out to 0.2% in the reaction mixture below). Tris-HCl buffer (400µl) was warmed in a water-bath at 37°C, to which was added 100µl (20 to 100 µg of protein) of the sample. Three minutes were allowed for equilibriation. Meanwhile the substrate mixture prepared as described above was also warmed at 37°C. At zero time the whole substrate mixture was added to the sample mixture in a 10ml glass tube. The contents were thoroughly mixed and allowed to incubate at 37°C for 5 to 20 minutes. The reaction was stopped by adding 500µl of 20% trichloroacetic acid followed by 500µl of 16% Triton X-100. After thorough mixing 5 ml of n-Hexane was added and the

contents were further mixed by inversion for 15 seconds at room temperature. A blank control was identically processed using 500ul of Tris-HCl buffer without the sample. The tubes were centrifuged at 600xg for fifteen minutes at room temperature. From the upper n-Hexane layer 200 ul was withdrawn and placed into scintillation vials containing 5ml aquasol for B-emission counts using tri-carb 2660 scintillation counter (phospholipases are identified as illustrated in figure 2).

Calculation of phospholipase activities.

To convert radioactivity counts per minute (CPM) to units of enzyme activity, the quantity of radioactive substrate (nmole) used in each assay and the equivalent radio-activity (CPM) of this quantity was considered.

One unit of enzyme activity (U) was defined as the apparent and free amount of enzyme that was able to transform one picomole (pmole) of the radiolabelled substrate to products.

The values given in this report are specific activites (Us) expressed as pmoles/min/mg protein. The following formula was used for the calculations:-

$$Us = \frac{S \times CPM^{o} \times 10^{6}}{P \times CPM^{t} \times T}$$

where S = substrate concentration in nanomoles (nmole)

CPM^{o} = Total counts per minute in the n-Hexane layer

CPM^{t} = Total counts per minute given by S

P = Amount of sample protein used in the assay in microgrammes.

T = Time in minutes.

2.7.0 Characterisation of the haemolytic activity of T.evansi.

2.7.1 Inactivation of HA by heat.

Immediately after the parasites were isolated from infected rat blood and homogenized as described previously, they were aliquoted into three equal parts. One aliquot was subaliquoted and exposed to various temperatures for fifteen minutes. The second part was heated at 89°C (boiling water temperature at Nairobi) for fifteen minutes. The third part was kept frozen at -80°C. All the three aliquots were assayed for HA as previously described.

2.7.2 Effect of KCN and E.D.T.A. on HA.

Immediately after purification, the parasites were pelleted down by spinning at 600xg for five minutes and then resuspended in PSG containing 5mM KCN or 5mM E.D.T.A. (disodium salt) to a concentration of 1×10^9 parasites/ml. Both suspensions were freeze-thawed three times using liquid nitrogen (-196°C) and 37°C water-bath. They were then assayed for HA.

2.7.3 Sedimentation of the fraction with HA at 100,000xg.

T.evansi lysates prepared as described previously in 2.4, 2.7.1 and 2.7.2 were ultracentrifuged at 100,000xg for 90 minutes and both pellets and supernatants were assayed for HA by various incubation periods at 37°C.

2.7.4 Extraction of HA by organic solvents: effect of heat and incubation time on extracts.

Organic solvent extraction was carried out on the following samples:

i) T.evansi lysate that was incubated at 37°C for sixteen hours.

ii) Freshly homogenized T.evansi lysate.

iii) T.evansi lysate that was heat inactivated (89°C, 15min) and then incubated at 37°C for 16h.

iv) T.evansi lysate that was incubated at 37°C for 16h then heat inactivated (89°C).

One ml of each of the above in a stoppered glass test tube was mixed with 3.75 ml of methanol-chloroform mixture (2:1,v/v). The mixture was shaken intermittently for 90 minutes and centrifuged. The supernatant extract was transferred to a clean glass tube and the residue re-extracted with methanol-chloroform-water mixture (2:1:0.8) and the mixture centrifuged. The combined supernatant extracts were diluted with 2.5ml each of chloroform and water and centrifuged. The lower chloroform phase was withdrawn and diluted with an equal volume of benzene and dried under a stream of nitrogen at 30°C. The residues were each resuspended in 1ml of PSG. One ml of PSG was treated in the same way as the lysates and this provided a negative control for haemolytic activity assay. All five preparations were tested for haemolytic activity as described previously.

2.7.5 Regeneration of the HA from heat-inactivated T.evansi lysate by commercial phospholipase A_2 or 100,000xg T.evansi lysate supernatant.

Commercial phospholipase A_2 (Sigma; Naja naja or bee venom extract) was first suspended in PSG buffer to a concentration of 1mg/ml. Both the phospholipase A_2 (PLA2) and T.evansi 100,000xg lysate supernatant were tested for absence of HA. The PLA2 (250µl) or T.evansi 100,000xg supernatant (250µl) was added to the heat-inactivated T.evansi lysate (250µl) and the mixture was tested for HA.

2.8.1 Inactivation of phospholipase activity by heating.

T.evansi lysate aliquots were exposed to various temperatures up to and beyond 60°C and then assayed for phospholipases A_2 and B activities (and also HA) as previously described.

2.8.2 Inhibition of phospholipase activity by KCN and E.D.T.A.

T.evansi lysate samples previusly prepared as described in 2.7.2 were also assayed for phospholipase A_2 and B activities by the methods already described to establish possible inhibition of these enzymes by these ions.

2.9.1 Preparation of rabbit anti-T.evansi lysate (RaTe).

From each of two adult rabbits forty millilitres of pre-immune blood was taken. Each rabbit was immunized by inoculating 30µg of crude T.evansi lysate in Freund's complete

adjuvant via three sites: first subcutaneously at the back of the neck then second and third intramuscularly into gluteal muscles. The pre-immune blood was allowed to clot and serum was recovered and stored at -80°C until needed. After two weeks each rabbit was given a boost inoculum of 50µg of lysate through the same sites in incomplete Freund's adjuvant. A third inoculum of 75ug lysate was given after another two weeks and the rabbits were test bled after the following ten days.

2.9.2 Effect of RaTe serum on haemolytic and phospholipase activities of T.evansi lysate.

T.evansi lysate and complement inactivated (56°C/30min) RaTe serum were initially mixed in a volume ratio of 1:1. The mixture was pre-incubated on ice for thirty minutes and then aliquots of the mixture were removed for HA and phospholipases A_2 and B assays as previously described. Pre-immune serum was similarly complement inactivated and added to T.evansi lysate in the ratio 1:1 and the mixture was processed in the same way as the test mixture above. The pre-immune serum provided the control of this assay.

2.10.0 Intravenous injections of T.evansi Lysate and lysate extracts, commercial phospholipase A_2, lysolecithin and palmitic acid into AJ mice to study their effects on packed cell volume (PCV) of the mice.

2.10.1 Experiment 1

Seven groups of AJ mice (4 mice, 6-8 weeks old per group) were given intravenous injections of the following extracts respectively:

A: T.evansi crude lysate

B: 180xg T.evansi pellet

C: 6000xg T.evansi pellet

D: 22,500xg T.evansi pellet

E: 100,000xg T.evansi pellet

F: T.evansi heat-inactivated lysate.

One group of the mice was given only PSG, a medium for the above fractions. Each fraction injected in each mouse was equivalent to 2.5×10^8 T.evansi parasites and was delivered in 0.2ml for each mouse. Pre-injection packed cell volume (PCV) was done for each mouse before the experiment was started. The PCV for each mouse was monitored 1h, 10h and 34h after injections. For each group PCV values of the four mice were averaged. PCV versus time curves were finally constructed. PSG group provided a negative control for all the groups.

2.10.2 Experiment 2

Five groups of AJ mice (6-8 weeks old) were each given 0.2ml intravenous injections containing one of the following:

A. 100,000xg pellet of 2.5×10^8 T.evansi parasite equivalent from T.evansi crude lysate in PSG.

B. 100,000xg supernatant of 2.5×10^8 T.evansi parasite equivalent from T.evansi crude lysate in PSG.

H. 200µg of palmitic acid in PSG.

G. 200µg of phospholipase A_2 in PSG.

I. 200µg of Lysolecithin in PSG.

Each group consisted of four AJ mice. Preinjection PCV for each mouse was taken before the experiment was started. Change in PCV was monitored at 16h., 24h., 36h. and 66h. intervals after the injections. PCV values were averaged for the four mice in each group. Change in PCV was plotted against time.

2.11 Determination of the lymphocytolytic activity (cytotoxicity) of T.evansi lysate using normal bovine peripheral lymphocytes.

Normal peripheral lymphocytes were purified from a normal cow's blood, collected in a sterile syringe containing heparin, by gradient centrifugation at 3000xg for thirty minutes: five ml of whole blood was layered on five ml of Ficoll Paque (Pharmacia Fine Chemicals, Upsalla, Sweden) and spun at 3000xg for thirty minutes. The interface between the lower Ficoll Paque and the upper plasma layers contained

peripheral blood lymphocytes. This interface was withdrawn and placed into a centrifuge tube containing Leibovitz (L-15) medium (Flow Laboratories, U.K.). The cells were washed in this medium three times by spinning at 600xg for ten minutes. Finally the cells were resuspended in L-15 medium supplemented by 10% Foetal calf serum (v/v), glutamine and gentamycin (0.5 µg/ml). The cell suspension was labelled, in a small volume, with 200 micro curies of radio-active Chromium-51 (Amersham-U.K.) per 1×10^7 total lymphocytes. For lymphocytolytic studies the labelled lymphocytes were mixed with T.evansi lysate appropriately diluted in supplemented L-15 medium and incubated at 37°C for twelve hours. The incubated mixture was spun at 600xg for ten minutes to pellet intact lymphocytes. Supernatants were withdrawn into separate tubes for gamma counts. Lysis, expressed as a percentage of total counts in the tube mixture was calculated as shown in the formula below: The lysis values were expressed as percentages of total chromium-51 content of the chromium-51 used in each single assay less background, non-specific chromium-51-release calculated as shown below:

$$C = \frac{(S - B) \times 2}{(D - B) + (S - B)} \times 100$$

where C = cytotoxicity expressed as percent cell lysis.

S = supernatant counts per minute (CPM)

B = background control CPM

D = deposit CPM

2.12.0 Partial purification of phospholipases A_2 and B from T.evansi lysate.

2.12.1 Partial purification of phospholipases A_2 and B by gelfiltration.

T.evansi lysate in PSG suspension was first sonicated on ice. Gentamycin was then added to the sonicated sample. The sample was now incubated at 37°C for four hours. This was an attempt to facilitate release of some particle bound phospholipases. Gentamycin prevented bacterial multiplication during this incubation period. The sample was then spun at 6000xg for five minutes to sediment particulate material. The supernatant was recovered and 10ml of it was loaded onto a G-100 column (Pharmacia column K26/70) equilibriated in PSG buffer containing gentamycin. The sample was eluted with the same buffer at a rate of 37.5mls per hour. Timed collections of 5mls were made on an LKB model fraction collector. Absorbance of each fraction was measured on a Gilford model 260 spectrophotometer at 280nm. Haemolytic and phospholipase activity in the fractions was determined using the methods described privously.

CHAPTER THREE

RESULTS

3.1 Haemolytic and phospholipase activities of trypanosome lysates and extracts.

Haemolytic activity was detected only in the crude trypanosome lysates and 100,000xg pellets prepared from same lysates of T.evansi. No haemolytic activity was detected in the 100,000xg supernatants of the T.evansi lysates. Only trace HA (8mg Hb/ml) was detected in relatively non-pathogenic T.musculi; while T.evansi, a pathogen in camels, horses and donkeys contained maximum HA (220mg/ml). Phospholipases A_2 and B activities were detected in both the 100,000xg pellets and supernants of T.evansi although the activities were higher in the 100,000xg supernatants than in the pellets (about four times higher). T.musculi does not contain detectable phospholipase B activity although it contains similar amounts of phospholipase A_2 activity compared to that of T.evansi. The haemolytic and phospholipases A_2 and B activities were completely heat inactivated by exposing freshly prepared samples to $89^{o}C$ for ten to fifteen minutes. The haemolytic activity was completely inhibited by 5mM Potassium cyanide or 5mM ethylenediamine-tetra-acetate (E.D.T.A.) disodium salt. These chemicals also inhibited phospholipase B (but not A_2) activity. Table 1 gives a summary of the results described above.

Table 1

Haemolytic and phospholipase activities of T.evansi lysate prepared under various conditions.

Haemolytic and phospholipase activities of T.evansi samples prepared in various ways: ultracentrifugation, heat inactivation at 89°C, treated with Potassium cyanide or E.D.T.A. The haemolytic and phospholipase activities of relatively non-pathogenic T.musculi are also presented for comparison with T.evansi. Methods for determination of haemolytic and phospholipase activities have been described under chapter 2.5.1 and 2.6.

TABLE 1

SAMPLE	HAEMOLYTIC ACTIVITY (mg Hb released per) ml	PHOSPHOLIPASE ACTIVITY TYPE B (pmoles/min/mg protein	PHOSPHOLIPASE ACTIVITY TYPE A_2 (pmoles/min/mg protein
T.EVANSI CRUDE LYSATE	220 ± 3.2	96 ± 3.5	6.5 ± 1.2
T.EVANSI 100,000xg PELLET	220 ± 2.8	24 ± 1.3	8.6 ± 1.5
T.EVANSI 100,000xg SUPERNATANT	0.4 ± 0.06	70 ± 2.6	28.1 ± 5.9
T.EVANSI CRUDE LYSATE (HEAT INACTIVATED)	0.32 ± 0.05	0	0
T.EVANSI CRUDE LYSATE IN 5mM KCN	0.34 ± 0.05	0	9.4 ± 1.4
T.EVANSI CRUDE LYSATE 5mM E.D.T.A.	0.34 ± 0.05	0	8.4 ± 1.3
T.MUSCULI CRUDE LYSATE	8.4 ± 1.2	1.3 ± 1.01	6.8 ± 1.2
PSG BLANK CONTROL	0	0	0

3.2 A time course profile of haemolytic activity generated by T.evansi 100,000xg lysates prepared under various conditions.

Figure 3 presents the results of a time course study of the HA generation by T.evansi extracts under various conditions. A sigmoidal curve with a steep gradient was obtained when HA generated by 100,000xg pellet was plotted against time. A similar curve was obtained with 100,000xg pellet which contained penstrep and was not previously heat-inactivated but maximum HA was not generated within the maximum incubation period. The heat-inactivated pellet without penstrep, the pellet containing 5mM potassium cyanide, the heat inactivated supernatant without penstrep and the supernatant containing penstrep but not previously heat-inactivated did not generate HA over the entire range of incubation periods. However, the supernatant that was not heat-inactivated previously and had no penstrep showed some capacity to generate HA. This was not an expected result but it demonstrated that bacterial contamination not blocked by the antibiotic could cause non-specific haemolysis.

3.3. Effect of temperature on the haemolytic and phospholipase activites.

Haemolytic and phospholipase activities remained constant between temperatures of 25°C and 58°C. However, beyond 58°C all the HA, phospholipases A_2 and B activities were completely inactivated resulting in a precipitous drop in activities between 58 and 65°C (fig .4)

'igure 3. Time course profile of haemolytic activity (HA) generated by T.evansi lysate extracts depicting, also, heat inactivation and Potassium cyanide inhibition of the HA and the effect of pencillin-streptomycin (penstrep) in the parasite lysate.

.00Kxg pellet (NB-NP) = 100,000xg pellet extract from T.evansi lysate without penstrep and not previously heat inactivated.

100Kxg pellet (NB-P) = 100,000xg pellet extract from T.evansi lysate with penstrep but not previously heat inactivated.

100Kxg pellet (BB-NP) = 100,000xg pellet extract from T.evansi lysate previously heat inactivated at 89°C without penstrep.

100Kxg supernatant NB-NP = 100,000xg supernatant extract from T.evansi lysate without prenstrep and not previously heat-inactivated.

100Kxg supernatant (NB-P) = 100,000xg supernatant extract from T.evansi lysate in penstrep but not previously heat inactivated.

100Kxg supernatant (BB-NP) = 100,000xg supernatant extract from T.evansi lysate previously heat inactivated at 89°C without penstrep.

100Kxg pellet in 0.005M KCN = 100,000xg pellet extract obtained from T.evansi lysate prepared in PSG containing 5mM Potassium cyanide

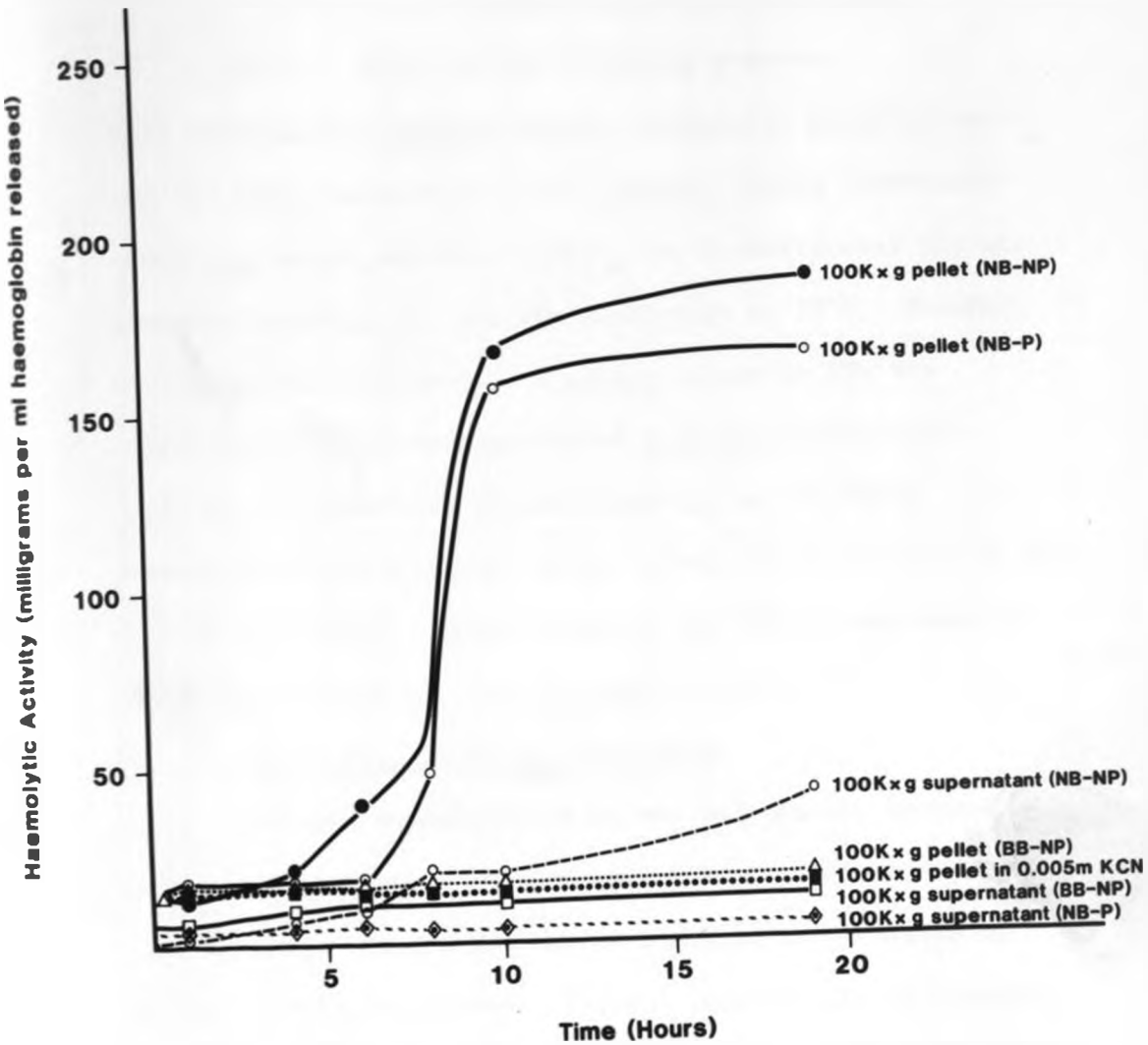

Haemolytic Activity (milligrams per ml haemoglobin released)
250
200
150
100
50
5
10
15
20
Time (Hours)
100K x g pellet (NB-NP)
100K x g pellet (NB-P)
100K x g supernatant (NB-NP)
100K x g pellet (BB-NP)
100K x g pellet in 0.005m KCN
100K x g supernatant (BB-NP)
100K x g supernatant (NB-P)

FIGURE 4

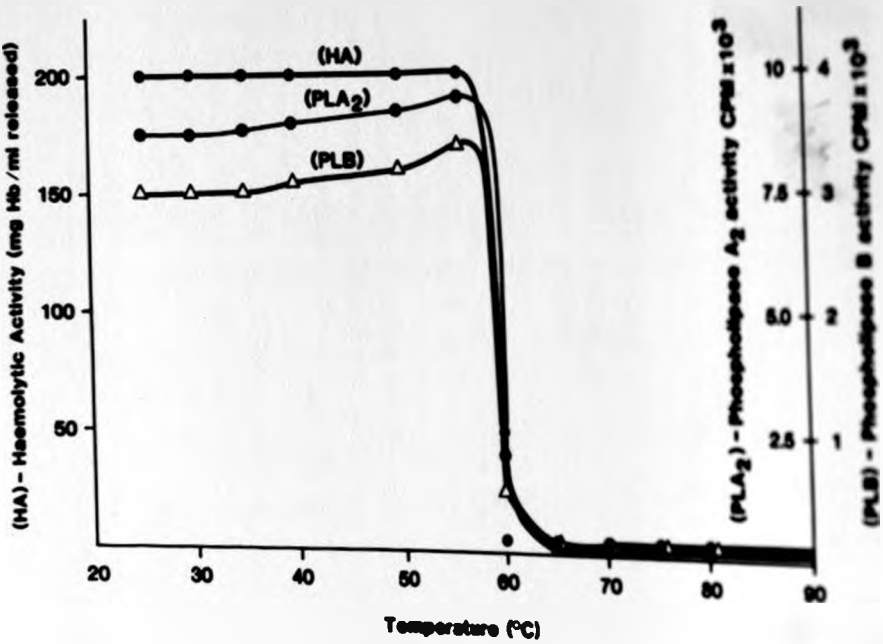
Effect of heat on haemolytic and phospholipases A_2 and B activities.

Eight aliquots of T.evansi crude lysate were exposed to 20°C, 30°C, 50°C, 60°C for thirty minutes and 70°C, 80°C and 90°C for fifteen minutes respectively. Haemolytic and phospholipase A_2 and B activities were then determined on each sample exposed to a different temperature using methods already described. The different activities were then plotted against temperature as shown in the figure. Assays were carried out in triplicates and each point on the curve represents an average of three determinations.

HA.....haemolytic activity

PLA_2.....phospholipase A_2

PLB.....phospholipase B


(HA)
(PLA2)
(PLB)
(HA) - Haemolytic Activity (mg Hb/ml released)
200
150
100
50
20
30
40
50
60
70
80
90
Temperature (°C)
(PLA2) - Phospholipase A2 activity CPM x 10^3
10
7.5
5.0
2.5
(PLB) - Phospholipase B activity CPM x 10^3
4
3
2
1

previously incubated (16h/37°C) lysate which was then heat-inactivated at 89°C for fifteen minutes retained HA.

3.6 Lysis of bovine peripheral lymphocytes by T.evansi lysate and 100,000xg T.evansi supernatant.

T.evansi crude lysate and its supernatant obtained after ultracentrifugation at 100,000xg caused lysis of bovine peripheral lymphocytes as measured by radio-active chromium release of chromium-51-labelled lymphocytes. Commercial phospholipases A_2, C and D caused similar lysis of the lymphocytes . Phospholipase C (previously shown to be directly haemolytic) caused the greatest lysis compared to the other two enzymes and had the same lytic activity as the T.evansi lysate and its 100,000xg supernatant. Table 4 presents the results of such lytic studies. The results are compared with the result of 10% nonidet-40 detergent which caused about 50% lysis of the lymphocytes.

3.7 Neutralisation of the HA by rabbit anti-T.evansi lysate serum (RaTe) and its effect on phospholipases A_2 and B activities.

A rabbit anti-T.evansi crude lysate completely neutralised the HA as shown in table 5. The same RaTe was unable to block the phospholipases A_2 and B activities. Pre-immune serum obtained from the rabbit used for RaTe production had no effect on HA of T.evansi crude lysate . RaTe serum alone had no HA. Thus the pre-immune serum and RaTe tested separately provided the controls of the experiment.

TABLE 2

Regeneration of haemolytic activity in heat inactivated T.evansi lysate by commercial phospholipase A_2 or 100,000Kxg supernatant.

First of all phospholipase A_2 (purified from Naja naja or bee venom and supplied commercially by Sigma Chemical Company) was tested for haemolytic activity. The enzyme was dissolved in PSG to a concentration of 1mg/ml. Then 250ul of the solution was mixed with 25ul of washed rat erythrocytes and incubated at 37°C for sixteen to forty eight hours to assess whether the enzyme alone contained measurable haemolytic activity. There was no detectable haemolytic activity up to 48 hours of incubation at 37°C. Heat inactivated T.evansi lysate (250ul) was mixed with 250ul of the enzyme solution. Then 50ul of washed rat erythrocytes were added and the mixture incubated at 37°C for sixteen hours. Haemolysis was observed after this period. A negative control comprising of 250ul of enzyme and 250ul of PSG did not show any significant haemolysis. Interestingly enough HA was also regenerated by addition of 100,000xg T.evansi supernatant after 16h incubation, reaching a maximum after 48h incubation.

TABLE 2

SAMPLE	HAEMOLYTIC ACTIVITY (milligrams Hb released/ml) After 16h	After 48h
1. HEAT INACTIVATED T.EVANSI LYSATE	0.88 ± 0.08	0.96 ± 0.009
2. PHOSPHOLIPASE A_2 IN PSG	0.78 ± 0.06	0.88 ± 0.07
3. HEAT INACTIVATED T.EVANSI LYSATE PLUS PHOSPHOLIPASE A_2 IN PSG	201 ± 1.05	202 ± 1.02
4. HEAT INACTIVATED T.EVANSI LYSATE PLUS 100,000xg T.EVANSI SUPERNATANT	10 ± 0.22	201 ± 1.00
5. T. EVANSI LYSATE IN PSG	220 ± 1.08	220± 1.08
6. 100,000xg T.EVANSI SUPERNATANT	0.90 ± 0.47	0.92 ± 0.41
7. PSG BLANK CONTROL	0.70 ± 0.47	0.84 ± 004

Table 3 HA of products of organic solvent extraction of T.evansi lysates pretreated in various ways.

Table 3 presents the results of HA distribution in various fractions following organic solvent extractions carried out on T.evansi lysate previouly exposed to various conditions. HA was present only in the chloroform extracts from T.evansi lysates previously incubated at 37°C for 16h and 37°C for 16h followed by heat-inactivation at 89°C for fifteen minutes. No HA was detected in the aqueous extracts from the above samples or chloroform and aqueous extracts from T.evansi fresh or heat-inactivated (89°C/15minutes) lysates.

TABLE 3

T.EVANSI LYSATE SAMPLE PRETREATMENT	HAEMOLYTIC ACTIVITY (milligrams Hb released/ml)	
	Chloroform Extract	Aqueous Extract
1. Incubated at 37°C/16h and then extracted by organic solvent	215 ± 1.09	0.84 ± 0.07
2. Extracted by organic solvents immediately	4 ± 0.12	5.00 ± 0.15
3. Heat inactivated (89°C) then incubated at 37°C/16h and extracted by organic solvents	0.84 ± 0.07	1.00 ± 0.12
4. Incubated at 37°C/16h then heat inactivated (89°C) and finally extracted by organic solvents	209 ± 1.10	2.00 ± 0.21

3.8 Induction of anaemia in mice

In figures 5(a) and 5(b) are presented similar anaemia induction curves in AJ mice. In figure 5(a) it was observed that intravenous injections of T.evansi crude lysate (A), 180xg pellet (B), 6000xg pellet (C), 22,500xg pellet (D) and 100,000xg pellet (E) caused a packed cell volume (PCV) drop of about 6% in ten hours. T.evansi heat-inactivated lysate (5,F) caused only 3% PCV drop in the same period while a 5% PCV drop was caused by 100,000xg supernatant whose results are depicted in figure 5(b). No anaemia was induced by PSG injections (Fig. 5(a)). Commercial palmitic acid caused a PCV drop of six and a half percent in 15 hours (Fig. 5(b),H) but the mice completely recovered within 24 hours. 100,000xg T.evansi pellet (Fig. 5(b), A) and lysolecithin (Fig. 5(b), I) caused PCV drop of 8% and 23% respectively within 15 hours and the mice showed a tendency to recover within 68 hours. In constrast commercial phospholipase A_2 (figure 5(b), G) caused a PCV drop of 19% after 36 hours instead of 15 hours.

3.9 Haemolytic and phospholipase activities in plasma recovered from rats infected with T.evansi.

Figure 6 depicts changes in PCV with the progression of parasitaemia (PARAS) in rats infected with T.evansi and correlates these changes with haemolytic activity (HA) of the plasma recovered from the infected rats on different days

Table 4: Lymphocytolysis (^{51}Cr-release) induced by T.evansi lysate and extract.

Lysis of bovine peripheral lymphocytes by T.evansi lysate and its (100,000xg) supernatant compared to that of commercial phospholipases. Although the 100,000xg supernatant was shown to be non-haemolytic, it had lyphocytolytic activity equivalent to that of the lysate. The supernatant, however, possessed phospholipases A_2 and B (see table 1)

TABLE 4

SAMPLE	$\%^{51}Cr$-RELEASED
T.EVANSI LYSATE	40
T.EVANSI 100,000xg SUPERNATANT	40
PHOSPHOLIPASE A_2	12
PHOSPHOLIPASE C	44
PHOSPHOLIPASE D	22
10% NONIDET - 40	50
L-15 medium	8

Table 5 Neutralisation of HA by RaTe.

RaTe was able to neutralize HA of T.evansi lysate but the same antiserum was unable to block the T.evansi phospholipases A_2 and B activities detectable in the lysate using small synthetic radio-labelled substrates as previously described in chapter 2.6.

TABLE 5

SAMPLE	HAEMOLITIC ACTIVITY (mg Hb/ml)	PHOSPHOLIPASE ACTIVITY TYPE B (pmoles/min/mg protein)	PHOSPHOLIPASE ACTIVITY TYPE A_2 (pmoles/min/mg protein)
T.EVANSI LYSATE + ANTI-*T.EVANSI* LYSATE SERUM	0.62 ± 0.04	86 ± 2.2	7.0 ± 1.1
T.EVANSI LYSATE + PRE-IMMUNE SERUM	219 ± 1.6	80 ± 1.8	8.0 ± 1.6
T.EVANSI LYSATE	220 ± 1.8	91 ± 2.1	7.4 ± 1.24
ANTI-*T.EVANSI* LYSATE SERUM	0.54 ± 0.12	0	0
PRE-IMMUNE SERUM	0.52 ± 0.14	0	0
PSG BLANK	0.42 ± 0.12	0	0

after infection. A drop in PCV was observed on day 3 after infection when the parasitaemia was about 1 x 10^8 parasites per ml. At the same point there was generation of HA. At a parasitaemia of 1 x 10^9 parasites per ml and a PCV drop of 16% (observed on day 6 after infection) HA reached a maximum of 60mg Hb/ml. These results are comparable to those of a separate experiment shown in table 6 where HA of about 60 mgHb/ml was detected in rat plasma recovered from a rat infected with T.evansi to a parasitaemia of 1.5 x 10^9 parasites per ml. In this plasma only phospholipase B activity was detected.

3.10 A comparison of phospholipase activities between T.evansi and T.brucei.

In table 7 the phospholipases A_2 and B activities of T.evansi are compared to those of highly pathogenic and virulent T.brucei 221 (MITat 1.2). Commercial phospholipase A_2 activity assayed under the same conditions is also presented as a reference. It was observed that the phospholipases A_2 and B actities of both parasites are inactivated by heating at 89°C for ten minutes. The activities of both phospholipases are about 50 times higher in T.brucei 221 than in T.evansi. Purified commercial phospholipase A_2 had substantially much higher activity than phospholipase A_2 of the crude trypanosome lysates. Commercial phospholipase A_2 did not exhibit phospholipase B activity by the procedure used in this investigation.

FIG 5: (a) Induction of anaemia in AJ mice using T.evansi lysate and its crude subcellular fractions.

Anaemia (reduction in packed cell volume: PCV) was induced in AJ mice given a single dose of T.evansi lysate, 180xg pellet, 6000xg pellet, 22,500xg pellet or 100,000xg pellet equivalent to 2.5×10^8 parasites by intravenous injection through the tail vein in a total volume of 0.2ml. Control mice given PSG or heat-inactivated T.evansi lysate showed no change in PCV.

A: T.evansi lysate

B: 180xg T.evansi pellet

C: 6000xg T.evansi pellet

D: 22,500xg T.evansi pellet

E: 100,000xg T.evansi pellet

F: T.evansi heat-inactivated lysate.

O——O PSG controls

●——● Experimental curves

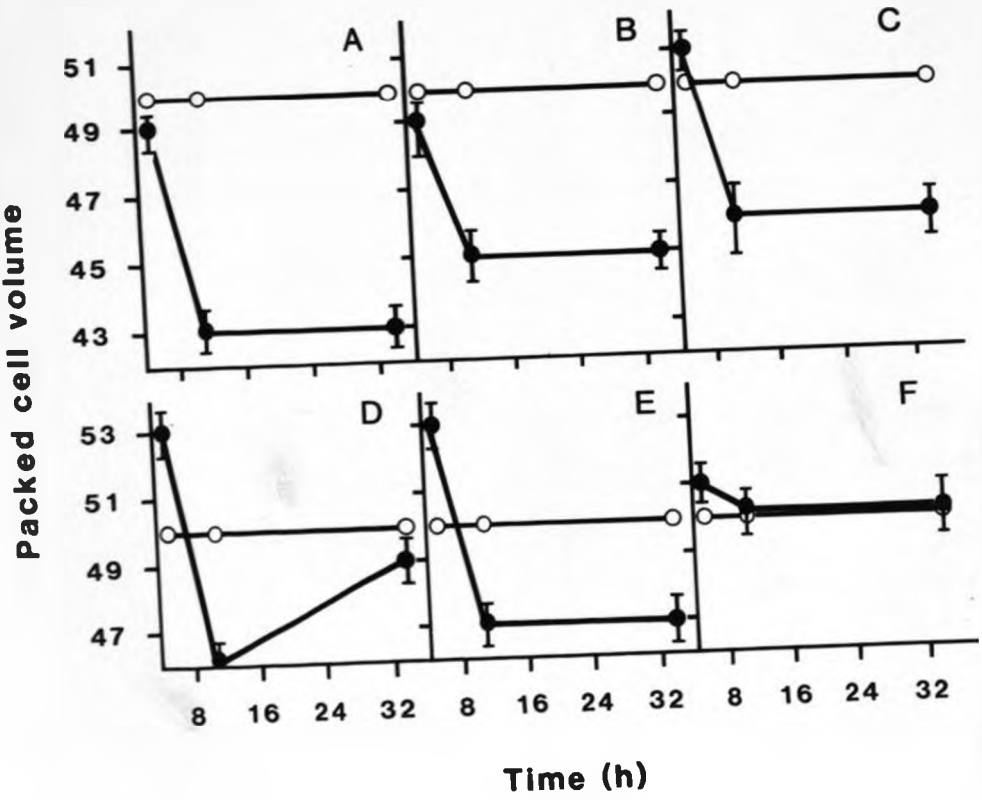
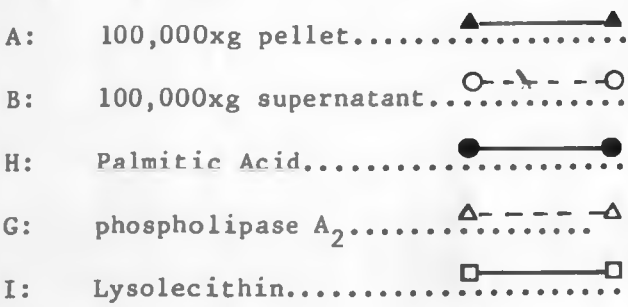
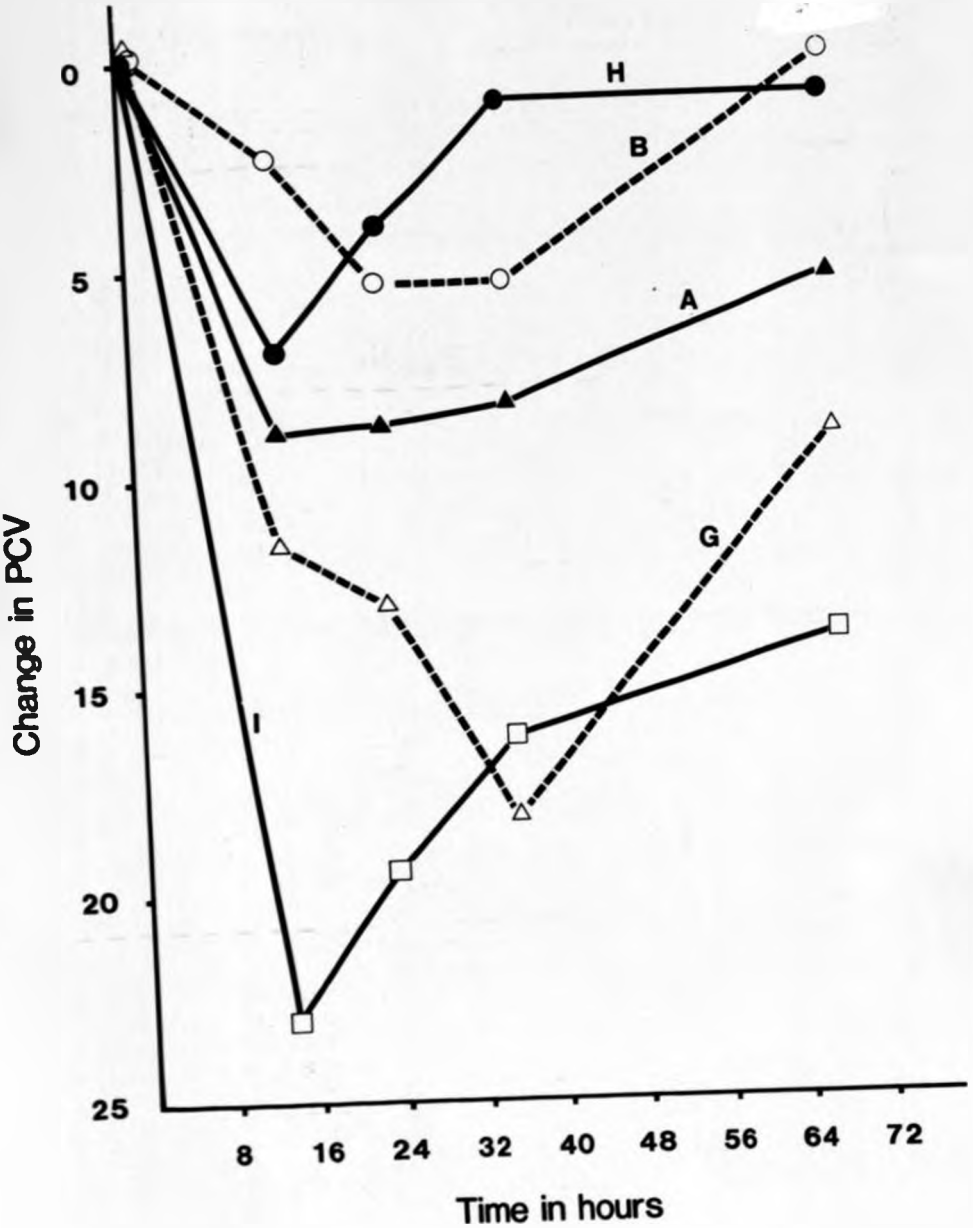

A
B
C
D
E
F
51
49
47
45
43
53
51
49
47
8
16
24
32
Packed cell volume
Time (h)

FIG 5(b) Induction of anaemia in AJ mice using T.evansi extracts compared with commercial phospholipase A_2, Palmitic acid and lysolecithin.

Anaemia (Drop in PCV) was induced in AJ mice given an intravenous injection of a single dose of 100,000xg T.evansi pellet (2.5×10^8 parasite equivalent), 100,000xg T.evansi supernatant (2.5×10^8 parasite equivalent), 200µg of bee venom commercial phospholipase A_2, 200µg of palmitic acid or 200µg of lysolecithin. The greatest PCV drop was observed with lysolecithin, followed by phospholipase A_2. Palmitic acid caused the least drop in PCV while 100,000xg T.evansi pellet caused a PCV drop that was greater than that of either palmitic acid or 100,000xg T.evansi supernatant. Each point on the curve represents an average of 4 assays.





In vivo effect of purified phospholipase A_2 Lysolecithin, Palmitic acid, 100,000g T.evansi homogenate pellet and 100,000g supernatant in AJ mice given IV injections

A. 100,000g T.evansi pellet

B. 100,000g T.evansi supernatant

H. Palmitic acid

G. phospholipase A_2

I. Lysolecithin.

Each point in the graph represents an average value of four mice

Table 6 Haemolytic and phospholipases A_2 and B activities of plasma recovered from rats infected with T.evansi.

Only HA and phospholipase B were detected in plasma recovered from infected rats at a parasitaemia level of about 1×10^9 parasites/ml.

TABLE 6

SAMPLE	PARASITAEMIA (parasites per ml of whole blood)	HAEMOLYTIC ACTIVITY (after 40 hour incubation)	PHOSPHOLIPASE ACTIVITY (pmoles/min/mg protein)	
		mg Hb/ml released	TYPE A_2	TYPE B
T.evansi infected rat plasma	1.5×10^9	60 ± 4.40	0	32 ± 2.40
Normal rat plasma	0	0	0	NIL
PSG	0	4 ± 0.68	0	NIL

3.11 Relationship between HA and phospholipases A_2 and B.

Table 8 summarises the properties of HA and phospholipases A_2 and B studied in this investigation and simultaneously compares the phospholipases A_2 and B properties with those of the HA. Out of the 8 properties studied phospholipase B has 6 properties similar to HA while phospholipase A_2 has only 2 properties similar to HA.

3.12 Distribution of HA and phospholipases A_2 and B on Sephadex G-100 gel filtration profile.

Figure 7 is a gel filtration profile of T.evansi supernatant obtained by centrifugation of T.evansi crude lysate. Two major peaks were obtained: a major peak at the void volume and a double peak between 14,000-16,000 Mr. The first major peak at void volume comprised of white opalescent material while the double peak between 14,000-16,000 Mr is a clear solution. HA is detected only in the first peak. Phospholipases A_2 and B activities were distributed over both peaks as shown in the figure.

Table 7 A comparison of phospholipase activities between *T.evansi* and *T.brucei*.

T.evansi and *T.brucei* crude lysates were each divided into two aliquots. One aliquot from each was heat-inactivated at 89°C for fifteen minutes. All the aliquots were assayed for haemolytic activity as previously described. Purified commercial phospholipase A_2 from *Naja naja* venom was also assayed for phospholipase A_2 and B activity as a comparative control.

BB.....heat inactivated (89°C/15min)

NB.....not heat-inactivated.

TABLE 7

PHOPHOLIPASES A_2 AND B

SAMPLE	pMoles substrate transformed per minute per mg protein	
	A_2	B
T.evansi heat inactivated lysate -BB-	0	0
T.evansi lysate NB	8.2 ± 1.8	87.5 ± 2.1
T.brucei 221 heat inactivated Lysate -BB-	0	0
T.brucei 221 lysate NB	519 + 11.5	3737 ± 20.5
Commercial Phospholipase A_2 (2.5ul assayed)	11382 ± 62.8	0
Commercial Phospholipase A_2 (10ul assayed)	18387 ± 102.5	0

TABLE 8

Relationship between HA and phospholipases A_2 and B in T.evansi.

Different biological and biochemical properties of HA, phospholipase A_2 and phospholipase B characterised in this investigation have been compared. Phospholipase B correlates to HA in these properties more than phospholipase A_2.

(+).....Positive property

(-).....Negative property

TABLE 8.

PROPERTY	HAEMOLYTIC ACTIVITY	PHOSPHOLIPASE B	PHOSPHOLIPASE A_2
Heat inactivation (89°C, 15min)	+	+	+
KCN inhibition	+	+	-
E.D.T.A. inhibition	+	+	-
Sedimented by 100,000xg therefore, membrane bound	+	+	+
Present in infected rat plasma	+	+	-
Present in non-pathogenic T.musculi	-	-	+
Neutralized by Rabbit anti-T.evansi crude lysate (RaTe)	+	-	-
Present in 100,000xg supernatant	-	+	+
Total number of properties tested	8	8	8
Score of similarity to haemolytic principle	8	6	2

FIG. 7 Sephadex G-100 gelfiltration profile of 6,000xg *T.evansi* lysate supernatant showing the distribution of HA and phsopholipases A_2 and B.

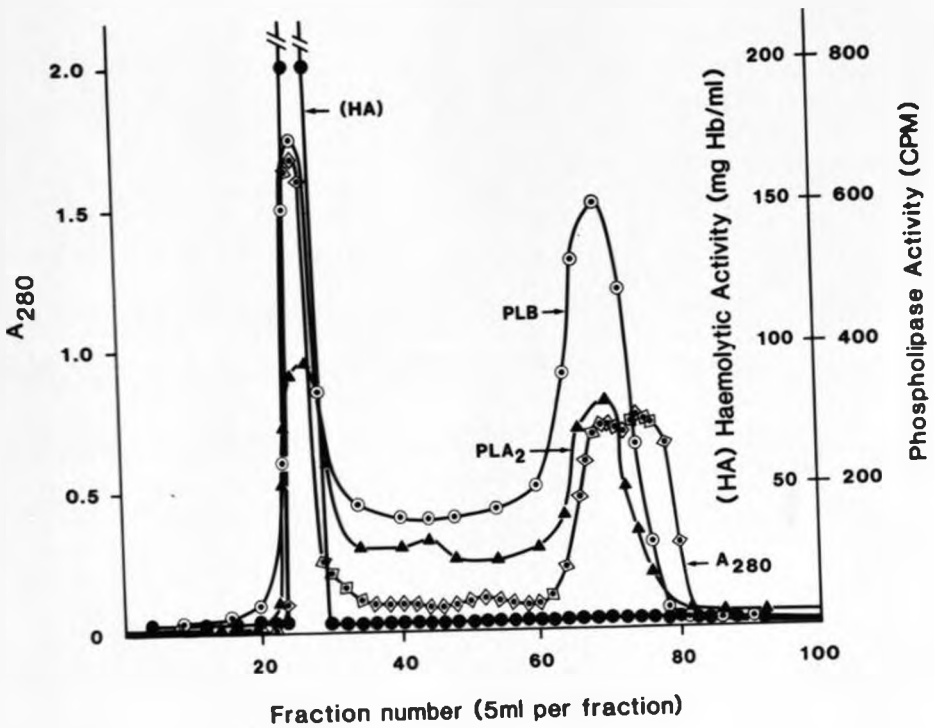
Two major peaks (280nm) are observed. HA is distributed only in the void volume while phospholipases A_2 and B are distributed in both the void volume and the double peak at about 15,000 Mr.

HA..... haemolytic activity.....

PLB..... phospholipase B activity.....

PLA_2....phospholipase A_2 activity.....

A_{280}....absorbance at 280nm.....


2.0
1.5
1.0
0.5
0
A_{280}
(HA)
PLB
PLA_2
A_{280}
200
150
100
50
800
600
400
200
(HA) Haemolytic Activity (mg Hb/ml)
Phospholipase Activity (CPM)
20
40
60
80
100
Fraction number (5ml per fraction)

CHAPTER FOUR

DISCUSSION:

The generation of trypanosome-derived haemolytic activity from samples of purified trypanosomes that were left to autolyse at room temperature for several hours has been demonstrated (Huan Chi *et al* 1975, Tizard *et al*, 1976, Murray *et al*, 1977 and 1979). In contrast, the present investigation used samples which were obtained by rapid freezing and thawing of freshly purified trypanosomes using liquid Nitrogen and 37^{o}C water bath, thus limiting the amount and number of products generated in the samples by stress induced parasite metabolism and degradative enzyme action *in vitro*. Thus it is more probable that the HA generated by these samples in this investigation would be more likely to be present in the living parasite.

Haemolysis caused by these samples was measured by haemoglobin release at its maximum absorbance at 415 nm. The reference was an artificially lysed erythrocyte sample (see Fig.1). This method was preferred to that employing chromium-51 release from labelled erythrocytes which gives very high background due to non-specific chromium leakage. Furthermore the haemoglobin release method is very simple and highly reproducible and comparable in sensitivity to the chromium release method. A serious *in vitro* artefact in the study of HA in such biological samples is that arising from

bacterial contamination of the sample. It was noted in this investigation that trypanosome lysates act as an excellent medium for bacterial growth. Premature HA was observed where bacterial growth occurred. This contamination can escape detection since the physiological buffer controls do not show it. This was first detected when an unpleasant malodorous smell was noted in tests that gave very high and premature haemolysis. On examination of these test mixtures using darkground-phase microscopy numerous motile and non-motile bacteria were noted. It was then decided to prepare all the samples in isotonic buffered saline glucose (PSG) containing 1µg/ml of the broad spectrum antibiotic gentamycin. In addition the target erythrocytes for haemolytic activity assay were collected aseptically in sterile isotonic PSG. The HA studied under the experimental conditions of this investigation can, therefore, be regarded as trypanosome-derived.

From time titration curves (Fig.3) haemolysis can be seen to be a gradual process. Maximum haemolysis occurs after about sixteen hours of incubation. This observation agrees with maximum PCV drop in anaemia induction curves (see Fig 5(a) and 5(b)). A possible explanation for the delayed appearance of haemolysis *in vitro* is that haemolysis does not occur until a threshold concentration of the haemolytic products is attained. At the threshold concentration the erythrocytes undergo immediate lysis as is indicated by a steep gradient in the sigmoidal curve (Fig. 3). However, *in vivo*, alteration in

the erythrocyte cell membranes by a subthreshold concentration of haemolytic products, or by enzyme action, may cause the erythrocytes to be recognized as foreign and thus removed by the phagocytic cells of the reticulo-endothelial system. As bilirubinaemia is not associated with African trypanosomiases, erythrophagocytosis mediated by such erythrocyte cell membrane alterations is probably the mechanism of anaemia in African trypanosomiases (see also Mackenzie and Cruickshank, 1973; Mackenzie et al and 1978). The latter proposed mechanism is in accord with the actions and effects of phosphilipases on cell membranes (Zwaal et al, 1975 and Renooij et al, 1976). These workers noted that phospholipases A_2, C and D from various sources caused 50-80% hydrolysis of membrane phospholipids on the outer surface of red cells without causing haemolysis but this resulted in morphological distortion of the cells. Cells thus affected acquired increased osmotic fragility.

An alternative explanation is that haemolytic free fatty acids released after phospholipase enzymatic action must reach a certain critical level of concentration before they can cause haemolysis. In parasite lysates used here the free and active phospholipases were probably in such a low concentration that the free fatty acids generated by the enzymatic action of these phospholipases were accordingly too low to cause immediate haemolysis. Enough time (see Fig 3) was therefore required for generation of sufficiently high levels of free fatty acids to cause haemolysis. It is likely that both the enzymatic

degradation of the cell membrane and the direct haemolytic action of free fatty acids operate simultaneously. It has been noted that sera or plasma from animals heavily infected with trypanosomes do not show bilirubinaemia which is usually a condition of direct intravascular haemolysis. This therefore argues against the involvement of free fatty acids in the direct erythrocytolysis leading to anaemia of trypanosomiasis and favours the mechanism of erythrophagocytosis brought about by alterations on the cell membranes by enzymatic action. The most likely enzymes are phospholipases and possibly proteases. It was pointed out earlier that the crude lysate used here may well contain phospholipase enzymes and their physiological inhibitors. This was part of the explanation for the low levels of these enzymes in the crude lysate samples assayed.

T.brucei, which is phylogenetically closely related to T.evansi but is much more pathogenic than T.evansi, in a rat host, had much higher phospholipase activity than T.evansi. These observations fit the argument that phospholipases and their products of enzymatic action, may be responsible for haemolytic anaemia in trypanosomiasis.

The HA studied here appeared to be more associated to phospholipase B activity (see table 8). However, haemolytic activity was present only in the 100,000xg T.evansi lysate pellet while phospholipase B activity was detected in both the pellet and supernatant. It therefore appears that there are two types of phospholipase B in T.evansi: the particulate or

membrane bound type and the free, soluble type. Only the membrane bound phospholipase B appeared to be associated with the HA (see table 1). Another possible explanation is that the 100,000xg supernatant does not contain membrane phospholipids which would generate haemolytic free fatty acids upon hydrolysis by the phospholipases. The membrane associated phospholipids were sedimented at 100,000xg into the pellet. Haemolytic and phospholipase B activities were not detected in Trypanosoma musculi, a non-pathogenic parasite in mice, rats and bovidiae. Phospholipase A_2 was, however, detected in this parasite. This lends further support to the association of the haemolytic activity with phospholipase B.

Membrane bound and soluble forms of phopholipase A_2 were also detected in T.evansi. Since phospholipases A and B often occur in association in nature the involvement of phospholipase A_2 in haemolysis cannot be ruled out. Commercial phospholipase A_2 was shown to induce mild anaemia in AJ mice (see Figure 6). The point of interest in this connection is that the maximum drop in PCV induced by this enzyme occurred after 36 hours compared to 16 hours for 100,000xg T.evansi lysate pellet. A probable explanation for this finding is that pure phospholipase A_2 does not cause sufficient hydrolysis of red cell phospholipids to predispose the red cell to early (sixteen hours) maximum haemolysis. Thus it takes longer for the red cell to be haemolysed. However, 100,000xg T.evansi lysate pellet which was shown to contain

both membrane bound phospholipases A_2 and B would cause greater cell distortion thus causing maximum drop in PCV within 16 hours. These observations suggested that membrane bound phospholipases A_2 and B may be responsible for haemolytic activity in T.evansi lysate and that this activity may well be significant and functional in an in vivo setting.

Soluble phospholipases A_2 and B in the 100,000xg T.evansi lysate supernatant did not cause significant PCV drop after 16 hours. This was in agreement with the previous results that the haemolytic activity was not detected in this sample. This difference suggests that both phospholipases and free fatty acids are involved in cell membrane perturbations. Mammalian cell membranes contain structural phospholipids and lysophospholipids (Parsons, 1975). Phosphatidylcholine, sphingomyeline and lysophosphatidylcholine are exposed on the outer cell surface of rat erythrocyte (Renooij et al, 1976). These are substrates for phospholipases A, B and sphingomyelinases. Additionally phospholipase B hydrolyses Lysophospholipids. Phospholipases A_1, C, D and sphingomyelinase (not studied here) may well be present in T.evansi and would most probably contribute to hydrolysis of these membrane phospholipids with resultant deleterious effects to various host cell types. These phospholipases and proteases are currently being investigated in T.brucei, T.vivax and T.congolense.

Haemolytic and phospholipase activities appear to be active in plasma recovered from heavily infected rats. No significant haemolytic and phospholipase activities were detected in uninfected rat plasma. Mild anaemia, of varying degrees, was induced in AJ mice given intravenous injections of 100,000xg pellet of T.evansi lysate, lysolecithin or commercial phospholipase A_2. No significant anaemia was induced by heat-inactivated 100,000xg T.evansi lysate pellet, suggesting that the anaemia is caused by heat-labile factors. Commercial palmitic acid was probably neutralized by albumin in the circulation and thus did not induce significant anaemia although it is haemolytic in vitro and would therefore tend to suggest that the release of free fatty acids in vivo may play a minor role in the induction of anaemia. Commercial phospholipase A_2 induced anaemia most likely by acting directly on red cell membranes and predisposing the red cells to the process of erythrophagocytosis. The 100,000xg T.evansi lysate supernatant, although it had high levels of phospholipases A_2 and B, did not induce significant anaemia, suggesting that some other heat sensitive factors in addition to the particle bound phospholipases A_2 and B are involved in inducing anaemia. Lysolecithin, a product of phospholipases A group is strongly haemolytic in vitro. It is also a substrate for lysophospholipase (phospholipase B) found in this organism. These results showed that products released by dead trypanosomes are pathogenic in vivo and may indirectly

or directly be responsible for pathophysiologic manifestations of cellular damage in trypanosomiasis. These results are further supported by the presence of haemolytic and phospholipase activities in plasma of infected rats (see Figure 6 and Table 6).

The haemolytic activity was neutralized by a rabbit anti-T.evansi crude lysate serum. However, neither phospholipase A_2 nor phospholipase B activities on small commercial phospholipid substrates were neutralized by this antiserum (see table 5). A possible explanation for this observation is that the antibodies were not directed against the active sites of phospholipases A_2 and B. This possibility was supported by the result of a separate experiment. In this experiment antibodies were raised, in a rabbit, against commercially purified phospholipase A_2 (results not published here). The antibodies did not inhibit phosphalipase A_2 activity using small synthetic radio-labelled substrates. It was quite apparent from these results that phospholipases are immunogenic but antibodies raised against them do not necessarily block their active sites. Neutralisation of the haemolytic activity by the rabbit anti-serum may be explained as follows: an antibody attached to epitope near the enzyme active site on the phospholipase molecule may obstruct the accessibility of the enzyme to the substrate molecule which is partially embedded

in the red cell membrane by steric hindrance. However, small synthetic substrates can easily reach the enzyme-active site and so be hydrolysed by the enzyme.

Depending on the source, phospholipases can be haemolytic and are generally heat labile (Osamu, 1955; Zwaal et al., 1975). Phospholipase B (lysophospholipase) from penicillium is inactivated by heat and inhibited by cyanide (Osamu, 1955). Phospholipase B detected in T.evansi in these studies is also cyanide and heat sensitive (cyanide inhibits metal-dependent enzymes). The phosphoplipase responsible for HA may also be a metal-dependent enzyme. This investigation has shown that trypanosome-derived HA is due to enzymatic action on trypanosome endogenous substrates (table 1 and figures 3 and 4). The involvement of trypanosome phospholipases in the generation of HA has been supported by the regeneration of HA from heat-inactivated T.evansi lysate by commercial phospholipase A_2 and the results of the organic solvent extraction of the lysates. The presence of haemolytic activity in the organic solvent phase of incubated T.evansi lysate suggested that the activity is caused by a product(s) of enzyme(s) action on a lipid substate(s).

The types of phospholipases detected in this study in T.evansi are generally in agreement with those reported by Hambrey et al, (1981); Sage et al,(1981); and Opperdoes and Van Roy, (1982), in T.brucei.

The presence of only trace amounts of phospholipase B in the relatively non-pathogenic T.musculi is compatible with the notion that phospholipase B is associated with the HA of the pathogenic trypanosomes studied in this investigation.

It is thus likely that the HA demonstrated in vitro in these studies may arise from enzyme reaction products of phospholipases acting directly on cell membranes. The erythrocyte membrane perturbations may cause these cells, in vivo, to become targets for erythrophagocytosis, another hallmark of African trypanosomiasis. This process contributes significantly to a decrease in PCV in this disease (Murray, 1974, Mackenzie and Cruickshank, 1973, and Mackenzie et al, 1978).

From work done by other investigators in the study of the mechanisms of the pathogenesis of African trypanosomiasis there is now accumulated evidence that pathogenicity of trypanosomes may operate through three main mechanisms; namely immune complex formation with resultant complement activation, (Assoku, 1975 and Ikede et al 1977); toxins and enzymes generated by in vivo autolysis of trypanosomes (Assoku, 1975 and Tizard et al 1976, 1977 and 1978), and generation of toxins from parasite metabolism; (Huan et al, 1975; Ackerman and Seed, 1976). Assoku (1975) argues that immune complexes coating erythrocytes can lyse the cells in the presence of complement and thus postulates that the anaemia observed in pathogenic trypanosomiasis is due to immune complexes. This is a possible mechanism but from the erratic reactions in the

Coomb's tests used in his detection of antibody and antigen coating of the red blood cells it would appear that the immunological parameters contribute only minimally to anaemia in this disease. The *in vitro* lysis of washed rat red cells by *T.evansi* particulate sample used in this investigation did not require the presence of complement.

It is generally agreed that anaemia in African Trypanosomiasis has its origin in the extravascular cell destruction and this implies, some structural cell membrane perturbations leading to red cell removal by phagocytic cells in RES. Anaemia, however, is not the only sign of trypanosomiasis. Specific organ damage with severe cell death in tissues has been observed (Jennings *et al* 1974). These authors also report swelling and degeneration of vessels in the microvasculature which occurs with associated perivascular oedema. Others have documented diffuse and chronic meningoencephalitis (Levandowsky and Hutner, 1979). These are pathophysiologic phenomena which may result from cellular damage secondary to some disorder of cell membrane function. Argued purely on the principle of cell membrane damage and the limited repertoire of molecules that can be released by trypanosomes it seems reasonable that the factors responsible for these tissue injuries would also be responsible for anaemia. It also seems likely, from work done by Tizard *et al*, (1976, 1977 and 1978), Hambrey *et al*, (1981) Sage *et al*, (1981) and Opperdoes and Van Roy, (1982) and from the results of this

investigation, that phospholipases and products released by their enzymatic action on host or parasite endogenous substrates would cause widespread damage in trypanosomiasis and thus appear to explain, in part, the mechanism of the pathogenicity of these parasites. Although the effects of the trypanosome metabolic products are not sufficiently studied so far, work by Ackerman and Seed (1976) offers some considerable evidence of the effects of some trypanosome metabolites in relation to immune suppression, hypersomnolence and also anaemia. Collectively, immunological factors, metabolic factors and phospholipases released by dying trypanosomes seem to be likely mechanism for the pathogenesis of African trypanosomiasis. However, the results from this investigation seem to bias our thinking that phospholipases and possibly proteases (John Lonsdale-Eccles - personal communication in reference to the ongoing work on trypanosome proteases) released in the infected host would be responsible for these deleterious effects in African trypanosomiasis. Prolonged parasitaemia may result in the accumulation of products from lysed parasites in quantities sufficient to biodegrade the host cells. The combined effect of phospholipase activity, parasite metabolic products and possibly antibody directed against altered host cell membranes would explain the range of signs seen in pathogenic trypanosome infections both in man and other hosts. Besides the biodegradation of the host cells, free phospholipase activity appears to induce lysis and

death of the still live and circulating trypanosomes, thus amplifying generation of pathogenic factors in an infected host (haemolytic T.evansi lysates have been shown in our laboratory to kill and lyse live T.evansi trypanosomes; results not published in this report).

From the results obtained in this investigation, and work done by others, it is concluded that the death per se of African trypanosomes generates a pot pourri of products that are deleterious to the host's cellular integrity. This appears to be responsible for the anaemia observed in trypanosomiasis and possibly for immune depression and may also explain the mechanism of the pathogenicity of these parasites. The haemolytic activity generated by trypanosome lysates appears to be derived from phospholipase activity directed against cellular membrane phospholipids while lipid products of phospholipase and lysophospholipase activity (possibly free fatty acids) are haemolytic at least in vitro in T.evansi studied here and other pathogenic trypanosomes studied elsewhere. From the results of this investigation the following specific conclusions can be made:

lysates of T.evansi are haemolytic to mammalian (human, bovid and rodent) erythrocytes; Sedimentation of fresh trypanosome lysates shows HA to be particulate (100,000xg pellet); this activity is heat labile (89^{o}C, 15min), and is inhibitable by EDTA or KCN; preincubation of trypanosome lysates yields chloroform soluble and heat stable products that are haemolytic;

based upon the hydrolysis of radio-labelled phospholipids, both phospholipases A_2 and B are present in T.evansi and T.brucei; differential inhibition by KCN suggests that HA is associated with phospholipase B.

Purification of phospholipases from pathogenic African trypanosomes is a subject of ongoing efforts with the objective of comparing their activities and levels in different trypanosome species. Purified and inactivated enzymes will also be used to immunize animals to study immunological protection against pathogenicity of these parasites. An attempt will also be made to demonstrate pathogenicity in vivo using phospholipases purified from pathogenic African trypanosomes.

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