ISOLATION AND CHARACTERIZATION OF TICK TOXINS

by

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LIST OF ABBREVIATIONS

A	absorbance
BAPNA	N-benzoyl-D-L-arginine 4-nitroanilide-HCl
BLP	buffered lactose peptone
BSA	bovine serum albumin
PBS	phosphate buffered saline
C	concentration of the cross-linker relative to the total concentration
c	*circa*
Cbz-Arg-AMC	7-carbobenzoxy-argininamido-4-methylcoumarin
Chaps	3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
DMSO	dimethyl sulfoxide
E	enzyme
E_t	total enzyme concentration
EI	enzyme-inhibitor complex
ELISA	enzyme-linked immunosorbent assay
xg	centrifugal force calculated at a distance from axis of rotation to mid point of tube
h	hour
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid
I	inhibitor
I_t	total inhibitor concentration
im	intramuscular
ip	intraperitoneal
iv	intravenous
IEF	isoelectric focusing
IFA	indirect fluorescent antibody
IgG	immunoglobulin G

IgM	immunoglobulin M
kdal	kilo-Dalton
K_i	general dissociation constant
$K_{i\ app}$	the apparent rate constant of dissociation
K_m	Michaelis constant
l	liter
LD_{50}	50 % lethal dose
min	minute
MW	molecular weight
P	product
PBE	polybuffer exchanger
P/N	infected to non-infected
S	substrate
sa	specific activity
sc	subcutaneous
SDS	sodium dodecyl sulfate
SDS PAGE	sodium dodecyl sulfate gradient gel electrophoresis
sec	second
SIP	iso-osmotic Percoll stock solution
SPNA	N-succinyl-L-phenylalanine p-nitroanilide
T	total gel concentration
$t_{½}$	half-time of inhibition
Tox	toxic
Tris	tris(hydroxymethyl)aminomethane
Tween	polyoxyethylene sorbitan monolaurate
UV	ultraviolet

v_i	internal volume
v_o	void volume
v_t	total volume
v	velocity

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CHAPTER 1

GENERAL INTRODUCTION

1.1. Scope of the tick problem

Ticks are the most important ectoparasites of domestic animals in the Republic of South Africa. They cause damage to livestock both directly and indirectly (Howell, Walker and Nevill, 1978).

The detrimental effects of ticks on their hosts was already mentioned on a papyrus scroll dated 1550 B.C. (Obenchain and Galun, 1982). The extent of the tick problem was even more apparent after the historic discovery by Smith and Kilbourne in 1893 that ***Boophilus*** ticks are the transmitters of the Texas fever pathogen ***Babesia bigemina*** (Smith and Kilbourne, 1893). This significant discovery was the first documented instance of a protozoan parasite transmitted by an arthropod. In subsequent years it has been shown that ticks are the vectors of other pathogenic protozoa, bacteria, rickettsiae, viruses and filaria (Chandler, 1961; Obenchain and Galun, 1982).

In all diseases of which ticks are the vectors, the transmitted pathogenic organisms have been established with the exception of tick toxicoses, where micro-organisms are apparently not involved (Howell, 1971).

Direct damage caused by ticks to their hosts is due to exsanguination, secondary infection by organisms at the attachment sites, damage of the hides, allergic effects and envenomation which may result in paralysis or other forms of toxicoses. Indirect damage results from the introduction of pathogenic organisms into the host during feeding (Howell, ***et al.***, 1978).

Numerous factors have to be considered for the reliable estimation of monetory losses incurred as the result of tick infestations. Data are required concerning mortality rates and control costs including labour, equipment and chemicals (Steelman, 1976). Furthermore, the costs involved in tick research, the construction of dipping facilities, the breeding of tick resistant cattle and costs involved due to loss of condition of livestock are difficult to estimate. However, conservative estimates have shown that the annual losses may add up to several hundred million Rand (Bigalke, 1976).

For these reasons, much research on ticks has been performed in many parts of the world for almost a century. In this thesis, some aspects concerning toxins produced by ticks were investigated. Included is research on the pathogen *Cowdria ruminantium*, the causative agent of heartwater which is transmitted by the *Amblyomma* tick. Methods for the purification and detection of this pathogen were investigated as a first step in the study of the assumed toxin produced by this pathogen (Neitz, 1968).

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1.2. Tick toxicoses

Numerous tick species secrete toxins which are present in their oral secretions. When introduced into a host a toxicosis may result which may be mild and ill-defined or extremely severe as with sweating sickness and tick paralysis (Stone and Wright, 1981).

Three possibilities regarding the origin of the toxin causing tick paralysis has been outlined by Gregson (1973). The toxin is either a product of tick tissues *per se*, or a product of metabolic breakdown of host tissues or a product of an organism within the tick. With this as a general basis, various possibilities (Fig 1.1) as to the origin of toxins causing toxicoses may be envisaged (Neitz, Bezuidenhout, Vermeulen, Potgieter and Howell, 1983).

Seven distinct types of toxicoses have been described in the literature. Neitz (1962) enumerated three forms of toxicoses as being induced by *Hyalomma truncatum* in Southern Africa namely: Sweating sickness, Mhlosinga and Magudu toxicoses. *Rhipicephalus appendiculatus* (Stone and Wright, 1981) and *Ornithodorus savignyi* (Neitz, Howell and Potgieter, 1969) are the cause of two other toxicoses. A sixth category is attributed to very heavy infestations of *Boophilus microplus* larvae on cattle in Australia and massive infestations of *Dermacentor albipictus* on yearling moose and elk in North America (Neitz, 1962). The seventh and most important form of toxicoses is tick paralysis (Gregson, 1973).

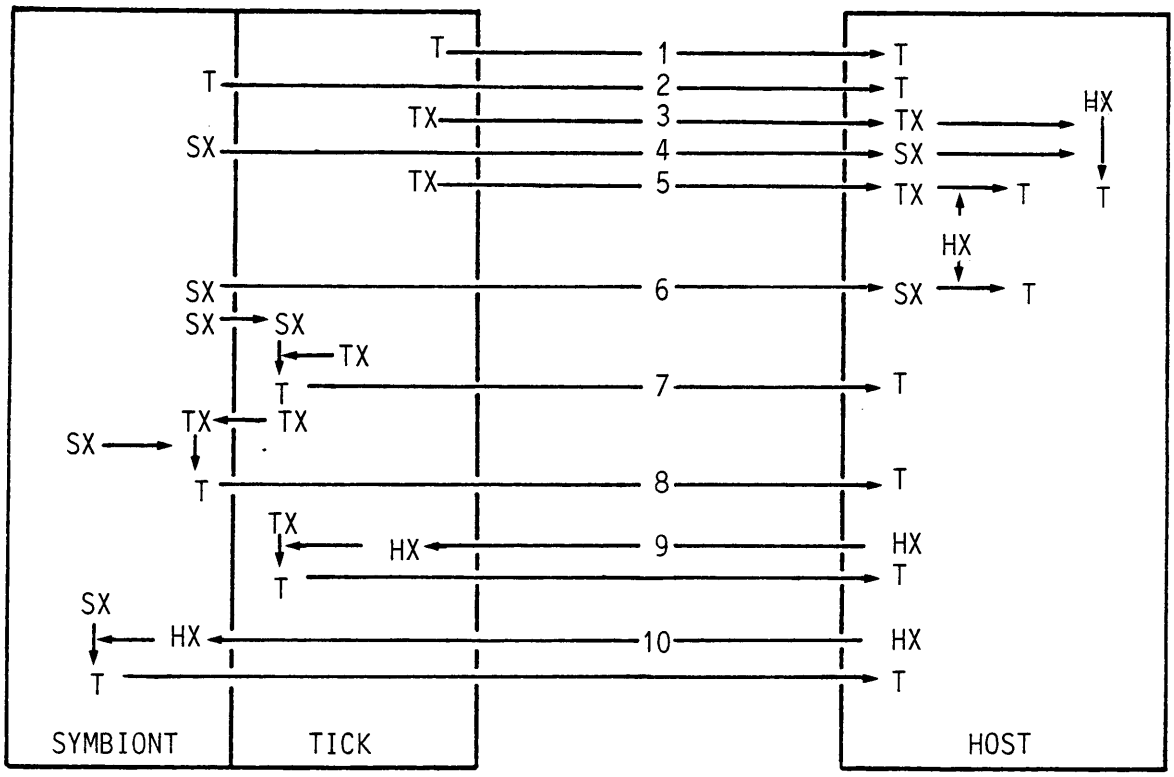

Fig 1.1. Some possible origins of tick toxins.
Toxin is a product of host tissue *per se* (1) or of a symbiont (2). Toxin is formed in host tissue as the result of the action of a product from tick tissue (3) or from a symbiont (4) on a host component. A non-toxic product from tick tissue (5) or from a symbiont (6) is converted to a toxin by a host tissue component. A non-toxic product of a symbiont is converted to a toxin by tick tissue (7). A non-toxic product of tick tissue is converted to a toxin by a symbiont (8). Ingested host component triggers tick (9) or symbiont (10) to produce toxin or causes conversion of non-toxic products of tick or symbiont to a toxin. TX, SX and HX: Products of tick, symbiont and host tissue respectively; T: toxin. (Neitz, Bezuidenhout, Vermeulen, Potgieter and Howell, 1983).

1.2.1. Tick paralysis

Paralysis caused by ticks is reputedly associated with more than 50 species of ticks belonging to 10 genera (Gothe, 1984). The tick species which appear to be of greatest importance in human paralyses are ***D. andersoni*** and ***D. variabilis*** in North America and ***Ixodes holocyclus*** in Australia. Of veterinary importance regarding paralyses are the same 3 species and also ***I. rubicundus***, ***R. evertsi evertsi*** and ***Argas (Persicargas) walkerae*** of Africa and ***A. (P.) radiatus*** of North America (Gothe, Kunze and Hoogstraal, 1979).

The onset of paralysis begins after a feeding period of approximately 5 days, (Gothe and Lämmler, 1982a). During this period there may be prodromal symptoms such as general malaise, lassitude, headache, vomiting and loss of appetite. The first signs of paralysis is noticeable in the lower limbs and are initially characterized by slight to pronounced incoordination, ataxia and muscular weakness. After a few hours these signs are intensified and extended to the upper limbs, torso, neck, throat and face. The typical syndrome is thus characterized as an ascending symmetrical, flaccid tetraplegia, with functional impediment to the reflexes of the superficial and deep tendons of the limbs and abdomen (Gothe, ***et al.***, 1979).

The pathomechanism of paralyses caused by ***D. andersoni***, ***D. variabilis***, ***I. holocyclus***, ***A. (P.) walkerae*** and ***R. evertsi evertsi*** have been studied using paralyzed animals. From these investigations it can be concluded that the mechanism of pathogenicity of ***I. holocyclus***-induced paralysis differs basically from that of the others. (Gothe, ***et al.***, 1979; Gothe and Kunze, 1981; Kunze and Gothe, 1971; Murnaghan, 1961; Murnaghan and O'Rourke, 1978). In contrast to the nerve action potential, which is unaffected in paralysis due to ***I. holocyclus*** ticks, the muscle action potential in response to nerve stimulation is dramatically reduced. The

slightly greater average depression of the hind-limb responses is consistent with the ascending nature of the paralysis. Furthermore, spontaneous release of acetylcholine appears to be unaffected. However, a temperature dependent depression of evoked acetylcholine release occurs (Cooper and Spence, 1976). It was hypothesized that the toxin interferes with the influx of calcium ions which seem to be essential before evoked release can occur. (Spence, 1979). Cooper (1975) could not demonstrate ultrastructural lesions in the nerve terminals.

The *Dermacentor*, *Argas* and *Rhipicephalus* paralyses on the other hand are essentially motor polyneuropathies with only limited participation of the afferent pathways. Thus a marked reduction in motor nerve conduction velocities, and a decrease in compound action potentials characterize the paralysis. The *Argas* paralysis in addition shows impairment in the distal nerve section or at the myoneural synapse. Ultrastructural defects in nerve fibres have not been observed. It has been postulated that the toxins responsible for the paralyses circulate humorally and have membranophilic properties with the primary point of attack possibly in the regions of the nodes of Ranvier (Gothe, *et al.*, 1979). The binding of the toxin to the membrane appears to be labile which is consistent with the observation that if ticks are removed from a host in which the paralysis has not progressed too far, immediate improvement results. In the case of *I. holocyclus* the symptoms do not necessarily abate upon timely removal of ticks and the animal may subsequently die (Stone, Doube, Binnington and Goodger, 1979).

Regarding the antitoxic immunity resulting from the afore mentioned paralyses, major differences have been reported. In *R. evertsi evertsi* paralysis, an effective immunity is induced at least two weeks after the primary tick exposure and lasts for 4 weeks. The antitoxic immunity can be extended to at least 8 weeks through a booster effect of re-infestation (Gothe and Lämmler, 1981). In the case of *A.(P.) walkerae* a partial

immunity after repeated challenge could be demonstrated. An immunity due to *I. holocyclus* is readily induced (Stone and Wright, 1981). Contradictory reports on immunity regarding paralyses due to *D. andersoni* and *D. variabilis* have been published. However, it is generally accepted that these 2 tick species do not produce immunity in their hosts.

The etiology of tick paralysis has been explained as being either an infectious agent or as a toxin produced by the tick. (Gothe, 1984). In support of toxin etiology numerous observations may be mentioned which singly can convincingly explain the causality of tick paralysis: (1) Negative results were always obtained after experimental inoculation of tissue extracts or body fluids from affected donors to susceptible recipients. (2) Regular failure to isolate micro-organisms associated with paralysis. (3) The symptoms of the disease disappear in most cases upon removal of the parasitizing ticks provided that the paralysis has not progressed too far. (4) A direct correlation between the severity of the clinical symptoms and the infestation rate (the number of parasitizing ticks) and thus the amount of toxin transferred to the host exists.

With the exception of *I. holocyclus*, most inoculations of whole tick body extracts or salivary gland extracts into animals have failed to induce paralysis (Kaire, 1966). This may be due to methodological inadequacies (Gothe, 1984): (1) The use of insensitive vertebrate hosts. (2) The application of insufficient doses of the extracts. (3) The use of extracts obtained from ticks outside the toxic phase. (4) Ineffective manner of preparation or storage of the test material resulting in the inactivation of the toxin. (5) The presence of an inhibitor liberated during the preparation of the extracts.

The production of the paralysis toxin has been related to oogenesis in ticks. It is postulated that the toxin is synthesized in the ovaries or eggs and transferred during embryogenesis to the host via the salivary

glands. This postulate is based on the early investigations of Regendanz and Reichenow (1931) and work of numerous other workers who showed that egg extracts of certain ticks show toxic properties, and when injected into test animals induce paralysis or paresis. Riek (1957) made an intensive study of tick eggs and showed toxins to be present in 17 species of ixodid ticks . They were found to be absent in 5 species of argasids. Because of the possible bearing of egg toxins on tick toxicoses they have been investigated in the present research program in the Department of Biochemistry at the University of Pretoria. Several toxins from 5 tick species were isolated in pure form and characterized. (Neitz, Prozesky, Bezuidenhout, Putterill and Potgieter, 1981; Viljoen, 1983; Viljoen, Neitz, Prozesky, Bezuidenhout and Vermeulen, 1985). Some aspects of this research are part of the present thesis.

The organ of synthesis and/or storage as well as the secretion of the toxin has been determined with certainty in *I. holocyclus*. In this tick species the toxin is associated with the salivary glands in definite periods during repletion (Kaire, 1966). Kaire (1966) isolated from homogenates of replete females of *I. holocyclus* a fraction capable of paralyzing mice. A fraction with a paralyzing effect was also obtained from salivary gland extracts of this tick species by ion-exchange chromatography, gel filtration and affinity chromatography (Stone, 1979; Stone and Wright, 1981). This fraction consisted of 2 major and 2 minor components according to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular weight range was between 40 kdal and 60 kdal and the pI in the range 4.5 - 5.0. The paralyzing fraction was very resistant to proteolysis by the enzyme pronase although non-toxic proteins were readily hydrolyzed. α-Chymotrypsin hydrolyzed about 30% of the undigested residue (MW 40 - 60 kdal) with a corresponding loss of toxicity. Kaire (1966) also showed that toxicity was retained after digestion of the paralyses fraction with proteases (pepsin, trypsin and papain). It was

concluded that the toxin was non-proteinaceous or that the toxin was resistant to digestion.

Despite extensive investigations on tick paralysis this toxicoses remains an enigma. In the present study the nature of the paralyses toxins of *R. evertsi evertsi* and *A.(P.) walkerae* were investigated as well as the kinetics of the previously isolated toxic anti-proteases from the eggs of various tick species.

1.3. Tick borne diseases caused by pathogens

Numerous diseases which are due to pathogens are transmitted by ticks. At least 15 such diseases are known to occur in the Republic of South Africa (Howell, *et al*, 1978). Economically important ticks and the diseases they transmit are shown in Table 1.1.

Since some aspects concerning *C. ruminantium*, the responsible agent of heartwater was investigated in this thesis, the disease is described in some detail below.

1.3.1. Heartwater

Heartwater or cowdriosis is a disease of ruminants, transmitted by ticks belonging to the genus *Amblyomma*. In the Republic of South Africa (Fig 1.2) the bont tick *Amblyomma hebraeum* (Fig 1.3), a 3 host tick, transmits the rickettsia, *C. ruminantium* which is the causal agent of heartwater (Cowdry, 1926; Neitz, 1968).

The disease is characterized by pyrexia, anorexia, malaise, nervous symptoms, dyspnoea, gastro-enteritis, hydrothorax and hydropericardium (Uilenberg, 1983). In typical cases, postmortem findings show accumulations of liquid in the pericardial sac and also in the thoracic and

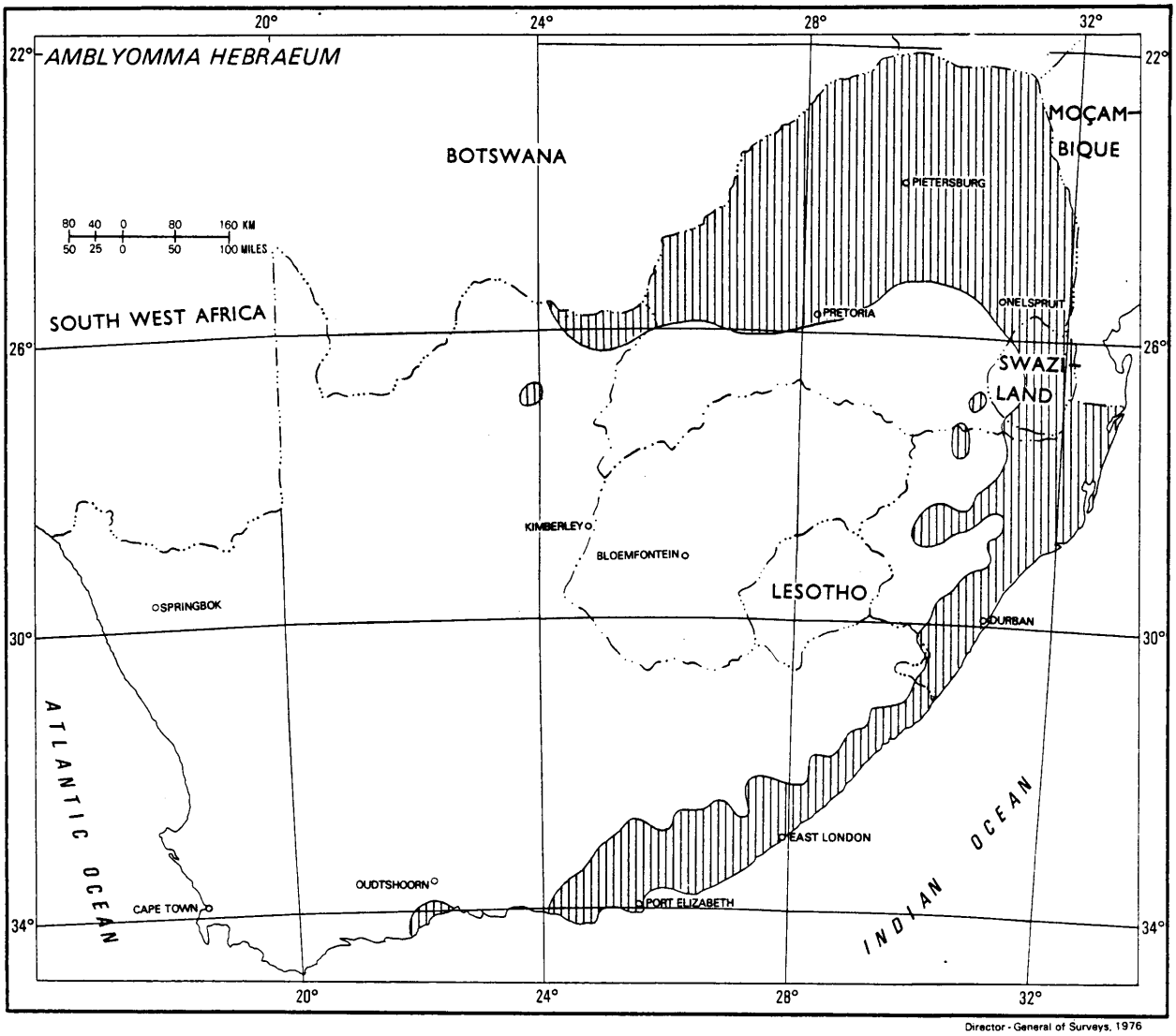

Fig 1.2. The distribution of *Amblyomma hebraeum*, the South African bont tick, in the Republic of South Africa. (Howell, Walker and Nevill, 1978).

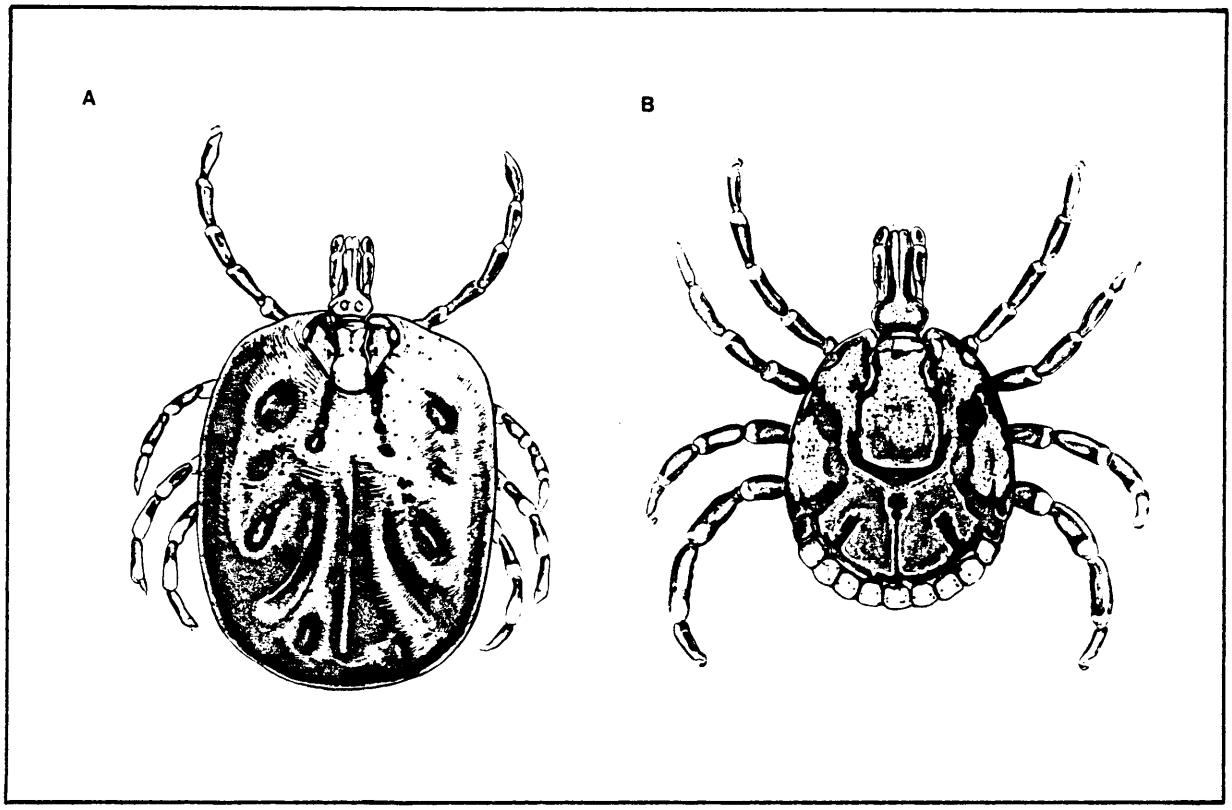

Fig 1.3. *Amblyomma hebraeum*, the South African bont tick.
(A) Female; (B) Male. (Howell, Walker and Nevill, 1978).

peritoneal cavities. The accumulation of the characteristic exudates may be explained by an increase in the permeability of the capillaries which in turn has been ascribed to a toxin. Toxins have also been involved to explain the nervous symptoms (Neitz, 1968).

Definite diagnosis of the disease in the living animal is difficult. The lack of suitable antigen has hampered the development of serological tests for heartwater.

The infection is acquired by ticks in either the larval or nymphal stage, whilst feeding on infected animals. The ensuing stages transmit the disease to susceptible animals. A transovarial infection from one generation to the next does not take place (Neitz, 1962). Male ticks, although capable of transmission, are poor vectors (Ilemobade, 1976).

Cattle, sheep and goats are susceptible. However, calves up to 3 weeks and lambs up to 1 week show a high degree of resistance to heartwater. This resistance is independent of the immune status of the dam (Neitz, 1968).

C. ruminantium, a pleomorphic Gram-negative organism (Cowdry, 1925), 0.25 - 1.0 µm in diameter (Pienaar, 1970), is usually coccoid in shape. Jackson (1931) and Haig (1955) described also ring-, comma- and horseshoe-shaped forms. The organisms occur in colonies within intracytoplasmic vacuoles with a distinct halo around them (Pienaar, 1970). Pienaar (1970) also confirmed the presence of small, intermediate and large forms of *C. ruminantium* in capillary endothelial colonies by electron-microscopy.

The organism has also been shown to occur in blood, and in various cell types including macrophages, monocytes, Kupffer's cells, reticulum cells

of the lymph nodes, fibroblasts and connective tissue and cells of various organs (Uilenberg, 1983).

In the tick vector the organism has been found only in the epithelial cells of the intestine and in the lumen of the gut (Cowdry, 1925; 1926).

The *in vitro* cultivation of *C. ruminantium* has repeatedly failed (Alexander, 1931; Ramisse and Uilenberg, 1971; Uilenberg, 1983). Haig (1955) was able to show that 9 day old heartwater infected chicken embryo tissue was infective for sheep, but attempts at serial passage of the agent failed.

The difficulties experienced in the *in vitro* cultivation of *C. ruminantium*, have seriously hampered the isolation and characterization of the heartwater agent.

Alexander (1931) reported that there is variation in the virulence of the different strains of *C. ruminantium* as the heartwater agent. The variation in virulence is inconsistent and is subject to extreme variation (Alexander, 1931), and can occur for the same strain during serial passage in sheep. Du Plessis (1982) was able to demonstrate common antibodies, cross-protection and common antigens for two of these strains, the mouse agent (*C. ruminantium*, Kumm strain) and the sheep agent (*C. ruminantium*, Ball 3 strain), with an indirect fluorescent antibody technique.

Ilemobade (1976) demonstrated sheep blood to be infective up to 50 days after recovery of heartwater or challenge. Neitz (1939) however, could not transmit heartwater from organ emulsions and endothelial scrapings of sheep recovered after more than 60 days. *C. ruminantium* was shown to persist in the brain of goats, up to 9 weeks after oxytetracycline treatment, but the blood was not infective (Ilemobade, 1976).

C. ruminantium is anextremely labile organism. Infected blood, stored at 4°C, loses its infectivity within 24 h. Much work has been performed regarding the preservation of the organism. The results are conflicting but it appears that rapid freezing in the presence of not more than 10% dimethyl sulfoxide (DMSO) as cryoprotectant preserves infectivity (Uilenberg, 1983). Glycerol, an alternative cryoprotectant was found to be toxic to the organism (Alexander, 1931).

Ilemobade (1976) and Alexander (1931) reported that laboratory and field animals (sheep, cattle and goats), are capable of developing a resistance towards heartwater. The nature of this immunity is obscure. Antibodies are detectable with the capillary flocculation test one to four weeks after treatment, clinical recovery or challenge (Ilemobade and Blotkamp, 1976), whereas animals are immune to challenge even after 4 months. On the other hand, Du Plessis (1981) found that sheep developed heartwater in spite of high levels of antibody, which was determined by an indirect immunofluorescent test. Alexander (1931) suggested that the protection against heartwater was probably cellular. Serum or large quantities of γ-globulins from hyperimmune serum do not effect the course of the disease whether injected before, after or simultaneously with infective blood (Du Plessis, 1970a). It is also not known whether the immunity is linked to the persistence of *Cowdria* in the host (premunition) or whether a sterile immunity is involved.

In spite of intensive investigations into heartwater, many important questions remain unanswered especially with respect to the characteristics of *Cowdria* organisms, the pathogenesis on a molecular level and immunological aspects. In this thesis some aspects of heartwater were investigated. These included developing methods for the purification of *C. ruminantium* organisms and their detection in the vector and the host as well as a study of antibody development in the host during infection. The purpose was to devise serodiagnostic methods and to acquire in-

formation regarding characteristics of the organism, their distribution in the host and vector, the infection rate of tick populations as well as the nature of the immunity to heartwater.

CHAPTER 2

ISOLATION OF A NEUROTOXIN FROM THE SALIVARY GLANDS OF SPRING LAMB PARALYSIS CAUSING FEMALE *RHIPICEPHALUS EVERTSI EVERTSI*

2.1. INTRODUCTION

It is generally accepted that tick paralyses is caused by toxins present in tick salivary glands which are transferred to the host during the process of feeding (Neitz, 1962). Spring Lamb Paralysis, which is caused by the red-legged tick, ***Rhipicephalus evertsi evertsi*** (Fig 2.1) (Gothe and Bezuidenhout, 1986) occurs in certain parts of the Republic of South Africa (Fig 2.2). Apparently all strains of this tick has a paralysis producing capacity (Gothe and Bezuidenhout, 1986).

The toxicoses is characterized as an ascending symmetrical, flaccid tetraplegia with functional impediment of the reflexes of the superficial and deep tendons of the limbs and abdomen (Gothe, Kunze and Hoogstraal, 1979). The symptoms of tick paralysis subside if ticks are removed providing the paralysis has not progressed too far (Neitz, 1956).

Extensive ***in vivo*** investigations by Gothe and Lämmler (1982a) showed that a definite relationship exists between toxicity of female ***R. evertsi evertsi*** and their state of repletion which is confined to a narrow body weight range of 15-21 mg. When male and female ticks commence feeding simultaneously, this range is normally reached between the 4 th and 5 th day of feeding. Therefore, salivary glands containing a maximum quantity of toxin may be obtained. A similar relationship exists also for other ixodid ticks (Gothe, 1984). In the case of ***Ixodes holocyclus***, maximum toxicity is reached on the 5 th and 6 th feeding day but unfed

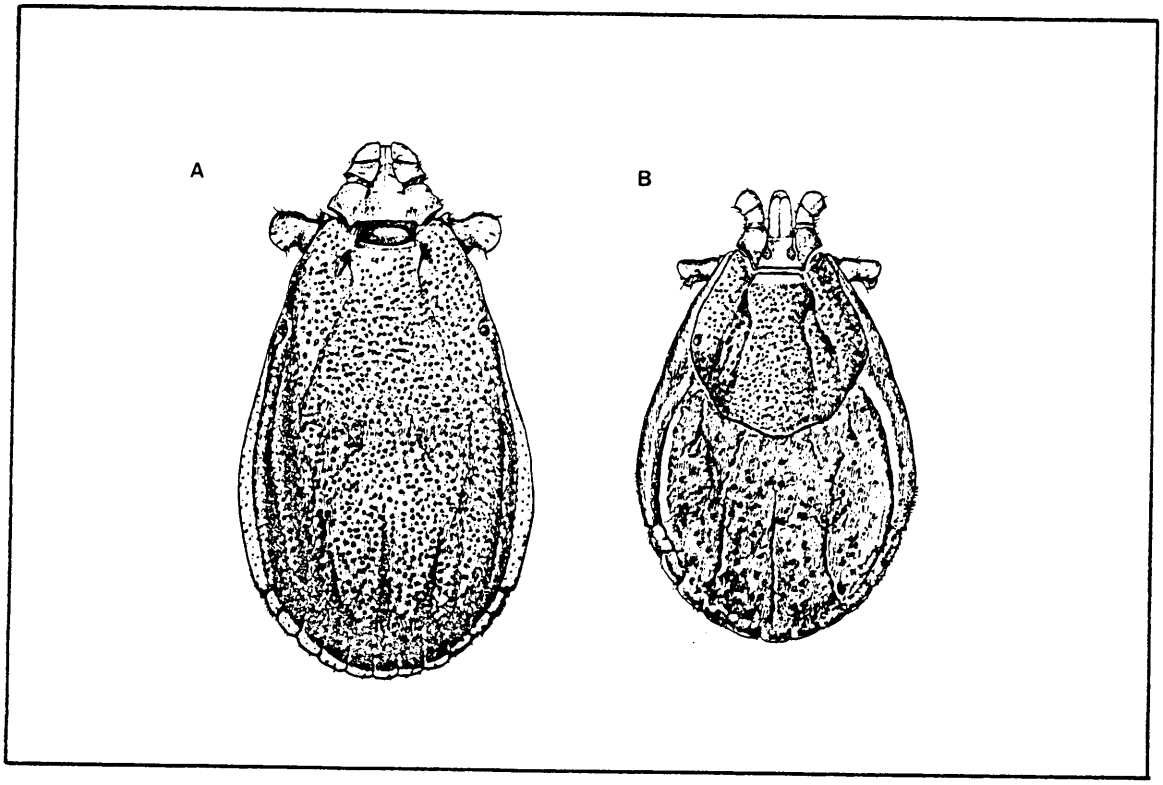

Fig 2.1. *Rhipicephalus evertsi evertsi*, the red-legged tick. (A) Male; (B) Female. (Hoogstraal, 1956).

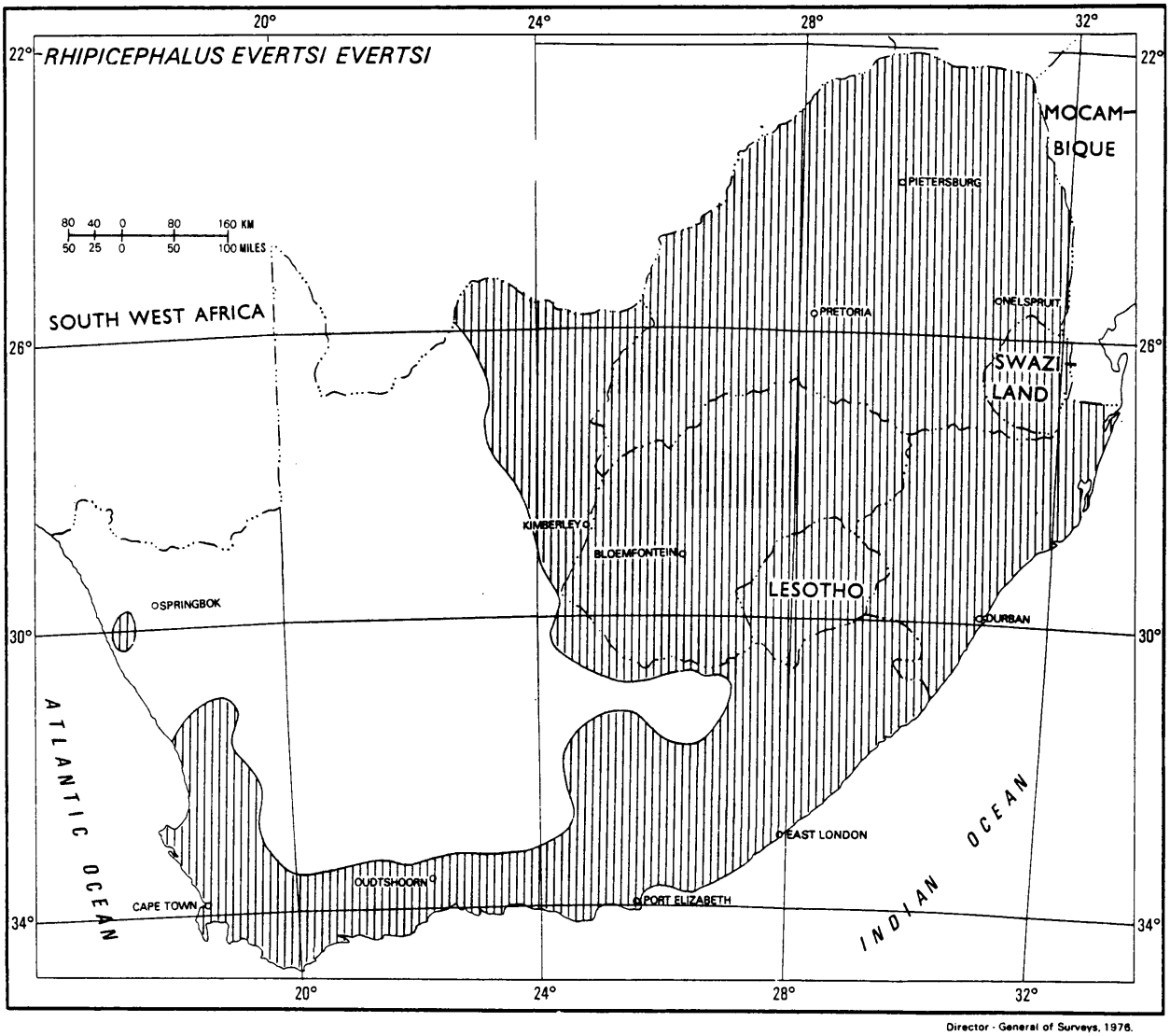

Fig 2.2. The distribution of *Rhipicephalus evertsi evertsi* in the Republic of South Africa. (Howell, Walker and Nevill, 1978).

as well as fully engorged ticks also contain toxin (Stone, Doube, Binnington and Goodger, 1979).

When host animals are infested with male *R. evertsi evertsi*, 7 days before females, the females attain average body weight up to the 3 rd feeding day, corresponding to those attained when both sexes commence feeding simultaneously. However, in the ensuing days, a steeper rise in body weight is attained and the first clinical symptoms appears earlier (Gothe and Budelmann, 1980). In infestations with exclusively female imagines, the period until the onset of clinical manifestations is always lengthened. Of particular interest is that under these conditions, the symptoms are considerably less pronounced (Gothe and Budelmann, 1980).

In view of these results, it has been postulated that increased feeding and toxicity of female ticks is initiated by copulation and the successful transfer of spermatophores. In male *R. evertsi evertsi*, spermatogenesis is complete after 4 - 5 days of infestation (Londt and van der Bijl, 1977). Thus when male and female ticks commence feeding simultaneously, copulation cannot occur before approximately the 5 th day. However, when male ticks start feeding 7 days before the females, copulation can take place earlier with immediate intensification of feeding by females. They thus pass the toxic phase faster resulting in earlier, but less severe manifestation of symptoms. In contrast, virgin females reach the toxic phase much later and presumably only marginally.

To explain the epidemiology of the paralysis caused by *R. evertsi evertsi*, the above findings together with ecological factors may be considered (Gothe and Budelmann, 1980). Adult ticks appear seasonally and first at the beginning of spring. Sheep are thus infested simultaneously by both sexes and as a consequence are paralyzed severely. The toxicosis becomes clinically manifest in lambs primarily because a direct interrelationship exists between infestation rate and body weight of animals.

Therefore, this type of paralysis is described as 'Spring lamb paralysis'. Because of the longer parasitism by male ticks, animals are gradually infested asynchronously by the sexes resulting in earlier copulations, a faster passage of the toxic phase and weak or clinically not always overt signs. Furthermore, it may be anticipated that animals that survive the early seasonal attacks in spring are protected by an effective immunity (Gothe and Lämmler, 1981) in the ensuing months.

Neurophysiological investigations by Gothe and Kunze (1982) on the exposed ***Nervus tibialis*** in paralyzed sheep revealed that paralysis due to ***R. evertsi evertsi*** is mainly a motor polyneuropathy. The amplitudes of nerve action potentials and evoked compound muscle action potentials were slowed down with 46.3% and 88.3% respectively. The maximum motor conduction velocity was only slightly reduced. It was concluded that this tick paralysis is associated with functional impairment of slow conducting motor nerve fibres. Since the blockage is readily reversible, it has been proposed that the toxin has membranophilic properties with a primary point of attack at the nodes of Ranvier (Gothe and Kunze, 1981).

An investigation into the qualitative changes in the protein pattern of the salivary glands of paralysis inducing female ***R. evertsi evertsi*** during the entire repletion period, revealed 2 proteins which first started to appear on the 5th day of feeding when ticks had attained a body weight of 15 - 21 mg (Neitz and Gothe, in press). Since both proteins were present during the remainder of the feeding phase, it was concluded that changes in the protein pattern ***per se*** were not associated with the toxic phase but rather an increased production and/or transmission of toxin during this phase. A quantitative study of the salivary gland proteins during feeding could therefore be of value for the identification of the toxin.

The isolation of the paralysis toxin from the salivary glands of *R. evertsi evertsi* has met with difficulties. These include the lack of strains of this tick species which do not cause paralysis (Gothe and Bezuidenhout, 1986). The availability of a negative strain could be exploited for the development of immunoadsorbents (Lowe, 1979; Sunderland, McMaster and Williams, 1979) for toxin isolation as well as toxin identification by protein blotting following electrophoresis (Gershoni, 1985).

A further problem is the failure to elicit paralysis in common small laboratory animals when infested with *R. evertsi evertsi* of known high paralysis inducing capacity. A suitable laboratory model for this toxicosis is thus excluded (Gothe and Lämmler, 1982b).

This chapter describes a quantitative study of the changes in the protein pattern in the salivary glands during the entire repletion process of the paralysis producing female *R. evertsi evertsi*. In addition, a sensitive *in vitro* toxin assay is reported as well as a simple isolation method for obtaining a homogeneous toxic fraction according to isoelectric focusing, sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis (SDS PAGE) and gel permeation chromatography

2.2. MATERIALS AND METHODS

Analytical quality reagents were used in all the experiments. All the glassware and equipment was sterilized with 70% (v/v) ethanol and the buffers by filtration through 0.22 µm filters (Millipore).

2.2.1. Origin and rearing of ticks

Laboratory colonies of the paralysis inducing Mkuze strain of *R. evertsi evertsi* (Gothe and Bezuidenhout, 1986) were maintained at 27 °C and 75-80% relative humidity. Equal numbers of male and female ticks were simultaneously allowed to attach freely and feed on sheep at an infestation rate of two ticks per kg body weight. They were removed after attainment of specific body weight (Gothe and Budelmann, 1980).

To obtain immune serum, paralyzed sheep were allowed to recover and 4 weeks later, ticks were fed on them in the same manner as described above. No paralysis was observed after the second tick infestation. Four weeks after the challenge, 100 ml blood was obtained and left to clot for 4 h at room temperature. The coagulated blood was centrifuged for 10 min at 300xg in a Piccolo bence top centrifuge (Heraeus-Christ). The serum was siphoned off, divided into 1 ml batches and stored at -75 °C.

2.2.2. Preparation of salivary glands

Salivary glands were dissected from female ticks fed in the presence of males for 4-9 days. These ticks had body weight ranges of < 15 mg, 15-21 mg, 21-40 mg, 40-120 mg and 120-400 mg. In addition, salivary glands of unfed females and 5 day fed males (3-7 mg) were dissected. Glands were removed within 3 h after collection with the aid of a Model Forty binocular stereo-microscope (American Optical Corporation) in ice cold 0.154 M NaCl and suspended in 100 µl ice-cold 0.154 M NaCl. Special care was taken to avoid contamination by hemolymph, gut contents and tick tissues. Dissection of a female ixodid tick to show the position of the salivary glands is shown in Fig 2.3 (obtained from Entomology section, Veterinary Research Institute, Onderstepoort). The gland suspension was then frozen at -75 °C or in liquid nitrogen in the gas phase.

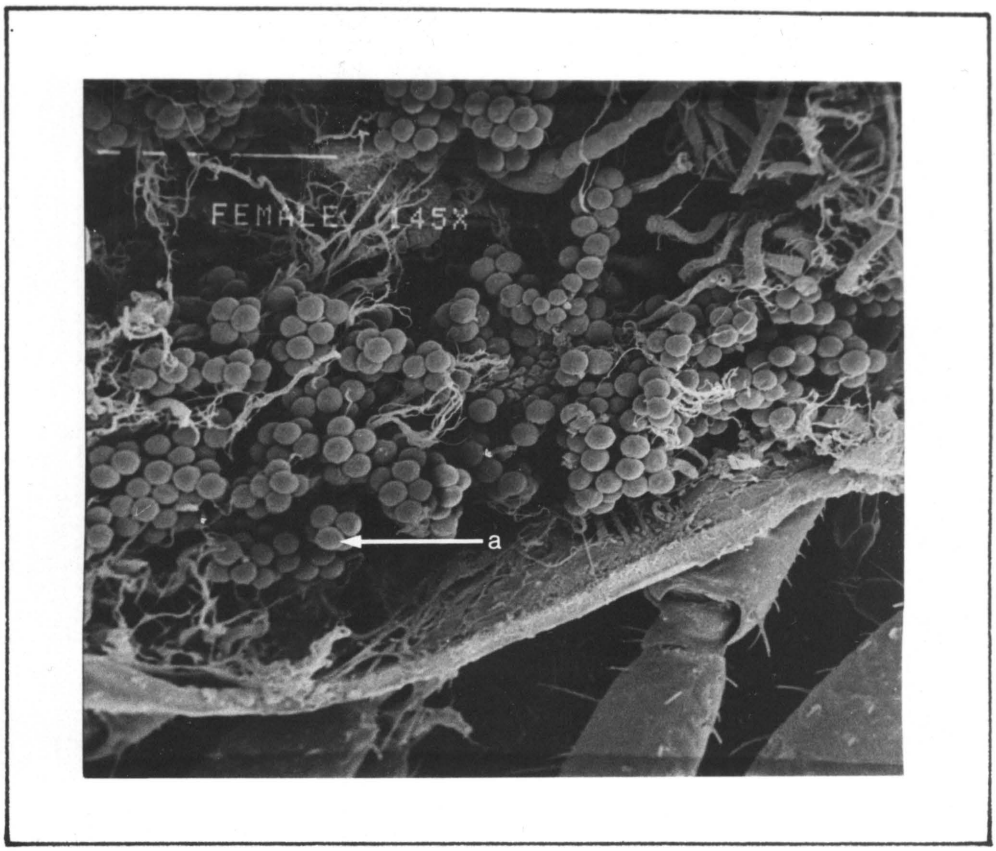

Fig 2.3 Dissection of female ixodid tick to show position of salivary glands. (a) Salivary gland acinar. (145x)

To prepare crude extracts, 30-100 glands were thawed and homogenized with a 2 ml Wheaton micro tissue grinder (Wheaton Scientific) for 10 min or sonified by means of a Branson sonifier Model B-30 (Branson Sonic Power Company) on ice for 15 seconds (3 second bursts) with the output set at 5. Samples were then centrifuged in a Beckman Model B microfuge for 10 min at 8 000xg and 4 °C and the supernatants used immediately or freeze-dried in a Virtis Freeze-mobile Twin 6 freeze-drier (Virtis Company). These freeze-dried samples were kept under vacuum at -75°C.

2.2.3. Isolation of a neurotoxin

For chromatofocusing separations in the pH range of pH 9 to pH 4, a Pharmacia fast protein liquid chromatograph (FPLC) system was used, consisting of a Mono P HR 5/20 (200 X 5 mm) column, two P-500 pumps, a V-7 valve injector with a 100 µl loop, a GP-250 gradient programmer, a UV-1 monitor with a 280 nm filter, a FRAC-100 fraction collector and a REC-482 recorder. Crude extracts, representing 30-100 freeze-dried salivary glands, were made up to 110 µl with starting buffer.

Starting buffer was 0.025 M Triethanolamine, pH 9.0, iminodiacetic acid and the eluting buffers were (a)Polybuffer 96 (9% ,v/v), Pharmalyte 8-10.5 (0.21%,v/v), iminodiacetic acid, pH 6.0 and (b)Polybuffer 74 (10%,v/v), iminodiacetic acid, pH 4.0.

The samples were eluted at a flow rate of 60 ml/h and peak fractions were collected with the fraction collector set in the peak fraction mode. The pH of each fraction was determined with a Model 26 pH meter (Radiometer). The fractions were dialyzed for 48 h against 40 l of distilled water at 4 °C. The retentates were freeze-dried.

2.2.4. Protein determination

Protein was determined by the Coomassie Brilliant Blue G-250 dye-binding (Bradford, 1976) assay as described in the Bio-Rad protein assay instruction manual. Bovine serum albumin (Merck) was used as standard in the concentration range of 5-4 000 µg/ml. All determinations were performed at least in triplicate and mean values as well as standard errors were calculated.

2.2.5. Determination of toxicity

2.2.5.1. *In vivo* determinations

Crude salivary gland extracts, obtained from ticks having 15-21 mg body weight, were injected sc or iv into 4 merino sheep, 6 mice or 1 chicken.

For the first 2 experiments, the salivary glands of 45 and 21 female ticks were removed and the remnants of these ticks were homogenized and injected subcutaneously (sc) into a 12 kg sheep and intravenously (iv) into a 15 kg sheep respectively.

Sheep that showed no paralysis symptoms were challenged with equal amounts of male and female ticks. The ticks were allowed to attach freely using a 2 ticks per kg sheep body weight ratio to ensure sufficient females in the toxic phase to potentially cause paralysis.

2.2.5.2. Determination with a nerve-muscle preparation

The crude extracts and the various isolated fractions were also tested using *Xenopus laevis* sciatic nerve-gastrocnemius muscle preparations. The dissection of the nerve-muscle was carried out as follows: in order to destroy the central nervous system, a blunt needle was pressed

through the foramen magnum to destroy the brain and then down the spinal canal to destroy the spinal cord. The skin was removed from the back of the frog and the animal laid in the prone position on a dissection board. The ***Nervus ischiadicus*** was exposed at the urostyle and a thread was bound around the three roots of the nerve. Then a cut was made through the urostyle at its base and the nerve was dissected out cleanly through the abdominal cavity, through the body wall and down the upper leg as far as the knee joint. Care was taken not to stretch the nerve or let it dry out. The ***Musculus gastrocnemius*** was identified and the muscle was cut at the distal end of the nerve (Fig 2.4(A)). A thread was bound around this end. The nerve-muscle preparation was handled only by means of the threads and placed into the 100 ml muscle bath (Fig 2.4(B)), (Bell, 1959) containing 50 ml frog Ringer solution (McKenzie and Dawson, 1969), (0.64% (w/v) sodium chloride, 0.02% (w/v) sodium bicarbonate, 0.02% (w/v) calcium chloride, 0.02% (w/v) potassium chloride and 0.1% (w/v) glucose). The nerve was stimulated electrically at 20 sec intervals by means of three 1.5 volt batteries connected in series. Muscular contractions were registrated on a chymograph (Palmer, England) (Fig 2.4(C)), (Harris, 1947). Solutions to be tested were applied onto a small portion of the nerve which was submerged in a specially constructed nerve bath (Fig 2.4(D) and Fig 2.4(E)) with 60 µl fluid capacity.

2.2.6. Analytical isoelectric focusing

Analytical isoelectric focusing with polyacrylamide as supporting medium was performed with a Pharmacia Fine Chemicals Model FBE 3 000 flat bed electrophoresis apparatus and a Pharmacia Fine Chemicals Model ECPS 2 000/300 power supply. Acrylamide and N, N'-methylene bisacrylamide were purified by means of Amberlite MB-1 ion exchanger resin. Polymerization was achieved under nitrogen in the presence of sodium sulfite, glycerol, carrier ampholytes (Pharmalytes, pH range 3-10) and

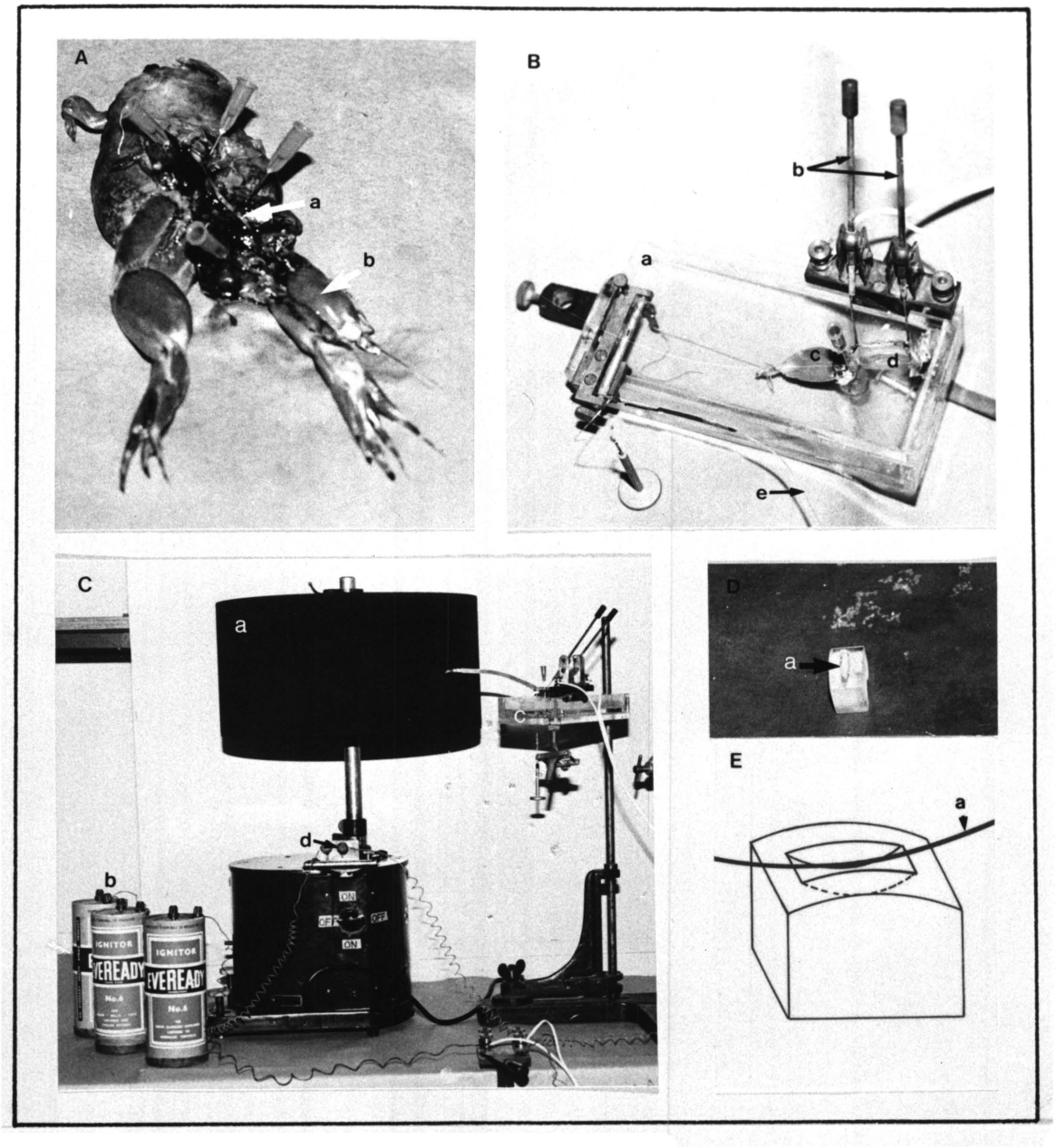

Fig 2.4 Apparatus employed for the *in vitro* detection of a neurotoxin present in the salivary glands of the female *Rhipicephalus evertsi evertsi*.

(A) Dissection of the nerve-muscle. (a) Sciatic nerve, (b) gastrocnemius muscle;

(B) Muscle bath, (a) 100 mℓ perspex bath, (b) electrodes, (c) gastrocnemius muscle, (d) nerve bath with sciatic nerve, (e) mechanical lever;

(C) Chymograph consisting of (a) rotating drum, (b) batteries connected in series, (c) muscle bath, (d) switch;

(D) Nerve bath (a) indicating the nerve holder well with 60 µℓ fluid capacity:

(E) Presentation of the nerve bath, (a) nerve.

potassium persulfate on silane-treated glass plates. The final total gel concentration (T) was 5.17% and the percentage concentration of the cross-linker relative to the total concentration (C) was 3.23%.

Samples containing approximately 20-50 µg protein were applied onto the gel with a mask. Standard proteins with known isoelectric points (Pharmacia Fine Chemicals) were run simultaneously with samples.

Isoelectric focusing was performed at a constant power of 30 Watt. Fixing of separated bands was achieved with 10% (w/v) trichloro-acetic acid (Merck) and 5% (w/v) sulfosalicylic acid (Merck) for 1 h and staining with 0.2% (w/v) Coomassie Brilliant Blue R-250 (Merck) in 45% (v/v) methanol and 10% (v/v) acetic acid (Fazekas de St. Groth, Webster and Datyner, 1963) for 1 h. Destaining was achieved with 22.5% (v/v) methanol and 7.5% (v/v) acetic acid and 5% glycerol (v/v) for approximately 12 h.

2.2.7. Sodium dodecyl sulfate gradient gel electrophoresis (SDS PAGE)

Acrylamide and N, N'-methylene bisacrylamide were used to prepare a 5 to 15% polyacrylamide gradient gel (Laemmli, 1970). The gel concentrations in the solutions were: T = 5%, C = 2.6% and T = 15%, C = 2.6%. The gradient was formed with a Pharmacia Fine Chemicals GM-1 gradient mixer and a Pharmacia Fine Chemicals peristaltic pump P-1 in 1.5mmx8cmx8cm glass plates.

Samples containing 30-50 µg protein were disaggregated in a boiling water bath for 5 min in the presence of 1.6% (w/v) 1,4 Dithiothreitol, 2.0% (w/v) SDS and 0.02 M Tris-HCl, pH 7. Electrophoresis was carried out in a Pharmacia Fine Chemicals GE-4 apparatus with a voltage of 150 Volt and 0.025 M Tris, 0.2 M glycine, 0.1% SDS buffer, pH 8.3 as running

buffer. Standard proteins with known molecular weight (Pharmacia Fine Chemicals) were run simultaneously with samples.

Fixing and staining of the protein bands was performed with 0.25% (w/v) Coomassie Brilliant blue R-250 in 5:1:5 methanol:acetic acid:water for 12 h and destained in 5:1:5 methanol:acetic acid:water. The gel was then stored in a solution containing 7% (v/v) acetic acid and 5% (v/v) methanol.

2.2.8. Gel permeation chromatography

A high performance liquid chromatography system was employed consisting of a Beckman Model 100A pump, a Beckman Model 160 detector at 214 nm, an Altex vent sample loader with a 20 µl sample loop, a Hitachi Model QD25 recorder with chart speed at 5 mm/min and 0.1 absorbance span and a Waters Protein Pak 300 sw column (7.5 mm x 30 cm) with v_o = 5 ml and v_t = 16.6 ml was used. The experiment was executed at room temperature. The flow rate was 1 ml/min.

The Waters Protein Pak 300 sw column was equilibrated with 15 column volumes of a 0.05 M sodium sulfate solution. Toxin, at a concentration of 5 and 2.5µg was applied to the column. Standard proteins consisting of catalase (MW = 232 kdal), lactate dehydrogenase (MW = 140 kdal), bovine serum albumin (MW = 67 kdal) and ovalbumin (MW = 45 kdal), all from Pharmacia Fine Chemicals or Boehringer Mannheim, were run in duplicate at similar concentrations as was used for the toxin.

The average retention time and standard deviation was calculated for each standard protein and the best least squares fit of the line was plotted. The toxin molecular weight was obtained from this plot.

2.2.9. Amino acid determinations

Samples were hydrolyzed with 0.5 ml constant boiling HCl for 24 h at 110 °C. The hydrolyzate was dried under nitrogen at room temperature and made up to 0.25 ml with a 0.2 N lithium citrate buffer, pH 2.2. A Beckman Model 121M amino acid analyzer was employed for the separation and quantitative estimation of the amino acids. A Beckman program, for the analysis of amino acids in physiological fluids was used.

2.2.10. Pronase digestion of the toxin

The digestion mixture consisted of 60 µl of a 1% (w/v) pronase (Boehringer Mannheim), (Glaser, Delange and Sigman, 1975) solution in 0.05 M HEPES buffer, pH 7.3 to which 100 µg of the toxin was added. The mixture was incubated for 12 h at 27 °C. As controls a 1% (w/v) pronase solution in 0.05 M HEPES buffer, pH 7.3 as well as a solution containing 100 µg toxin in 60 µl of 0.05 M HEPES Buffer, pH 7.3 were incubated under the same conditions. The samples were tested with the nerve-muscle preparation.

2.2.11. Effect of immune antiserum on toxicity of the toxin

To 100 µg toxin, 60 µl immune antiserum, obtained as described in paragraph 2.2.1., p. 24 , was added. This solution as well as 60 µl immune serum, as control, was incubated for 12 h at 27 °C and tested on the nerve-muscle preparation.

2.3. RESULTS

2.3.1. Toxicity of crude salivary extracts

Crude salivary gland extracts obtained with the tissue grinder, caused no paralysis symptoms when inoculated into sheep (Table 2.1). Also no paralysis was observed for the tick extracts prepared from ticks from which the salivary glands had been removed. On subsequent challenge with competent adult ticks, all the artificially inoculated sheep showed typical symptoms of paralysis. All the mice and one chicken failed to show paralysis symptoms upon injection with crude salivary gland extracts (Table 2.1).

Toxicity determinations using the frog nerve-muscle preparation revealed that the crude extracts obtained from ticks with body weight in the 15-21 mg and 21-40 mg ranges caused total inhibition of muscle contraction 10 min after application of concentrations of 6,5 mg and 16,4 mg protein/ml, respectively (Table 2.2).

2.3.2. Quantitative changes in the protein pattern in the salivary glands during infestation

The total protein content in the salivary glands of females increased approximately 7 fold during the early stages of feeding and remained relatively constant throughout the entire repletion period. This was also observed by Neitz and Gothe (in press). In comparison, salivary glands of males contained a low protein concentration even after several days of feeding (Table 2.2).

Chromatofocusing and isoelectric focusing of crude extracts of female salivary glands revealed the presence of at least 14 UV positive (280 nm)

TABEL 2.1. Effect of crude salivary gland extracts, obtained from *Rhipicephalus evertsi evertsi* ticks in the toxic phase, on laboratory animals.

Quantity of glands inoculated	Extraction method	Protein quantity inoculated (mg)	Laboratory animal	Inoculation route	Observation
90	Homogenization	3.528[1]	sheep[3] (10 kg)	sc	nr
42	Homogenization	1.646[1]	sheep[3] (12 kg)	iv	nr
70	Homogenization	2.744[1]	sheep[3] (15 kg)	iv	nr
66	Homogenization	2.587[1]	sheep[3] (10 kg)	iv	nr
40	Homogenization	1.578[2]	mouse[3] (15 g)	sc	ir
64	Sonification	2.498[2]	mouse[3] (15 g)	sc	ir
64	Sonification	2.517[2]	mouse[4] (1 g)	sc	ir
43	Sonification	1.672[2]	mouse[4] (1 g)	sc	ir
48	Sonification	1.897[2]	chicken[3] (20 g)	sc	ir

[1] - mg inoculated in one 1 ml dose per animal
[2] - mg inoculated in one 100 µl dose per animal
[3] - only one animal was inoculated
[4] - duplicate animals were inoculated
nr - no paralysis symptoms on initial inoculation, but showed paralysis when subsequently challenged
ir - no paralysis symptoms when inoculated, but were not challenged

fractions corresponding to isoelectric points of between 8.75 to 4 (Fig 2.5 and 2.6, Table 2.2).

These fractions may conveniently be grouped according to their distinct concentration changes during the repletion phase (Fig 2.5 and 2.7; Table 2.2). Group A (fractions No. 4, 5 and 6) are absent in ticks which have fed for 4 days and appear only after mating. The concentration of these fractions with pI 8.2 - 8.4 increase as feeding proceeds. The remaining groups are present in all ticks from at least the 4 th day of feeding. The concentration of group B (fraction 13) increases during feeding and reaches a maximum on the 7th day. Group C (fractions 2, 3, 7 and 12) reach a maximum concentration soon after attachment, before mating, while Group D (fractions 1 and 9) likewise show concentration maxima

TABLE 2.2 Paralysis inducing capacity and concentration of crude salivary gland extracts[1] of *Rhipicephalus evertsi evertsi*, before and during feeding.

Fraction no.	Group	pI	Relative protein concentration[2]						
			Male	Female					
			3 - 7 mg	Unfed	<15 mg	15 - 20 mg	21 - 40 mg	40 - 120 mg	120 - 400 mg
1	D	8.75 ± 0.05	28.3	48.7	436.9	203.9	204.1	234.2	378.6
2	C	8.65 ± 0.05	10.4	60.7	105.7	24.6	27.2	26.5	28.3
3	C	8.42 ± 0.1	18.2	-	182.1	34.4	31.7	21.9	19.9
4	A	8.30 ± 0.05	-	-	-	60.7	75.5	78.4	68.5
5	A	8.20 ± 0.1	-	-	-	22.6	24.3	155.5	181.2
6	A	8.15 ± 0.05	-	-	-	60.6	57.2	66.9	78.1
7	C	8.10 ± 0.15	3.1	-	126.8	45.3	52.9	44.0	49.8
8	E	8.05 ± 0.1	184.2	146.1	480.2	780.2	946.3	1 085.7	757.5
9	D	7.50 ± 0.1	12.4	-	71.4	46.1	65.3	72.7	103.7
10	F	7.14 ± 0.15	-	-	21.9	19.0	43.9	28.3	41.7
11	G	6.00 ± 0.1	-	-	38.2	369.2	93.4	48.1	20.5
12	G	5.10 ± 0.1	-	-	144.8	12.4	14.5	16.2	18.6
13	B	4.24 ± 0.15	27.1	-	72.7	81.4	109.1	112.3	127.8
14	G	4.00 ± 0.1	85.9	-	171.6	198.1	127.7	106.7	106.1
Protein concentration in sample (ug/100ul)			367 ± 9.3	255 ± 4.8	1 852 ± 38.3	1 959 ± 48.2	1 891 ± 53.4	2 097 ± 51.1	1 980 ± 54.2
Protein content (ug/gland)			7.4 ± 0.4	5.1 ± 0.6	36.8 ± 0.7	39.2 ± 1.5	37.9 ± 1.7	41.3 ± 1.7	39.8 ± 1.3
Paralysis assay: ug protein per 60 ul used in assay Result:			444 N	306 N	1 104 N	390 I	984 I	1 236 N	1 194 N

[1]- In each case extracts from 50 salivary glands were applied to the column

[2]- Expressed in terms of peak area, as percentage of total protein. Calculated as width at half height X height

I- Inhibition of muscle contraction

N- No inhibition of muscle contraction

Coefficient of variation was at all times<5 %

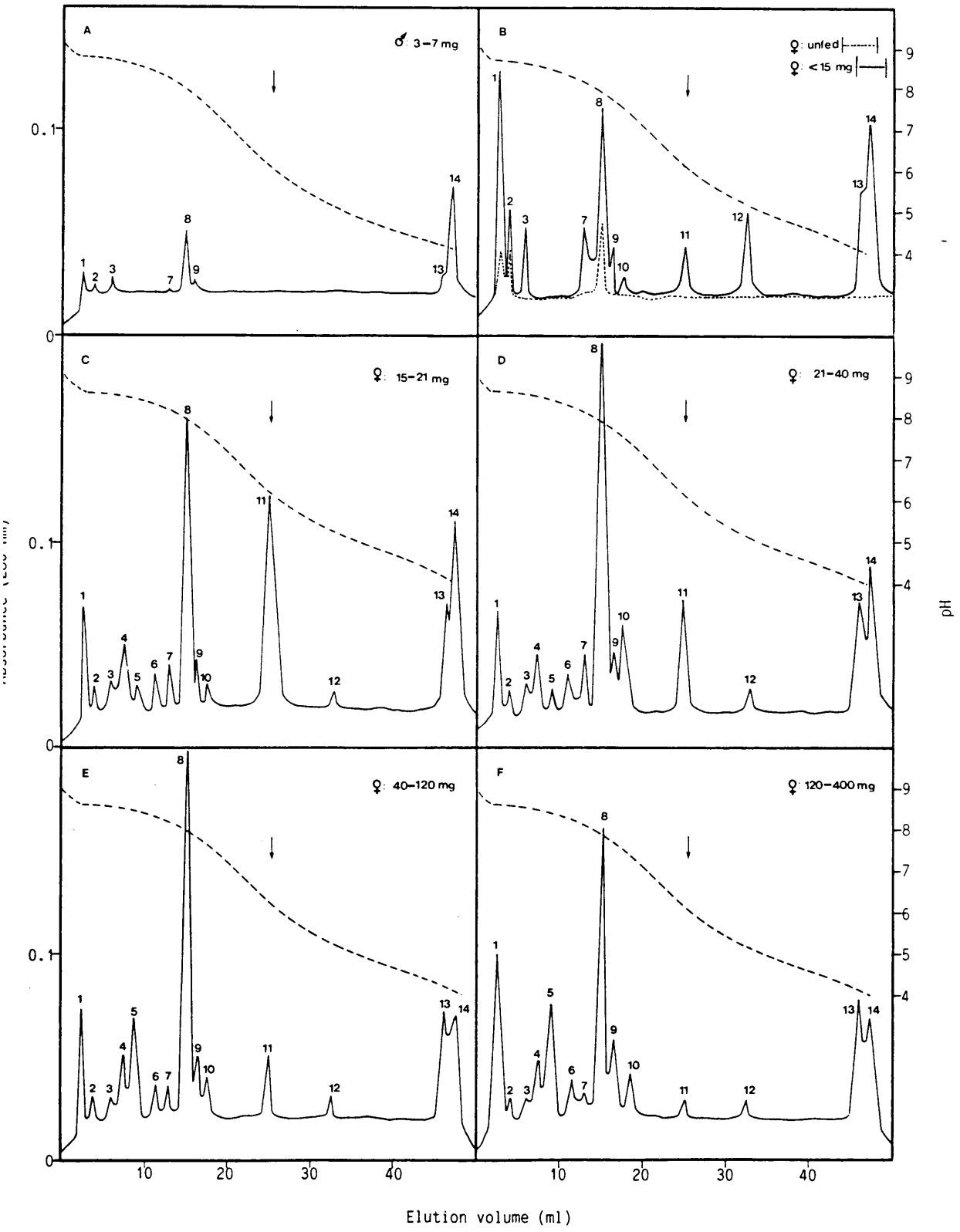

Fig 2.5. Elution pattern of *Rhipicephalus evertsi evertsi* crude salivary gland extracts on a Mono P chromatofocusing column. Concentration: 50 salivary gland extracts/ 100µl starting buffer. Span: 0.2 A, flow rate: 1 ml/min, chart-speed: 0.5 cm/min, absorbance (———), pH gradient (- - -). Arrow indicates buffer change. (A) Males; (B) unfed females and <15 mg weight range females; (C) 15-21 mg weight range females; (D) 21-40 mg weight range females; (E) 40-120 mg weight range females; (F) 120-400 mg weight range females. The numbers of the proteins correlate to those in Table 2.2.

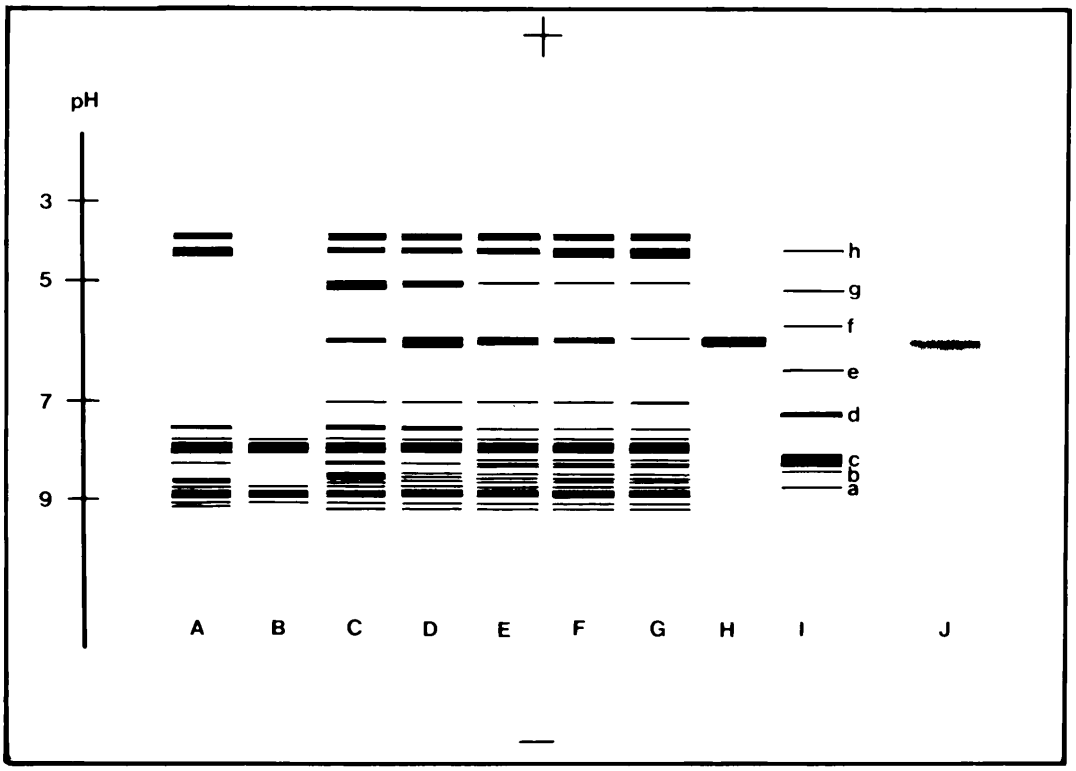

Fig 2.6 Analytical isoelectric focusing of the *Rhipicephalus evertsi evertsi* crude salivary gland extracts and isolated paralysis toxin.

(A) Male crude salivary gland extract, 3-7mg weight range;

(B) Female unfed;

(C) Females, < 15 mg weight range;

(D) Females, 15-21 mg weight range;

(E) Females, 21-40 mg weight range;

(F) Females, 40-120 mg weight range;

(G) Females, 120-400 mg weight range;

(H) Isolated toxin;

(I) Isoelectric focusing standards: (a) lentil lectin basic band, pH 8.65, (b) lentil lectin middel band, pH 8.45, (c) lentil acid band, pH 8.15, (d) myoglobin, pH 7.35, (e) human carbonic anhydrase B, pH 6.55, (f) bovine carbonic anhydrase B, pH 5.85, (g) β-lactoglobulin A, pH 5.20, (h) soyabean trypsin inhibitor, pH 4.55;

(J) Isolated toxin, 25µg applied (photograph).

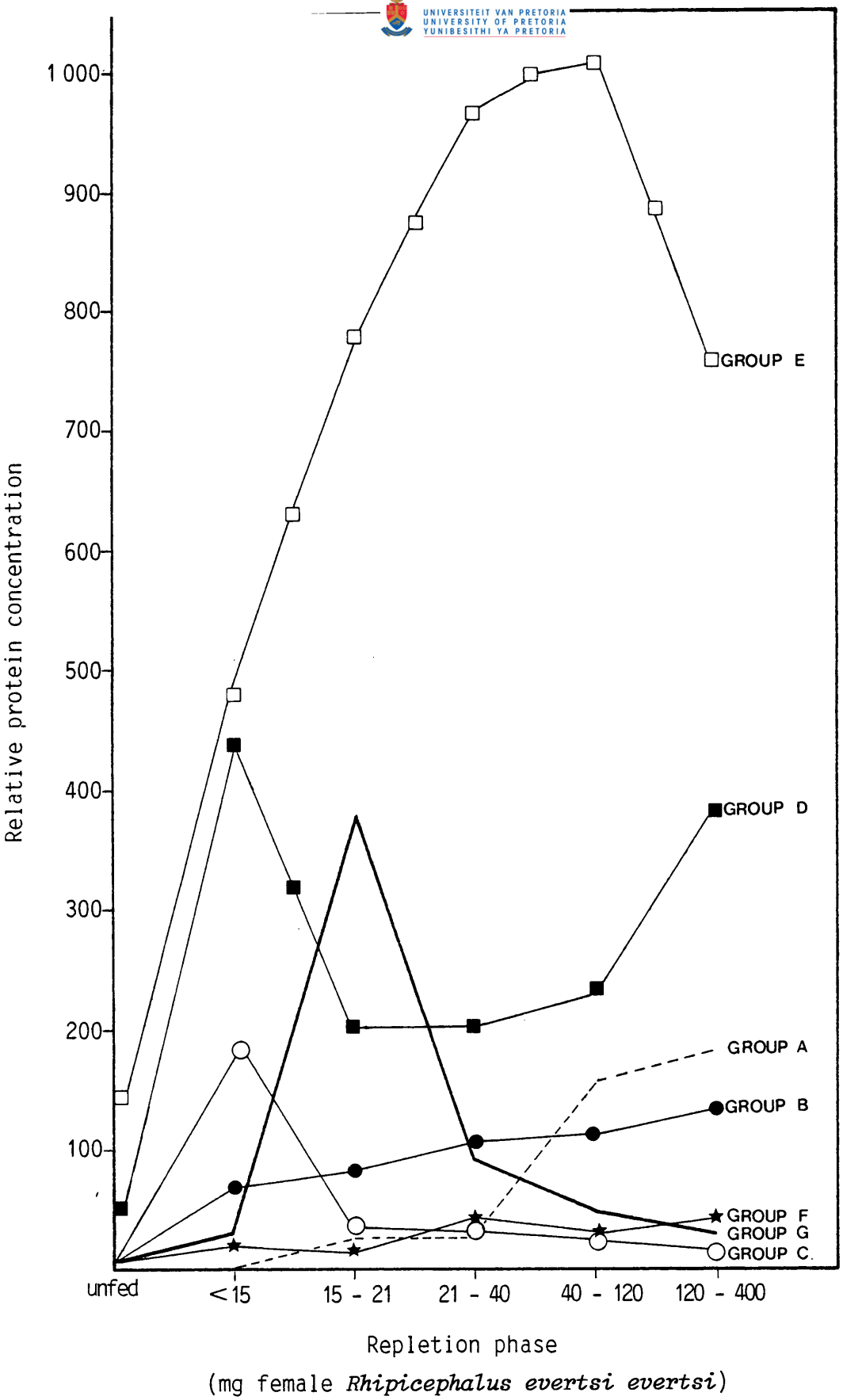

Fig 2.7. Representative concentration changes during the repletion phase of *Rhipicephalus evertsi evertsi* ticks. Group A: protein nr. 5 (------); group B: protein nr. 13 (—●—●—); group C: protein nr. 3 (—○—○—); group D: protein nr. 1 (—■—■—); group E: protein nr. 8 (—□—□—); group F: protein nr. 10 (—★—★—) and group G: protein nr. 11 (———). For each group only one representative protein is shown. Data obtained from Table 2.2.

before mating but also again during the final feeding phase. Group E (fraction 8) is present in all ticks analyzed i.e. fed males, unfed and fed females and constitutes approximately 30-50% of the total salivary protein. This fraction reaches a maximum concentration in females with body weight of 40-120 mg. Group F (fraction 10) is present in low fluctuating concentration in the salivary glands of all fed females. Group G (fractions 11 and 14) reach a maximum concentration during the toxic phase with fraction 11 exhibiting the most pronounced increase.

2.3.3. Toxicity determination of fractions obtained from ticks in the toxic phase

Of the 14 fractions obtained from ticks within a body weight range of 15-21 mg by means of chromatofocusing only fraction 11 with a pI of 6 (Fig 2.6 and Table 2.2) caused total inhibition of muscular contraction after 10 min at a concentration of 73,8 µg/ml in the nerve bath (Fig 2.8). All other fractions, tested in groups at considerable higher individual concentration as for the toxin, caused no inhibition during a test period of 120 min.

The inhibitory action of fraction 11 was shown to be reversible since washing of the nerve in the nerve bath with the frogs Ringer solution resulted in a resumption of contraction to a normal level after 5 min.

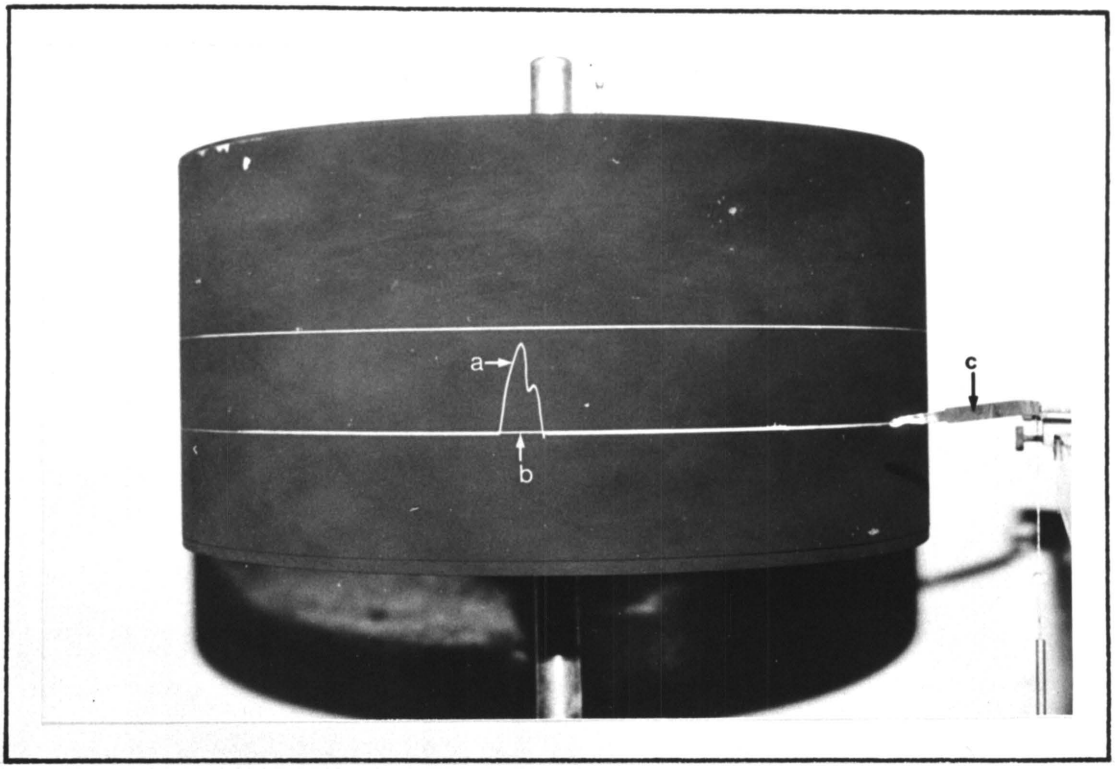

Fig 2.8 Effect of the isolated toxin from the salivary glands of female ***Rhipicephalus evertsi evertsi*** on muscle contraction. (a) Normal muscle contraction, (b) inhibition of muscle contraction by the application of the isolated toxin onto the sciatic nerve, (c) mechanical lever.

TABLE 2.3. Amino acid composition of the toxic protein present in *Rhipicephalus evertsi evertsi* salivary glands

Amino acid	Mole ratios[1]
lys	1
his	1
met	1
arg	1
asp	3
thr	2
ser	7
glu	6
pro	1
gly	7
ala	3
val	1
ile	1
leu	1
tyr	1
phe	1
cys(1/2)	1
minimum MW	4 kdal
multiple min. MW	68 kdal

[1] - experimental duplicates were obtained, mole ratios were calculated with respect to histidine

2.3.4. Homogeneity, molecular weight and amino acid composition of toxic fraction

The toxin was found to be homogeneous according to analytical isoelectric focusing at 25 µg toxin applied to the gel (Fig 2.6). SDS PAGE (Fig 2.9) at 30 µg toxin applied to the gel, showed a single band at a position corresponding to a molecular weight of 74 kdal. The minimum molecular weight calculated from the amino acid composition (Table 2.3) is 4 kdal and the nearest multiple molecular weight in agreement with that obtained by SDS PAGE is 68 kdal. A single peak (Fig 2.10) was observed after gel permeation chromatography of the toxin. The retention time corresponded to a molecular weight of 68 kdal.

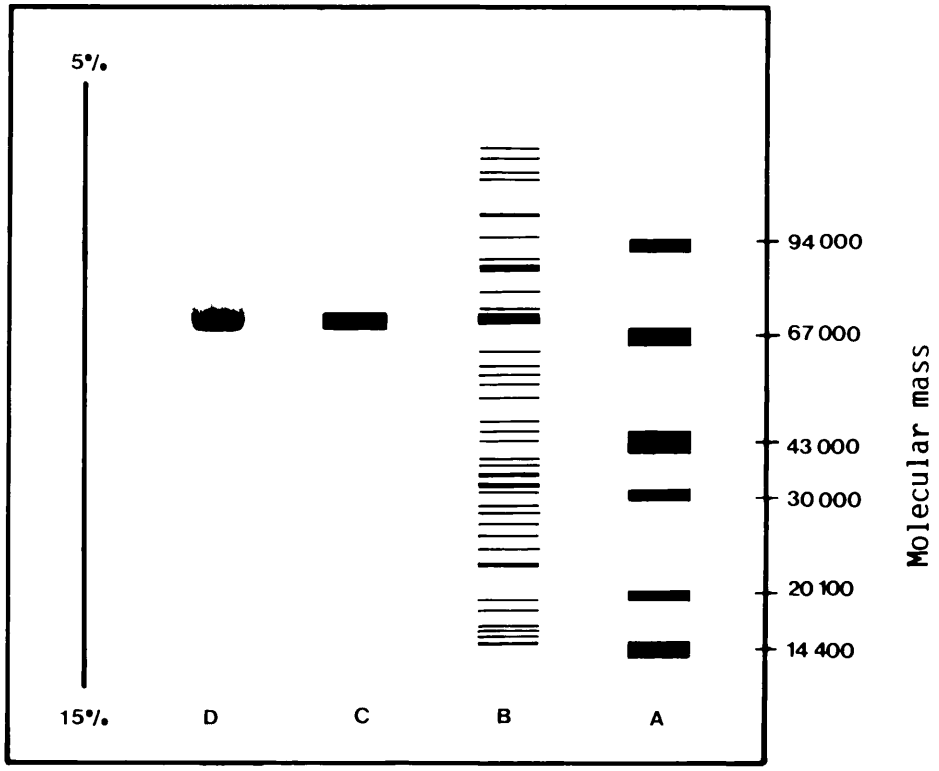

Fig 2.9 Determination of the molecular mass of the *Rhipicephalus evertsi evertsi* paralysis toxin according to SDS - polyacrylamide gradient gel electrophoresis.

(A) Low molecular mass calibration kit: phosphorylase b (94 000), bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100) and ∝- lactalbumin (14 400);

(B) Crude salivary gland extract, 15-21 mg weight range;

(C) Isolated toxin;

(D) Isolated toxin, 30µg applied (photograph).

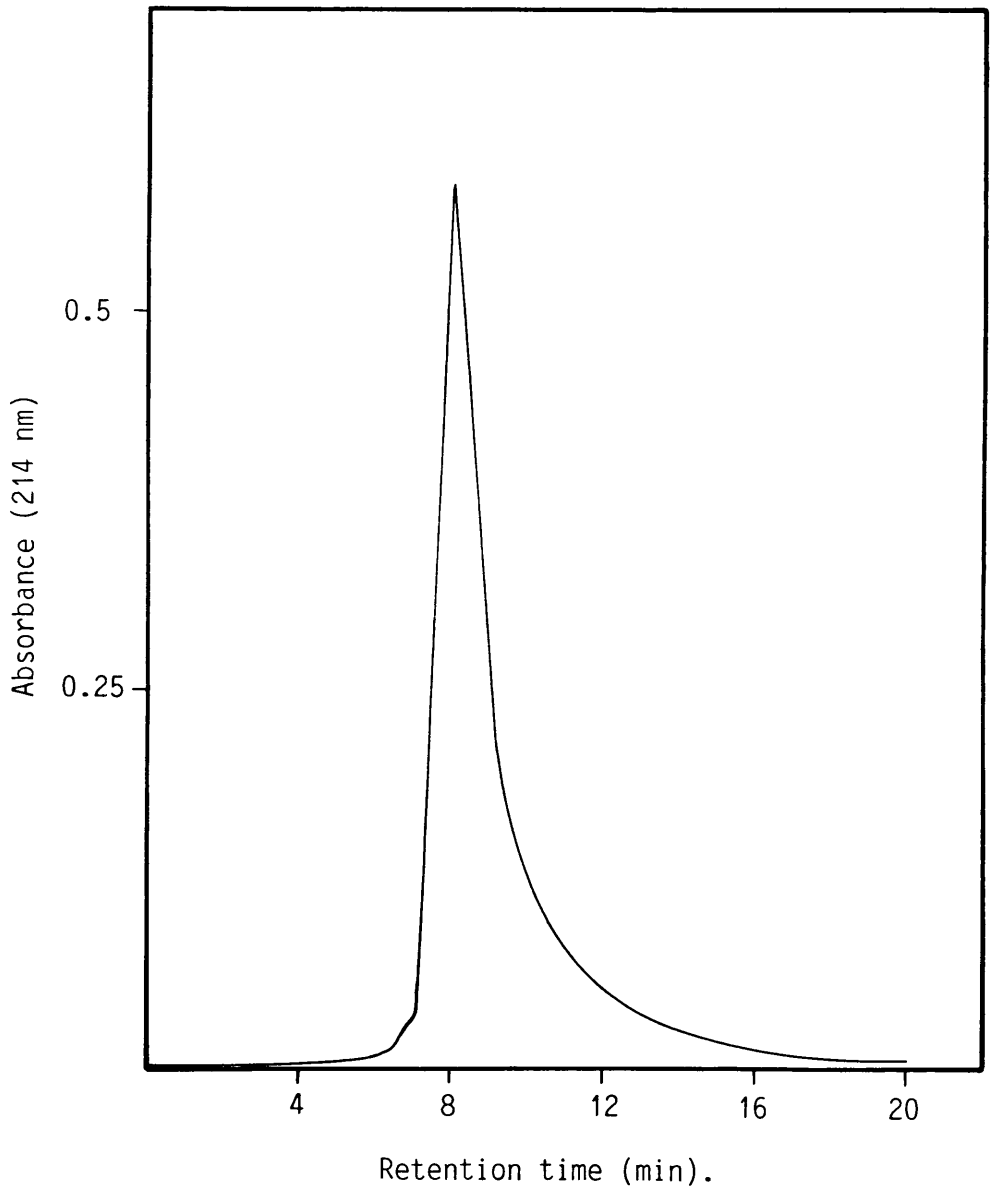

Fig 2.10. Gel permease chromatography of the salivary paralysis toxin on a Protein Pak 300 sw column. The toxin concentration was 5 µg applied with a 20 µl loop. Elution was performed with 0.05 M sodium sulfate at room temperature at a flow rate of 1 ml/min. Span at 0.1 A, chart speed: 5 mm/min and wavelenth: 214 nm.

2.3.5. Effect of pronase digestion and antiserum on toxicity

Pronase inactivated the toxin as was shown by the absence of inhibition of muscle contraction during the 120 min test with the pronase-toxin digestion mixture. The control, consisting of incubated toxin in the absence of pronase showed full retention of activity. A further control in which the effect of pronase on muscle contraction was investigated showed that this enzyme had no effect on nerve conduction.

The toxin was completely neutralized by the antiserum as indicated by the absence of inhibition of muscle contraction during the test period of 120 min. The control, consisting of antiserum alone, likewise had no effect on the muscle contraction.

2.4. DISCUSSION

The procedure described in this chapter, offers a simple method for the isolation of an electrophoretically homogeneous toxin from the salivary glands of *R. evertsi evertsi* (with a quantity of 30 µg toxin on SDS PAGE and 25 µg toxin on isoelectric focusing). The gel permeation chromatography showed the isolated toxin to be 99% homogeneous with respect to peak area. The *in vitro* toxicity assay method proved to be sensitive and reliable, requiring microgram quantities of test material.

Crude salivary gland extracts obtained from female *R. evertsi evertsi* in the toxic phase by sonification causes no overt symptoms when introduced subcutaneously into mice or chickens. Gothe and Lämmler (1982b) have also shown that infestation of several common laboratory animals with female *R. evertsi evertsi* develop no apparent clinical symptoms even at supraoptimal infestation rates based on sheep as experimental animals. It thus appears that these animals are insensitive to the paralysis toxin when introduced either during tick feeding or sc inoculation.

Sheep which were injected either sc or iv with up to 90 homogenized salivary gland extracts which were obtained from 45 female *R. evertsi evertsi* in the toxic phase also failed to show symptoms and developed typical paralysis when subsequently challenged by tick infestation.

The failure to induce paralysis in sheep with crude salivary extracts, is probably due to too low dosages administered, although infestation by approximately 5 ticks would have been sufficient to cause paralysis. This discrepancy is likely due to the fact that salivary glands contain only that amount of toxin present at the time of excision. The toxin content of these glands represents that present at that particular instant. During tick feeding in the toxic phase, toxin synthesis is probably high

and fairly large amounts are transferred to the host in the 15 - 21 mg tick body weight.

It should also be borne in mind that under natural conditions toxin is probably transferred in small amounts during several hours. The effect of continuous sc inoculation of the paralysis inducing crude extract over extended periods thus needs to be investigated. Gregson (1973) has used this approach in the investigation into the effects of saliva collected from paralysis inducing *Dermacentor andersoni* ticks. A marmot showed impaired motility and a hamster paralytic symptoms as the result of continuous sc injection, for about 16 h, of the saliva of female ticks. The paralysis in the hamster began to subside 3 h after the parenteral application of the saliva was discontinued. After 8 h the animal had recovered.

Because of the above mentioned failures and those of Gothe and Lämmler (1982b) with *in vivo* toxin assay procedures involving infestation by ticks using common laboratory animals, a sensitive *in vitro* assay was developed. This was based on neurophysiological investigations involving paralyzed animals. The latter studies showed that in paralysis caused by *R. evertsi evertsi* (Gothe and Kunze, 1981; Gothe and Kunze, 1982), *D. andersoni* (Murnaghan and O'Rourke, 1978) and *Argas (Persicargas) walkerae* (Gothe *et al.*, 1979), a reversible impairment of impulse propagation in peripheral nerve fibres occurred. It was concluded that the toxins circulate humorally and possess membranophilic properties with a possible primary target of attack in the region of the nodes of Ranvier. Consequently, in our assay procedure, fractions to be tested for toxicity were applied directly onto the sciatic nerve of a frog nerve-muscle preparation. The response on the muscle contraction was recorded. By using a specially constructed micro-nerve bath, it was possible to investigate extremely small fraction quantities.

It should be stressed that this test system involves an ***in vitro*** assay but may well indicate the actual paralysis toxin. This possibility resides in the fact that the results obtained from the assay are in accordance with those expected from the neurophysiological investigations of paralyzed animals.

Furthermore, the observation that salivary gland extracts of ticks in the body weight range of 15-21 mg exhibit the highest inhibitory activity with respect to muscle contraction is in agreement with the observations of Gothe and Lämmler (1982a) concerning the toxic phase of ***R. evertsi evertsi***. Also in accordance with these observations is that the toxic fraction, obtained from the crude extracts by chromatofocusing, reaches a maximum concentration and activity during the toxic phase. Final proof will only be possible if inoculation of the toxic fraction into sheep, preferably by continuous infusion over extended periods, causes paralysis symptoms.

The molecular weight of the toxin is similar to that of the heterogeneous paralysis inducing fraction isolated from ***I. holocyclus*** (Stone, ***et al.***, 1979). The fractions from both tick species also have a pI in the acid pH range. The ***I. holocyclus*** toxic fraction, which has been called holocyclotoxin, was isolated from ticks in the feeding phase of day 5 and day 6 during which time the toxicity was found to be at a maximum. It is associated with proteins having a molecular weight in the 40 kdal to 60 kdal range and an isoelectric point between 4.5 and 5.0. Unlike the ***R. evertsi evertsi*** toxin, holocyclotoxin is resistant to digestion by pronase (Stone, 1979). Kaire (1966) also showed that the toxin was unaffected by pepsin, trypsin and papain. He concluded that the toxin was non-proteinaceous or that the toxin was indeed proteinaceous but resistant to digestion. However, Stone (1979) found that digestion with α - chymotrypsin caused a decrease in toxicity. The results concerning protease digestion of holocyclotoxin and the toxin from ***R. evertsi evertsi***

are difficult to compare since neither Stone (1979) nor Kaire (1966) mentioned the digestion conditions.

Pronase digestion of the toxin resulted in a loss of toxicity indicating that this toxic component is a protein. The neutralization of the toxin with immune serum, indicates the presence of specific antibodies, developed in the host as the result of tick feeding directed against the toxin. This result suggests that the toxin isolated from the salivary glands may be immunologically similar to the toxin transferred into the host during natural feeding.

For *I. holocyclus* a relationship between changes in salivary gland morphology and toxicity during feeding has been described (Binnington and Stone, 1981). A similar relationship is likely to exist for *R. evertsi evertsi* and would explain the fact that the toxin reaches a maximum concentration within a distinct period during feeding.

Changes in granular salivary cells during feeding have been correlated with specific functions during attachment and repletion, e.g. secretion of cement, anticoagulants and enzymes (Binnington and Kemp, 1980). This may also explain the observed concentration changes of the various protein fractions during the feeding process. McSwain, Essenberg and Sauer (1982) have reported changes in the concentration of proteins in the salivary glands of *Amblyomma americanum* before and during feeding. As in the case of *R. evertsi evertsi* (Neitz and Gothe, in press) they observed numerous proteins which are synthesized only after tick attachment and feeding and a few after mating had occurred. In addition, proteins present in glands before feeding diminished during the feeding process and either increased or continued to decrease towards the end of feeding.

For *A. americanum* the overall results were interpreted to suggest that attachment and commencement of feeding are the primary stimuli for the production of new proteins and that mating and subsequent increased feeding was the primary stimulus for increased protein synthesis. In *R. evertsi evertsi*, the production of numerous new proteins as well as increased protein synthesis occur before mating.

The functions of the various proteins have not yet been investigated but the chromatofocusing method described in this paper has made it possible to isolate them. Their characterization thus becomes feasible, so that their function during tick feeding and their effects on the host or transmissible pathogens could be further explored.

CHAPTER 3

THE DETECTION AND ISOLATION OF A PARALYSIS TOXIN PRESENT IN *ARGAS (PERSICARGAS) WALKERAE*

3.1. INTRODUCTION

Argas (Persicargas) walkerae causes paralysis in poultry during infestation. This tick, also called Walker's fowl tampan (Fig 3.1) is the most common fowl tick in the Republic of South Africa (Howell, Walker and Nevill, 1978). It has been recorded in the Pretoria area and at Christiana in the Transvaal, in the Cape Province at Fort Beaufort and Queenstown, in Lesotho at Maseru and in South West Africa at Omaruru.

Gulyás (1952) attempted to explain the pathogenesis of the paralysis by an anaemic state caused by blood-loss during infestations by the ticks. He excluded the possibility that the symptoms were due to a toxic effect. Gothe, Kunze and Alt, (1970) however, showed that no hematological changes occurred during the course of the paralysis even in very extensive cases which terminated in death. The paralysis can thus rather be defined as a toxicosis (Gothe, 1971) which shows the typical signs of a generalized affection of the peripheral nervous system (Gothe, Kunze and Hoogstraal, 1979). Partial immunity is also produced in some hosts after repeated infestations (Gothe, 1971).

From the earlier literature, conflicting opinions exist as to which stage or stages of the tick are capable of causing paralysis during feeding. According to Lounsbury (1904), nymphae as well as adult ticks are capable of causing paralysis. Emmel (1945) and Neitz (1962) were of the opinion that all postembryonal stages may cause this toxicosis, whereas Coles (1959) reported that paralysis is caused by the simultaneous feeding of both larvae and adults. The imbroglio was eventually unravelled through intensive studies by Gothe, ***et al.***, (1970). They unambiguously

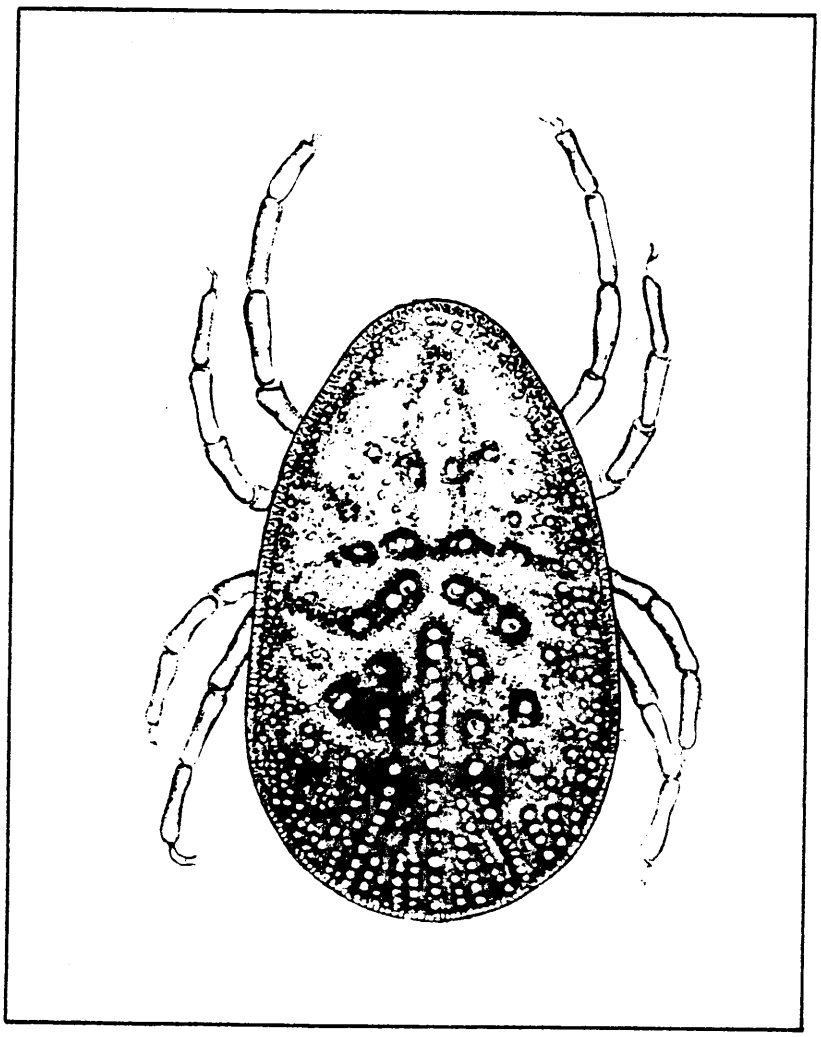

Fig 3.1. Female *Argas (Persicargas) walkerae* (Howell, Walker and Nevill, 1978).

showed that only larvae caused paralysis after at least 4 days of feeding. Of interest is that the larval body weight increases abruptly from approximately the 3 rd feeding day. It was concluded that the onset of symptoms may be correlated with increased blood sucking behaviour. The incubation time of at least 5 days was found to be independent of the number of infesting ticks and the age of the host. The degree of paralysis however, was dependent on these factors.

The inability of post larval ticks to cause paralysis during infestation is well established. It is not known however, if this is due to the absence of toxin production or due to an inability of these ticks to transfer the toxin to the host during their short feeding time. It is well known that larval ticks of this species imbibe blood during several days whereas all the other stages are fully engorged within 1-2 h or less (Howell, *et al.*, 1978). To help solve this enigma the paralysis inducing capability of all post-larval stages were investigated in the present study by inoculation of crude extracts into chickens.

Neurophysiological studies on animals paralyzed by *A. (P.) walkerae* have shown that the afferent as well as fast conducting motor nerve fibres and also neuromuscular transmission are affected (Gothe and Kunze, 1971; Gothe *et al.*, 1979). Neuropharmacological investigations on these animals (Gothe and Kunze, 1974) demonstrated that neuromuscular impulse propagation was markedly improved by acetylcholine as well as inhibitors of its splitting enzyme. It was inferred that acetylcholine liberation is reduced and the receptor sensitivity is altered in the myoneural synaps. Electron-microscopic investigations of peripheral nerves of completely paralyzed fowls, showed no morphological lesions of the parenchyma (Gothe, Hager, Jehn, Kunze and Thoenes, 1971).

As in paralysis caused by *Rhipicephalus evertsi evertsi* (Gothe, *et al.*, 1979) it was concluded from further studies that the toxin from *A. (P.)*

walkerae has membranophilic properties with a probable point of attack on nerve fibres in the region of the nodes of Ranvier. The notion that the toxin-membrane interaction is labile is consistent with the observation that if parasitizing ticks are removed from paralyzed animals in time, they recover completely (Gothe, *et al.*, 1979).

The combined results together with investigations on the partial pressure of CO_2 and O_2 and the pH of blood of paralyzed fowls (Gothe and Riethmüller, 1972) has led to the following proposal as to the cause of death (Gothe, Kunze and Mechow, 1971). Functional impairment during paralysis also affects the efferent nerves serving the breathing muscles. As the degree of paralysis increases, more and more nerve fibres are affected until innervation of the intercostal musculature and of the auxiliary breathing muscles finally ceases. The hypoventilation during the paralysis leads to an increased CO_2 retention and a lowering of the partial O_2 pressure and pH of the blood. The respiratory acidoses may result in impairment of important organs.

In the present investigation an attempt was made to isolate the responsible toxin. Toxicity was tested by sc inoculation into one day old chickens. Salivary glands could not be used as toxin source since their excision from large numbers of ticks is impracticable due to the small size of the larvae. Their average body weight is only approximately 1.3 mg and their average body diameter less than 1.5 mm after full engorgement.

3.2. MATERIALS AND METHODS

All the glassware and equipment was sterilized with 70% (v/v) ethanol and the buffers by filtration through 0.22 µm filters (Millipore).

3.2.1. Origin and rearing of *Argas (Persicargas) walkerae* ticks

Laboratory colonies of *A. (P.) walkerae*, named the Pretoria strain since they were collected in the region of Pretoria on different farms, were maintained and reared in an Forma Scientific Model 3149 incubator at 27°C and at least 80% relative humidity. Three weeks after the hatching of eggs, the larval batch of 1 female, was allowed to attach freely under the wings and feed on a approximately 7 week old white Leghorn chicken as described by Alt (1971). The replete larvae were collected on the mornings of the 5 th,6 th, 7 th and 8th day after attachment. To obtain fed nymphae, the larvae were allowed to moult and the resulting unfed nymphae were fed overnight on 7 month old white leghorn hens using approximately 100 ticks per animal. The morning following the attachment, the replete stage 1 nymphae were collected. The same pattern of feeding was followed for the stage 2 and stage 3 nymphae.

3.2.2. Preparation of *Argas (Persicargas) walkerae* crude egg, larval, nymphal and adult extracts for the detection of the paralysis toxin

A. (P.) walkerae eggs, larvae, nymphae and adult ticks were homogenized (quantities are given in Table 3.1) in 2 ml distilled water with an Ultra Turrax (Janke und Künkel) at low speed. Eggs and larvae were homogenized for 2 min and the other stages for 5 min at 4°C. The homogenates were then sonified for 15 sec with a Branson Model B-30 sonifier with the output set at 5 (continuous cycles). The sonified homogenates were centrifuged in a Beckman Model B microfuge for 10 min at 8 000xg and 4°C. The resulting supernatants were freeze-dried

TABLE 3.1. Determination of the presence of *Argas (Persicargas) walkerae* paralysis toxin in the various stages

Developmental stage	Number of ticks (crude extract)
freshly laid eggs	2 000
unfed larvae[1]	2 000
unfed larvae[2]	2 000
replete larvae[3]	3-400
unfed nymphae stage 1[4]	2 000
replete nymphae stage 1[3]	400
unfed nymphae stage 2[4]	400
replete nymphae stage 2[3]	200
unfed nymphae stage 3[4]	200
replete nymphae stage 3[3]	200
unfed adult ticks[4] *	200
replete adult ticks[3] *	200
Chicken normal blood (control)	1 ml

[1] - extracts prepared 3 days after hatching
[2] - extracts prepared 2 weeks after hatching
[3] - extracts prepared within 3 h after collection
[4] - tested 2 weeks after moulting
* = either males or females

and suspended in c. 0.1 ml 0.154 M NaCl and injected sc in the neck region of one day old white leghorn chickens. The chickens were kept under observation for at least 4 days. The degree of paralysis observed in one day old white Leghorn chickens was evaluated according to Gothe and Englert (1978) as is described in Table 3.2.

3.2.3. Toxin isolation

3.2.3.1. Preparation of *Argas (Persicargas) walkerae* crude larval extracts

Preliminary studies showed no differences in toxicity when extracts were prepared in the presence of either distilled water or 0.154 M NaCl. Therefore the isolation of the toxin was conducted in either of these solvents.

TABLE 3.2. Description of the clinical symptoms in chickens due to inoculation of crude extracts of *Argas (Persicargas) walkerae* larvae (Gothe and Englert, 1978).

Paralysis	Clinical symptoms
N	No symptoms
Wings	
F 1	Slight degree of paresis
F 11	Medium degree of paresis
F 111	High degree of paresis
Legs	
B 1	Slight degree of paresis
B 11	Medium degree of paresis
B 111	High degree of paresis
VP	Total paralysis
VP(t)	Total paralysis ending in death

For the isolation of the paralysis toxin, 100 replete larvae were homogenized in 1 ml either 0.154 M NaCl or distilled water depending on whether the further isolation was to be executed in distilled water or 0.154 M NaCl, respectively. The homogenates were sonified and centrifuged (as described in paragraph 3.2.2., p. 54) whereafter the resulting supernatants were stored at -75°C in a Specht Scientific refrigerator.

For gel permease chromatography and chromatofocusing the freeze-dried crude larval extracts were made up to 12 ml with either 0.154 M NaCl or distilled water, and centrifuged in a Beckman Model L5-65 ultracentrifuge for 5 h at 80 700xg in a Rotor 40 at 5°C. The supernatants were freeze-dried and the salt containing samples were made up to 2.5 ml with distilled water and desalted with a Sephadex PD-10 column whereafter these samples were also freeze-dried.

3.2.3.2. Preparative column isoelectric focusing

For preparative column isoelectric focusing a LKB column of 440 ml capacity in a glycerol gradient was used. Glycerol and pH gradients (Moreno, Ochoa, Gascon and Villanueva, 1975) were prepared as described in the LKB instruction manual for the Electrofocusing 8 100 system. A pH gradient from 3-10 or 7.5-9 pH units (Pharmacia Fine Chemicals) was prepared with Pharmalyte carrier ampholytes. The column isoelectric focusing was performed at 5°C with a final Pharmalyte concentration of 1.5%. The anode electrolyte solution was at the bottom of the column and the cathode electrolyte solution at the top. The sample consisting of 200 freeze-dried larvae (obtained as described in paragraph 3.2.2. ,p. 54) was mixed with 2 ml heavy and light solution from the middle of the sucrose gradient. Electrofocusing was performed for 24-36 h with a starting power of 6 Watt and a final power of 3 Watt. The column was emptied from the anode solution side (bottom) by means of a Pharmacia Fine Chemicals Model M-1 peristaltic pump at a flow rate of 120 ml/h. Fractions of 3 ml were collected and monitored at 280 nm with a Beckman Model 25 spectrophotometer at 280 nm. The pH value of each fraction was determined with a pH meter, Model 26 (Radiometer Copenhagen). The pooled fractions were then dialyzed[1] for 36 h against 50 l distilled water. The retentates were freeze-dried. These samples were reconstituted to 2 ml and tested for toxicity.

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[1] A preliminary study using gel permeation chromatography showed that the toxin was associated with a fraction with molecular weight in excess of 100 kdal, see also paragraph 3.3.2.3., p. 64

3.2.3.3. Preparative flat bed isoelectric focusing

Since this method is not generally used a more detailed description of the procedure is included. For preparative flat bed isoelectric focusing 15 g Sephadex IEF (Pharmacia Fine Chemicals) was swollen in 225 ml distilled water at 10°C for c.12 h. Before use 12 ml Pharmalytes pH 5-8 or pH 7.5-9 (Pharmacia Fine Chemicals) or Ampholytes pH 6-8 (LKB) were added (Pharmacia Fine Chemicals Manual). The gel was thoroughly mixed and degassed under vacuum for c.1 h. The gel slurry was then poured into the gel frame (Pharmacia Flat Bed Apparatus FBE 3 000) which was pre-cooled to 5°C with a B.Braun Melsungen AG cooling bath and a Model 1 441 Thermomix. As electrode solutions, 0.1 M H_3PO_4 and 0.1 M NaOH were used. Dry Sephadex IEF was poured on top of the swollen Sephadex layer to thicken the gel. Pre-focusing was performed for 45 min at 8 Watts with a Bio-Rad Model 3 000/300 power supply. To apply the sample, the gel at approximately pH 7 was removed with a fractionation grid (7 mm wide) and spatula and mixed with 2 ml crude larval extract prepared from 200 larvae. The sample containing gel was then replaced. Focusing was resumed for 6 h at 30 Watt. Fractions were then removed in 1 cm zones parallel to the electrodes using the fractionation grids and a spatula. The gel fractions were then transferred to small empty Bio-Rad glas columns (1.6x10 cm) and the samples were eluted with 0.154 M NaCl and freeze-dried. The fractions were reconstituted to 2 ml and tested for toxicity.

3.2.3.4. Gel permeation chromatography

Upward-flow elution was used in all the gel chromatographic separations. The Sephadex G-100 (Pharmacia Fine Chemicals) and Sephacryl S-200 Superfine (Pharmacia Fine Chemicals) columns were calibrated with standard proteins (Pharmacia Fine Chemicals calibration kit) under the same elution conditions as for the samples. The proteins, 5 mg ribonuclease A (MW 13.7 kdal), 5 mg chymotrypsinogen A (MW 25 kdal), 5 mg ovalbumin (MW 43 kdal) and 5 mg bovine serum albumin (MW 67 kdal), were dissolved in 1 ml distilled water or 0.154 M NaCl.

3.2.3.4.1. Sephadex G-100 gel permease chromatography

Sephadex G-100 gel was swollen in distilled water for 5 h at 90°C, and after that for 20 h at 10°C. The gel was packed into a Pharmacia Fine Chemicals K26/40 column. The column volumes were: v_t, 210 ml; v_o, 74 ml and v_i, 125 ml.

A 2 ml crude larval extract representing 200 larvae in 2 ml of 0.154 M NaCl was applied onto the column and separated using a Pharmacia Fine Chemicals peristaltic pump, Model P-1, 0.154 M NaCl as eluent (flow rate 30 ml/h). Fractions of 3 ml were collected with a LKB Redirac fraction collector Type 2112 and absorbancies read at 280 nm with a Beckman Model 25 spectrophotometer. The fractions were divided into peaks , dialyzed against 25 l distilled water with water changes every 6 h for c. 38 h and then freeze-dried.

3.2.3.4.2. Sephacryl S-200 Superfine gel permeation chromatography

Pre-swollen Sephacryl S-200 Superfine (Pharmacia Fine Chemicals) was packed in a Pharmacia Fine Chemicals K26/40 column with column volumes: v_t, 193 ml; v_o, 65 ml and v_i, 126 ml.

The crude larval extracts obtained from 200 larvae in 2 ml of appropriate eluent were separated on the Sephacryl S-200 column, using the same apparatus as for the Sephadex G-100 chromatography, with either distilled water; 0.154 M NaCl; 0.154 M NaCl, 0.05% Tween 20; 0.154 M NaCl, 0.1% SDS or 0.154 M NaCl , 0.1% Chaps as eluent. Fractions were collected as described for Sephadex G-100 chromatography and the fractions were divided into peaks according to absorbancies taken at 280 nm. The fractions were freeze-dried. When necessary the freeze-dried fractions were desalted through a Sephadex PD-10 (Pharmacia Fine Chemicals) column and freeze-dried.

3.2.3.4.3. Sephadex PD-10 gel permeation chromatography

Pharmacia Fine Chemicals Sephadex PD-10 columns, are supplied pre-packed with Sephadex G-25 with a bed height of 5 cm and a bed volume of 9.0 ml. The gel was equilibrated with approximately 25 ml distilled water. The freeze-dried samples, made up to 2.5 ml with distilled water, were run into the gel and eluted with 3.5 ml distilled water. This procedure was performed in order to reconstitute the sample in distilled water before use in the further isolation procedures.

3.2.3.5. Chromatofocusing

The main advantage of this technique compared to conventional ion- exchange chromatography is due to the focusing effect and short separation time. Furthermore, an internal pH gradient is formed and the sample is not exposed to extremes of pH. (Sluyterman and Elgersma, 1978; Sluyterman and Wijdenes, 1978). Hence exceptional high resolution may be obtained.

A PBE 94 ion-exchanger (Pharmacia Fine Chemicals), which is supplied pre-swollen as a suspension in 24% ethanol was used. The gel was degassed under vacuum for c.1 h and packed into a Pharmacia Fine Chemicals K15/10 column at 10°C. The column was washed with 3 bed volumes of 1 M NaCl and equilibrated with 0.025 M Tris-acetate, pH 9.00 (starting buffer).

The eluting buffer was prepared as follows: 10% (v/v) Polybuffer 96 (Pharmacia Fine Chemicals) was made up with distilled water to a volume of 200 ml. The pH was adjusted to pH 7 with 1 M acetic acid. The buffers were degassed with Argon gas (Afrox) for 2 min before use.

The freeze-dried crude larval extract (obtained as described in chapter 3.2.2., p. 54), dissolved in 2 ml starting buffer, was applied after 1 ml eluent had been pumped through the column. Thereafter pumping with eluent was continued. The solutions were pumped with a Pharmacia Fine Chemicals peristaltic pump P-1 at a flow rate of 30 ml/h. Fractions of 3 ml were collected with a LKB Redirac fraction collector Type 2 112 and monitored at 280 nm with a Beckman Model 25 spectrophotometer. The fractions were divided into peaks and freeze-dried.

3.2.4. Protein determination

The determination of protein was performed as described in paragraph 5.2.6., p. 117 .

3.3. RESULTS

3.3.1. Determination of toxicity of crude tick extracts

Of all the tick stages tested, either fed or unfed, at extremely high dosages, only replete larvae induced paralysis in one day old chickens (Table 3.3); extracts obtained from 2 000 unfed larvae failed to show paralysis symptoms (Table 3.3).

Further investigations regarding the toxicity of replete larval crude extracts showed that extracts prepared from 3 larvae caused a F 1 paresis whereas extracts from 50 caused a total paralysis (Table 3.4). All the paralyzed animals recovered within 48 h.

No difference in toxicity between the crude larval extracts in either distilled water or 0.154 M NaCl was observed. The addition of detergent at room temperature, whether Tween 20, SDS or Chaps, had no effect on the toxicity. Heating in the presence of detergent however, resulted in total loss in toxicity. This was also observed for the heated crude extract in the absence of detergent.

TABLE 3.3. Determination of the presence of paralysis toxin in crude extracts from various stages of *Argas (Persicargas) walkerae*

Developmental stage	Amount	Protein quantity inoculated (mg)	Clinical symptoms
Freshly laid eggs	2 000	100.3 ± 12.8	N
unfed larvae[1]	2 000	87.7 ± 3.2	N
unfed larvae[2]	2 000	82.4 ± 8.1	N
replete larvae[3]	300	32.3 ± 3.1	VP[5]
unfed nymphae stage 1[4]	2 000	217.4 ± 17.4	N
replete nymphae stage 1[3]	400	123.1 ± 9.7	N
unfed nymphae stage 2[4]	400	90.7 ± 10.5	N
replete nymphae stage 2[3]	200	97.3 ± 14.9	N
unfed nymphae stage 3[4]	200	82.6 ± 11.3	N
replete nymphae stage 3[3]	200	101.5 ± 18.4	N
replete adult male ticks[3]	200	89.9 ± 17.6	N
replete adult female ticks[3]	200	203.1 ± 23.7	N
chicken normal blood control	1 ml	0.9 ± 0.1	N

[1] - extracts prepared 3 days after hatching
[2] - extracts prepared 2 weeks after hatching
[3] - extracts prepared immediately after collection
[4] - extracts prepared 2 weeks after moulting
[5] - Further investigated in Table 3.4

3.3.2. Isolation of the paralysis toxin

3.3.2.1. Preparative column isoelectric focusing

Preparative column isoelectric focusing of larval extracts resulted in a whole series (±10) of hemoglobin bands (identified by their reddish coloured appearance). The best resolution was obtained at a pH range 6-9.5.

All the fractions between pI 6.0-8.5 were shades of red. It was further observed that 2 fractions with a pI at c. 6.5 and another at a pI of c. 8.0 (i.e. fractions IEF_1 and IEF_3 from Fig 3.2) were toxic.

On *in vivo* testing of the reconstituted dialyzed and freeze-dried fractions, it was found that c. 50% of the total toxicity was lost. The toxin with pI c. 8.0 represented 80% while the other component represented 20% of the recovered toxicity.

TABLE 3.4. Toxicity determinations of crude extracts prepared from replete *Argas (Persicargas) walkerae* larvae

Amount inoculated	Protein content inoculated (mg)	Clinical symptoms[1]
3 larvae	0.32 ± 0.05	F 1
5 larvae	0.51 ± 0.07	F 11
10 larvae	0.94 ± 0.06	F 111/B 1
15 larvae	1.39 ± 0.11	B 1/B 11
20 larvae	1.97 ± 0.15	B 11
25 larvae	2.53 ± 0.13	B 111
50 larvae	5.04 ± 0.17	VP

[1] - see Table 3.2

3.3.2.2. Preparative flat bed isoelectric focusing

On *in vivo* testing of the reconstituted freeze-dried fractions, (i.e. 2 ml) it was found that c. 90% of the toxicity was lost. The only toxic fraction was the one collected nearest to the cathode, which contained 10% of the toxicity (Fig 3.3).

3.3.2.3. Gel permeation chromatography

With the Sephadex G-100 column, 100% of the protein and 90% of the toxicity was recovered, The toxin co-eluted (fraction G_1) with the hemoglobin at the void fraction (Fig 3.4) representing a molecular weight in excess of 150 kdal.

Elution of the crude *A. (P.) walkerae* larval extract on the Sephacryl S-200 column gave no separation of the toxic component from the hemoglobin fraction when using 0.154 M NaCl (fraction SN_1 in Fig 3.5) or 0.154 M NaCl, containing 0.05% Tween 20, 0.1% SDS or 0.1% Chaps, as eluting buffer (fraction SND_1 in Fig 3.6). Identical elution patterns were observed for all these detergents and therefore only one is given in Fig 3.6. In all the cases virtually 100% of the protein as well as the toxicity was obtained.

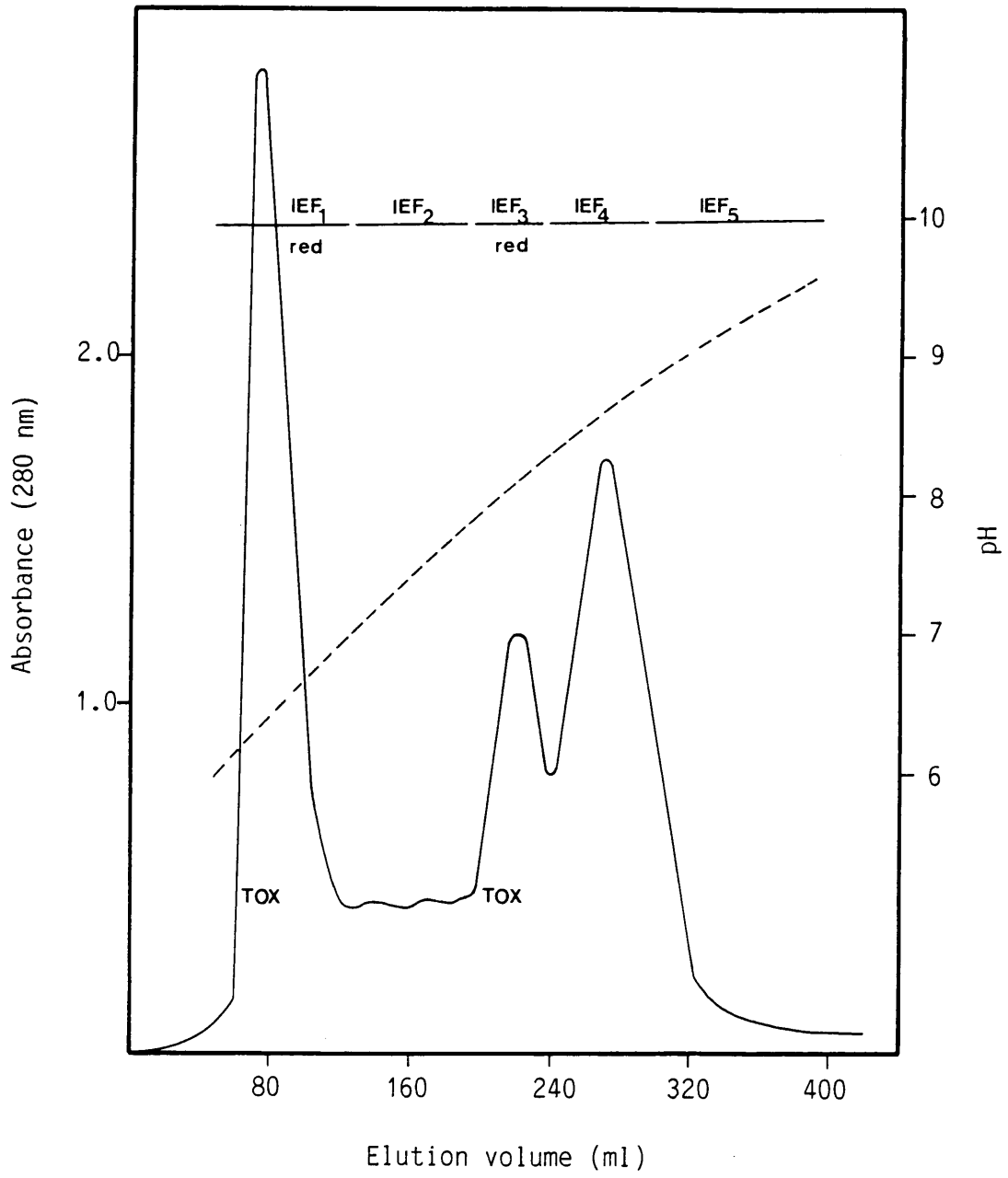


Fig 3.2. Preparative column isoelectric focusing of crude *Argas (Persicargas) walkerae* larval extract in a pH gradient from pH 6-9.5. Electrofocusing was performed for 24-36 h with a starting power of 6 Watt and a final power of 3 Watt. Fractions of 3 ml were collected and analysed. Absorbance (———) at 280 nm, pH (-----).

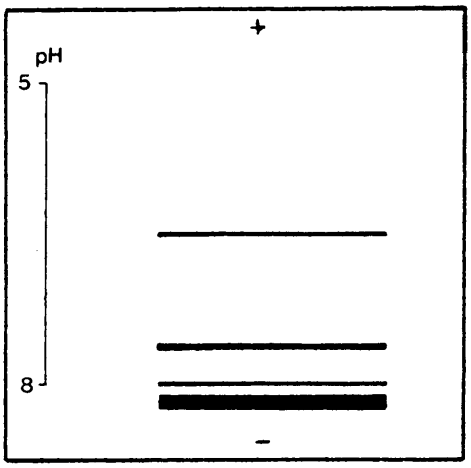


Fig 3.3. Line diagram of preparative flat bed isoelectric focusing of crude *Argas (Persicargas) walkerae* larval extract. Pre-focusing was performed for 45 min at 8 Watt and focusing for 6 h at 30 Watt. Fractions were removed in 1 cm zones parallel to the electrodes. Only the visual reddish bands are shown.

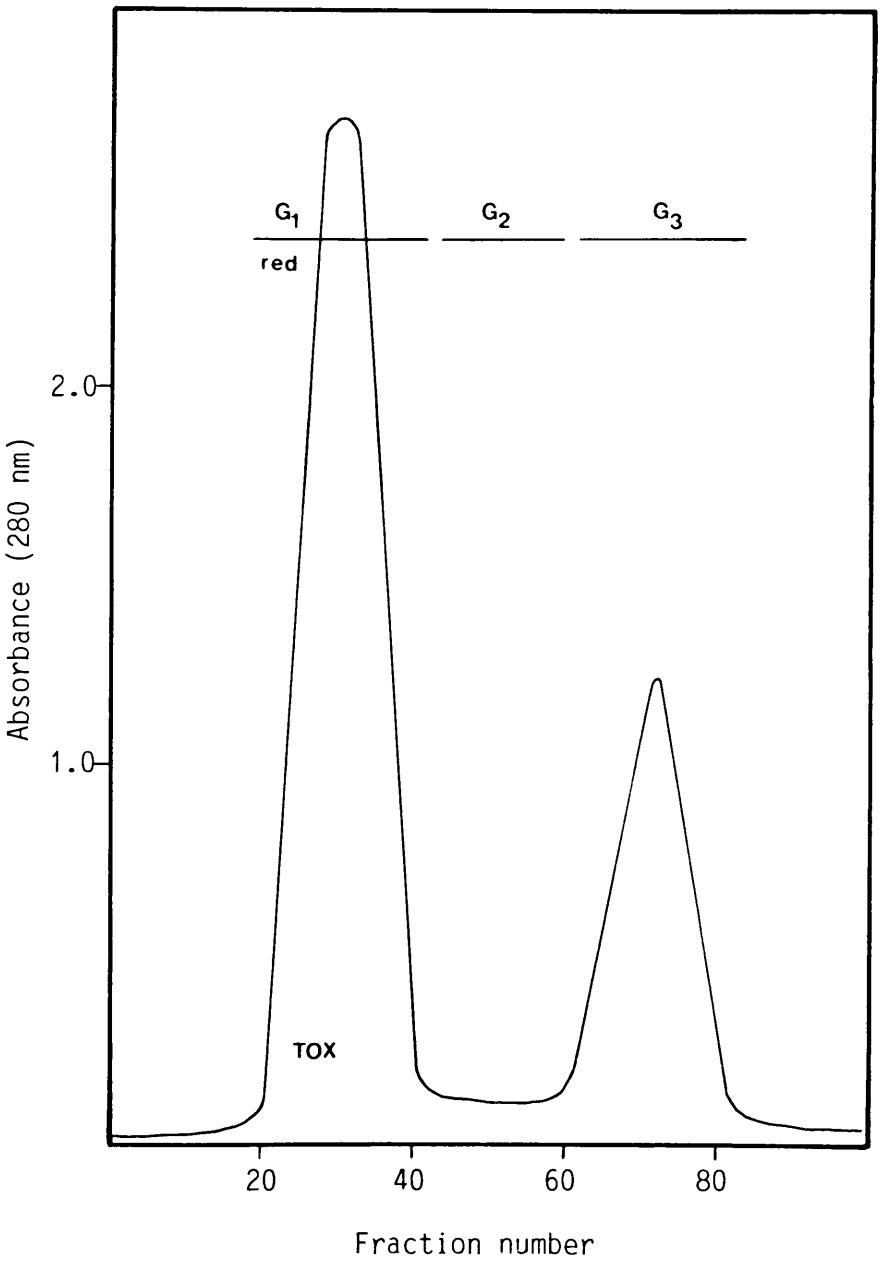


Fig 3.4. Sephadex G-100 gel permeation chromatogram of *Argas (Persicargas) walkerae* crude larval extract. The column was eluated with 0.154 M NaCl. Fractions of 3.5 ml were collected and monitored at 280 nm.

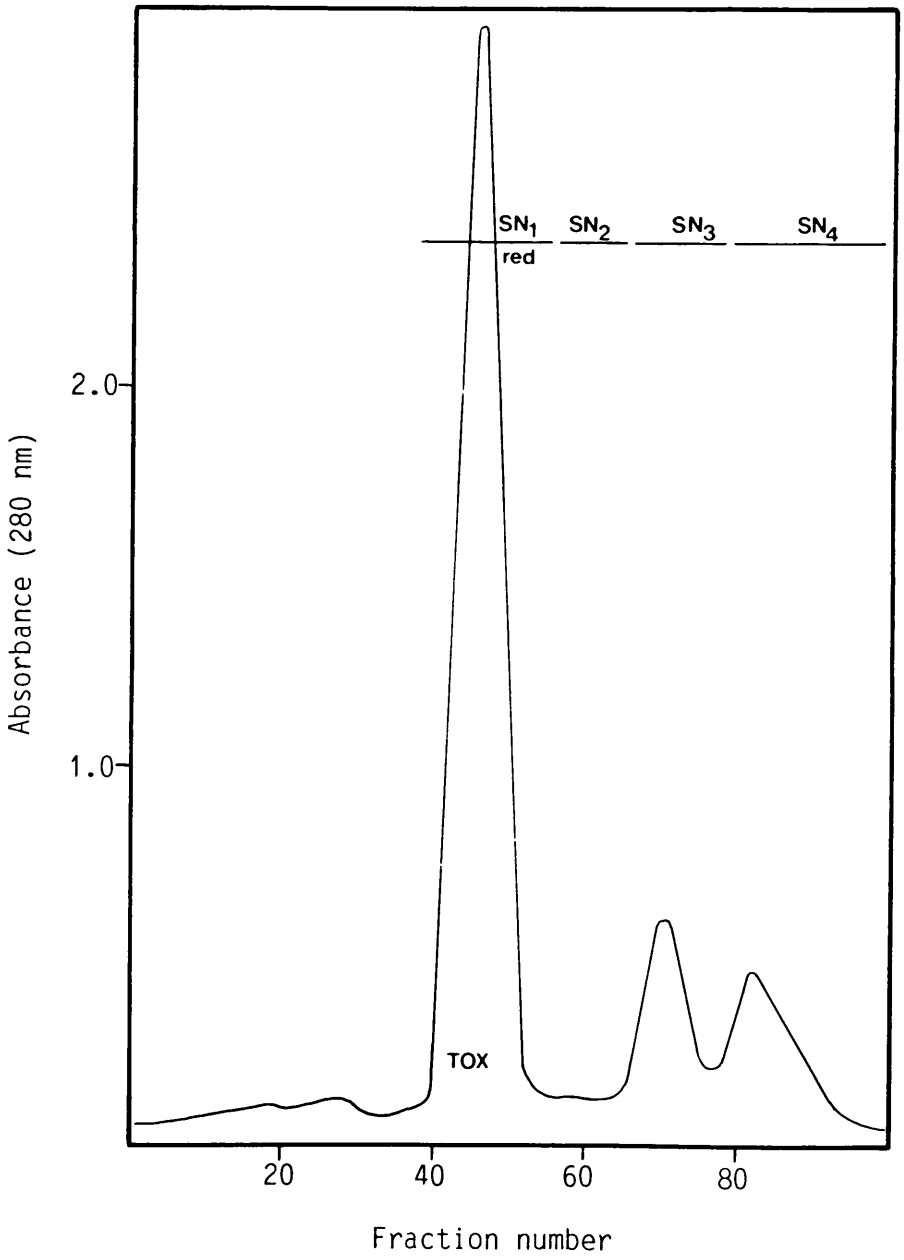


Fig 3.5. Sephacryl S-200 gel permeation chromatogram of ***Argas (Persicargas) walkerae*** crude larval extract. The column eluted with 0.154 M NaCl. Fractions of 3.5 ml were collected and monitored at 280 nm.

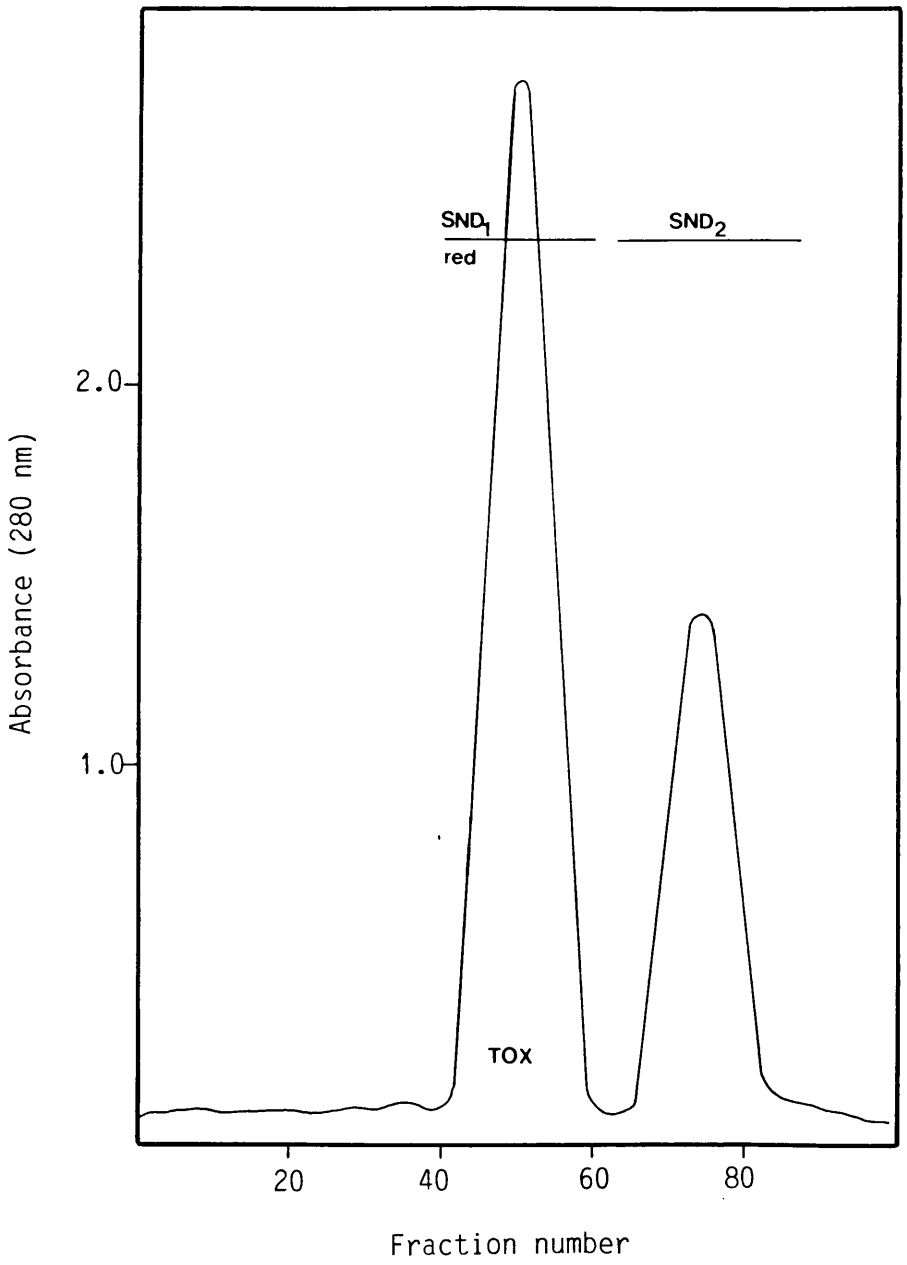


Fig 3.6. Sephacryl S-200 gel permeation chromatogram of *Argas (Persicargas) walkerae* crude larval extract. The column was eluted with 0.154 M NaCl containing either 0.05% Tween 20 (v/v), 0.1% SDS (w/v) or 0.1% Chaps (w/v). Fractions of 3.5 ml were collected and monitored at 280 nm.

The Sephacryl S-200 column gave reasonable resolution when the column was eluted with distilled water (Fig 3.7). With the Sephacryl S-200 column, a colourless peak (SH_2) was obtained which represented 30% of the original protein and 40% of the original toxicity. This peak eluted at a volume corresponding to a molecular weight of 80 to 100 kdal. A red fraction (SH_4) adsorbed onto the column which could be eluted with 0.154 M NaCl. This fraction represented approximately 65% of the sample protein and 70% of the sample toxicity.

3.3.2.4. Chromatofocusing

This method gave good recoveries with respect to protein and toxicity (above 85%). As in the case with the preparative column isoelectric focusing, 2 toxic fractions (C_1 and C_3) with pI of approximately 7.6 and 8.3, each associated with hemoglobin, were observed (Fig 3.8).

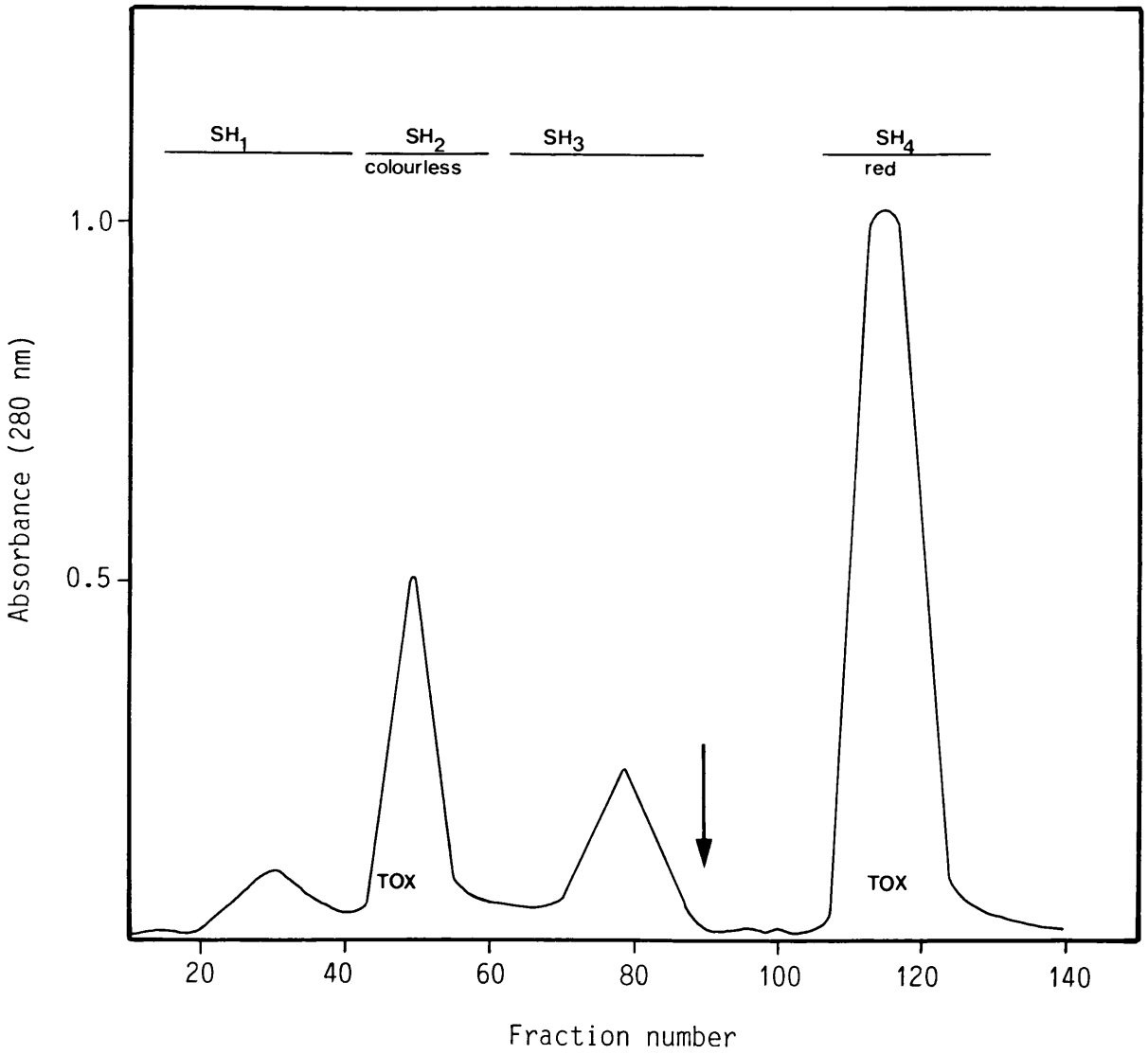


Fig 3.7. Sephacryl S-200 gel permeation chromatogram of *Argas (Persicargas) walkerae* crude larval extract. The column was eluted with distilled water. Fractions of 3.5 ml were collected and monitored at 280 nm. Arrow indicates an elution buffer change to 0.2 M NaCl.

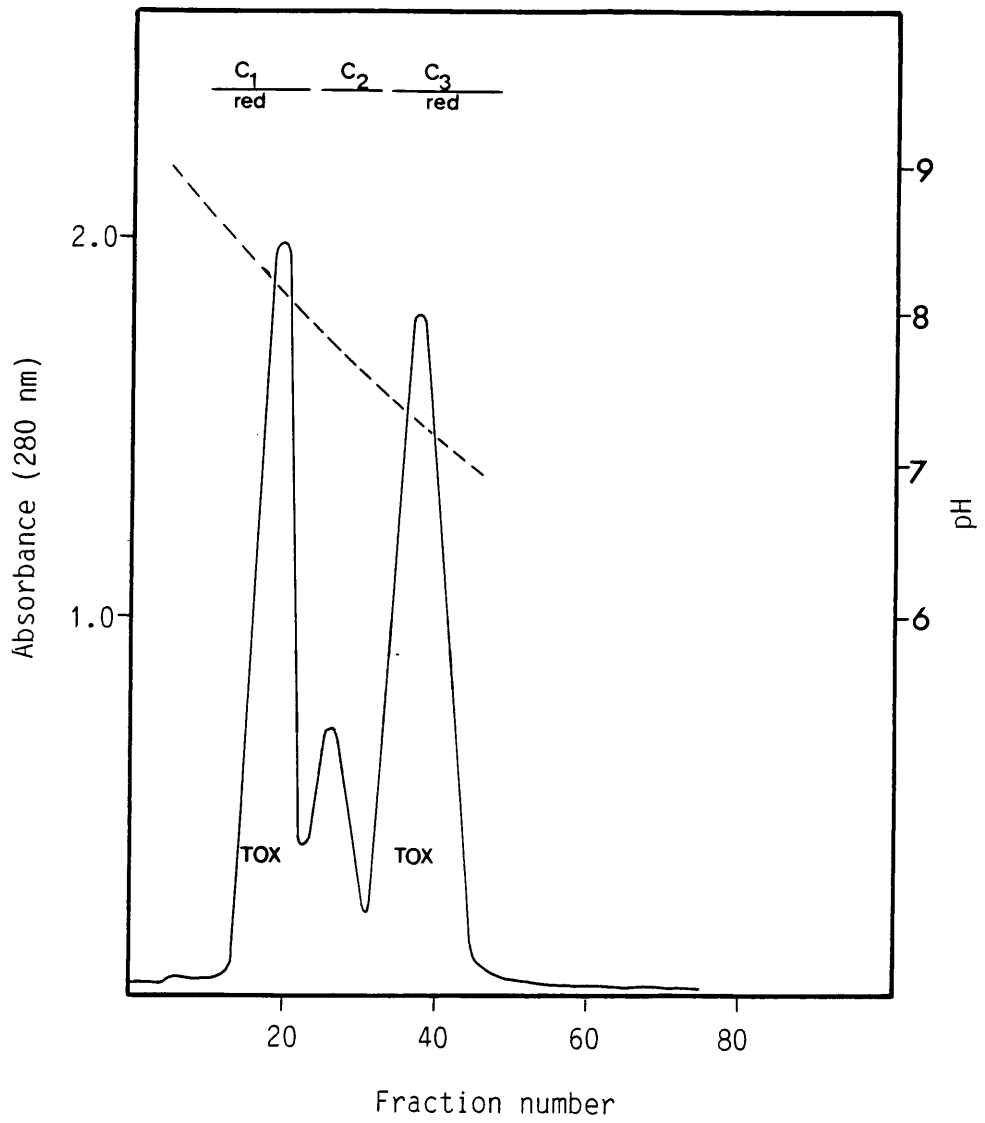


Fig 3.8. Chromatofocusing chromatogram of *Argas (Persicargas) walkerae* crude extract eluted with a pH gradient ranging from pH 9 - 7. Fractions of 3.5 ml were collected and the pH (--------) and 280 nm absorbance (——) were recorded.

3.4. DISCUSSION

One day old leghorn chickens proved to be suitable for determining the toxicity of crude *A. (P.) walkerae* tick extracts and fractions obtained during the isolation procedures. With the use of this *in vivo* test system, it was found that only replete *A. (P.) walkerae* larval crude extracts produced paralysis. Unfed larvae as well as all the other stages either fed or unfed showed no overt effect when inoculated at high dosage rates into one day old chickens.

These observations are in agreement with those of Gothe (1971) regarding the paralysis inducing capabilities of the larval, nymphal and adult ticks during feeding. He showed that only larvae, after at least 4 days of infestation caused paralysis. All other stages were incapable to cause this toxicosis.

The present results also substantiate the finding of Gothe (1971) that the paralysis is due to a toxin and does not develop as a result of blood loss in the host caused by feeding ticks as suggested by Gulyás (1952). Furthermore, it has been reported that the timely removal of ticks from paralyzed animals leads to a subsidence of symptoms and complete recovery (Gothe, *et al.*, 1979). Likewise, with the toxicity tests of crude larval extracts it was observed on many occasions that animals, paralyzed by a single inoculation of a supra-optimal paralysis inducing dosage, recovered within 1-2 days.

The combined results of the present investigations and those of Gothe, *et al.* (1970) thus show that toxin production and transfer occurs only after several days of feeding of specifically larval ticks. Post-larval stages do not produce toxin in amounts detectable by the present toxicity assay.

It is clear that the main obstacle encountered during the isolation of the toxin is the presence of extremely high hemoglobin concentrations in the extracts prepared from fed larvae. Excision of salivary glands from hundreds of larvae is impracticable due to their small size. Thus the use of salivary glands to circumvent this problem was not possible.

Excellent resolution was obtained with the preparative column isoelectric focusing procedure as seen by at least 10 sharp red bands, probably due to hemoglobin and hemoglobin degradation products. However, upon emptying the column mixing and diffusion occurred resulting in the recovery of only 3 detectable bands. Furthermore, it was found that two peaks with pI's of 8.0 and 6.5, both in association with hemoglobin, were toxic. The presence of two toxic peaks probably does not indicate 2 separate toxins but one toxin associated with 2 different hemoglobin variants. The low recovery of toxicity is probably as the result of the extensive dialysis required to remove ampholytes and glycerol.

Preparative flat bed isoelectric focusing was used to circumvent the problems of collection of resolved bands encountered with the column method. It was found however, that the resolution was much less satisfactory and that a pronounced electro-endosmotic flow caused some of the bands to be transferred into the cathode region. This was probably the reason for the low recovery of toxin. Here too a toxic fraction was recovered which was associated with hemoglobin.

Gel permease chromatography of crude larval extracts showed that elution in the presence of saline resulted in a toxic fraction which eluted at the void volume of the Sephadex G-100 as well as a Sephacryl S-200 column. These fractions were reddish to brown in colour. Recovery of protein and toxicity were in excess of 90%. The inclusion of 0.05% Tween 20, 0.1% SDS or 0.1% Chaps to the sample and eluent had no effect on the elution diagram as well as on protein and toxicity recoveries.

Elution of the Sephacryl S-200 column with distilled water resulted in the recovery of a colourless toxic fraction representing 40% of the original toxicity at a volume corresponding to a molecular mass of approximately 100 kdal. Under these conditions, the hemoglobin adsorbed onto the column. Elution of this fraction was achieved with 0.154 M NaCl. It contained approximately 70% of the original toxicity. These results indicate the possibility that two toxins are present. More likely however, is that the toxin is associated with hemoglobin through hydrophobic interaction. At low ionic strength, toxin is partially liberated from a hemoglobin-toxin complex with the result that some free toxin is present. Also at low ionic strength, the hemoglobin-toxin is adsorbed to the column while the free toxin is eluted at a volume according to its molecular size and conformation.

It is envisaged that with further refinement of this method a toxin should be obtainable in high yield and free from hemoglobin. In working towards this goal different conditions with respect to ionic strength and pH are presently being investigated. The results have not been included in this chapter since the work has not been completed.

Chromatofocusing was also tested as a means of toxin isolation starting with the crude larval extracts. Good resolution of peaks was obtained as well as high recoveries with respect to protein and toxicity. However, the same problem was experienced: toxicity was associated with hemoglobin. As with the isoelectric focusing procedures, two toxic peaks were obtained with pI's of approximately 8.3 and 7.6. The peak with pI of 8 almost corresponds to a peak obtained from the preparative isoelectric focusing column while the one with pI 7.6 differs from the peak with the lower pI obtained through preparative isoelectric focusing. This indicates that several hemoglobin variants and probably their breakdown products are present in the crude extracts and that more than one of these may associate with the toxin.

Although little information concerning the characteristics of the paralysis toxin of *A. (P.) walkerae* has been obtained through these investigations, much has been learnt as to the behaviour of the toxin during the various isolation attempts. It is hoped that a homogeneous toxic fraction will be obtained in the near future so that its structure and neuropharmacological properties may be investigated.

CHAPTER 4

KINETIC PROPERTIES OF TOXIC PROTEASE INHIBITORS ISOLATED FROM TICK EGGS

4.1. INTRODUCTION

The biochemical characterization of tick toxins is a prerequisite for a study regarding their possible symbiotic origin (Houk and Griffiths, 1980). Such investigations of toxins from tick eggs may provide an insight into selective symbiont-tick interrelationship (Koch, 1960; Martin, 1979). Furthermore, these toxins may have a bearing on toxins associated with tick toxicoses (Regendanz and Reichenow, 1931; De Meillon, 1942).

Several proteinaceous components, present in tick eggs have been found to have protease inhibitory activity (Vermeulen, Neitz, Potgieter and Bezuidenhout, 1984; Willadsen and Riding, 1980; Neitz, Prozesky, Bezuidenhout, Putterill and Potgieter, 1981). Protease inhibitors have also been found to be present in the venom of certain snakes (Joubert and Strydom, 1978), insects (Shkenderov, 1976) and in the toxic salivary secretion of the tick *Ornithodoros savignyi* (Neitz, 1976). In addition, protease inhibitors are associated with toxins isolated from snake venom (Strydom, 1973; Kondo, Narita and Lee, 1978) and tick eggs (Neitz, *et al*, 1981).

Ray, Guho and Sinha (1982) suggested that the physiological rol of anti-proteases present in the white of *Geomyda trijuga trijuga* (Scharigger) reptile eggs, is to protect the egg against microbial and viral invasions. Kuvcer and Turner (1981) showed extracts of the eggs

of the range caterpillar (***Hemileuca oliviae***) to inhibit trypsin, kallikrein and papain. They proposed the possibility that these inhibitors play an important role in embryogenesis.

Extensive investigations by Neitz, ***et al***. (1981), Viljoen (1983) and Viljoen, Mills, Neitz, Potgieter and Vermeulen (1984) have shown that the egg toxins isolated from 5 species of ixodid ticks exhibit protease inhibitory activity. The toxin isolation procedures involved gel permease chromatography and chromatofocusing. They were shown to be pure by isoelectric focusing and SDS PAGE. The toxins cause histopathological lesions in guinea-pigs including focal areas of necrosis in the liver, with mineralization and oedema of the mucosa of the urinary bladder, and vacuolation of the lining epithelium. The genesis of the lesions in the various organs appears to be vascular. Some properties of these toxins are summarized in Table 4.1.

A knowledge of the detailed inhibition mechanism of anti-proteases is important since the physiological role of these inhibitors may then possibly be predicted (Bieth, 1980), remembering that an inhibitor, although efficient ***in vitro***, may be physiologically irrelevant if its ***in vivo*** concentration is very low (Baici and Gyger-Marazzi, 1982).

Protein inhibitors of proteolytic enzymes are widely distributed in nature (Feinstein, Hoffstein and Sokolovsky, 1974). They can be divided into two groups, those that bind only one class of protease, namely a thiol, carboxyl, metallo or serine protease. The other group, the non-class specific inhibitors inhibits more than one class of protease.

The serine group of inhibitors can be divided into three subgroups based on their mode of action. The Kazal (pancreatic trypsin inhibitor composed of 56 amino acids with 3 inter-disulfide bonds) and Kunitz (soybean trypsin inhibitor, composed of 181 amino acids with 2 inter-disulfide

TABLE 4.1 Comparison of egg toxins(Viljoen,1983).

Tick species	Specific activity (sa)	Molecular weight (kdal)	Yield^{Y}	pI^{f}
Rhipicephalus evertsi evertsi	0.3	5^{uc}, 6^{sds}	3	6
Hyalomma truncatum	2.3	26^{uc}, 28^{sds}	2	8.3
Boophilus microplus	1.2	31^{uc}, 36^{sds}	2	9.1
Boophilus decoloratus	1.4	40^{uc}, sds	2	9.2

uc, sds: determined by sedimentation equilibrium centrifugation, SDS-PAGE and amino acid analysis respectively, y: mg toxin/gram wet eggs, f: determined by analytical isoelectric focusing, sa: MLD/mg toxin (dose expressed/kg body mass).

bonds) types of anti-proteases have similar heat and acid stability with only one reactive site for the binding of trypsin or chymotrypsin (Laskowski and Kato, 1980; Ikenaka, Odani and Koide, 1974). The third type, Bowman-Birk (soyabean trypsin inhibitor, composed of 71 amino acids with 7 inter-disulfide bonds) anti-proteases have 2 independent reactive sites (double headedness) for the binding of trypsin at the one and chymotrypsin at the other site (Vogel, Trautschold and Werle, 1968). Some anti-proteases such as the Japanese quail ovoinhibitor, exhibits multi-headedness (i.e. more than two independent reactive sites) inhibiting trypsin and chymotrypsin simultaneously (Laskowski and Kato, 1980).

The reactive site of the inhibitor is that part of the protein which has direct contact with the active centre of the enzyme, forming a complex. During the course of inhibition, a peptide bond at the reactive site of the inhibitor may be hydrolized, giving rise to modified inhibitor. Inhibitor in which this peptide bond is intact, is referred to as virgin inhibitor (Travis and Salvessen, 1983)

In this chapter the kinetic properties of the previously isolated protease inhibitors associated with egg toxins isolated from the tick species, ***Rhipicephalus evertsi evertsi***, ***Boophilus decoloratus***, ***B. microplus*** and ***Hyalomma truncatum*** (Viljoen, 1983; Viljoen, Neitz, Prozesky, Bezuidenhout and Vermeulen, 1985) are described. Furthermore, their immunogenic and antigenic inter-relationships were investigated since all the tick egg toxins showed similar histopathology and clinical symptoms.

4.2. MATERIALS AND METHODS

Biochemicals were obtained from Merck except for bovine pancreatic trypsin, bovine pancreatic chymotrypsin,

benzoyl-D,L-arginine-p-nitroanilide (BAPNA) and N-succinyl-L-phenylalanine-p-nitroanilide (SPNA) (Boehringer-Mannheim) and 7-carbobenzoxy-argininamido-4-methylcoumarin-HCl (Cbz-Arg-AMC.HCl), from Bachem Feinchemikalien AG (Switzerland).

Tick egg toxins from *R. evertsi evertsi* (Mkuze strain), *H. truncatum* (South West Africa x Kaalplaas strain), *B. decoloratus* (Ethel strain) and *B. microplus* (Onderstepoort strain) were isolated as described by Viljoen, *et al.* (1985).

4.2.1. Enzyme kinetics of the tick egg toxins

Enzyme inhibition studies were done at least in triplicate on three separate isolates.

4.2.1.1. Fast-binding inhibition

Determination of inhibition of trypsin and chymotrypsin by the tick egg toxins was performed according to the methods described by Fritz, Trautschold and Werle (1977) and Erlanger, Edel and Cooper (1966) respectively. The concentration of trypsin was determined by active-site titration with p- nitrophenyl-p'-guanidinobenzoate as described by Chase and Shaw (1970).

A solution of 0.51 mM BAPNA was used for trypsin inhibition studies and 35 µM SPNA for chymotrypsin inhibition studies in 3 ml Beckman quartz cuvettes. The formation of p-nitroaniline was monitored at 405 nm with a Beckman Acta MVI spectrophotometer at room temperature (20°C). Assays were performed in a 0.1 M sodium phosphate buffer, pH 7.4 containing 15 mM $CaCl_2$ at room temperature. The buffer, enzyme and inhibitor was mixed before the addition of substrate. Hydrolysis of

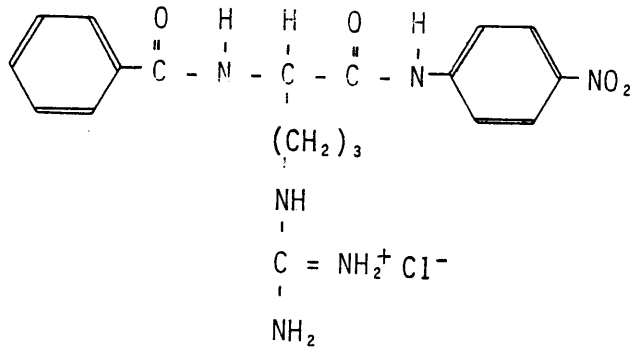
BAPNA and SPNA by trypsin and chymotrypsin respectively is shown in Scheme 1 and 2.

4.2.1.2. Slow-binding inhibition

Preliminary studies (Viljoen, 1983) on the toxins from *B. decoloratus*, *B. microplus* and *H. truncatum* showed slow-tight binding inhibition when BAPNA was used as a substrate. The ratio [I]/[E] was approximately 1 according to these studies. By substituting BAPNA with Cbz-Arg-AMC.HCl, the conditions could be changed such that [I]/[E] was larger than 10, making it possible to study these inhibitors as slow-binders

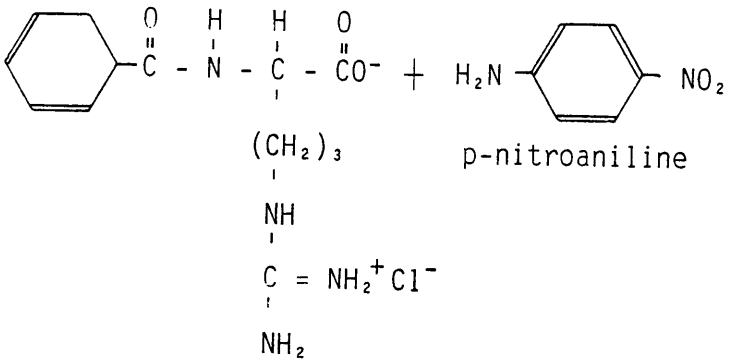
Inhibition experiments with the egg toxins were performed at fixed concentrations of trypsin and variable concentrations of substrate and inhibitor. For these studies Cbz-Arg-AMC.HCl was used and the progress of the reaction was followed fluorometrically by monitoring the release of 7-amino-4- methylcoumarin from the substrate with a recording FOCI spectrofluorometer from Farrand Optical Co. Inc.(New York). Trypsin assay with Cbz-Arg-AMC as substrate is shown in Scheme 3. The excitation and emission wavelengths were 380 and 460 nm respectively (Zimmerman, Ashe, Yurewicz and Patel, 1977) and the recorder scale was calibrated with 7-amino-4-methylcoumarin solutions of known concentration. The absorption coefficient for this substance at 342.5 nm is 9770 $M^{-1}cm^{-1}$ (Baici and Gyger-Marazzi, 1982).

The assays were performed in a 0,08 M Tris, 0.01 M $CaCl_2$, 1% DMSO (w/v) buffer, pH 8 at room temperature. The method and nomenclature described by Baici and Gyger- Marazzi (1982), was used to calculate K_i.



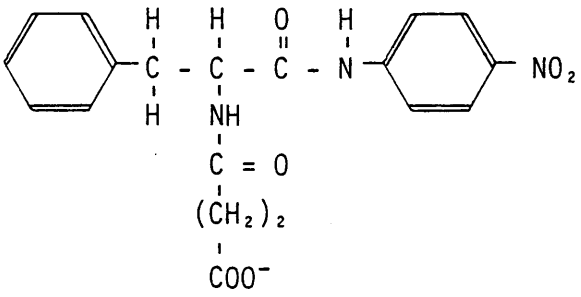
Benzoyl-D,L-arginine-p-nitroanilide

Trypsin

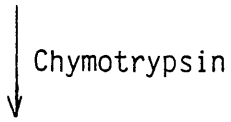


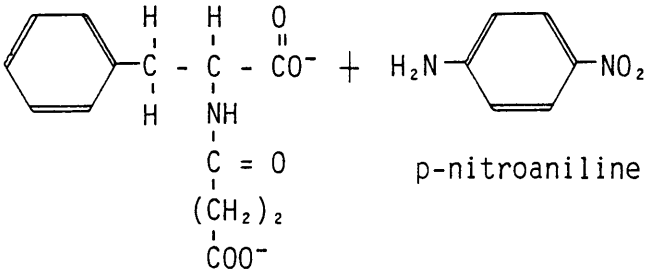
benzoyl-D,L-arginine

Scheme 1. Trypsin assay with BAPNA as substrate



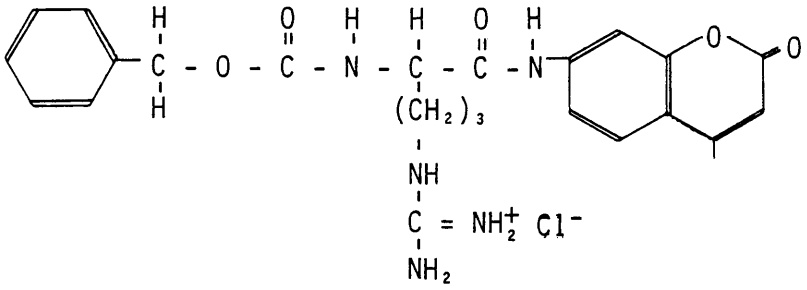
N-succinyl-L-phenylalanine-p-nitroanilide





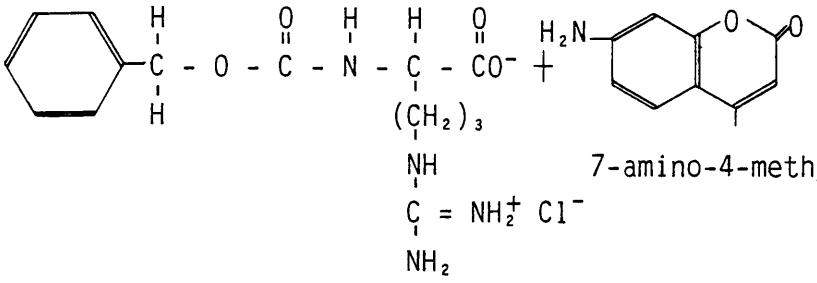
N-succinyl-L-phenylalanine

Scheme 2. Chymotrypsin assay with SPNA as substrate



7-carbobenzoxy-argininamido-4-methylcoumarin-HCl

Trypsin



7-amino-4-methylcoumarin

Carbobenzoxy-arginine

Scheme 3. Trypsin assay with Cbz-Arg-AMC.HCl as substrate

Thus interaction between enzyme and inhibitor is as follows:

$$E + S \rightleftarrows ES \longrightarrow \ldots\ldots \longrightarrow E + \text{products}$$

$$+$$

$$I$$

$$k_{off} \uparrow\downarrow k_{on}$$

$$EI$$

Scheme 4. A model for fully competitive, slow-binding inhibition. (Baici and Gyger-Marazzi, 1982).

The rate of product formation in the presence of inhibitor can be described as

$$[P] = \frac{v_o k_{off}}{\lambda} t + \frac{v_o k_{on}[I]}{(1+\sigma)\lambda^2}(1 - e^{-\lambda t}) \quad \ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where $\sigma = [S]/K_m$ and λ = an apparent pseudo-first order rate constant which describes the exponential approach to steady state.

$$\lambda = \frac{k_{on}}{1+\sigma}[I] + k_{off} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

After attainment of the steady sate the exponential terms in equation 1 becomes negligibly small and therefore

$$[P] = \frac{v_o k_{off}}{\lambda} t + \frac{v_o k_{on}[I]}{(1 + \sigma)\lambda^2} \quad \text{.......(3)}$$

A plot of Product [P] *versus* time (Fig 4.1) gives a straight line with slope = v_1 = $(v_o k_{off})/\lambda$, and respective intercepts on the P and time axes of

$$\pi = \frac{v_o k_{on}[I]}{(1 + \sigma)\lambda^2} \quad \text{.......(4)}$$

and

$$\tau = \frac{-k_{on}[I]}{k_{off}(1 + \sigma)\lambda} \quad \text{.......(5)}$$

It can be shown that

$$k_{off} = \frac{v_o - v_i}{v_o} \frac{1}{|\tau|} \quad \text{.......(6)}$$

and that rearangement of equation (5) gives

$$\frac{1}{|\tau|} = K_i k_{off} (1 + \sigma) \frac{1}{[I]} + k_{off} \quad \text{.......(7)}$$

K_i, k_{on} and k_{off} can also be calculated with the different equations given. Extrapolation back to the linear steady-state part of the curves (Fig 4.1) permitted the determination of π and τ. With the aid of the Guggenheim (1926) procedure as described by Baici and Gyger-Morazzi

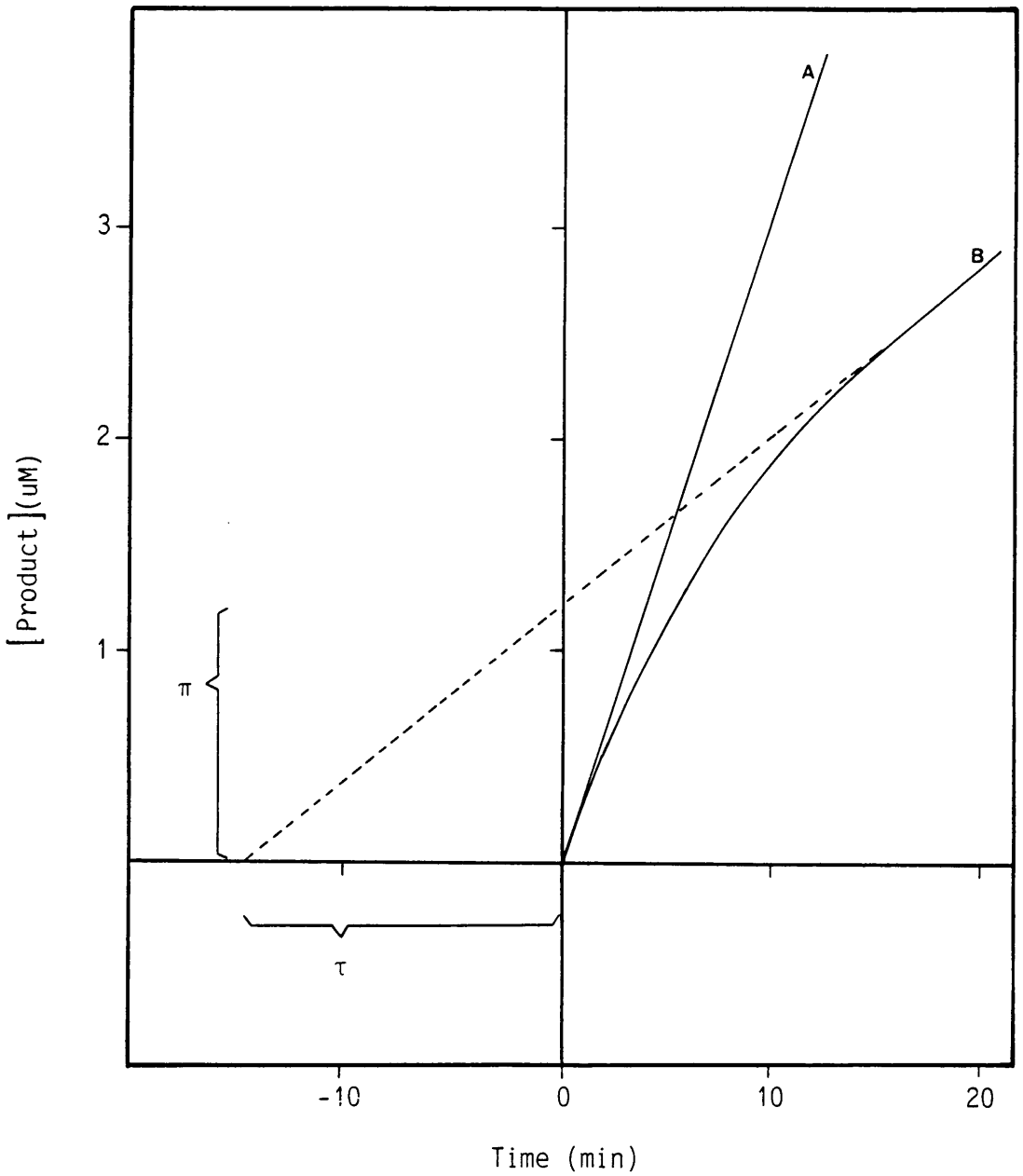


Fig 4.1. Progress curve for slow-binding inhibition. (A) No inhibition; (B) Inhibitor and enzyme. (Baici and Gyger-Marazzi, 1982).

(1982), π as well as λ can be determined, according to equations (8) and (9).

$$\pi = e^{int}/1 - e^{-\lambda\Delta t} \quad \text{.....(8)}$$

and

$$\lambda/2.303 = \text{slope of Guggenheim plot} \quad \text{.....(9)}$$

4.2.2. Preparation of tick egg toxin antiserum

Antisera against ***R. evertsi evertsi***, ***H. truncatum***, ***B. microplus*** and ***B. decoloratus*** egg toxins were prepared as described by Eshar (1982). BALB/c female white mice (4 for each egg toxin) were inoculated intradermally (id) in the hind paw foot pads with 50 µl (25 µl in each) aliquots containing 50 µg toxin. The toxic suspension was made up as follows: 250 µl 0.154 M NaCl containing 750 µg toxin, was added to 500 µl Freund's complete adjuvant (Difco Laboratories), mixed and stored at 4 °C till used. Four weeks later each animal received its second and final booster of 100 µl each, sc in the neck region with the same suspension as was used for the first inoculation. Two weeks after this final booster the mice were bled from the tail (***Vena caudalis***). Approximately 500 µl of blood was bled from each mouse. The blood was allowed to clot for 4 h at 10°C, and then centrifuged at 8 000xg in a Beckman Model B microfuge for 10 min and stored in 50 µl quantities at -30°C.

4.2.3. Micro-immunodiffusion

Ouchterlony double micro-immunodiffusion (Ouchterlony, 1958) was carried out using 1% agarose (Miles Laboratories) in Dulbecco's phosphate

buffered saline. The agarose was heated in the PBS buffer until dissolved (± 54°C) and cast onto microscope plates to a thickness of approximately 1 mm. Once the agarose had set, wells were made with a flat head 17 gauge needle at a distance of 2-2.5 mm from one another. Into the centre well, 3 µl of 10 µg tick egg-toxin per 1 ml PBS buffer was pipetted and into the outer wells, 3 µl of serum (obtained as described in chapter 4.2.2., p. 89) 1:10 diluted with PBS buffer. The precipitin lines were allowed to develop overnight at room temperature in a desiccator at relative humidity of at least 80%. The wells were filled with PBS buffer and the lines were photographed with a Wild Heerburgg photo-microscope Model M400 using a Ilford FP4 film with a matt red background.

4.2.4. Enzyme-linked immunosorbent assay (ELISA)

The ELISA involving the egg toxins was performed as described in Chapter 6 using Peroxidase conjugated IgG as indicator. The optimum serum solution concentration was determined as described in chapter 5. The frozen serum solutions were thawed and diluted 100X with 0.05 M Tris, 0.154 M NaCl buffer, pH 7.4. The tick egg toxins were applied into the microtiter wells (0.5 µg toxin per well). Antiserum against each toxin was tested with respect to each of the toxin antigens.

4.3. RESULTS

4.3.1. Fast-binding inhibition

4.3.1.1. *Rhipicephalus evertsi evertsi*

The egg toxin from ***R. evertsi evertsi*** exhibited fast-binding inhibition of trypsin with BAPNA as substrate. A Dixon plot (Dixon, 1972 and Segal, 1975) and a replot of K_i app ***versus*** [S] (Fig. 4.2(A) and Fig 4.2(B) showed typical competitive inhibition. K_i was calculated as 16.3

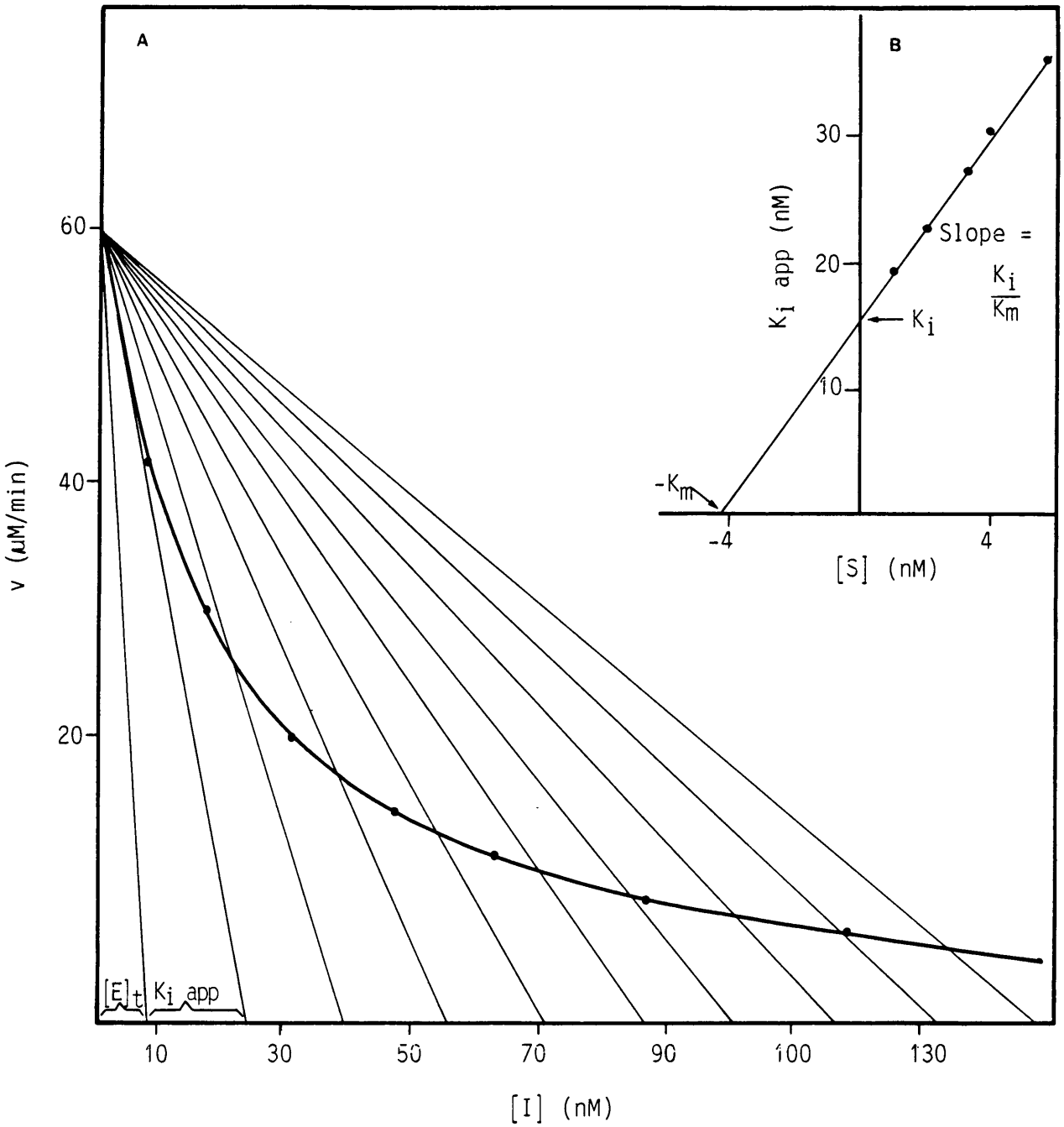


Fig 4.2. (A) The relationship of velocity *versus* inhibitor concentration, of *Rhipicephalus evertsi evertsi* egg toxin in [BAPNA] = 0.5 mM and [Trypsin] = 6.1 nM. (B) Replot of K_i app *versus* substrate concentration.

inhibit on of enzyme activity could be detected, even when the inhibitor and e zyme were pre-incubated for 30 min before assaying.

4.3.1.1. *Boophilus microplus*

At a *B. microplus* egg toxin concentration of 42 µM and a chymotrypsin concen ration of 0.34 µM, no inhibition was observed. Pre-incubation of en: yme and inhibitor for 30 min also gave no inhibition.

4.3.1.3. *Boophilus decoloratus*

The egg toxin of *B. decoloratus* showed fast binding inhibition of chy otrypsin (Fig 4.3(A) and Fig 4.3(B). K_i was calculated as 36.2 nM (T ble 4.2) by methods described (in paragraph 4.3.1.1., p. 90) for the fast-binding inhibition by *R. evertsi evertsi*.

4.3.1.4 *Hyalomma truncatum*

H. tr ncatum egg toxin exhibited fast-binding inhibition of chymot ypsin. K_i was found to be 23.1 nM (Fig 4.4, Table 4.2) using the sa ne approach as described (in paragraph 4.3.1.1., p. 90) for the fa t-binding inhibition by *R. evertsi evertsi*.

4.3.2. Slow-binding inhibition

Incuba on of the tick egg toxins from *B. microplus* (Fig 4.5), *B. decolor tus* (Fig 4.6) and *H. truncatum* (Fig 4.7) with trypsin, showed slow-bi ding inhibition when Cbz-Arg-AMC.HCl was used as substrate (Table 4.2). When BAPNA was used as a substrate, slow, tight-binding inhibiti n was observed (Fig 4.8). Only the inhibition exhibited by *B. micropl s* is shown in Fig 4.8.

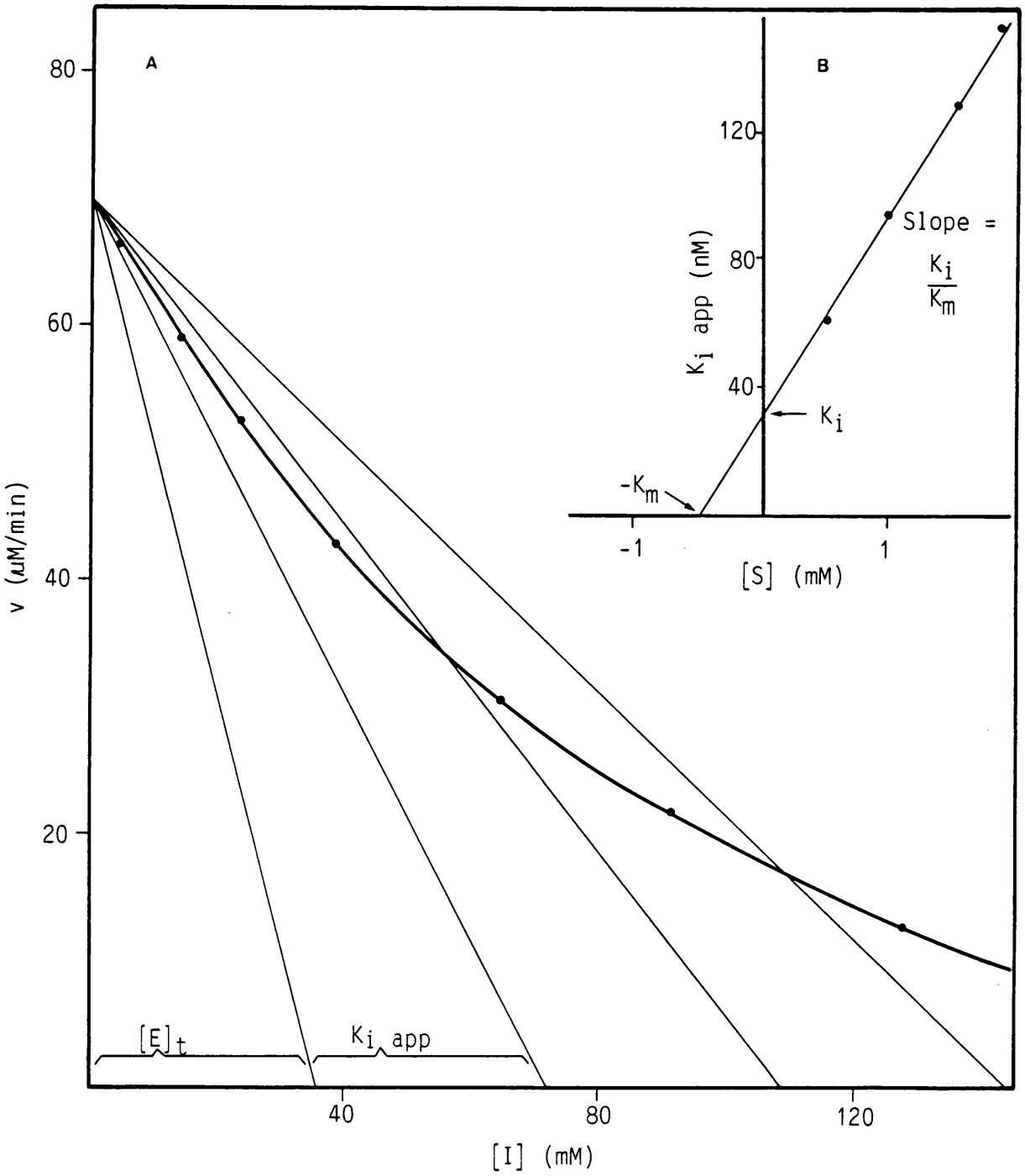


Fig 4.3. (A) The relationship of velocity *versus* inhibitor concentration, of *Boophilus decoloratus* egg toxin in [SPNA] = 0.5 mM and [Chymotrypsin] = 33 nM. (B) Replot of K_i app *versus* substrate concentration.

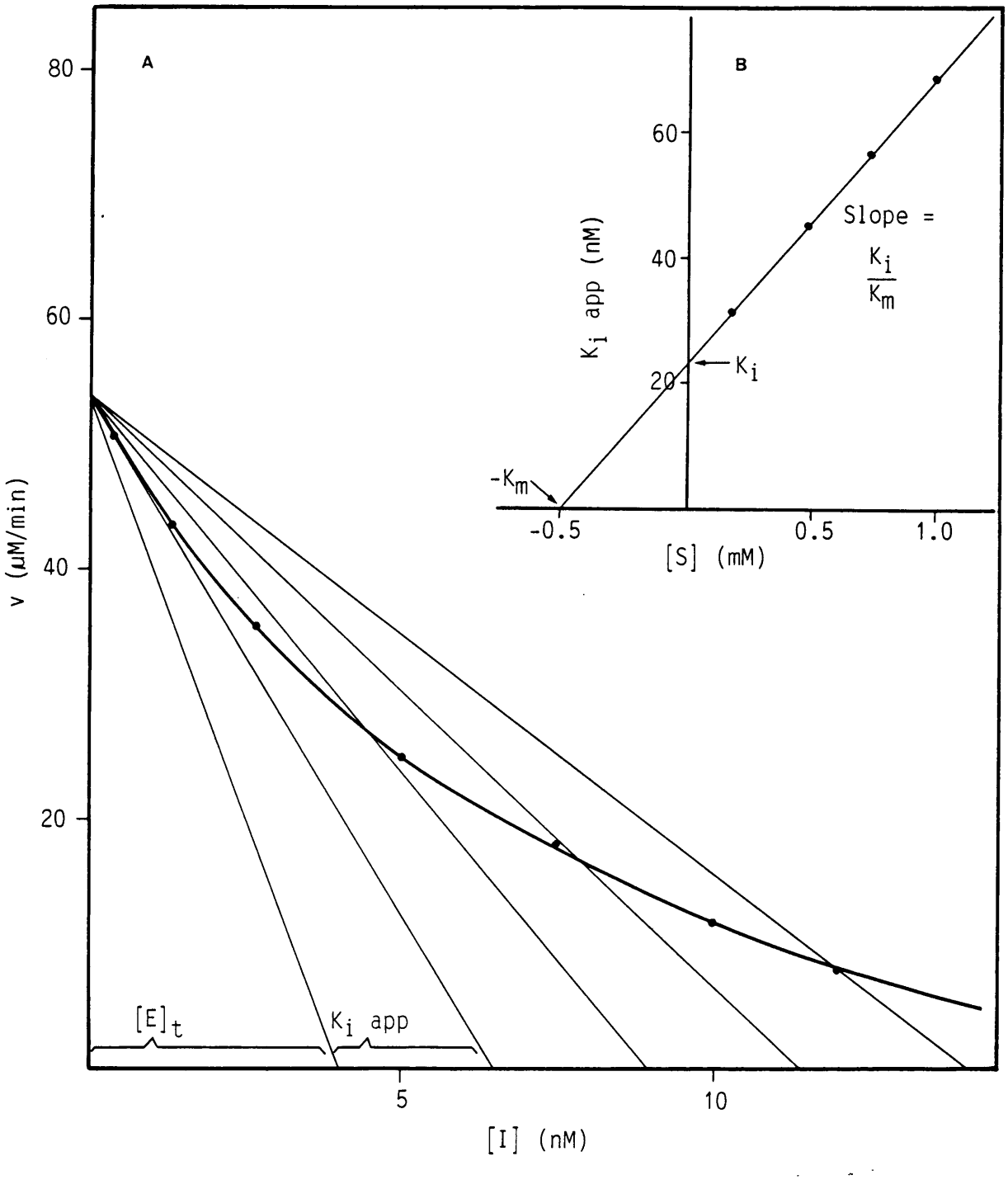


Fig 4.4. (A) Relationship of velocity against *Hyalomma truncatum* egg toxin concentration with chymotrypsin. [SPNA] = 0.5 mM, [Chymotrypsin] = 4.1 nM. (B) Replot of K_i app *versus* substrate concentration.

TABLE 4.2 Kinetic constants for *Rhipicephalus evertsi evertsi*, *Hyalomma truncatum*, *Boophilus microplus* and *Boophilus decoloratus* egg toxins.

Enzyme	Substrate	Inhibitor	Type of inhibition	Pre-steady state			Steady state		
				k_{on} $M^{-1}sec^{-1}$	k_{off} sec^{-1}	K_i	K_i	K_m mM	K_i*
TRYPSIN	BAPNA	*R. evertsi evertsi*	Fast				16±0.3 nM	4.5±0.2	
	Cbz-Arg-AMC	*B. decoloratus*	Slow	$4.17x10^5$	$1.53x10^{-3}$	3.6±0.2 nM*	4.1±0.1 nM	0.25±0.07	
	Cbz-Arg-AMC	*B. microplus*	Slow	$5.43x10^5$	$2.17x10^{-3}$	4.0±0.1 nM*	4.6±0.2 nM	0.25±0.07	
	Cbz-Arg-AMC	*H. truncatum*	Slow	$4.4x10^6$	$2.82x10^{-3}$	0.64±0.1 nM*		0.25±0.07	0.34±0.2 nM[1]
CHYMO= TRYPSIN	SPNA	*R. evertsi evertsi*	No-inhibition						
	SPNA	*B. decoloratus*	Fast				36.2±0.8 nM	0.5±0.1	
	SPNA	*B. microplus*	No-inhibition						
	SPNA	*H. truncatum*	Fast				23.1±0.4 nM	0.5±0.1	

* $K_i = \frac{k_{off}}{k_{on}}$ (Baici and Gyger-Marazzi, 1982)

K_i* = overall dissociation constant (Baici and Gyger-Marazzi, 1982)

[1] $k_5 = 0.00151^{sec^{-1}}$ $k_6 = 0.00171^{sec^{-1}}$

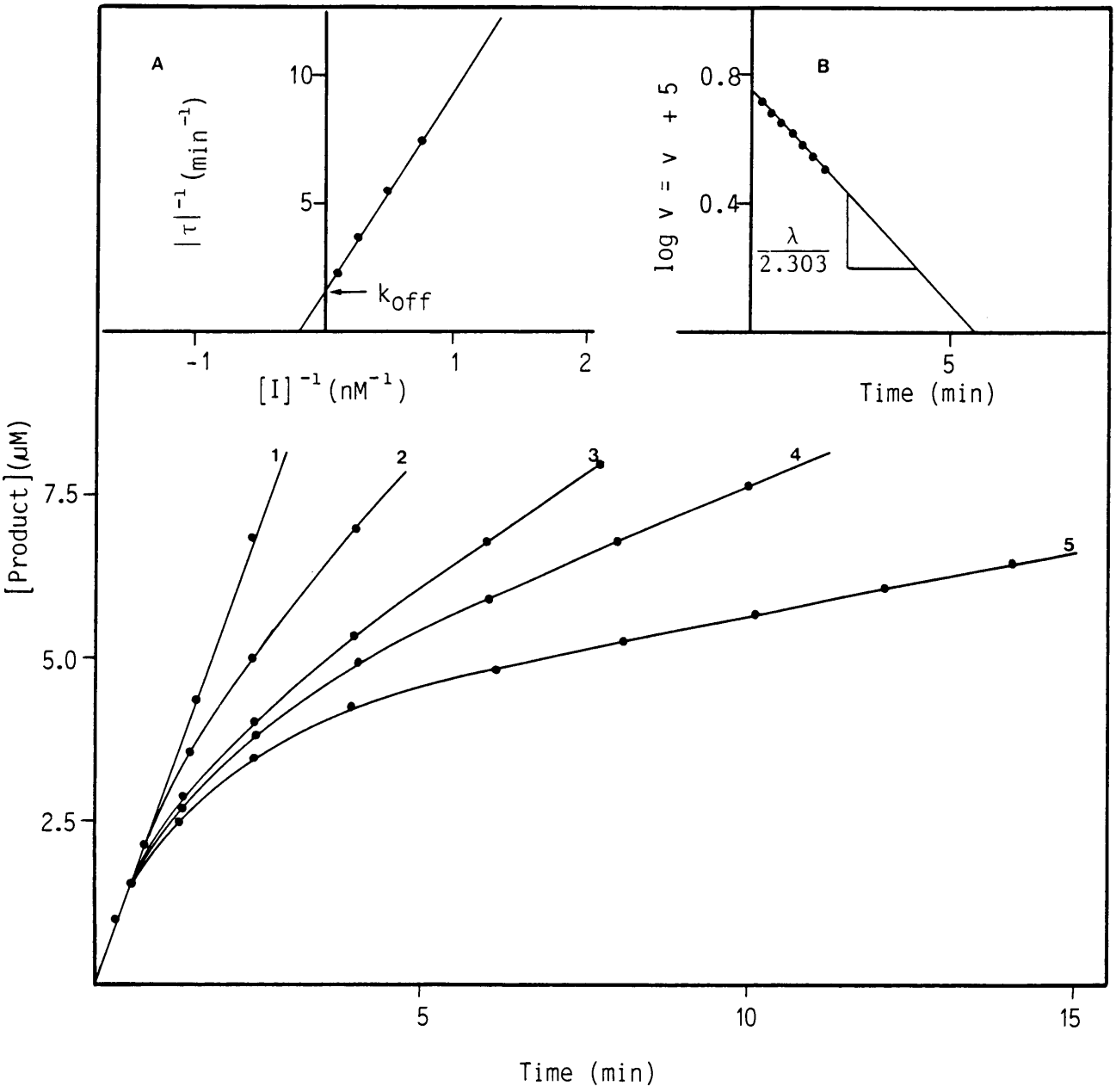


Fig 4.5. Inhibition of trypsin by ***Boophilus microplus*** egg-toxin. [Cbz-Arg-AMC] = 2 mM, [Trypsin] = 0.42 nM and [*B. microplus*] were (nM): 0(1),2.5(2), 7.5(3), 10.0(4), 15.0(5). The insert (A) is a replot according to

$$\frac{1}{|\tau|} = K_i k_{off}(1 + \sigma)\frac{1}{[I]} + k_{off} \quad \text{(Eqn 7)}$$

and the insert (B) the Guggenheim plot.

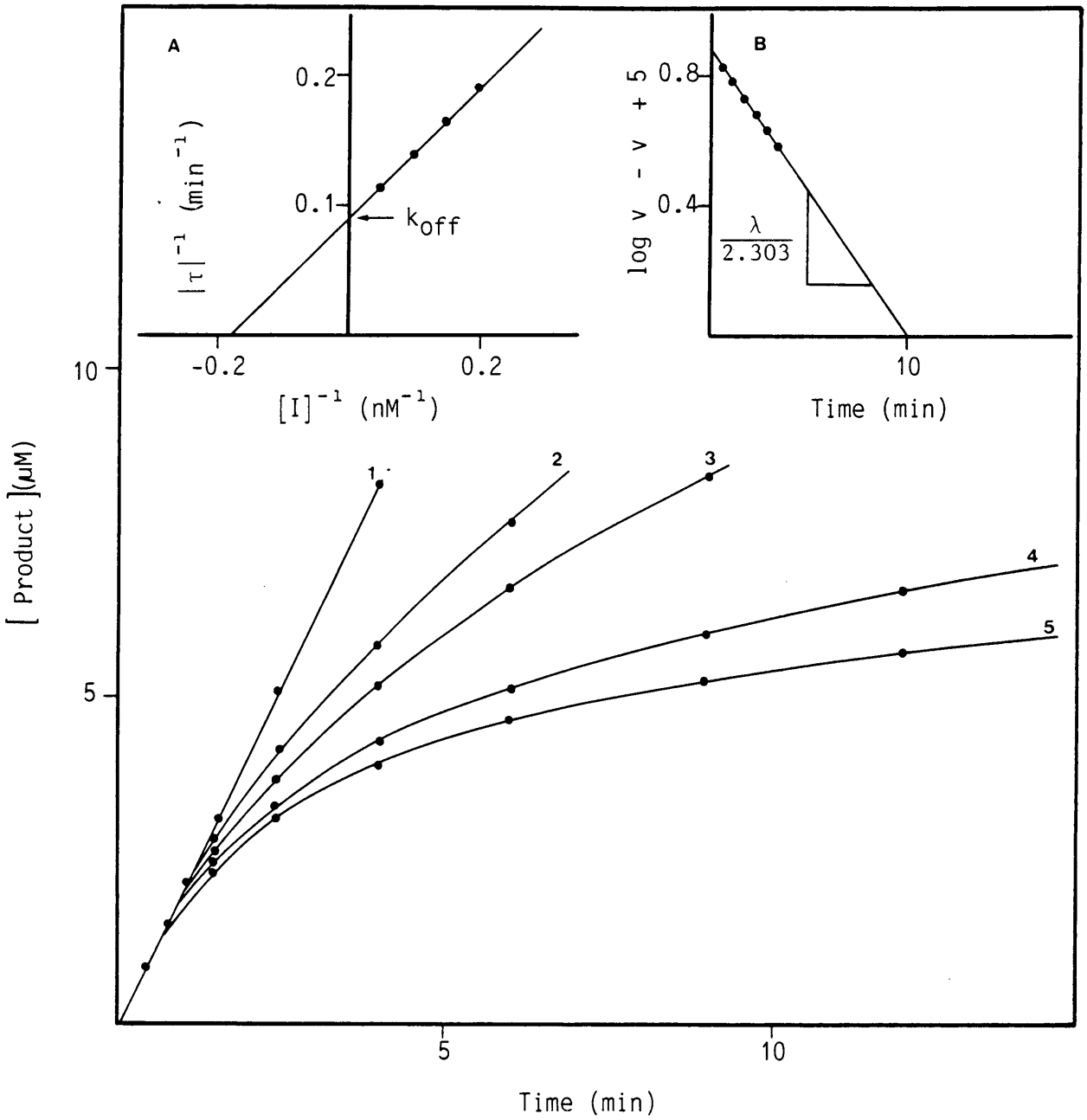


Fig 4.6. Inhibition of trypsin by ***Boophilus decoloratus*** egg-toxin. [Cbz-Arg-AMC] = 3.6 mM, [Trypsin] = 0.42 nM and [***B. decoloratus***] were (nM): 0(1), 1.25(2), 6.25(3), 9.4(4), 12.4(5). The insert (A) is a replot according to

$$\frac{1}{|\tau|} = K_i k_{off} (1 + \sigma) \frac{1}{[I]} + k_{off} \quad \text{(Eqn 7)}$$

and the insert (B) the Guggenheim plot.

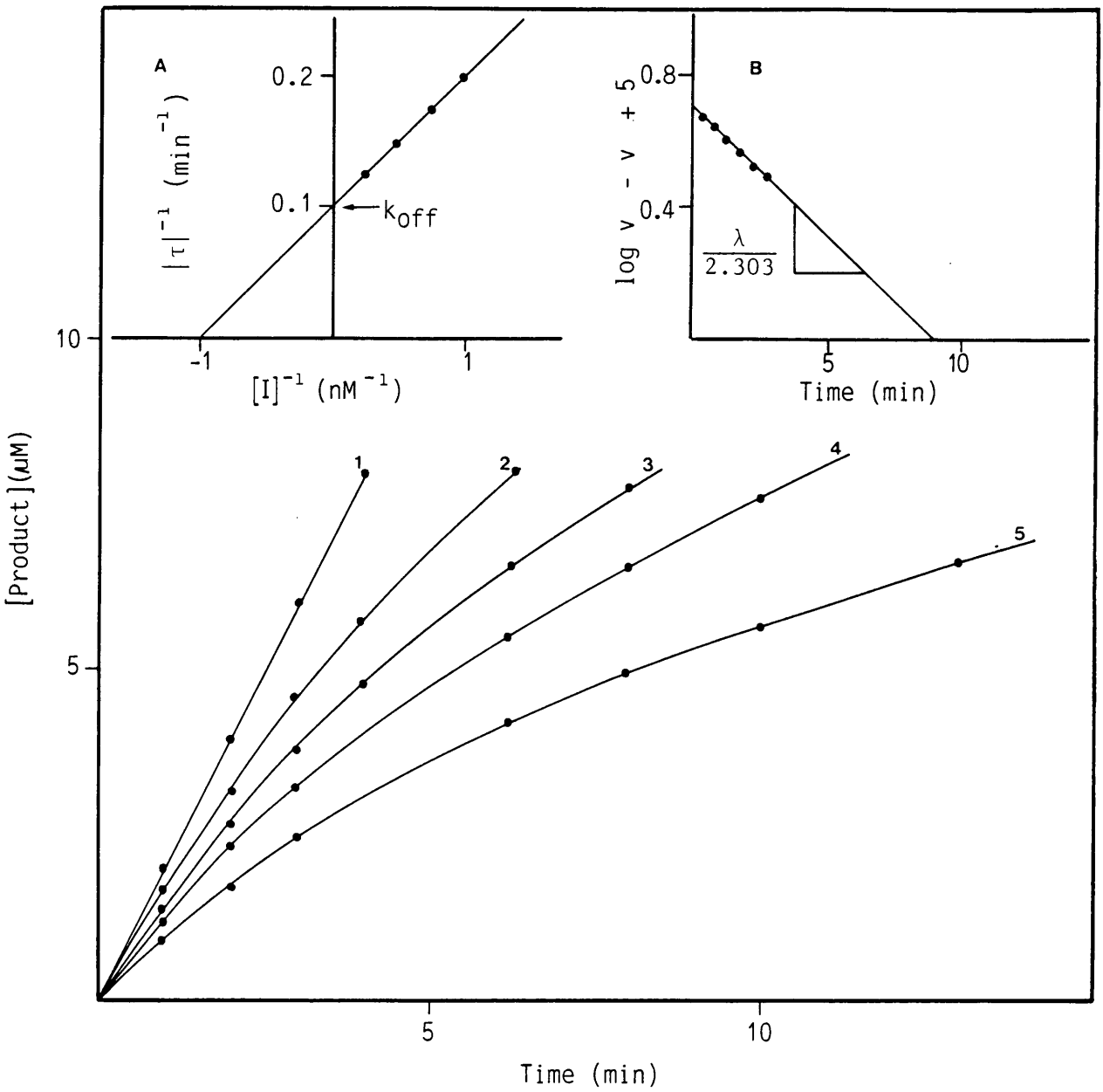


Fig 4.7. Inhibition of trypsin by *Hyalomma truncatum* egg toxin. [Cbz-Arg-AMC] = 0.76 nM, [Trypsin] = 0.1 nM and [*H. truncatum*] where (nM): 0(1), 0.9(2), 1.1(3), 2.3(4), 7.0(5). The insert (A) is a replot according to

$$\frac{1}{|\tau|} = K_i k_{off} (1 + \sigma) \frac{1}{[I]} + k_{off} \quad \text{(Eqn 7)}$$

and the insert (B) the Guggenheim plot.

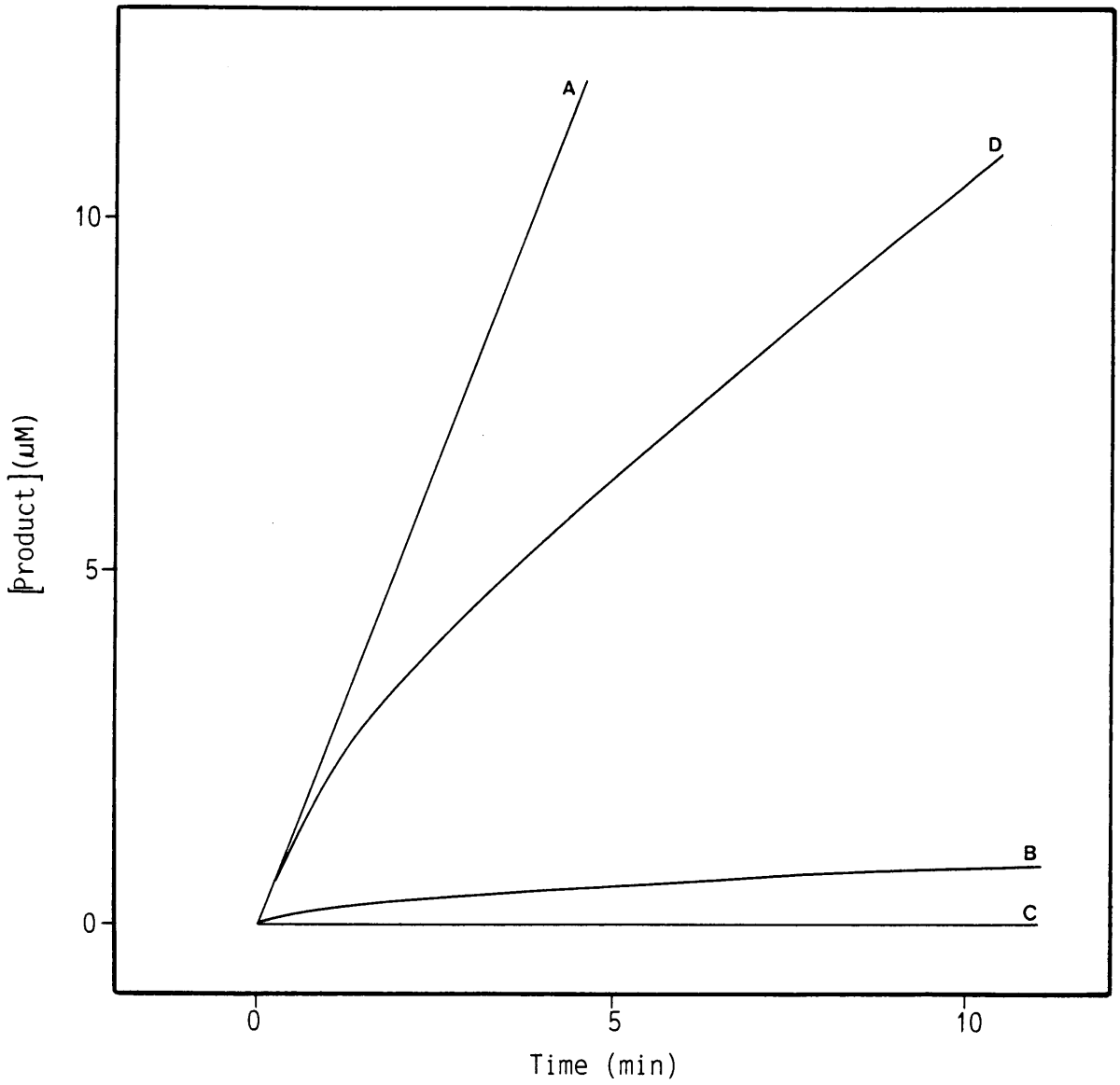


Fig 4.8. Progress curve for ***Boophilus microplus*** egg toxin inhibition of trypsin. (A) No inhibitor, [Trypsin] = 0.42 nM, [Cbz-Arg-AMC] = 2 mM; (B) [I]/[E] ≈ 1, [Trypsin] = 6.1 nM, [*B. microplus*] = 7.5 nM, [BAPNA] = 0.5 mM; (C) [I]/[E] ≈ 10, [Trypsin] = 6.1 nM, [*B. microplus*] = 75 nM, [BAPNA] = 0.5 mM; (D) [I]/[E] > 10, [Trypsin] = 0.42 nM, [*B. microplus*] = 7.5 nM, [Cbz-Arg-AMC] = 2 mM.

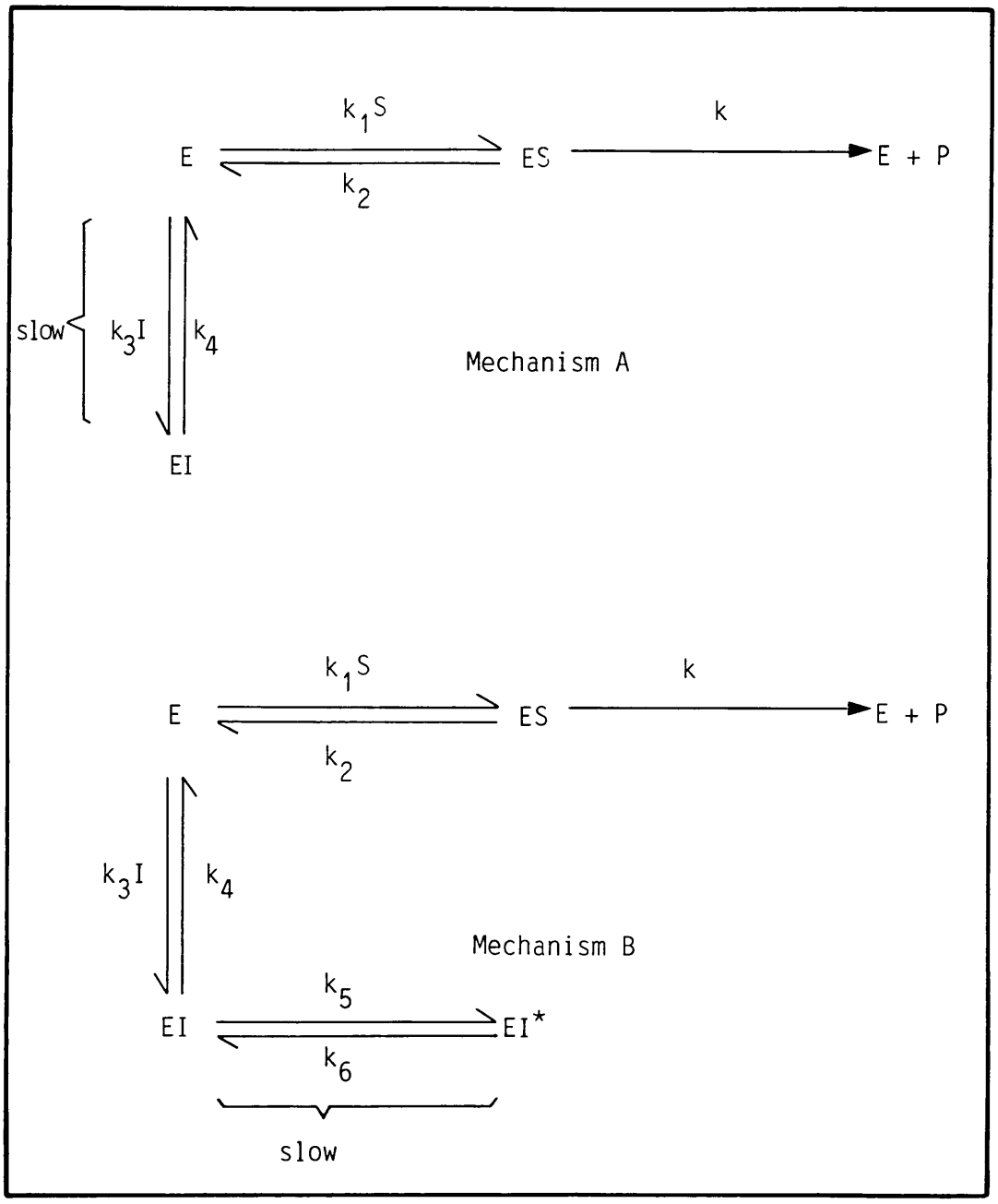


Fig 4.9. Mechanisms that describe reversible slow-binding and slow, tight-binding inhibition. (Morrison, 1982).

4.3.3. Micro-immunodiffusion and ELISA

The Ouchterlony double diffusion showed a reaction of absolute identity between ***B. microplus*** and ***B. decoloratus***. These two toxins however, exhibited no cross identity with the other two toxins. ***R. evertsi evertsi*** and ***H. truncatum*** on the other hand demonstrated no identity with each other as well as with the toxins from ***B. microplus*** and ***B. decoloratus*** (Fig 4.10). From the ELISA results (Table 4.3), it is evident that ***B. microplus*** and ***B. decoloratus*** show cross identity with each other. These two egg toxins however, exhibited no cross identity with ***R. evertsi evertsi*** and ***H. truncatum***.

TABLE 4.3 ELISA signal to background rations of absorbance values to determine cross reaction between *Rhipicephalus evertsi evertsi*, *Hyalomma hebraeum*, *Boophilus microplus* and *B. decoloratus* egg toxins.

Antigen source	Antibody source	P/N ratio[1]
R. evertsi evertsi	*R. evertsi evertsi*	5.32 ± 0.81
	H. truncatum	1.14 ± 0.07
	B. microplus	1.09 ± 0.10
	B. decoloratus	1.11 ± 0.13
H. truncatum	*H. truncatum*	7.19 ± 0.95
	R. evertsi evertsi	1.17 ± 0.10
	B. microplus	1.02 ± 0.07
	B. decoloratus	1.19 ± 0.11
B. microplus	*B. microplus*	6.48 ± 0.75
	R. evertsi evertsi	1.10 ± 0.10
	H. truncatum	1.06 ± 0.14
	B. decoloratus	5.93 ± 0.62
B. decoloratus	*B. decoloratus*	6.83 ± 0.87
	R. evertsi evertsi	1.12 ± 0.07
	H. truncatum	1.09 ± 0.10
	B. microplus	6.13 ± 0.93

[1] - P-specific anti-toxin serum, N-normal serum

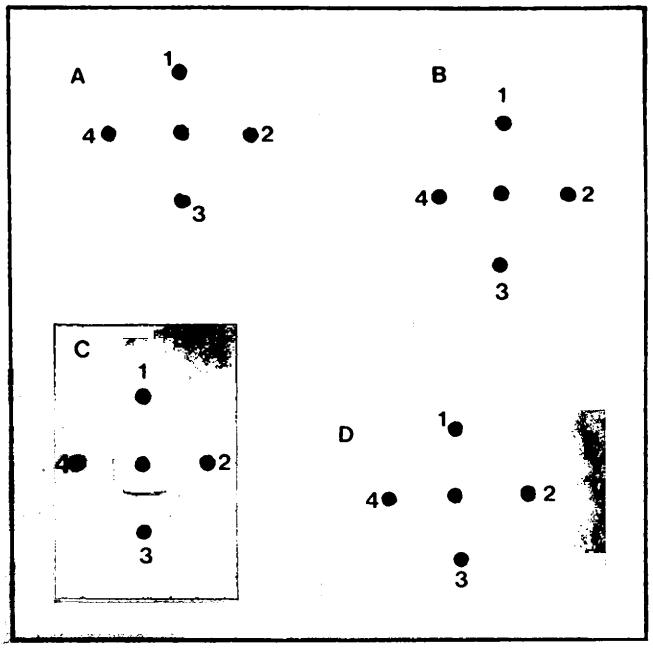


Fig 4.10 Ouchterlony micro-immunodiffision of egg toxins.

(A) Centre well: ***Rhipicephalus evertsi evertsi*** egg toxin; well 1: mouse anti-***R. evertsi evertsi*** egg toxin serum; well 2: mouse anti-***Hyalomma truncatum*** egg toxin serum; well 3: mouse anti-***Boophilus microplus*** egg toxin serum; well 4: mouse anti-***B. decoloratus*** egg toxin serum.

(B) Centre well: ***H. truncatum*** egg toxin; well 1: mouse anti - ***R. evertsi evertsi*** egg toxin serum; well 2: mouse anti-***H. truncatum*** egg toxin serum; well 3: mouse anti-***B. microplus*** egg toxin serum; well 4: mouse anti-***B.decoloratus*** egg toxin serum.

(C) Centre well: ***B. microplus*** egg toxin; well 1: mouse anti-***R. evertsi evertsi*** egg toxin serum; well 2: mouse anti-***H. truncatum*** egg toxin serum; well 3: mouse anti-***B. microplus*** egg toxin serum; well 4: mouse anti-***B. decoloratus*** egg toxin serum.

(D) Centre well: ***B. decoloratus*** egg toxin; well 1: mouse anti- ***R. evertsi evertsi*** egg toxin serum; well 2: mouse anti-***H. truncatum*** egg toxin serum; well 3: mouse anti-***B. microplus*** egg toxin serum; well 4: mouse anti-***B. decoloratus*** egg toxin serum.

Enlargement: 2X

4.4. DISCUSSION

In general, studies with reversible inhibitory substrate analogues are performed under steady-state conditions where the concentration of enzyme is very much less than that of the inhibitor and where all the equilibria involving the enzyme and reactants are set up rapidly. It was, however, recognized that there are also compounds which inhibit enzyme catalyzed reactions at concentrations comparable to that of the enzyme and under conditions where the equilibria are set up rapidly (Morrison, 1969). As the strength of interaction between an enzyme and a tight-binding inhibitor increases, a stage is reached when the equilibrium of the reaction cannot be determined experimentally, unless lower [I] and [E] are employed (Cha, 1975). Morrison (1982) reported that reversible enzyme inhibitors can be grouped into four categories on the basis of the relative concentrations of inhibitor and enzyme and whether the equilibria are established rapidly or slowly (Table 4.4). Morrison also further assumed that all slow-binding inhibitors give rise to reversible, competitive inhibition.

Many protein and peptide protease inhibitors of the fast, tight-binding type have been described (Bieth, 1974). The toxins isolated from the different tick species not only show typical fast-binding inhibition of trypsin and chymotrypsin, but also slow-binding inhibition of trypsin as defined by Morrison (1982). Fast-binding competitive inhibition of trypsin was observed with a Dixon plot when the toxin of *R. evertsi evertsi* was studied at different substrate concentrations. A plot for one concentration only is shown in Fig 4.2(A). A replot of K_i determined at different substrate concentrations is given in Fig 4.2(B). A dissociation constant of 16 nM could be determined from the replot. This phenomenon was observed for all the fast- binding tick egg toxins investigated. With chymotrypsin as enzyme, *R. evertsi*

Table 4.4 Classification of reversible enzyme inhibitors (Morrison, 1982)

Characteristics		
Class of Inhibitor	Relationship between E_t and I_t	Rate of establishment of equilibrium between E, I and EI
Classical	$I_t > E_t$	fast
Tight-binding	$I_t \simeq E_t$	fast
Slow, tight-binding	$I_t \simeq E_t$	slow
Slow-binding	$I_t > E_t$	slow

evertsi and *B. microplus* showed no inhibitory activity. *B. decoloratus* and *H. truncatum*, however, showed fast- binding inhibition of chymotrypsin with inhibition constants 36 nM and 23 nM respectively.

B. decoloratus, *B. microplus* and *H truncatum* showed slow-binding inhibition with trypsin (Fig's 4.5, 4.6 and 4.7). Inhibition was observed when the enzyme and inhibitor concentrations were the same ($[E]_t = [I]_t$), i.e. 1:1 inhibition kinetics with BAPNA as substrate. It was therefore necessary to employ the computer programs developed by Williams, Morrison and Duggleby (1979) or to use fluorometric substrates (Baici and Gyger-Marazzi, 1982) or higher concentrations in order to obtain the necessary 10:1 inhibitor to enzyme ratio, which is a pre-

requisite for deriving the velocity equation used for this type of inhibition. The necessary computer programs were obtained from Williams, J. W. (John Curtin School of Medical Research, Australian National University) and co-workers. However, at present there is difficulty in running their Fortran dialect on the computer system available to us. Therefore fluorometric substrates were used.

From Table 4.2 it can be seen that the K_i for the three slow-binding inhibitors vary from 0.91 to 3.6 nM, calculated from pre-steady state data. These values compare well with those calculated from steady-state data (0.93 to 4.1 nM). They are comparable to similar results obtained for leupeptin inhibition of cathepsin B (Baici and Gyger-Marazzi, 1982).

B. decoloratus and *H. truncatum* egg toxins are also slow-binding inhibitors of trypsin (Fig's 4.6 and 4.7 and Table 4.2) These toxins are thus probably double-headed anti- proteases, i.e. acting on both trypsin and chymotrypsin with different types of kinetics. Double headed protease inhibitors have also been found to be present in turkey ovomucoid as well as in human α_1-inter trypsin (Vogel, Trautschold and Werle, 1968; Laskowski and Kato, 1980; Tschesche, 1972).

It can be seen from Fig's 4.5, 4.6 and 4.7 that the initial velocities in the case of *B. microplus* and *B. decoloratus* egg toxins are independent of inhibitor concentrations, while in the case of *H. truncatum* egg toxin the initial velocity is dependent of the inhibitor concentration. Morrison (1982) ascribes such behaviour to two different types of inhibition patterns.

The two mechanisms described in Fig 4.9, assume that slow- binding inhibition is not due to the slow isomerization of the inhibitor to a more potent species. The two mechanisms exhibit different characteristics. In the one the initial velocity is independent of the inhibitor

concentration [I] (mechanism A). In the other, the initial velocity varies as a function of [I] (mechanism B) (Morrison, 1982). The slow-binding properties of *B. decoloratus*, *B. microplus* and *H. truncatum* and the equations (Morrison, 1982) accounting for the kinetic features are very similar to those of the hysteretic enzyme described by Frieden (1970). These enzymes respond slowly to a rapid change in ligand concentration, be it substrate or inhibitor. We assume as Baici did for cathepsin B (Baici and Gyger-Marazzi, 1982), that trypsin belongs to this category of enzymes with respect to these tick egg toxins. It is possible that the binding of these egg toxins induces a slow conformational change in the enzyme, analogous to that associated with the formation of the transition state during catalysis. This hypothesis, suggested by Baici and Gyger-Marazzi (1982), is also supported by studies entailing the inhibition of the cysteine protease, papain, by aldehydes (Lewis and Wolfenden, 1977).

The mechanism for slow-binding inhibition has not yet been established (Morrison, 1982). Perhaps slow-binding inhibitors are such good analogues of the substrate that they induce a conformational change in the enzyme which is analogous to that associated with the formation of the transition state in enzymic catalysis (Morrison, 1982 and Baici, 1981). The forward isomerization reaction would then be slow because the inhibitor does not have all the essential structural features of the transition state of a substrate while the reverse isomerization reaction would be even slower because that rate is not enhanced through product formation. The final effect is that inhibitors subject to slow-binding inhibition hold on to the enzymes so as to hinder their release from enzyme-inhibitor complexes (Morrison, 1982). It was suggested (Rich, 1985) that the slow-binding inhibition observed when pepstatin binds with aspartic proteases, is probably caused by the slow displacement of the water hydrogen bound to the aspartic residues in the active site. No or little conformational change is observed during the

slow-binding process. It is also suggested that as water is displaced by pepstatin, the "tightened complex" forms and is stabilized further by the favourable entropic process, returning water to bulk solvent.

At this stage of the investigations, there is no evidence available to connect the anti-proteolytic activity of the tick egg toxins to the toxic symptoms or the histopathological lessions (Viljoen, *et al.*, 1985).

Furthermore, it is not known whether these toxins function as inhibitors *in vivo*. Bieth (1980) has shown that inhibitors active *in vitro* are not necessarily active *in vivo* (i.e. that they play a physiological role). However, the physiological function may be inferred from kinetic parameters of the protease- inhibitor interaction (Bieth, 1980). This involves determining the rate of complex formation between inhibitor and protease. According to Bieth (1980) the *in vivo* half-time of inhibition is as follows:

$$t_{1/2} = 1/k_{on}[I]$$

A $t_{1/2}$ of 100 msec is the maximum time that should be allowed for effective control of a particular enzyme (Travis and Salvessen, 1983). A further parameter to be considered is the K_i value. This should indicate a sufficiently high affinity (i.e. a high $1/K_i$) for the protease. The overall efficiency of the inhibitor depends not only on K_i but also upon its concentration. The important factor being the ratio $[I]/K_i$. An inhibitor is efficient only if $[I]/K_i > 10$ (Bieth, 1974; Bieth, 1980).

In Table 4.5, the $t_{1/2}$ and $[I]/K_i$ values of the tick egg toxin inhibitors are shown. It is evident that the values indicate physiological significance with respect to the animal proteases tested. Further work regarding the inhibition of proteases in tick eggs (Mills, 1985) by the toxins need to be investigated.

TABLE 4.5 The half-time of inhibition ($t_{\frac{1}{2}}$) and (I)/K_i for slow-binding egg toxin inhibitors

Tick egg toxin	Conc (1) µM	k_{on} M^{-1} sec^{-1}	$t_{(\frac{1}{2})}$ (msec)			Enzyme (3)	
			I = *	I = K_i	I = 10^4 K_i	Trypsin (I)/K_i	Chymotrypsin (I)/K_i
R. evertsi evertsi	20	-	-	-	-	38750	-
B. decoloratus	45	0.42×10^6	53	661×10^3	66.1	12500	1243
R. microplus	101	0.54×10^6	18	463×10^3	46.3	25500	-
H. truncatum	61	4.4×10^6	462.7 (2)	814×10^3 (2)	458.9 (2)	95313	2684

1 - concentration in tick eggs from Viljoen, Neitz, Prozesky, Bezuidenhout and Vermeulen (1985).

2 - $t_{\frac{1}{2}} \simeq \frac{1}{(I).k_{on}} + \frac{0.693}{k_5}$ It is assumed that the individual $t_{\frac{1}{2}}$'s calculated from the association constants k_{on} and k_5 are additive (Bieth, 1980).

* - $t_{\frac{1}{2}}$ calculated for toxin concentrations in second column.

3 - Ratios calculated at inhibitor concentrations present in the tick eggs.

Inhibition of the animal proteases is most probably only incidental. They are similar in structure and function to some microbial proteases (Davis, Zahnley and Donovan, 1969). The inhibition of the latter enzymes may be the natural function of these ovoinhibitors. Hereby the multiplication of some species of invading organisms could be prevented (Board and Faller, 1974). In addition, they could be partially responsible for specific symbiotic associations in tick eggs and act as primitive humoral defence agents which does not depend on specific recognition of antigens (Lackie, 1980).

The Ouchterlony double-diffusion showed absolute identity between the egg toxins of ***B. decoloratus*** and ***B. microplus***. This toxins however,

were non-identical to *H. truncatum* and *R. evertsi evertsi* toxins which were in their turn also non-identical.

It is evident that no deductions can be made at this stage regarding the functions of these toxic anti-proteases. Most probably the protease inhibitory activity is not the sole activity associated with the toxin but merely serves to enhance or preserve the toxicity of a toxic domain (Rayn, 1979). Such a suggestion was also made by Habermann and Breithaupt (1978) for a non-toxic sub-unit complexed with phospholipase A_2 (Thesleff, 1979).

CHAPTER 5

PURIFICATION OF *COWDRIA RUMINANTIUM* BY CELLULAR AFFINITY CHROMATOGRAPHY AND PERCOLL DENSITY GRADIENT CENTRIFUGATION AND DETECTION BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

5.1. INTRODUCTION

A study of the presumed toxin produced by *Cowdria ruminantium* (Neitz, 1968) depends principally upon the availability of pure preparations of the organism. Pure preparations are also essential for investigations into the biochemical, antigenic and immunogenic properties of the organism. The purification of *C. ruminantium* is furthermore important for the preparation of a suitable vaccine, free of extraneous antigens (Wilson, 1967).

Through such studies, information concerning the pathogenesis of heartwater could be gained. In addition, methods for sensitive specific serodiagnosis could possibly be developed, the nature of the immunity to heartwater disease studied, the taxonomic position of the organism more accurately described and morphological studies extended (Pienaar, 1970; Du Plessis, 1970b; Uilenberg, 1981). The study of the developmental cycles and distribution in the vertebrate and invertebrate hosts would also be facilitated.

The isolation of sufficient amounts of viable pure *C. ruminantium* has for many years been hampered by their extremely labile nature and the difficulties encountered in the cultivation of the organism in chicken yolk sacs and tissue culture (Uilenberg, 1983). The propagation of this pathogen in laboratory animals has also met with problems (Du Plessis, 1982). Mason and Alexander (1940) were able to show 5 serial passages

of *C. ruminantium* in ferrets as experimental animals, but were unable to infect guinea-pigs, rats, rabbits or mice, with the agent. *C. ruminantium*, inoculated intraperitoneally (ip) (from infected brain material) into white mice, retained the infection for 90 days, without showing clinical signs of the disease. Recently, Du Plessis (1982) and Mackenzie and McHardy (1984) have succeeded in the proliferation of certain strains of *C. ruminantium* in mice. Hereby a sufficient source of the organisms for further purification could be provided.

Various methods for the purification of rickettsial organisms have been reported. These include differential centrifugation (Bell and Theobald, 1962), sucrose (Wang and Grayston, 1967), Renografin (Howard, Orenstein and King, 1974), or Percoll (Tamura, Urakami and Tsuruhara, 1982) density gradient centrifugation, continuous flow zonal centrifugation (Anacker, Gerloff, Thomas, Mann, Brown and Bickel, 1967),celite-treatment (Weiss, Rees and Hayes, 1967), fluoro-carbon extraction (Dubois, Cutchins, Berman, Lowenthal and Timchak, 1972) and anion- (Hoyer, Bolton, Ormsbee, Le Bouvier, Ritter and Larson, 1958) and cation-exchange chromatography (Hara, 1958). Many of these methods are time-consuming and have detrimental effects on the organisms (Weiss,Coolbaugh and Williams, 1975). Apparently no attempts utilizing these or alternative techniques for the purification of *C. ruminantium* have been described in the literature.

In the present work the suitability of lectin affinity chromatography and Percoll density gradient centrifugation for the purification of *C. ruminantium* was investigated.

Affinity chromatography with specific lectins is a quick and mild procedure for the isolation of a variety of cells (Sharma and Mahendroo, 1980). Since *C. ruminantium* organisms show staining characteristics similar to gram-negative bacteria (Cowdry,1925) an attempt was made to purify

viable *C. ruminantium* by means of wheat germ lectin cellular affinity chromatography (Sharma and Mahendroo 1980). This lectin shows specificity towards N - acetyl - D - glucosamine (Nagata and Burger, 1974), which is a characteristic constituent of the cell wall of gram-negative micro-organisms (Salton, 1964). The use of Concanavalin A and Helix pomatia lectin cellular affinity chromatography was also investigated. Concanavalin A and Helix pomatia lectins show specificity preferentially towards α-D-glucose (Plow and Resnick, 1970) and N-acetyl-D-galactosamine (Sharma and Mahendroo, 1980) respectively. Lectin affinity chromatography is unlikely however, suitable for discriminating between various forms and sizes of the organism since separation is based on the presence of specific sugars of the cell membrane.

For the separation of heterogeneous populations of *C. ruminantium*, Percoll density gradient centrifugation was investigated. Density gradient centrifugation in general has been employed for large scale isolation of cells, subcellular particles and viruses under mild conditions (Anderson, 1966). The use of Percoll (Pertoft and Laurent, 1977) as density gradient media offers many advantages and has been applied to the isolation of a variety of cells with complete retention of morphological integrity and biological activity (Schumacher, Schafer, Holstein and Hiltz, 1978). Percoll consists of colloidal silica particles of 15 - 30 nm diameter which have been coated with polyvinylpyrrolidone (Pertoft and Laurent, 1977). One of the advantages of Percoll resides in the fact that it does not penetrate biological membranes. In addition, gradients of Percoll can be adjusted to give precise ionic strength, pH and osmolality compatible with cells. Furthermore, the low viscosity facilitates very rapid isopycnic banding of cells. Thus, self-generated gradients at moderate g-forces may be formed. Percoll can easily be removed from purified materials (Pharmacia Fine Chemicals Manual on Percoll, Methodology and applications, 1982).

For both lectin affinity chromatography and Percoll density gradient centrifugation, *C. ruminantium* infected brain, liver or spleen tissue and *Amblyomma hebraeum* nymphae were used as source of the organism. Corresponding non-infected material was used as controls. An ELISA was developed, which together with electron-microscopy and *in vivo* infectivity determinations were employed for the identification of the organism.

5.2. MATERIALS AND METHODS

Analytical quality reagents were used in all the experiments. All the glassware and equipment was sterilized with 70% (v/v) ethanol and the buffers by filtration through 0.22 µm filters (Millipore).

5.2.1. Preparation of crude sheep or goat brain, mouse liver and spleen and nymph extracts

In order to obtain *C. ruminantium* infected brain material, sheep or goats were infected by iv inoculation of the Onderstepoort, Ball 3 heartwater vaccine (Bezuidenhout, 1981). The disease was allowed to run its course. Immediately after the death of the animals their brains were removed and frozen in liquid nitrogen and transported on dry ice from Onderstepoort and used within 1 h. In an attempt to increase the number of organisms in the brains, two sheep were treated with an arsenic containing tonic (Acetarsonic acid (5%), Vetoquinol) (Neitz,1940) for 18 days prior to inoculation. The dosage regime was 5 ml iv every 3 rd day.

C. ruminantium infected engorged *A. hebraeum* nymphae were obtained by feeding the larvae on sheep reacting to inoculation with the Onderstepoort, Ball 3 nymph vaccine as described by Bezuidenhout (1981). The nymphae were then fed on either heartwater susceptible sheep or on sheep reacting to vaccination as described above. The Spes

Bona strain of *A. hebraeum* was used in all cases as it has been found to be free of any rickettsial organisms other than Wolbachia- like symbionts. Nymphae were used within 3 days after dropping. All further work on these sources of *C. ruminantium* was performed at 4°C. The crude brain extracts were prepared using 89 - 224 g of frozen infected and non - infected brain. These were quickly thawed and homogenized at 4°C in a Waring Blender (Waring Products Division) in 50 - 120 ml of a 0.05 M HEPES, 0.154 M NaCl, pH 7.4 buffer for 5 min at low speed. Infected and non-infected *A. hebraeum* nymphae were homogenized with an Ultra Turrax at a dilution of 10 nymphae per 5 ml buffer.

The *C. ruminantium* Kumm strain infected mouse liver and spleen tissue (Du Plessis, 1982), were homogenized at low speed, immediately after dissection, with a Waring Blender in buffered lactose peptone (BLP), consisting of 0.6 g (w/v) Na_2HPO_4, 0.08 g (w/v) KH_2PO_4, 2% (w/v) Difco peptone and 10% (w/v) lactose, for 5 min.

All homogenates were centrifuged for 30 min at 1 000xg in a Rotor 19 or Rotor 40 in a Beckman L5-65 ultracentrifuge. This centrifuge was also used in all subsequent centrifugations with half maximum acceleration and braking. The supernatants were then centrifuged for 30 min at 10 000xg with a Rotor 30 or Rotor 40. The resultant supernatants were centrifuged at 30 000xg for 30 min in a Rotor 30 or Rotor 40. The sediment was resuspended in 12 ml of a 0.05 M HEPES, 0.154 M NaCl, pH 7.4 buffer. This represented the crude extract.

5.2.2. Lectin chromatography

Wheat germ lectin-Sepharose 6 MB (10 ml) (Vretblad and Hjorth, 1977), (carbohydrate specificity N-acetyl-D-glucosamine), Concanavalin A lectin-Sepharose 4B (10 ml) (Plow and Resnick, 1970), (carbohydrate specificity α-D-glucose) and Helix pomatia lectin-Sepharose 6 MB (10 ml) (Sharma and Mahendroo, 1980), (carbohydrate specificity N-acetyl-D-galactosamine), were packed into Pharmacia columns K 10/20 (10mmx13.5cm). The lectins and columns (fitted with 80 µm mesh nylon nets) were obtained from Pharmacia Fine Chemicals. The void volume of the columns was c. 10 ml. The wheat germ lectin gel was regenerated with 100 ml of 0.1 M Tris-HCl, 0.5 M NaCl, 0.02% NaN_3 (w/v), pH 8.5 and 100 ml of 0.1 M sodium acetate, 0.5 M NaCl,0.02% NaN_3 (w/v) ,pH 4.5 and equilibrated with 100 ml of 0.05 M HEPES,0.154 M NaCl,0.02% NaN_3 (w/v), pH 7.4. The column was also stored in the latter buffer. Before use the column was washed with 500 ml of 0.05 M HEPES, 0.154 M NaCl, pH 7.4 to remove the preservative. In the case of the other two lectin columns, the above buffers and procedures were used but 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$ were included in each buffer.

The crude extracts (10 ml) were applied onto the column and incubated for 2 h. The non-adsorbed material was eluted from the column with the respective HEPES buffers before a pulse of N-acetyl-D-glucosamine, α-D-glucose or N-acetyl- D-galactosamine was applied (20 ml of the respective HEPES buffers containing 2 g of carbohydrate). The column fractions were analyzed for their protein content, infectivity and antigenicity. Only wheat germ lectin column fractions and the corresponding crude extracts were investigated electron-microscopically.

In order to enlarge the binding capacity of the 10 ml wheat germ lectin, the lectin was poured into a 250 ml glas beaker. A 10 ml infected crude brain extract was added, mixed and incubated for 1 h with gentle mixing

every 10 min. The lectin was then packed into the already mentioned column and the procedure was repeated as described.

5.2.3. Percoll density gradient centrifugation

An iso-osmotic Percoll (Pharmacia Fine Chemicals) stock solution (SIP) with density of 1.125 g/ml was prepared by adding 9 ml of Percoll to 1 ml of 1.54 M NaCl (Jackson, Dench and Hall, 1979). The resuspended crude extracts (0.5 ml) in 0.154 M NaCl, were then layered on top of the gradient forming material prepared by mixing 15 ml SIP with 14.5 ml of 0.154 M NaCl to form a 50% SIP solution. As reference, a tube containing 50% SIP solution and density marker beads (Pharmacia Fine Chemicals) was used (Pharmacia Fine Chemicals Percoll Manual, 1982).

Centrifugation was performed for 15 min at 30 000xg in a Rotor 30 employing maximal acceleration and half maximal braking. Hereafter the gradient was collected from the bottom of the tube with a fraction recovery system (Beckman). Ten fractions of 3 ml each were collected and used immediately or stored at -30°C.

5.2.4. Percoll density gradient centrifugation of wheat germ lectin-Sepharose 6 MB chromatographic fractions

To determine the density and antigenic difference between organisms present in fractions obtained after wheat germ lectin chromatography from *A. hebraeum* nymphae, they were centrifuged on Percoll density gradients (as described in chapter 5.2.3., p 116). Antigenic properties were analyzed with ELISA.

5.2.5. Determination of infectivity

Sheep of c. 40 kg body weight of either sex were injected iv at a dosage rate of 2 ml per animal with the Ball 3 strain crude extracts and 0.5-6 ml per animal with fractions obtained by lectin chromatography (3-6 ml) and density gradient centrifugation (0.5 ml). The injection needle was dipped into an 1% adrenalin solution (Centaur) prior to the injection. This reduced the initial shock of the injection. The Kumm strain crude extracts as well as subsequent lectin fractions obtained by chromatography and density gradient centrifugation were injected ip into mice at a dosage rate of 200 µl per animal. Their 50% lethal dose (LD_{50}) was statistically calculated according to Reed and Muench (1938). Daily rectal temperatures of the injected sheep were obtained and all the test animals were kept under observation for at least 24 days. In the case of no reaction, the sheep were challenged (iv) 24 days after the initial inoculation with 5 ml (1 dose) Onderstepoort heartwater Ball 3 vaccine. Reacting sheep as well as mice were allowed to die after which a complete necropsy was performed. A diagnosis of heartwater was made only after Giemsa stained brain smears were found to be positive, for typical *C. ruminantium* colonies (Purchase, 1945).

5.2.6. Protein determinations

The protein content was determined as described by the heated biuret-Folin method described by Dorsey, McDonald and Roels (1977). This method is conducted under conditions which rupture cells and is therefore suitable for determination of total protein of cells. To 100 ml 2.0% Na_2CO_3 (w/v) and 100 ml 0.1 M NaOH diluted with 800 ml distilled water, 10 ml each of 20.0% sodium potassium tartrate (w/v) and 5.0% $CuSO_4$ solutions was added. This reagent was prepared immediately before use. After 0.2 ml of a 100-400 µg protein - HEPES solution had been

added to 5 ml of the freshly made solution, in a test tube stoppered with glass marbles, it was heated together with albumin standards and blanks for 100 min at 100°C. Immediately after the heating period, 0.5 ml Folin-Ciocalteau reagent (Merck), diluted 1:1 with distilled water, was added and mixed quickly with a VM 20 vortex mixer (Chiltern Scientific Enterprise). The tubes were then placed into a vigorously stirred bath at 10 °C for 15 min after which the samples were allowed to reach room temperature. The colour developement was monitored at 660 nm in a Beckman Model 25 spectrophotometer against a blank, containing 0.1 ml of HEPES buffer in place of the sample. The protein content served as a means of estimating sample concentration.

5.2.7. Electron-microscopic studies

Aliquots (0,5 ml) of samples to be examined were made up to 10 ml with 0,154 M NaCl and centrifuged for 30 min at 30 000xg in a Rotor 40 to sediment the organisms. The supernatants were removed and the sediment of each fraction was mixed with c. 0.1 ml of 2% (w/v) agar (Difco) at 45 °C and siphoned into a glass capillary tube. The content of the tube was streaked across filter paper, which was then cut into narrow strips with a Swan-Morton Scalpel. The strips were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 for 5 h, followed by post-fixing with 2% osmium- tetroxide in 0.1 M cacodylate buffer, pH 7.3 in a fume cupboard for 3 h. The preparation and embedding of these sections were performed as described by Glauert (1965). Dehydration was achieved with stepwise addition of 30%, 50%, 70%, 90% and 2 x 100% ethanol for 15 min each. The sections were embedded in 100% Spurrs (Polaron Equipment Ltd) solution and thin sections (0,1 µm) were cut with a Reichert-Jung Ultracut Microtome. These were stained for 45 min in a 4% aqueous solution of uranyl acetate and for 10 min in lead citrate, at room temperature on G-300 copper grids (Polaron

Equipment Ltd). The grids were investigated with a Hitachi H600 or a Phillips P350 electron – microscope.

5.2.8. Isolation of IgG from sheep serum

The isolation procedure was performed at room temperature. To 100 ml heartwater infected sheep serum, drawn 3 weeks after treatment of the diseased sheep, 18% (w/v) sodium sulfate (Johnstone and Thorpe, 1982) was added and stirred until dissolved. This solution was incubated for 30 min and centrifuged at 5 000xg in a Rotor 30. The sediment was suspended to give a final volume of 50 ml. To this , 4.4% (w/v) sodium sulfate was added, stirred, incubated for 30 min and centrifuged as described above. The precipitate was resuspended in distilled water to give a final volume of 10 ml and dialyzed against 50 l, 0.01 M sodium phosphate buffer, pH 8.00, for 36 h. Whatman DE 52 (DEAE cellulose anion-exchanger) gel was thoroughly washed with the buffer and packed into a Pharmacia Fine Chemicals K26/40 column. The sample (17 ml) was applied onto the column, eluted with 0.01 M sodium phosphate buffer, pH 8.0, collected in 10 ml fractions and monitored at 280 nm with a Beckman Model 26 spectrophotometer. The presence of IgG in the first peak was confirmed in an ELISA in which the immunoglobulin fraction was adsorbed onto the microtiter plate and Protein A-alkaline phosphatase conjugate added. The peak 1 was dialyzed for 58 h against 54 l distilled water and freeze-dried.

5.2.9. Enzyme-linked immunosorbent assay (ELISA)

A modification of the ELISA method described by Notermans, Timmermans and Nagel (1982) was used. The various crude extracts as well as the fractions obtained from lectin chromatography and Percoll density gradient centrifugation were screened for antigenic properties.

Sera from heartwater infected and non-infected sheep and mice and infected goats were drawn 3-4 weeks after treatment of the diseased animals, and used as antiserum which were prepared as follows: blood samples (10 ml and in the case of mice 0.5 ml) were left to clot and after 2 h the coagulated blood was centrifuged for 10 min at 300xg in a Piccolo bench top centrifuge at room temperature. The serum was siphoned off, divided into 0.2-1 ml batches and stored at -30°C. A serum solution was made up as follows: 1 ml serum was diluted 1:30 with a 0.05 M Tris, 0.154 M NaCl, buffer, pH 7.4. In some cases isolated reconstituted IgG was used at the same dilutions. IgG was also isolated from serum by the method of Johnstone and Thorpe (1982) (as described in paragraph 5.2.8., p.119). Serum was used as antibody source for testing brain, liver and spleen, and nymph material and the isolated IgG for the detection of antigen for brain material.

Linbro microtiter plates (Flow Laboratories) were coated with 100 µl antigen at a concentration of either 3,10 or 100 µg prot per ml of a 0.05 M Tris, 0.154 M NaCl buffer, pH 7.4. The antigen was sonified beforehand for 5 seconds with a micro-tip at 40% continuous duty cycles and a setting of 5 on the output control.

The plates were incubated for 2 h while gently shaking on a Titertek (Flow Laboratories) and washed twice with 0.05 M Tris, 0.154 M NaCl, 0.05% Tween 20 (v/v), pH 7.4 and 3 times with distilled water. The washing solution was siphoned off after each wash. The blocking buffer,

3% bovine serum albumin (BSA) (w/v) in Dulbecco's phosphate buffered saline (PBS), was applied in 200 µl quantities to block all the vacant sites. After gentle shaking for 1 h, the blocking buffer was siphoned off and the serum containing 0.1 mg protein or 0.1 mg isolated IgG in 30 ml of 0.05 M Tris, 0.154 M NaCl buffer, pH 7, was then added to the microtiter plate in 100 µl quantities to each well and gently shaken for 1 h. The plate was washed once with a solution containing 0.05% (v/v) Tween 20 in PBS and once with 500 ml of 1% BSA (w/v) in PBS according to the method of Conradie, Voster and Kirk (1981) or manually twice with 1% BSA/PBS, 0.05% Tween 20 (v/v) and 4x with 1% BSA/PBS. A stock solution of 0.5 mg Protein A-alkaline phosphatase (Sigma), in 50 ml distilled water was prepared. Of this solution, 5 µl was diluted to 7.5 ml with 1% BSA (w/v) in PBS of which 50 µl was added to each well and incubated at room temperature for 40 min while shaking. The plate was washed 6 times with 100 µl per well of a 1% BSA (w/v) PBS solution.

The substrate buffer, contained 3.16 g of 2-amino-2-methyl-1:3-propanediol and 40.6mg $MgCl_2$ in 100 ml distilled water. To 10 ml of the freshly made substrate buffer, 35 mg p-nitro phenylphosphate (Sigma) was added. Of the substrate solution, 50 µl was added to each well and the colour development was monitored with a Titertek Multiscan MC (Flow Laboratories) at 690 and 406 nm for 30-60 min.

As controls for the ELISA, either non-infected material was used as antigen or antisera obtained from sheep injected with non-infected nymph homogenates.

5.2.10. Micro-immunodiffusion

In order to substantiate the cross reaction between the Kumm strain and the Ball 3 strain organisms, micro-immunodiffusion was employed.

Ouchterlony double micro-immunodiffusion (Ouchterlony, 1958) was carried out using 1% agarose (Miles Laboratories) in Dulbecco's phosphate buffered saline and 0.05% Tween 20 (v/v) (Merck). The agarose was heated in the PBS/Tween buffer until dissolved (±54°C) and cast into Petri dishes or onto microscope plates to a thickness of approximately 1 mm. Once the agarose had set, wells were made with a flat head 17 gauge needle at a distance of 2-2.5 mm from one another. Into the centre well, 3 µl serum, diluted 1:10 with the PBS/Tween buffer, was pipetted and into the outer wells, 3 µl antigen, sonified for 5 sec with a Branson Model B-30 sonifier at output set of 5, at a concentration of 0.1 mg/ml in the PBS/Tween buffer. In some cases antibodies were introduced into the outer wells and antigen into the centre well. The precipitin lines were allowed to develop overnight (± 16 h) at room temperature in a desiccator at a relative humidity of at least 80%. The wells were then filled with PBS and photographed with a Wild Heerburgg photo-microscope M-400 using a Ilford FP4 film with a matt red background.

5.3. RESULTS

5.3.1. Purification of *Cowdria ruminantium*

5.3.1.1. Lectin chromatography

Giemsa staining showed that the brains of the animals injected with arsenic, were not notably more infected with heartwater organisms than those where no arsenic was injected.

Except for protein quantity, no difference could be observed between the peak patterns of the chromatograms of sheep, goat, mouse organs or nymph crude extracts after chromatography on a wheat germ lectin-Sepharose 6 MB column (Fig 5.1(A)) and a Concanavalin A lectin-Sepharose 4B column (Fig 5.1(B)). A significant difference in the amount of protein bound to the column (peak 2) was observed, however, between extracts from infected and non- infected material. The Helix pomatia lectin-Sepharose 6 MB chromatogram (Fig 5.1(C)) showed only a slight difference with respect to protein quantity in peak 2 for infected and non-infected material. The heated biuret-Folin protein assay of Dorsey, *et al*. (1977) was found to be as reliable as the method of Lowry (Maddy, 1976). The protein content of heartwater infective and non-infective material is shown in Table 5.1.

5.3.1.2. Percoll density gradient centrifugation

Protein determinations of fractions obtained after Percoll density gradient centrifugation, revealed proteins in all the fractions with the content significally higher in the heartwater infected material in comparison to the controls. In all cases the protein content showed 3 peaks in the lower, middle and higher density regions respectively (Table 5.2).

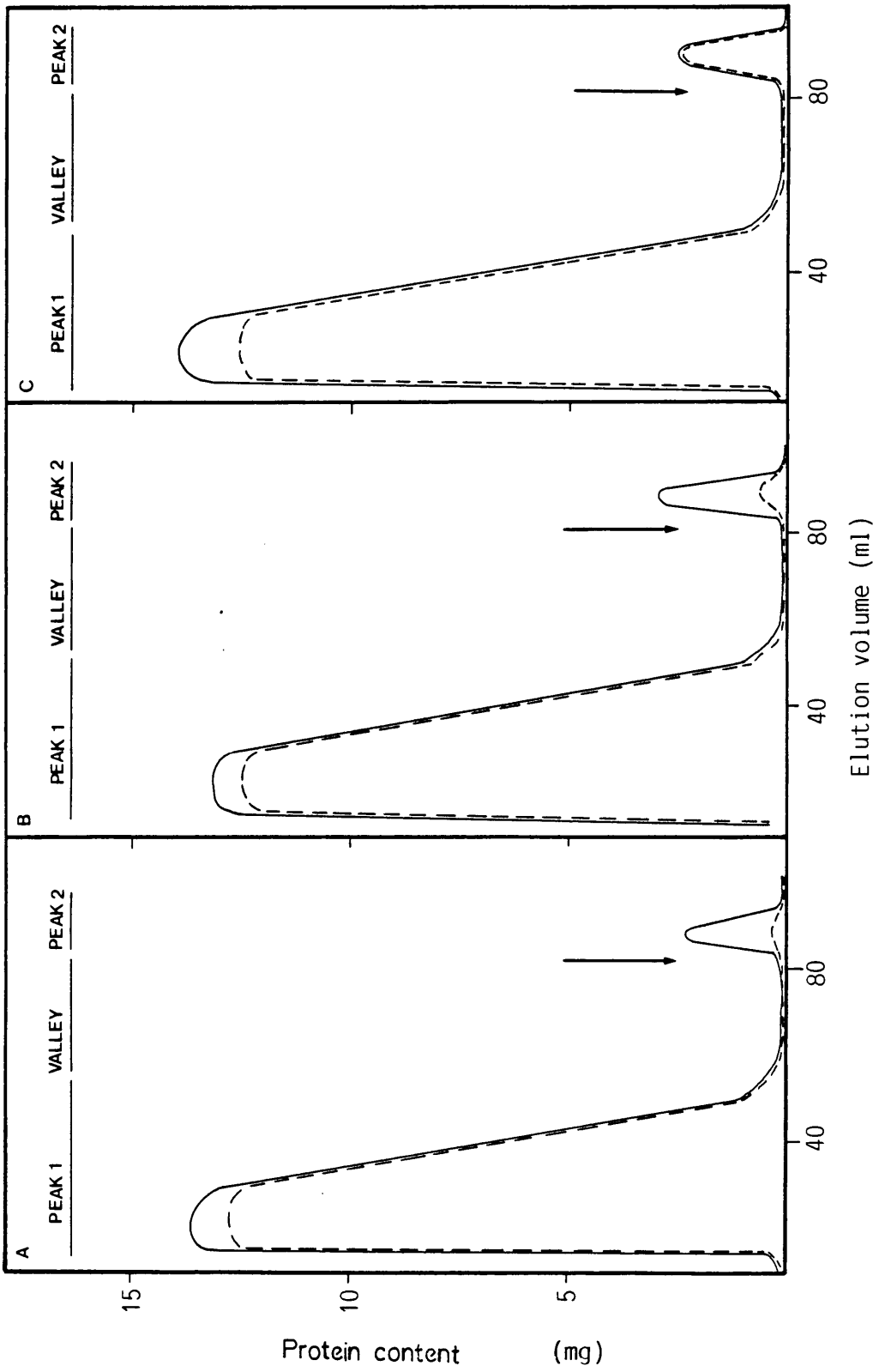


Fig 5.1. Representative chromatograms of infected (———) and non-infected (------) nymph crude extracts on (A) wheat germ lectin-Sepharose 6 MB, (B) Concanavalin A lectin-Sepharose 4B and (C) Helix pomatia lectin-Sepharose 6 MB. The arrows indicate the application of elution buffer containing the applicable carbohydrate at 1 g per 10 ml HEPES buffer. The Flow rate was 30 ml/h.

TABLE 5.1. Properties of heartwater infected and non-infected crude extracts and column fractions.

Source Amount	Protein content (mg)			
	Crude extract	Peak 1	Valley	Peak 2
Wheat germ lectin:				
(Ball 3 strain)				
Nymph :2400 NN +	3600[1]	3582[1]	0	13.5[1]
400 NN +	1919[3]	1860[3]	0	12.6[3]
200 NN +	843[1]	828[1]	0	11.7[1]
200 NN -	758[3]	750[3]	0	2.7[3]
200 NN -	712[3]	678[3]	0	2.1[3]
Brain :224g S +	1728[2]	1716[2]	0	11.0[5]
207g S +	1476[2]	1452[2]	0	12.3[5]
122g S +	864[4]	846[3]	0	12.7[3]
134g S +[7]	912[3]	892[3]	-	15.6[3]
187g G +	996[4]	982[2]	0	11.3[2]
89g G +	708[2]	694[2]	0	12.9[2]
214g S -	1452[3]	1446[3]	0	2.6[3]
197g S -	1164[3]	1158[3]	0	2.7[3]
(Kumm strain)				
Liver/				
spleen :12g M +	29[6]	24[6]	0	4.5[6]
9g M -	27[3]	25[3]	0	1.4[3]
15g M +	32[3]	25[3]	0	6.2[3]
Concanavalin-A lectin:				
(Ball 3 strain)				
Nymph : 100 NN +	387[1]	362[2]	0	21.3[1]
25 NN +	95[1]	85[2]	0	7.2[1]
100 NN -	329[3]	325[3]	0	4.1[3]
Helix pomatia lectin:				
(Ball 3 strain)				
Nymph : 100 NN +	362[1]	346[1]	0	9.5[2]
25 NN +	89[1]	81[3]	0	3.1[2]
100 NN -	297[3]	281[3]	0	8.9[3]
Total volume/ fraction:	10 ml	60 ml	30 ml	9 ml

M - Mouse liver and spleen material
NN - Engorged *A. hebraeum* (Spes Bona strain) nymphae
S - Sheep brain material
G - Goat brain material
\+ - Heartwater infected material
\- - Heartwater non-infected material
[1] - Heartwater infective as determined in sheep
[2] - Heartwater non-infective as determined in sheep
[3] - Biologically not tested
[4] - Died within 24 hours after injection
[5] - Sheep showed for one day high rectal temperature, but had no heartwater protection when challenged
[6] - Heartwater infective as determined in mice
[7] - Batch method (peak 1 represent the non-adsorbed fraction and peak 2 the adsorbed fraction)

TABLE 5.2. Protein content of heartwater infected and non-infected brain or nymph material after Percoll density gradient centrifugation

	Protein content (mg)			
Percoll fractions	Brain material P[1]	N[2]	Nymph material P[1]	N[2]
Crude extracts	9.0±0.2	1.5 ±0.15	8.0±0.3	0.7 ±0.1
1	1.3±0.1	0.08±0.02	0.9±0.1	0.02±0.01
2	0.2±0.1	0.06±0.03	0.3±0.1	0.01±0.01
3	0.7±0.15	0.09±0.03	0.4±0.05	0.02±0.01
4	0.4±0.05	0.07±0.03	0.4±0.03	0.03±0.01
5	1.1±0.1	0.10±0.03	0.9±0.05	0.08±0.02
6	1.2±0.2	0.11±0.04	1.2±0.1	0.1 ±0.01
7	0.6±0.15	0.05±0.03	0.7±0.07	0.05±0.01
8	0.5±0.2	0.04±0.03	0.6±0.03	0.02±0.01
9	1.4±0.15	0.28±0.04	1.1±0.1	0.1 ±0.02
10	1.5±0.2	0.42±0.04	1.3±0.2	0.2 ±0.03

P - Heartwater infected (experiment II and V from Table 5.3)
N - Hearwater non-infected
[1] - All fractions obtained from heartwater infected fractions contained *Cowdria ruminantium*-like organisms according to electron-microscopy
[2] - No heartwater suspected organisms were found in the non-infected material according to electron-microscopy

5.3.2. Biological tests

5.3.2.1. Lectin chromatography

Sheep injected with crude brain extracts, showed no heartwater symptoms during the entire observation period of 24 days. Sheep injected with column fractions (Fig 5.1) from brain material (Table 5.1) however, exhibited a high body temperature in two cases only, one on the 8 th and one on the 20 th day after injection. The high temperature lasted in both cases for 24 h only (Table 5.1). These animals were not protected against heartwater when challenged. On the other hand, the infected nymph crude extracts and column fractions (peak 1 and 2 in Fig 5.1) were highly infective (Table 5.1). Sheep injected with infected nymph column fractions developed a high constant rectal temperature from as early as the 10 th day after injection. Typical heartwater colonies were

observed with Giemsa staining in the endothelial cells of brain smears obtained from these animals.

Sheep injected with the valley fraction obtained from infected as well as crude extracts and column fractions obtained from non- infected nymphae (Table 5.1) failed to show a temperature reaction and became diseased when challenged and died. Mice inoculated with the Kumm strain fractions reacted to heartwater as indicated in Table 5.1. The Kumm strain crude extracts showed a LD_{50} at a dilution of 1/790, and peak 2 at a dilution of 1/630. With the addition of metals, at the same concentration as was required for the other two lectin columns, wheat germ lectin exhibited the same binding capacity with respect to protein content of the bound peak.

5.3.2.2. Percoll density gradient

All infected Ball 3 strain crude brain extracts and subsequent gradient fractions were infective after iv inoculation (Table 5.3) into sheep. The Kumm strain infected crude liver and spleen extracts and density gradient fractions were infective after ip inoculation. The Kumm strain fractions were titrated using mice. For practical reasons the original 10 Percoll density gradient fractions were pooled (Table 5.3). The infectivity (LD_{50}) of the starting material, titrated at 1/790 and 1/10 000, correlated well with the infectivity of the isolated density gradient fractions (Table 5.3), as titrated in mice.

TABLE 5.3. Infectivity of Percoll density gradient fractions

		Ball 3 strain					Kumm strain	
Percoll fractions	Density g/ml	Sheep brain material		*Amblyomma hebraeum* Spes Bona strain			Mouse liver and spleen	
		I	II*	III	IV	V*	VI[1]	VII[1]
Starting material		A	A	A	A	A	10 000	790
Crude extract		+ (14)	+ (17)	A	A	A	A	
1	1.134± 0.008	+ (12)	+ (11)	+ (11)	+ (18)	+ (10)	2512	166
2	1,109± 0.017	+ (11)	+ (10)	+ (8)	+ (18)	+ (8)		
5	1.078± 0.013	+ (13)	+ (11)	+ (8)	+ (17)	+ (7)		
4	1.062± 0.003	-	+ (12)	+ (10)	-	+ (7)	3163	398
5	1.058± 0.001	-	+ (10)	+ (10)	-	+ (8)		
6	1.056± 0.001	-	+ (11)	+ (8)	-	+ (9)	2512	158
7	1.054± 0.002	-	+ (12)	+ (8)	-	+ (8)		
8	1.050± 0.002	+ (13)	+ (12)	+ (11)	-	+ (9)	1585	106
9	1.045± 0.004	+ (10)	+ (10)	0	+ (17)	+ (8)		
10	1,034± 0.007	+ (11)	+ (10)	0	-	+ (8)		

The day on which the first heartwater symptoms (constant high rectal temperature) appeared, is given in parentheses
I to V1 - Experiment number; I - V: tested in sheep as experimental animals, VI and VII: in mice as experimental animals
+ - Positive reaction (raised temperature)
- - No reaction in sheep (temperature normal during incubation period). Heartwater positive reaction on challenge
A - Not biologically tested
0 - Sheep died c.15 min after injection, probably as a result of anaphylactic shock
* - Investigated electron-microscopically (see Table 5.2)
[1] - Fractions were pooled as follows: fractions 1,2 and 3, fractions 4 and 5; fractions 6 and 7 and fractions 8,9 and 10 and titrated in mice. Dilutioned factors of the pooled fractions which caused 50% mortality in mice are shown in the table

5.3.3. Enzyme-linked immunosorbent assay

The ELISA method using Protein A-alkaline phosphatase as indicator reagent, was optimized with respect to coating efficiency of antigen by sonification (Table 5.4), concentration manipulation (Table 5.5) and lectin affinity enrichment (Table 5.6). Absorbance signals were found to be more than twice the background with 1/30 dilutions of antiserum (Table 5.6). Ratios obtained for infected material to reagent background were at all times larger than 6:1. Purified IgG fractions obtained from immune sera further improved this signal to almost thrice the background (Table 5.6).

ELISA screening of fractions derived from the lectin column and its starting material, tending to all the above-mentioned modifications resulted in data which were in accordance with what can reasonably be expected from such an affinity purification (Table 5.6).

After Percoll density gradient centrifugation of crude heartwater suspensions, *C. ruminantium* organisms and antigen were dispersed widely in the gradient according to biological tests (Table 5.3) and ELISA methods (Table 5.7). With ELISA (Table 5.7), the highest infected to non-infected (P/N) ratios were observed at densities of 1,109 g/ml, 1,058 g/ml and 1,050 g/ml.

No difference was observed, according to ELISA between wheat-germ lectin peak 1 and peak 2 after Percoll density gradient centrifugation.

TABLE 5.4. Determination of the effect of sonification on adsorption of the antigens[1] to Linbro microtiter plates by ELISA

	Origin of antigen material[2]			
	Brain[3]		Nymph[3]	
	Non-sonified	Sonified	Non-sonified	Sonified
Crude extract	1.39±0.38	1.74±0.47	1.21±0.31	1.46±0.32
Lectin column Peak 1	1.64±0.34	1.97±0.37	1.43±0.37	1.70±0.29
Lectin column Peak 2	2.06±0.42	2.55±0.41	1.81±0.34	2.28±0.31

[1] - n=6
[2] - Ratios for infected sheep and nymph material to non-infected material, tested with infected sheep serum.
[3] - Antigen concentrations: 10µg protein/ml

Table 5.5. Determination of optimal antigen adsorption[1] to a Linbro microtiter plate by ELISA

	Origin of antigen material[2]					
Fraction	Brain (µg protein/ml) 3	10	100	Nymph (µg protein/ml) 3	10	100
Crude extract	1.42± 0.37	1.74± 0.47	1.53± 0.41	1.22± 0.37	1.46± 0.32	1.32± 0.4
Lectin column Peak 1	1.62± 0.32	1.97± 0.37	1.72± 0.44	1.47± 0.33	1.70± 0.29	1.60± 0.34
Lectin column Peak 2	2.23± 0.39	2.55± 0.41	2.40± 0.43	2.02± 0.38	2.28± 0.31	2.14± 0.38

[1] - n=5
[2] - Ratios for infected to non-infected material, tested with infected sheep serum

TABLE 5.6. Determination of *Cowdria ruminatium* in lectin affinity chromatography by ELISA[1].

Antigen source	Antibody source	Crude extract	Peak 1	Valley	Peak 2
Wheat germ:					
Ball 3 strain					
brain	Isolated IgG	1.83 ± 0.4	1.89 ± 0.4	1.00 ± 0.1	2.80 ± 0.5
	Infected serum[2]	1.74 ± 0.5	1.97 ± 0.4	1.00 ± 0.1	2.55 ± 0.4
	Non-infected serum[3]	1.00 ± 0.1	1.00 ± 0.1	1.00 ± 0.1	1.00 ± 0.1
Ball 3 strain					
nymph	Infected serum[2]	1.46 ± 0.3	1.36 ± 0.3	1.02 ± 0.1	2.28 ± 0.3
	Non-infected serum[3]	1.00 ± 0.1	1.00 ± 0.1	1.00 ± 0.1	1.01 ± 0.1
Ball 3 strain					
nymph[7]	Infected serum[4]	1.43 ± 0.2	1.32 ± 0.2	1.00 ± 0.1	2.49 + 0.1
Kumm strain					
liver/spleen[5]	Infected serum[4]	1.30 ± 0.2	1.33 ± 0.3	1.05 ± 0.1	2.78 ± 0.2
	Infected serum[6]	1.23 ± 0.1	1.28 ± 0.1	1.01 ± 0.1	2.32 ± 0.3
Concanavalin-A:					
Ball 3 strain					
nymph	Infected serum[2]	1.31 ± 0.1	1.12 ± 0.2	1.05 ± 0.1	3.15 ± 0.3
	Non-infected serum[3]	1.00 ± 0.1	1.01 ± 0.1	1.00 ± 0.1	1.03 ± 0.1
Helix pomatia:					
Ball 3 strain					
nymph	Infected serum[2]	1.42 ± 0.1	1.52 ± 0.2	1.00 ± 0.1	1.12 ± 0.2
	Non-infected serum[3]	1.02 ± 0.1	1.01 ± 0.1	1.00 ± 0.1	1.00 ± 0.1

1 - n=6

2 - Serum from Onderstepoort Ball 3 vaccine, inoculated sheep.

3 - Serum from non-infected Ball 3 nymphae homogenate, inoculated sheep.

4 - Serum from infected Kumm strain mouse organ homogenates, inoculated sheep.

5 - Kumm strain infected liver and spleen homogenates as signal and non-infected liver and spleen homogenates as background.

6 - Serum from infected Kumm strain mouse organ homogenates, inoculated mice.

7 - Ball 3 strain infected nymphae as signal and non-infected nymphae as background.

Table 5.7. ELISA detection[1] of heartwater organisms after Percoll density gradient centrifugation.

Percoll fraction	Density g/ml	Brain material	Nymph material
		P/N ratio[2]	P/N ratio[2]
Crude extracts		1.53 ± 0.6	1.82 ± 0.5
1	1.134 ± 0.008	1.36 ± 0.3	1.48 ± 0.2
2	1.109 ± 0.017	2.30 ± 0.3	2.72 ± 0.5
3	1.078 ± 0.013	1.54 ± 0.4	1.84 ± 0.5
4	1.062 ± 0.003	1.70 ± 0.3	1.61 ± 0.4
5	1.058 ± 0.001	2.21 ± 0.5	2.36 ± 0.4
6	1.056 ± 0.001	1.1 ± 0.2	1.88 ± 0.5
7	1.054 ± 0.002	1.1 ± 0.1	1.55 ± 0.2
8	1.050 ± 0.002	2.1 ± 0.4	2.54 ± 0.4
9	1.045 ± 0.004	1.2 ± 0.4	1.55 ± 0.3
10	1.034 ± 0.007	1.2 ± 0.5	1.42 ± 0.6

[1] - n = 6
[2] - Heartwater infected to heartwater non-infected ratio.

5.3.4. Electron-microscopy

5.3.4.1. Lectin chromatography

In the Peak 1 fraction of 2 batches infected nymphae and in Peak 2 fraction of 3 batches infected sheep brain, a low concentration of suspected *C. ruminantium* organisms were noted. They were oval to coccoid in shape, and ranged in size from 0.36 - 0.8 µm in diameter. Each organism was enveloped by a double membrane. The inner electron-dense membrane was surrounded by an electron-transparent layer (c. 20-30 nm) which often had a rippled appearance (Fig 5.2(A). The outer membrane appears to be less electron-dense. The inner structure of the organism consisted of electron-dense and electron-transparent areas and no specific distribution pattern was evident. Occasionally fine fibrillar material was visible in the electron-translucent areas. Organisms undergoing binary fusion were infrequently seen (Fig 5.2(B) and Fig 5.2(C).

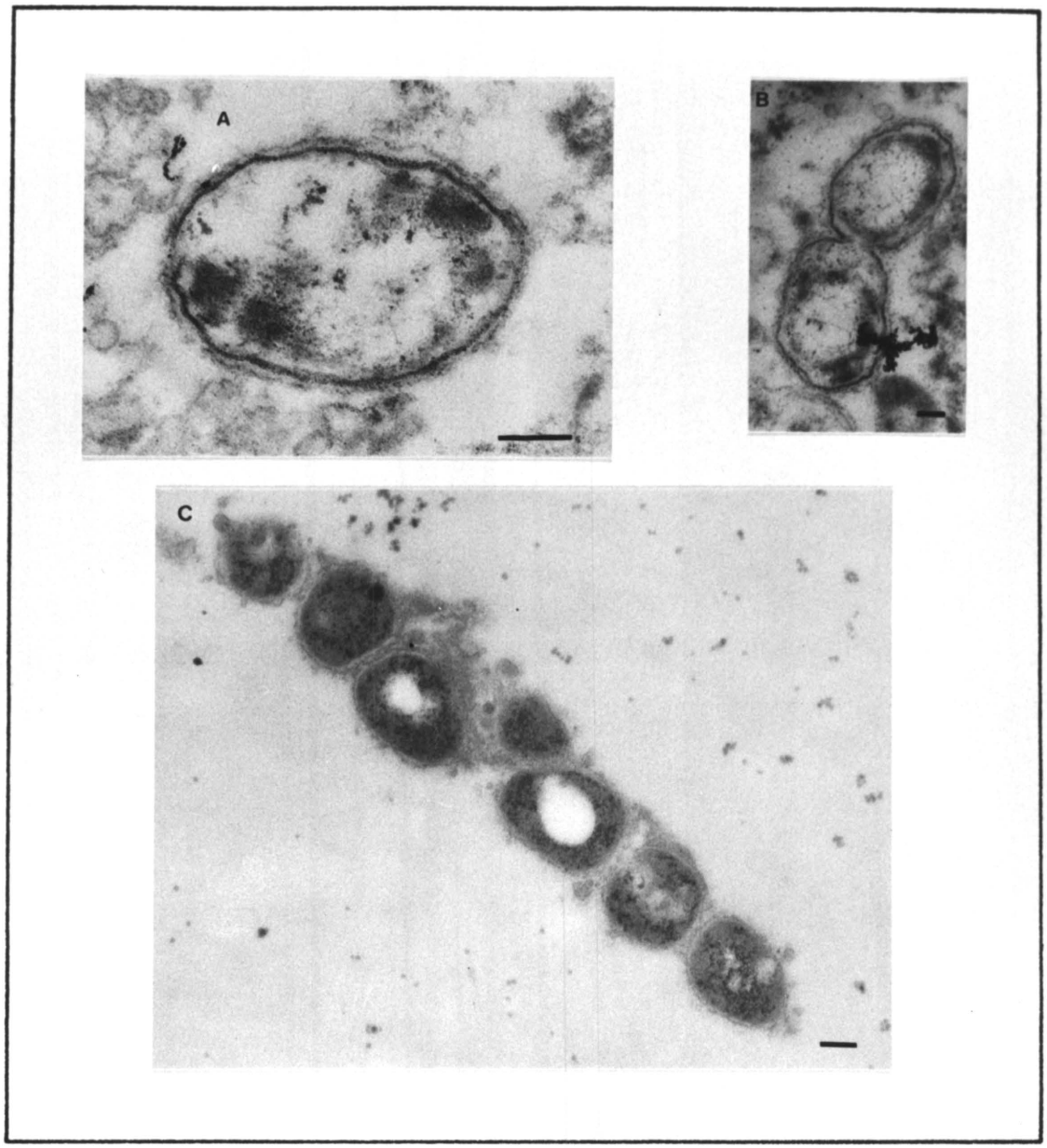


Fig 5.2 Electron micrographs of typical *Cowdria ruminantium* organisms isolated on a wheat germ lectin Sepharose 6 MB column.

(A) Infected *Amblyomma hebraeum* nymphae as starting material;

(B) Infected *A. hebraeum* nymphae as starting material;

(C) Infected sheep brain as starting material.

Bar scale = 0.1 µm

Although the batch method, which involved incubating the sample with the wheat germ lectin gel in a glass beaker, gave a higher protein yield (Table 5.1), it was not suitable as an isolation method. The electron-microscopic investigation revealed myelin sheaths to be present in the bound peak.

5.3.4.2. Percoll density gradient centrifugation

Suspected *C. ruminantium* organisms were seen in all density gradient fractions according to electron-microscopic investigations (Fig's 5.3 and Fig 5.4 and Table 5.3). In non-infected brain material very little or no background was observed electron-microscopically. In the case of non-infected nymph material, unidentified rickettsial organisms other than *C. ruminantium* were observed.

Although a limited number of organisms were studied, it appeared that different sizes of heartwater organisms were present at different densities: An increase in organism size was observed with a decrease in density. The smaller ones (0.45 ± 0.15 µm) at a density of 1.134 g/ml and the larger (1.00 ± 0.2 µm) at a density of 1.034 g/ml. No significant ultra- structural differences were observed between the different organisms sizes. It is however interesting to note that ultrastructural differences were observed between the organisms present in infected brain and nymph material. The infected mouse material was not studied electron-microscopically. Organisms obtained from brain tissue demonstrated the presence of defined electron-transparent regions in the electron micrograph (Fig 5.3), whereas, organisms isolated from infected nymph material, displayed several dispersed electron- transparent areas (Fig 5.4).

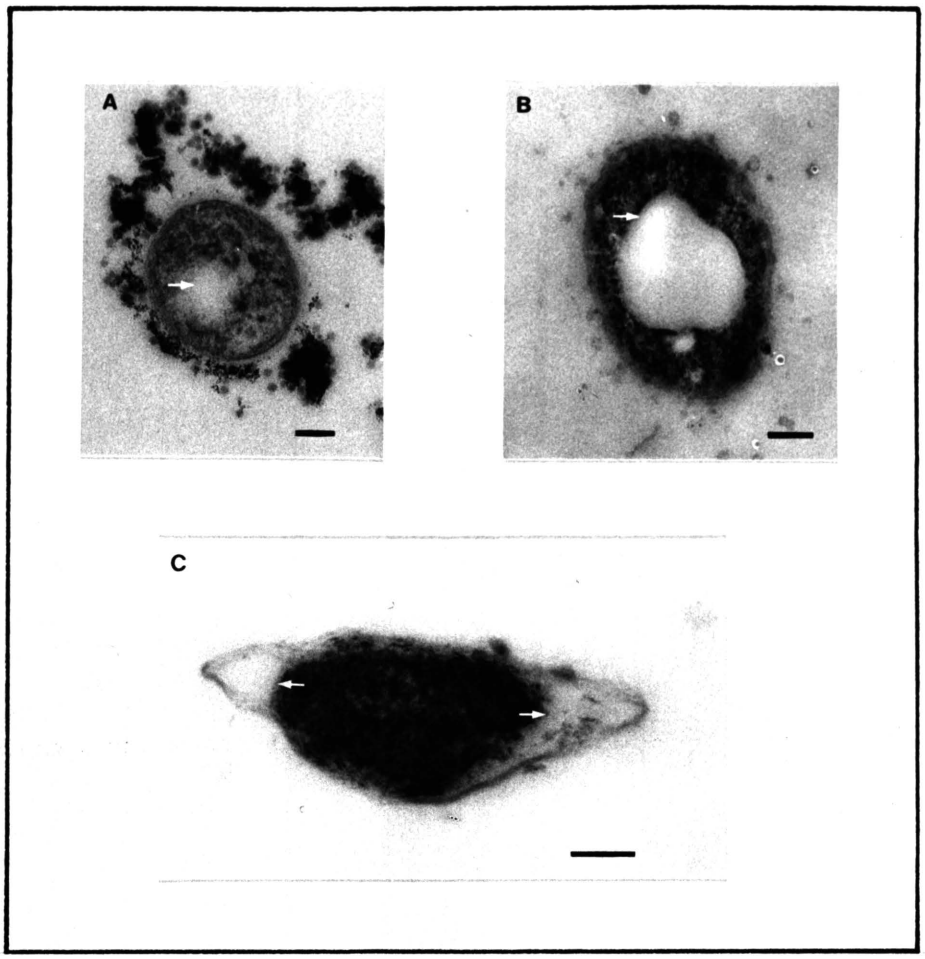


Fig 5.3 Electron micrographs of suspected *Cowdria ruminantium* organisms isolated on a Percoll density gradient starting with heartwater infected sheep brain material.

(A) Fraction 2 (1.109 ± 0.017 g/ml);

(B) Fraction 5 (1.058 ± 0.001 g/ml);

(C) Fraction 8 (1.050 ± 0.002 g/ml)).

Arrows indicate defined electron-transparent areas.

Bar scale = 0.1µm

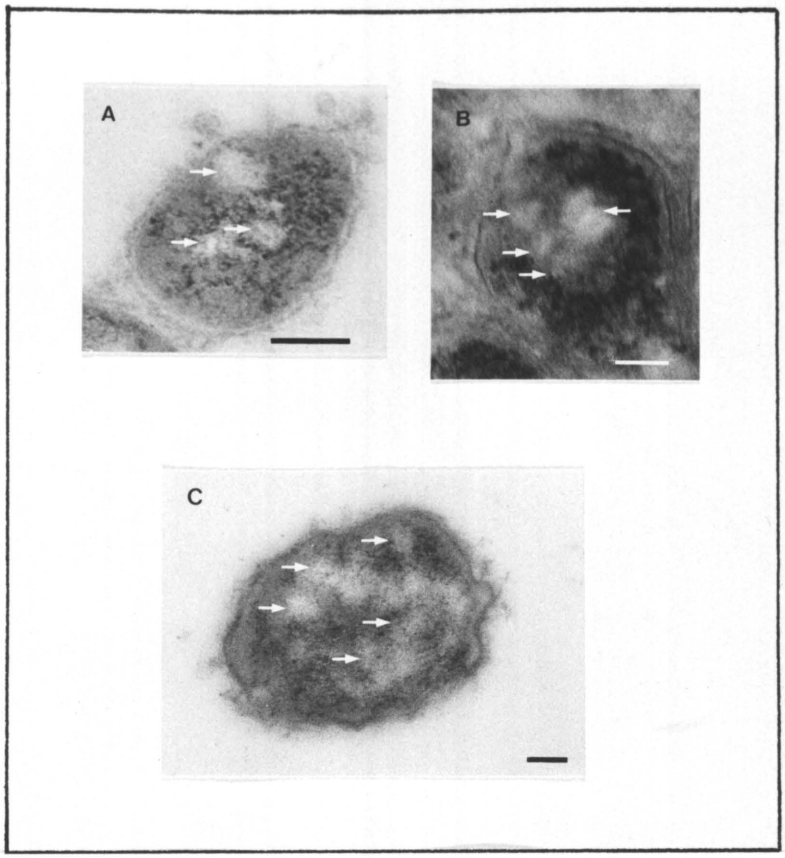


Fig 5.4 Electron micrographs of typical *Cowdria ruminantium* (Ball 3 strain) organisms isolated on a Percoll density gradient starting with heartwater infected *Amblyomma hebraeum* nymphae material.

(A) Fraction 2 (1,109 ± 0,017 g/ml);

(B) Fraction 5 (1,058 ± 0,01 g/ml);

(C) Fraction 8 (1,050 ± 0,002 g/ml).

Arrows indicate some scattered electron-transparant areas.

Bar scale = 0.1µm

5.3.5. Micro-immunodifusion

Without the addition of the detergent Tween 20, the crude extracts and bound lectin affinity chromatography fractions were unable to penetrate the gel and no immunological relationships between the different antigens were observed (Fig 5.5(A)). The addition of detergent solubilized the membranes of the organisms, enabling them to penetrate the stabilizing gel (Fig 5.5(B)). The wheat germ lectin peak 2 fractions of Ball 3 strain and Kumm strain infected tissue showed absolute identity (Fig's 5.5(C) and 5.5(D)). Crude extracts from the Ball 3 strain and Kumm strain infected material showed no precipitin bands in the presence of Tween 20, most probably as the result of too low concentration.

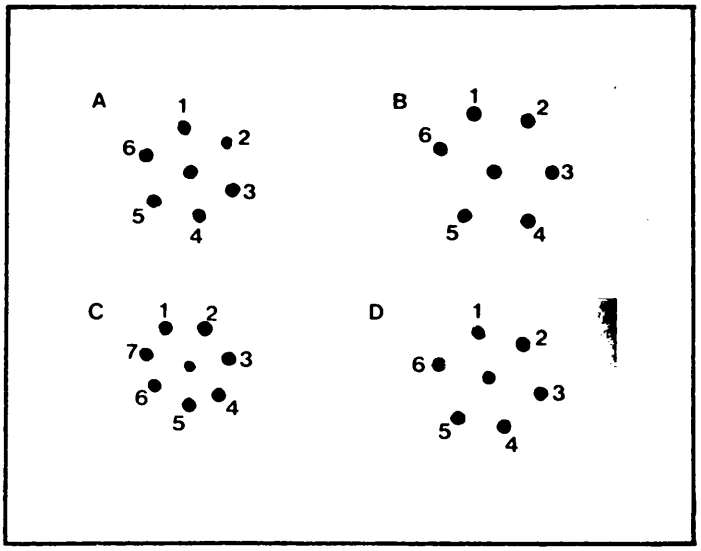


Fig 5.5 Ouchterlony double micro-immunodiffusion to test cross reaction between Ball 3 and Kumm strains of ***Cowdria ruminantium***. In all cases antigens were obtained by wheat germ lectin affinity chromatography.

(A) Centre well: Ball 3 strain infected sheep brain without Tween 20; wells 1,3 and 5: mouse anti-Kumm strain serum; wells 2,4 and 6: sheep anti-Ball 3 strain serum.

(B) Centre well: Ball 3 strain infected sheep brain in the presence of Tween 20; wells 1,3 and 5: mouse anti-Kumm strain serum; wells 2,4 and 6: sheep anti-Ball 3 strain serum.

(C) Centre well: sheep anti-Ball 3 strain serum; well 1: Ball 3 strain infected sheep brain as antigen; well 2: non-infected sheep brain as antigen (as control); well 3: Ball 3 strain infected ***Amblyomma hebraeum*** as antigen; well 4: non-infected ***A. hebraeum*** as antigen (as control); well 5: Kumm strain infected mouse liver and spleen as antigen; well 6: non-infected mouse liver and spleen as antigen (as control); well 7: Tween 20.

(D) Centre well: Kumm strain infected mouse serum; well 1: non-infected sheep brain as antigen (as control); well 2: Ball 3 strain infected sheep brain as antigen; well 3: non-infected ***A. hebraeum*** as antigen (as control); well 4: Ball 3 strain infected ***A. hebraeum*** as antigen; well 5: non-infected mouse liver and spleen as antigen (as control); well 6: Kumm strain infected mouse liver and spleen as antigen.

Tween 20 was always added to antigens in (C) and (D).

Enlargement : 2X

5.4. DISCUSSION

It is generally recognized that the purification of *C. ruminantium* is more difficult than the purification of other rickettsiae. This is due to *C. ruminantium* being a particularly labile organism (Uilenberg, 1983). The inability to count the individual organisms with standard procedures for example, by means of a coulter counter or a light-microscope, made it difficult to establish organism concentration. Protein content was used as a concentration indication.

Lectin chromatography proved to be a suitable isolation method for obtaining partially purified *C. ruminantium* in a short period of time. With the use of Percoll density gradient centrifugation on the other hand, it is possible to discriminate between the various forms and sizes of the organism present in infected material.

Brain material from heartwater infected sheep was initially used as source of *C. ruminantium* since it was reasoned that this organism would be the only rickettsia-like organism present in infected brain. However, variable infectivity of brain crude extracts as demonstrated by iv or in one case sc injection into sheep suggested that this starting material has its limitations. The inconsistent results could be due to too low concentrations or avirulent forms of the organism in the test material. Nevertheless, *C. ruminantium* organisms or derived antigens were shown to be present in these tissues by electron-microscopic investigations and ELISA respectively. In an attempt to increase the proliferation of *C. ruminantium* in the brains of experimental animals, some test animals were treated with an arsenic containing tonic prior to inoculation (Neitz, 1940). No enhancement of infectivity through this treatment could be obtained.

Of interest is that the present work has indicated infected crude brain extracts to be infective on several occasions when inoculated iv. Only on one occasion has this route of injection of brain material been proven to be successful in transmitting heartwater (Uilenberg, 1971). This is contrary to the results of Ilemobade and Blotkamp (1976) who were able to cause infection by infected brain homogenates through the sc route only. Iv inoculations either failed to cause disease or resulted in immediate fatalities. These contradicting findings could possibly be explained by the fact that our crude brain extracts were prepared by differential centrifugation of brain homogenates. This indicates that certain unknown components in brain homogenates may prevent infectivity by the iv route.

Because of the variable infectivity of the crude brain extracts, an alternative source of the organism was sought. Heartwater infected nymphae and mouse liver and spleen proved to be suitable. Crude extracts and column fractions obtained from these sources caused typical heartwater symptoms when injected iv into sheep or ip into mice.

Affinity chromatography using wheat germ lectin or Concanavalin A lectin as ligand exhibited no difference in the elution patterns of brain, liver and spleen or nymph material. A large quantitative difference in protein content of the bound peak of positive material in comparison to negative controls in the case of wheat germ lectin and Concanavalin A lectin affinity chromatography, indicates that these 2 columns are suitable for the purification of *C. ruminantium*. On the other hand, the elution patterns of infected as well as non-infected material on the Helix pomatia lectin affinity chromatography column were similar. Furthermore, the bound peak was negative with respect to ELISA and infectivity tests whereas the unbound peak was positive. The suitability of wheat germ lectin affinity chromatography for the isolation of *C. ruminantium* was substantiated by electron-microscopic investigations, ELISA and infectivity tests,

while the suitability of Concanavalin A lectin affinity chromatography was confirmed by ELISA and infectivity tests.

Wheat germ lectin was first employed on the grounds of its N-acetyl-D-glucosamine binding specificity. The study was further extended to Concanavalin A and Helix pomatia lectins (the only two lectins available at the time of this study) to investigate the possibility of an other lectin that binds *C. ruminantium* more effectivily. Concanavalin A demonstrated, according to protein content of the column bound peak, show a higher binding capacity towards *C. ruminantium*. This could however, also mean the binding of other material since this lectin binds α-D-mannopyranose, α-D- glycopyranose, D-fructofuranose, their glycosides and sterically related structures (Lis and Sharon, 1973).

The morphology and size of the organisms in the peak 1 fraction of 2 batches of infected nymphae and the peak 2 fraction of 3 infected sheep brain closely resembled those of *C. ruminantium* described in sheep and mice (Pienaar, 1970; Prozesky and Du Plessis, 1985).

The location of defined electron-transparent areas observed in electron micrographs of *C. ruminantium*-like organisms from brain has also been reported by Prozesky and Du Plessis (1985). In organisms from nymph material such defined, single electron-transparent areas were absent. Although caution should be exercised when identifying *C. ruminantium* on the basis of morphology of single organisms, the infectivity and ELISA of the nymphae fractions and the absence of the organisms in the controls, serve as additional evidence that the organisms are most probably *C. ruminantium*. The difficulty encountered in demonstrating heartwater organisms by ultrastructural methods in fractions of the infected peak 2 from nymphae may be attributable to a too low concentration of organisms. This could in part be due to dilution of the organism sample by the addition of agar. Without the stabilizing effect of the agar, the organisms

were disrupted and they disintegrated on the addition of glutaraldehyde. Furthermore, the presence of material of variable electron-density and morphology made the identification of suspected *C. ruminantium* organisms extremely difficult. The presence of *C. ruminantium* in the unbound peak is probably due to overloading of the column because the protein content of the bound peak remained reasonably constant irrespective of the number of nymphae used as starting material. Another consideration is the omission of mechanical agitation during affinity adsorption, which has been reported to optimize and enhance binding of the cells to the stationary phase (Kinzel, Richards and Kubler, 1977; Sharma and Mahendroo, 1980). Apart from inducing maximum contact between cells and the lectin, the time required for binding would possibly also be reduced. Such a modification in the purification procedure rendered a higher protein quantity in the bound peak. Electron-microscopical investigations showed the presence of myelin sheaths which may account for the higher protein content. The addition of metals to the elution buffer in the case of wheat germ lectin chromatography rendered no enhancement of the binding capacity of this lectin (Nagata and Burger, 1974). The other two lectin columns however required the addition of metal ions in the buffers (Lis and Sharon, 1973).

The low signal to noise values and titers obtained with ELISA- screening of infected tissues is probably, due to the complexity of the antigen. Nevertheless, the values obtained were reproducible and the technique was found useful as an additional and rapid assay for biological activity from the lectin affinity column. The results in Table 5.6 show evidence that at least some activity was retained on the column, which could be eluted as an enriched fraction. In addition to the protein determinations which indicated overloading of the column it is also possible that the column was unable to retain antigenic fragments lacking the carbohydrate moiety which is recognized by the lectin.

Lectin affinity purification of the infected tissue extracts therefore yields an improved antigen for the use in ELISA screening of sera for the detection of *C. ruminantium* specific antibodies. This may serve as base for the development of monoclonal antibodies to single *C. ruminantium* specific determinants, the application of which should further increase the specificity and sensitivity of this ELISA procedure. This should prove to be usefull in screening nymph material for the presence of *C. ruminantium* and in improving serodiagnostic identification of heartwater disease.

Absolute immunological identity between the *C. ruminantium* Ball 3 strain obtained from sheep brain and nymphae as well as the Kumm strain from mouse liver and spleen was observed according to Ouchterlony double micro-immunodiffusion. It was however, not expected to have only one precipitin band of identity between the two strains. This could nevertheless be the case if the migrating critical concentration of the antigens which react with both the more and the less specific antibodies, is ahead of that of the other antigens (Ouchterlony, 1958).

The isolation of *C. ruminantium* by means of Percoll density gradient centrifugation, permits the recovery of partially purified viable organisms possessing different densities in the range from 1.134 g/ml to 1.034 g/ml. This exemplifies the pleomorphism of this organism, or the presence of organisms in different stages of development. Similar observations were made for *Rickettsia tsutsugamushi* (Tamura, *et al.*, 1982).

The fractions obtained at densities of 1,109; 1,058 and 1,050 g/ml, showed an ELISA infected to non-infected ratio of more than 2, suggesting that the antigenic material was more concentrated at these levels. Protein content was found to be a maximum at densities of 1,134; 1,078; 1;058; 1,056; 1;045 and 1;034 g/ml. Infectivity incubation periods could not be correlated with ELISA colour development or

protein content. Protein determinations showed that the infected fractions had a higher protein content in comparison with the non-infected fractions, indicating that the protein content of these fractions relates to the infected state of the tissue. This observation is based on equal wet frozen weight of brain and liver and spleen material and equal amounts of infected and non-infected nymphae. However, a simple relationship could not be revealed between organism concentration (protein content), antigenicity (ELISA) and pathogenicity (incubation period).

Expectedly, electron-microscopy of the infected nymph material obtained from Percoll density gradient centrifugation showed contaminating micro-organisms, probably belonging to the *Rickettsiaceae* family described by Cowdry (1925). This makes brain material, where no contaminating organisms could be detected, the better source of starting material for the purification of *C. ruminantium*.

Both isolation procedures described in this chapter render quick and mild methods by which *C. ruminantium* organisms can be obtained in a partially purified and viable form as observed with electron-microscopic studies, ELISA and infectivity assays. Alternatively, such preparations have been employed for the detection of antibodies to *C. ruminantium* (as described in Chapters 6 and 7).

CHAPTER 6

THE DETECTION OF ANTIBODIES TO *COWDRIA RUMINANTIUM* IN SERUM AND *C. RUMINANTIUM* ANTIGEN IN *AMBLYOMMA HEBRAEUM* NYMPHAE BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

6.1. INTRODUCTION

To study the immunological aspects of heartwater, it is important to be able to detect antibodies to *Cowdria ruminantium* in the serum of infected hosts (Alexander, 1931; Neitz, 1939; Du Plessis, 1970a). This could also be of aid in the diagnosis of the disease. For epizootiological investigations of the disease, the antibodies may be employed to monitor the presence of the organism in the tick vector, *Amblyomma hebraeum*.

Both Du Plessis (1970b) and Ilemobade (1976) had negative or inconclusive results using an indirect fluorescent antibody (IFA) test with squashed brain material as antigen for the detection of antibody to *C. ruminantium* in serum. However, Du Plessis (1981) was successful with an IFA test employing peritoneal cells of mice infected with a mouse infective strain (Du Plessis and Kumm, 1971) as antigen. A simple, useful capillary flocculation test using crude brain material as antigen has also been reported (Ilemobade, 1976; Ilemobade and Blotkamp, 1976). They were able to demonstrate antibodies to *C. ruminantium* one to two weeks after clinical recovery or after treatment which persisted for periods varying between one and four weeks.

Since the sensitivity and specificity of serological tests depend on the source and the method employed for the preparation of the antigen as well as on the detection method for antibody- antigen interaction, alternative tests were investigated. In this chapter an ELISA is described using as antigen *C. ruminantium* from *A. hebraeum* nymphae, partially purified by wheat germ lectin affinity chromatography and anti-IgG peroxidase conjugate as indicator. In addition, the screening of *C. ruminantium* antigen in *A. hebraeum* nymphae by an ELISA was undertaken. The latter study could be of value in the study of the infection rate of tick populations, which is one of the factors governing the epizootiology of heartwater (Uilenberg, 1983). Furthermore, an indication of the infectivity of nymph suspensions used for vaccination (Bezuidenhout, 1981) could be obtained.

6.2. MATERIALS AND METHODS

Reagents and glassware were used as described in Chapter 5

6.2.1. Detection of antibodies to *Cowdria ruminantium* in serum

6.2.1.1. Preparation of antigen

C. ruminantium antigen was prepared from infected *A. hebraeum* nymphae using wheat germ lectin affinity chromatography (see paragraph 5.2.2., p 115). Heartwater non-infected *A. hebraeum* nymphae were used as control.

6.2.1.2. Preparation of sera

Sera from 41 heartwater-infected and 14 susceptible sheep (not infected) and 9 heartwater-infected cattle were used for serum screenings. Sheep were infected with 1 dose of the Onderstepoort Ball 3 heartwater blood vaccine (Bezuidenhout, 1981). Except for one sheep which had been inoculated sc the dosage was by iv injection. Animals which showed no rise in rectal temperature during the 24-day observation period, were challenged with the vaccine. Reacting sheep were treated by intramuscular (im) injection with long acting oxytetracycline (liquamycine LA) from Pfizer, at a dosage rate of 10 mg/kg. All the sheep were then boosted 17 weeks after the initial inoculation with the vaccine. Sera were collected 5 weeks after the final challenge.

Six lambs under 3 weeks of age were inoculated with the heartwater vaccine and sera was obtained 5 - 6 weeks later.

One-year-old cattle (9) were injected iv with 2 ml infective nymph suspension (Bezuidenhout, 1981), consisting of 1 nymph per dose. After 8 weeks they were challenged iv with 5 ml of heartwater infective sheep blood. Sera were collected 19 weeks after the initial inoculation.

The blood samples were left to clot and after 4 h the coagulated blood was centrifuged for 10 min at 300xg at room temperature in a Piccolo bench top centrifuge. The sera were siphoned off, divided into 1 ml aliquots and stored at -75 °C.

6.2.2. Detection of *Cowdria ruminantium* antigen in *Amblyomma hebraeum* nymphae

6.2.2.1. Preparation of nymph crude extracts

In order to screen *A. hebraeum* nymphae which had fed on infected sheep for the presence of *C. ruminantium* Ball 3 strain, nymphae were homogenized individually for 10 min in 1 ml of 0,05 M HEPES, 0,154 M NaCl buffer (pH 7,4), at low speed with an Ultra Turrax. All the preparation steps of the crude extracts were performed at 4 °C. The nymphal homogenates were centrifuged for 30 min at 1 000xg in a Rotor 40 in a Beckman L5-65 ultracentrifuge

The supernatants were then centrifuged for 30 min at 10 000xg in a Rotor 40. The resultant supernatants were centrifuged at 30 000xg for 30 min. The final sediment was resuspended in 1 ml of the HEPES buffer. Nymphae which were known to be non-infected were used as controls.

6.2.2.2. Preparation of sera containing antibodies to *Cowdria ruminantium*

Sera from Kumm strain *C. ruminantium*-infected white mice (Du Plessis and Kumm, 1971) and sera from Ball 3 strain-infected sheep were used to screen *A. hebraeum* nymph populations. Sera were prepared as described in paragraph 6.2.1.2., p.147.

6.2.3. Protein determinations

Protein was determined by the high temperature, biuret-Folin method described by Dorsey, McDonald and Roels (1977) as described in paragraph 5.2.6., p.117. The Folin-Ciocalteau (Merck) reagent was diluted 1:1 with distilled water. The colour development was monitored at 660 nm in a Beckman Model 25 spectrophotometer against a blank, containing 0,1 ml of HEPES buffer.

6.2.4. Enzyme-linked immunosorbent assay (ELISA)

A modification of the ELISA method described by Notermans, Timmermans and Nagel (1982) as employed in paragraph 5.2.9., p. 120, was used.

Serum solutions were made up as follows : 1 ml of serum was diluted 1:30 with a 0,05 M Tris, 0,154 M NaCl buffer, pH 7,6. As antigen, 10 µg of protein/ml of sonified infected or non-infected nymphal fractions or of wheat-germ lectin column Peak 2 was used. A 1 000x dilution of either rabbit-anti sheep IgG peroxidase (Miles Laboratories) rabbit-anti mouse IgG peroxidase (Miles Laboratories) or rabbit-anti bovine IgG peroxidase (Miles Laboratories) conjugates in 1% (w/v) bovine serum albumin/phosphate buffered saline was used for detection.

To 10 ml of the freshly made substrate buffer, containing 1 mg/ml of o-phenylenediamine (Sigma) in 0,1 M citrate buffer, pH 4.5, 5 µl 30%(v/v) peroxidase (Merck) was added immediately before use. Colour development was monitored with a Titertek Multiscan MC (Linbro Division) at 690 and 450 nm for 30-45 min.

The infected to non-infected (P/N) ratios reported for sera screenings were calculated by dividing the absorbance value obtained from the lectin

fraction (Peak 2) from infected nymphae by the value obtained from the corresponding lectin fraction from non-infected nymphae.

In the case of nymph screenings, P/N ratios were calculated by dividing the absorbance values obtained for nymphae which had been tested by the absorbance value for known non-infected nymphae.

6.3. RESULTS

6.3.1. Detection of antibodies in sera

From Table 6.1 it is evident that of the 41 sheep sera screened for antibodies to *C. ruminantium* after 2 - 3 inoculations with the Ball 3 strain, 100% showed P/N ratios of 1,8-2, of which 83% were above 2. The 6 lambs infected once at under 3 weeks of age and tested 5-6 weeks later, showed lower ratios of 1,6-1,8. All Ball 3 negative sheep sera exhibited P/N ratios of less than 1,05. All of the cattle sera screened exhibited P/N ratios higher than 2. No negative cattle sera were tested.

6.3.2. Detection of antigen in nymphae

The results obtained from infected nymphae (Table 6.2), whether tested with sera from Ball 3 strain-infected sheep or sera obtained from Kumm strain-infected mice, were virtually identical, with at least 74% of the infected nymphae showing ratios above 1,5 (>60% exhibited values above 2).

TABLE 6.1. ELISA screening of antibodies to *Cowdria ruminantium* in the sera of sheep and cattle

Material source			
Serum screened	Antigen	P/N ratio[1]	%[2]
41 Ball 3 + sheep	Ball 3 WG(P2)	>1.8	100
		>2	83
6 Ball 3 + sheep[3]	Ball 3 WG(P2)	1.6-1.8	100
14 Ball 3 - sheep	Ball 3 WG(P2)	<1.05	100
9 Ball 3 + cattle	Ball 3 WG(P2)	>2	100

WG(P2)- Peak 2 of wheat germ lectin column
\+ - Heartwater infected
\- - Heartwater susceptibile (non-infected)
[1] - Infected to non-infected antigen
[2] - % of animals showing indicated P/N ratios
[3] - Sheep infected only once as lambs

TABLE 6.2. ELISA screening for *Cowdria ruminantium* antigen in *Amblyomma hebraeum* (Spes Bona strain) nymphae

Material source			
Antigen screened	Serum	P/N ratio[1]	%[2]
50 Ball 3 + nymphae	Ball 3 + sheep	>1.5	78
		>2	60
		<1.1	22
50 Ball 3 - nymphae	Ball 3 + sheep	<1.1	100
30 Ball 3 + nymphae	Ball 3 + sheep	>1.5	80
		>2	66
		<1.1	20
	or		
	Kumm + mouse	>1.5	77
		>2	66
		<1.1	23
50 Ball 3 + nymphae	Kumm + mouse	>1.5	74
		>2	60
		<1.1	26
50 Ball 3 - nymphae	Kumm + mouse	<1.1	100

\+ - Heartwater infected
\- - Heartwater non-infected
[1] - Infected to non-infected antigen
[2] - % of ticks showing indicated P/N ratios

6.4. DISCUSSION

From the results it is evident that a clear indication of the absence or presence of antibodies to *C. ruminantium* in the serum of sheep or cattle is given by the ELISA employed in this study. Furthermore, since Peak 2 of wheat germ lectin (Chapter 5) from non-infected nymphae was used as control, the test is specific for *C. ruminantium*. The antigen, obtained from *A. hebraeum* nymphae and partially purified by lectin chromatography (Chapter 5), proved to be satisfactory for this assay.

The fact that sera for screening were obtained 5 weeks after the final inoculation suggests that the antibody being tested was IgG. Ilemobade (1976), on the other hand, found by the capillary flocculation test that antibodies were detectable for 1 - 4 weeks only after treatment, spontaneous recovery or challenge-inoculation of infected animals. He deduced that the antibodies involved were IgM, since they are the first antibodies produced in response to an immunogen and have a relatively short half life (Bauer, Mathies and Stavitsky, 1963). To clarify the involvement of IgM and IgG, a study was undertaken regarding the development of the immune response (see Chapter 7).

The screening of nymphae for the presence of *C. ruminantium* by the described ELISA showed that virtually identical results were obtained with either sera from Ball 3 strain-infected sheep and sera from Kumm strain-infected mice. This confirms the notion of Du Plessis (1981) that the 2 strains are immunologically closely related. It was also indicated by the micro-immunodiffusion results in Chapter 5. The observation that 22% - 26% of the infected nymphae showed absorbance ratios of less than 1.1 could either be due to the inability of the test to detect the organisms in these ticks or to their absence. Since the nymphae were infected in the larval stage and subsequently fed as nymphae on susceptible sheep (Bezuidenhout, 1981), either the larvae were refractory to infection

or the organisms were lost during moulting. Screening of larvae for *C. ruminantium* should be conducted to help solve this enigma. Bezuidenhout (1985), (personal communication) reported infection rates of between 1% - 20% using a fluorescent antibody test and mice as experimental animals. The present study on laboratory heartwater infected *A. hebraeum* nymphae and hosts, should be extended to field conditions where numerous different strains of *C. ruminantium* may be present. The detection of *C. ruminantium* in the different organs of adult *A. hebraeum* ticks should further give valuable information on the transmission mechanism, and on the developmental cycles of the organism in the tick. Results of a study to this effect are described in Chapter 7.

The ELISA reported in this chapter should prove to be of value in epizootiological investigations and in the immunodiagnosis of heartwater. The application of the assay in the determination of the earliest stage of detection of antibody in diseased animals, and the determination of the persistence of antibody are described in Chapter 7. These studies, in conjunction with the determination of immunity to challenge, could in turn substantiate the finding of Du Plessis that immunity to heartwater is mediated cellularly rather than humorally (Du Plessis, 1970a; 1981).

CHAPTER 7

THE DETECTION OF *COWDRIA RUMINANTIUM* ANTIBODIES DURING THE COURSE OF HEARTWATER DISEASE IN SHEEP AS WELL AS ANTIGEN IN BLOOD AND IN *AMBLYOMMA HEBRAEUM* IMAGINES BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY.

7.1. INTRODUCTION

The immunological aspects of heartwater need to be further investigated, since the duration or nature of the immunity have not been clarified. The duration has been reported to vary in sheep from 7-34 months after recovery (Neitz, 1939) or up to at least 4 years if sheep were challenged within 2 months of recovery (Neitz, Alexander and Adelaar, 1947). Immunity in cattle appears to abate earlier than in sheep (Haig, 1955).

Regarding the nature of the immunity, it is not known at present whether the immunity is dependent on the persistence of *Cowdria* in the host (premunition) or not (sterile immunity). Furthermore, it is not known with certainty whether the immunity to heartwater is mediated cellularly or humorally. In addition, more information regarding the development of IgG and IgM isotypes during the course of the disease would be of interest in the study of the immunity.

In order to help clarify some of these aspects a study was conducted with respect to the development of antibodies and the persistence of the organism in blood fractions of the host during the course of the disease.

Cowdria ruminantium occurs in the blood and vascular endothelial cells of ruminants. In the latter cells the organism is found in the cytoplasm as groups, also called colonies or clusters (Cowdry, 1925). Inconsistent

results have been reported by various workers regarding the appearance and localization of the organism in various blood fractions. Jackson and Neitz (1932) have claimed to have detected rare organisms free in the blood with the light-microscope. Using electron-microscopy, Stewart and Howell (1981) and Pienaar (1970) frequently found single organisms in the lumen of capillaries. Cowdry (1925; 1926) believed that single organisms in the blood enter endothelial cells, where they proliferate to form a colony. This causes the dissemination of the organisms into the blood after cell rupture with commencement of a new cycle.

Infectivity tests have implied that the organism is located in the white cell fraction (Ilemobade, 1976), the red cell fraction (Fawi, Karrar, Obeid and Campbell, 1969), both these fractions (Alexander ,1931), plasma (Ramisse, 1971) but not in the serum.

In order to help explain these conflicting results, the present study was extended in order to detect the organism by ELISA in various blood fractions. This may be of value in the elucidation of the developmental cycle of the organism, the mechanism of invasion of the organism in the host and the pathogenesis of heartwater.

Early studies by Cowdry (1925, 1926) on the development of *C. ruminantium* in the tick *Amblyomma hebraeum* revealed that the organism was present in the epithelial cells of the intestine and in the lumen of the gut. He was unable to find the organism in the salivary glands. The deduction was therefore made that the transmission might occur as the result of regurgitation during feeding. Bezuidenhout (1981) found that the infectivity of saliva collected from infected *A. hebraeum* females was very low. Further investigations regarding the localization of the organism in the tick would be of value in the study of the survival of the organism in the tick, its stage to stage (transtadial) transmission in the vector and its transmission from vector to host. In the present work

therefore, various organs of the tick were dissected and investigated for *C. ruminantium* by an ELISA.

7.2. MATERIALS AND METHODS

Reagents and glassware were used as described in Chapter 5.

7.2.1. Investigations into the development of antibodies to *C. ruminantium*

7.2.1.1. Preparation of antigen

As antigen, wheat germ lectin-Sepharose 6 MB Peak 2 obtained from infected and non-infected *A. hebraeum* nymph suspension, as described in Chapter 5, was used.

7.2.1.2. Preparation of serum.

The blood samples (10 ml), taken at 2-7 day intervals during the entire observation period, of 2 infected and 2 non-infected sheep (paragraph 7.2.2.1., p.157) were left to clot and after 4 hours the coagulated blood was centrifuged for 10 min at 300xg in a Piccolo bench top centrifuge at room temperature. The sera were siphoned off, divided into 1 ml batches and stored at -75 °C.

7.2.2. Detection of antigen in blood fractions

7.2.2.1. Preparation of blood fractions

Two sheep were injected iv with the heartwater Onderstepoort Ball 3 vaccine (Bezuidenhout ,1981) in order to obtain heartwater infected sheep. Two control sheep were injected iv with non-infected nymph homogenates, obtained in the same manner as for the vaccine. Blood samples (10 ml) were taken in the presence and absence of heparin, at 2-7 day intervals following the inoculation.

Blood samples collected in the presence of heparin, were centrifuged for 10 min at 300xg in a Piccolo bench top centrifuge at 8 °C after a 1 ml sample of whole blood was taken. The plasma, white blood cells and red blood cells, were siphoned off and stored at -75 °C. Samples collected without heparin were left to clot and centrifuged as described in chapter 7.2.1.2., p. 156 whereafter the serum was siphoned off and stored under the same conditions.

7.2.2.2. Preparation of antiserum

Blood from heartwater infected and heartwater non-infected sheep and serum prepared as described in paragraph 5.2.9., p. 120 were taken 4 weeks after the treatment of the diseased animals.

7.2.3. Detection of *Cowdria ruminantium* antigen in *Amblyomma hebraeum* imagines

7.2.3.1. Collection of tick tissue and hemolymph

A. hebraeum larvae were allowed to attach on laboratory infected sheep (Bezuidenhout, 1981). In the nymph or adult stage the ticks were allowed to feed on either heartwater infected or susceptible sheep. The adult ticks were immediately collected when they dropped off and dissected in ice cold BLP. Of the *A. hebraeum* Ball 3 strain infected males, the salivary glands, brain, Malpighian tubes, gut, rectal sac, hypodermis and intestinal remnants were dissected and collected. Of the *A. hebraeum* Kumm strain infected females, the salivary glands, brain, hypodermis, gut, Malpighian tubes, rectal sac, ovaries and intestinal remnants, were dissected and collected. Tissues obtained from 25 ticks were suspended in 0.5 ml BLP. Hemolymph was collected from the stumps of severed trochanters and the amount collected from 25 ticks was made up to 0.5 ml with BLP. Samples were stored in an liquid nitrogen freezer.

7.2.3.2. Preparation of tick tissue and hemolymph extracts

Samples were thawed and homogenized for 2 min with an Ultra Turrax at 4°C. The homogenates were then sonified for 10 seconds with a Sonifier using a micro-tip with output control at 3 and centrifuged for 30 min at 1 000xg in a Rotor 40 using a Beckman L5-65 ultracentrifuge. The supernatants were then centrifuged for 30 min at 10 000xg and the resultant supernatant centrifuged at 30 000xg for 30 min. The final sediment was resuspended in 0.5 ml of 0.154 M NaCl. This represented the crude extract.

7.2.3.3. Preparation of antiserum

Antiserum was obtained as described in paragraph 5.2.9., p.120 .

7.2.4. Protein determinations.

Protein was determined by the high temperature biuret-Folin method (paragraph 5.2.6., p.117).

7.2.5. Enzyme-linked immunosorbent assay (ELISA).

The ELISA method described in paragraph 6.2.4., p. 149 was used. Serum solutions were made up as follows: 1 ml serum was diluted 1:30 with 0.05 M Tris, 0.154 M NaCl buffer, pH 7,4. For the detection of IgM antibodies in serum a 1 000x dilution of rabbit-anti sheep IgM peroxidase (μ chain specific) (Cooper Biomedicals) conjugate in 1% (w/v) BSA/PBS was used. As antigens, 10 μg protein/ml either infected tick tissue and hemolymph, infected and non-infected blood fractions or peak 2 obtained from wheat germ lectin infected or non-infected nymphae were used.

The ratios reported for antibody screenings were obtained from infected and control sheep using peak 2 obtained from wheat germ lectin affinity chromatography as antigen source.

The infected to non-infected (P/N) ratios reported for the detection of *C. ruminantium* in blood fractions were calculated by dividing the absorbance values obtained from infected animals by the values obtained from the control animals (paragraph 7.2.2.1., p. 157). In the case of organ screenings, P/N ratios were calculated by dividing the absorbance values obtained from infected serum, by the absorbance value from non-infected serum.

7.3. RESULTS.

In the early stages during the course of the disease, insignificant infected to non-infected ratios of IgM and IgG were detected (Fig. 7.1). IgM antibodies were first observed in minute amounts 3 days after the inoculation. Their concentration reached a maximum on the 4th day after which these immunoglobulins disappeared on the 7th day. IgG antibodies first appeared on the 8th day and continued to increase during the remainder of the observation period of 28 days. Directly after treatment with oxytetracycline, a sharp rise in the IgM level occurred which reached a maximum on the 3rd day after treatment. Hereafter the level decreased.

The presence of *C. ruminantium* antigen in the blood fractions of heartwater infected sheep was demonstrated by ELISA (Fig. 7.2). Serum and the red blood cell and white blood cell fractions exhibited infected to non-infected ratios higher than 1.5. The earliest day of *C. ruminantium* antigen detection was in plasma and serum on day 4 after the inoculation. In the red blood cell fraction the antigen was first detected on the 6th day and in the white blood cell fraction 2 days later. Of all fractions investigated, erythrocytes showed the highest P/N ratio which reached a maximum of 2, two days after commencement of the febrile reaction. In both the erythrocytes and leukocytes, the antigen declined upon treatment with oxytetracycline and became undetectable on the 7 th day after treatment.

Investigations into the presence of *C. ruminantium* antigen in the various tick tissues and hemolymph of adult *A. hebraeum* ticks revealed that the organism invades a number of body parts (Table 7.1). In females, the gut and synganglion (brain) and in males the salivary glands and gut showed the highest signals.

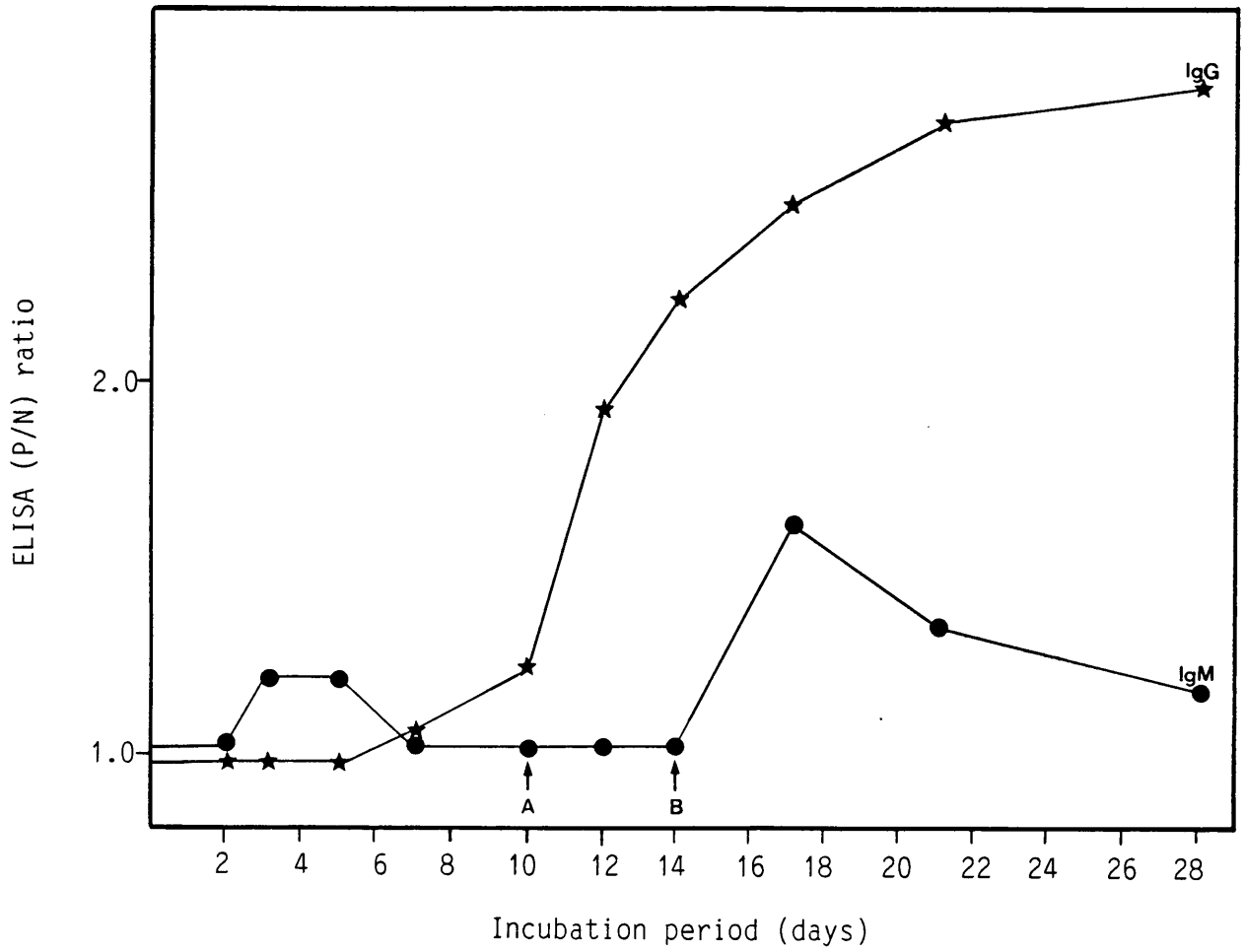


Fig 7.1. The detection of antibodies by ELISA to *Cowdria ruminantium* in heartwater infected sheep. (A) Day of reaction, (B) day of treatment. IgG (—★—★—) and IgM (—●—●—).

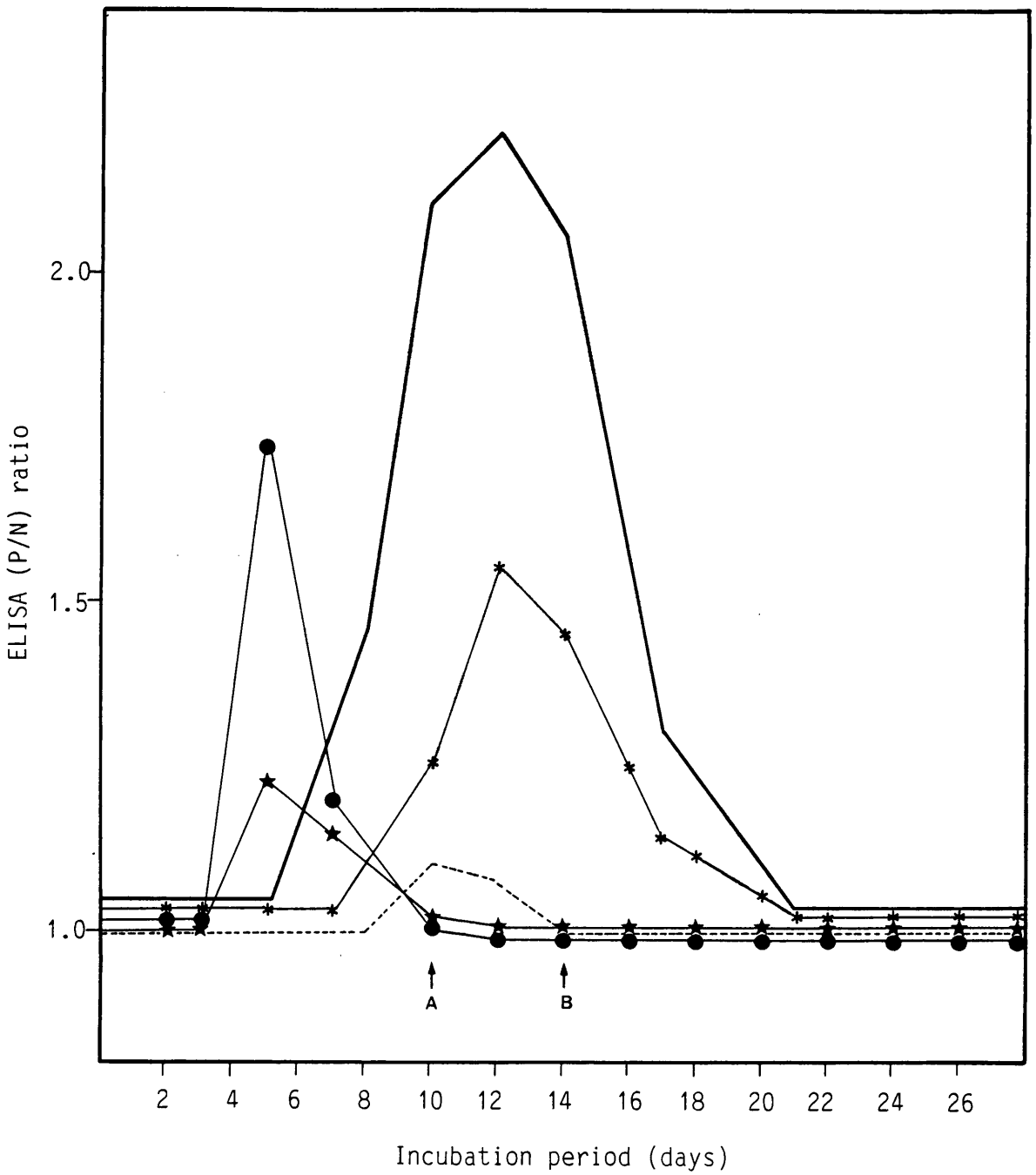


Fig 7.2. ELISA screening of *Cowdria ruminantium* antigen in blood fractions of heartwater infected sheep. (A) Day of reaction, (B) day of treatment. Serum (-★-★-), plasma (-●-●-), whole blood (------), red blood cells (——) and white blood cells (-*-*-).

TABEL 7.1. ELISA screening of *Cowdria ruminantium* (Ball 3 or Kumm strain) antigen in *Amblyomma hebraeum* males and females.

Material source		
Antigen screened	P/N Ratio[1]	
	Male[2]	Female[3]
Salivary glands	1.8±0.2	1.6±0.1
Synganglion	1.2±0.1	1.8±0.1
Hemolymph	1.4±0.2	1.3±0.2
Malpighian tubes	1.1±0.1	1.1±0.1
Gut	1.7±0.1	1.9±0.2
Ovaries	-------	1.1±0.1
Rectal sac	1.1±0.1	1.1±0.2
Hypodermis	1.4±0.2	1.6±0.1
Intestinal remnants	1.5±0.1	1.4±0.2

[1] - Antigen was screened with infected and non-infected sheep sera.
[2] - Ball 3 strain
[3] - Kumm strain

7.4. DISCUSSION

Significant antibody (IgG) levels were detected from the 10 th day after the inoculation. IgG levels increased onwards and started to plateau from day 21. The inability to detect IgM present in the initial stages of the disease, could be due to the binding of antigen to IgM resulting in the absence of free IgM in the serum in the initial stages of the immune response. This was also observed by Benacerraf and Gell (1959) in their studies on hypersensitivity, delayed and arthus-type skin reactivity to protein conjugates in guinea-pigs. This could explain the increase of free IgM upon treatment of the disease which inhibited the organism to proliferate.

It is evident from the results concerning the localization of *C. ruminantium* in the blood of the host that the organism firstly upon entering the bloodstream, is present in the plasma and serum. Thereafter the erythrocytes and slightly later the leukocytes are invaded with a

concomitant decrease of *C. ruminantium* levels in the plasma and serum. On day 21, i.e. 7 days after treatment of the diseased animals, no *C. ruminantium* antigen is detectable in any of the blood fractions. The low levels of antigen observed for whole blood is probably due to too low *C. ruminantium* antigen amidst the enormous total blood proteins.

It appears that the detection of *C. ruminantium* antigen and their antibodies in blood by an ELISA could be applied to the diagnosis of the disease. This method is not only suitable for identifying the etiological agent in blood in a highly specific manner but also for monitoring the time course of the immune response. Hereby the change in antibody titer, i.e. the appearance of antibody and their subsequent decline may be established.

Several organs and probably the hemolymph of both female and male *A. hebraeum* ticks seem to harvest *C. ruminantium* organisms. Their presence in the males, although they are poor vectors of the disease may be related to their slower feeding habits (Uilenberg, 1983). The presence of antigen in the salivary glands and in the gut suggest that the pathogen can be transmitted as the result of salivation and/or regurgitation during feeding. The latter mode was proposed by Cowdry (1925). Their absence in the ovaries is consistent with the notion that this organism is not transmitted transovarially from generation to generation (Neitz, 1968). It should be borne in mind that transmission of the pathogen by a vector to a host could be by means of salivation, regurgitation during feeding or by faeces, coxal or dermal secretions entering the wound made by the mouthparts of the vector.

Therefore, the study regarding the localization of *C. ruminantium* in the vector should be extended to include the possible presence of organisms in the salivary, coxal and dermal secretions.

The ELISA developed in the present work should prove suitable for future studies since it is sensitive and specific for *C. ruminantium* antigen. Furthermore, an investigation into the presence of antigen in spermatophores and tick eggs could help to clarify the possibility that females can be infected by males during sperm transfer or that the pathogen is transmitted transovarially.

CHAPTER 8

CONCLUDING DISCUSSION

During the present study some new knowledge has been gained concerning tick paralyses, tick egg toxins and the causal agent of heartwater. Some conclusions and perspectives are discussed below.

Chromatofocusing proved to be a rapid one-step technique for obtaining an electrophoretic homogeneous toxin from crude salivary gland extracts of female ***Rhipicephalus evertsi evertsi***. In addition, this technique could be applied for the estimation of quantitative changes in the concentration of proteins in salivary gland extracts during feeding.

The ***in vitro*** toxicity assay involving the application of test substances directly onto the sciatic nerve of ***Xenopus laevis***, proved to be sensitive and suitable for the detection of neurotoxic activity in salivary gland extracts.

The finding that the neurotoxin reaches its highest concentration in salivary glands of ticks specifically during the toxic feeding phase seems to indicate that this toxin may be associated with the toxin responsible for paralysis during natural infestations. To ultimately prove the identity of these 2 toxins, inoculation of the isolated toxin into sheep will be necessary. This has not been possible up to now because of lack of sufficient material.

The most important further investigations will therefore first of all involve the collection of sufficient salivary glands of ticks in the toxic phase, the purification of toxin and the sc or iv inoculation of crude extracts

and purified toxin over several hours into sheep. Continuous, instead of a single administration of the extracts or toxin is envisaged since by this method the transfer of toxin under natural infestation is probably more closely simulated.

The availability of purified toxin and thus potentially available polyclonal antibodies will make it possible to develop a specific ELISA so as to locate the locality of synthesis of the toxin as well as its distribution in the tick and host animal. Hereby the origin of the toxin in the vector and its distribution in the host should become known.

Further characterization of the toxin especially with respect to its structure and its mode of action on the nerve fibre will be of considerable importance. The toxin could prove to be useful in the study of the mechanism of impulse propagation in nerves (Narahashi, 1974). Of interest is that this toxin is a relatively high molecular weight protein acting on the nerve axon. It seems that known proteinaceous toxins with such properties are of low molecular weight (Narahashi, Moore and Shapiro, 1969; Beress, Beress and Wunderer, 1975; Bergman, Dubois, Rojas and Rathmayer, 1976; Meves, Rubly and Watt, 1982). Those with high molecular weight which cause inhibition of muscular contraction, appear to act on the neuromuscular junction (Narahashi, 1974; Spence, 1979).

A knowledge of the amino acid sequence of the toxin could lead to the synthesis of a probe to obtain the appropriate mRNA which in turn could lead to the synthesis of cDNA. By means of DNA cloning, sufficient toxin should be provided for preparation of a specific vaccine and for further characterization studies. Monoclonal antibodies would also lead to a rapid identification of the toxicosis in the field and would be of considerable importance to the farmer.

The results obtained from the chromatofocusing of crude salivary gland extracts, suggests that numerous proteins are synthesized only after tick attachment and commencement of feeding. The functions of the isolated proteins have not been investigated. Of particular interest would be to investigate their immunogenic properties, since this could have bearing on host resistance to tick infestations. The immuno-manipulation of tick hosts, so that they could become resistant to tick infestation in the field has been suggested as an alternative measure for tick control (Allen and Humphreys, 1979; Wikel and Allen, 1982). Wikel (1981) has shown that resistance to tick infestations can be induced by host immunization with salivary gland antigen.

The paralysis toxin present in ***Argas (Persicargas) walkerae*** larvae has not been isolated in a homogeneous form. The high hemoglobin concentration in the crude extracts was found to be the main obstacle hindering the final purification. However, several alternative approaches for the isolation are considered at present. These include hydrophobic interaction chromatography and the use of immunoadsorbents involving anti-chicken hemoglobin immunoglobulins. In addition, salt and organic solvent precipitation of hemoglobin is being considered.

The anti-protease activity of the egg toxins was determined with pancreatic trypsin and chymotrypsin. These are proteases with which the toxins certainly do not come into contact under natural conditions. However, tick eggs have been shown to contain proteases (Mills, 1985). The inhibitory activity determinations should be extended so as to include these enzymes. Through such studies, an insight into the function of these inhibitors in tick eggs, especially during embryogenesis, should be obtained.

It will be of considerable interest to establish if there is any relationship between the toxin present in ***R. evertsi evertsi*** eggs and the neurotoxin

isolated from the salivary glands of these ticks. By means of such a study it will be possible to investigate the possibility that the neurotoxin originates in the genital organs of the female (Regendanz and Reichenow, 1931). The toxin may then subsequently be incorporated into the eggs or in paralysis inducing ticks also transferred via the hemolymph to the salivary glands. Furthermore, the toxin concentration reaches a peak in these glands in ticks during the toxic phase of feeding and transferred into the host during this phase. Present indications are that, if this is indeed the case, the toxin is modified during its passage from the female genital glands to the salivary glands since the molecular weight of the egg toxin is 6 kdal whereas the neurotoxin isolated from the salivary glands is 68 kdal. Nevertheless, immunological investigations could reveal common epitopes and amino acid sequence determinations an overlapping sequence.

Regarding the kinetics of inhibition of the egg toxins investigated in the present study, it is evident that they exhibit fast-binding and/or slow-binding inhibition of either trypsin or chymotrypsin depending on the tick species from which they originate.

The *R. evertsi evertsi* egg toxin showed fast binding inhibition of trypsin whereas the *Boophilus decoloratus* and *Hyalomma truncatum* egg toxins exhibited fast-binding inhibition of chymotrypsin. The fast binding inhibition studies concerning trypsin was employed using D,L-BAPNA. D-arginine is a competitive inhibitor of trypsin (Tokura, Nishi and Noguchi, 1971) and for that reason L-BAPNA would have been the better substrate.

In addition, the toxins from *B. decoloratus* and *H. trancatum* as well as the toxin from *B. microplus* showed slow-binding inhibition of trypsin. The *B. decoloratus* and *H. truncatum* anti-proteases could possibly be

double headed inhibitors. To investigate this possibility, the inhibition of one enzyme in the presence of the other needs to be determined.

Although the mechanism for slow-binding inhibition has not yet been established (Morrison, 1982), it can be speculated that the enzymes subjected to slow-binding inhibition are held firmly by the inhibitors so as to hinder the release of the enzyme from the enzyme-inhibitor complex.

The physiological role of the egg toxins have not been elucidated. The anti-protease activity is probably not the sole activity of the toxins. However, their short ***in vitro*** half-time of complex formation with proteases and their high concentration in eggs with respect to their K_i values indicate that they have a role ***in vivo*** as protease inhibitors.

Approximately 60 years has passed since Cowdry identified the organism responsible for heartwater. During this time little further knowledge of the organism, beyond the resolving power of the electron-microscope, has been gained. Many aspects of the disease also remains unsolved. Biochemical studies on the organism are clearly only possible with pure preparations.

It appears that the purification of ***Cowdria ruminantium*** as described in the present work was successful with infected brain tissue as source. Further work is needed to improve the purity and yield of the organism obtained from nymphae and brain respectively before biochemical studies on the organism can be commenced. Sufficient quantities of pure organisms are also required to investigate the involvement of a presumed toxin of ***C. ruminantium*** in heartwater disease.

Cellular affinity chromatography was found to be suitable for the partial purification of the organism. Density gradient centrifugation with Percoll revealed the pleomorphism of the pathogen. Viable organisms of various

sizes could be obtained by this method. Furthermore, organisms from the host and the tick vector could be distinguished; isolated *C. ruminantium* from the host showed defined electron- translucent areas in electron micrographs whereas organisms from ticks exhibited several scattered electron-transparent areas.

The main problems encountered during the isolation of *C. ruminantium* organisms include the difficulty in obtaining a suitable source of the organisms, the lability of the organisms, the difficulty in identifying single *C. ruminantium* organisms by electron-microscopy and the lack of practical means by which the organism may be quantified.

Ideally, the source for the isolation of *C. ruminantium* should be readily obtainable and contain sufficient quantities of the organism and be free from rickettsia-like organisms. Infected brain material was initially chosen since it was likely to meet these requirements. However, the problem of inconsistent infectivity of this tissue should be circumvented before it can serve as source of organisms on a routine basis. Infected tick tissue on the other hand was found to be consistently highly infective but contained organisms which contaminated the isolated fractions.

The isolation of large amounts of pure, viable *C. ruminantium* should become possible once the problems encountered in their *in vitro* culture have been solved.

It thus appears that the successful isolation of *C. ruminantium* is at this stage dependent on an optimum source of the organism and that the described isolation methods should prove to be suitable.

Identification of the organism by electron microscopy should be facilitated by using gold- or ferritin-labelled antibodies (Rikihisa and Ito, 1981).

A suitable method for the quantification of the organism is essential for an evaluation of the effectiveness of the various isolation steps. The quantification of the organism in terms of infectivity is possible with the mouse strain (Kumm strain) of *C. ruminantium*. This illustrates one of the important applications of a strain infective to a small laboratory animal. Quantification in terms of antigenicity by ELISA has become possible during the course of the present study.

The ELISA may be regarded to be specific for *C. ruminantium* antigen since appropriate controls are employed. Thus non-infected sheep brain tissue and nymph material as well as non-infected mouse liver and spleen are used as control antigens. Alternatively, serum obtained from sheep inoculated with non-infected nymph material as well as serum from mice inoculated with non-infected mouse liver and spleen material were used as antibody controls. Both horse-radish peroxidase-IgG conjugate as well as Protein A-conjugate were found to be suitable as indicators in the assay. Protein A-conjugate is more specific but less sensitive than anti-IgG immunoglobulin-conjugates of peroxidase or alkaline phosphatase (Langone, Boyle and Borsos, 1978; Goding, 1978).

Conclusive evidence that the Kumm and Ball 3 strains have common antigens was obtained with the ELISA and Ouchterlony double micro-immunodiffusion technique. This cross reaction was also observed by Du Plessis (1982) by means of the indirect fluorescence antibody method. These two strains differ, however, with respect to their pathogenicity in sheep and mice.

The ELISA for the detection of *C. ruminantium* antigen as well as antibodies to the organism has important and far reaching applications. The assay has been applied for the detection of the organism in host brain and blood fractions and in *Amblyomma hebraeum* ticks. This should prove to be of value in immunodiagnosis of heartwater and epizootiological in-

vestigations. The studies need to be extended to determine the time course of the immunity and the migration of the organism in body fluids and tissue during infection of the host and the vector.

Furthermore, the application of the ELISA in the development of monoclonal antibodies to single *C. ruminantium* specific determinants common to all strains is envisaged. It is axiomatic that this extension of the present studies could lead to the production of a highly specific and efficient vaccine and the development of a sensitive serodiagnostic test.

Almost a century has now elapsed since a tick has been incriminated as being a vector of a pathological agent. During this period, numerous pathogens for which ticks are the vectors have been identified. Potentially, ticks may serve as vectors of many other pathogens. Furthermore, ticks may become infected and transmit disease in countries in which they are presently free of such pathogens. For this reason, tick borne diseases are looming world-wide and their intensive study on a global scale is therefore warranted.

When considering present and future strategies for tick research it seems inevitable that in addition to the conventional approaches of parasitologists and veterinary entomologists, the techniques of biochemistry and molecular biology need to be applied. This has been the approach while conducting the present investigations. It is hoped that this work has made a modest contribution towards a better understanding of tick-borne diseases.

SUMMARY

Ticks are the most important ectoparasites of domestic animals in the Republic of South Africa. Annual losses due to ticks amount to several hundred million Rand. They may cause disease since they may serve as vectors of pathogens. Certain tick species cause toxicoses which are generally believed to develop as the result of the transfer of toxins from tick to host.

In the present work, attempts were made to isolate and characterize toxins from *Rhipicephalus evertsi evertsi* and *Argas (Persicargas) walkerae* ticks. These ticks cause paralysis in sheep (Spring lamb paralysis) and poultry, respectively. Furthermore, tick egg toxins were investigated because they may have a bearing on the paralysis toxins. In addition, methods for the purification and detection of *Cowdria ruminantium* were investigated as a first step in the study of the assumed toxin produced by this pathogen. *C. ruminantium* is the causative agent of heartwater and transmitted by *Amblyomma* ticks.

A toxin was isolated by chromatofocusing from the salivary glands of female *R. evertsi evertsi* in the toxic feeding phase. The toxin was shown to reach a maximum concentration during this phase. The toxin was found to be homogeneous according to isoelectric focusing, sodium dodecyl sulfate polyacrylamide gradient gel electrophoresis and gel permeation chromatography. Toxicity was assayed by means of a *Xenopus laevis* nerve muscle preparation. The toxin exhibited a reversible inhibition of muscle contraction when applied directly onto the nerve. The molecular weight was found to be 68 kdal by gel permease chromotography and the pI equal to 6.

Attempts were also made to isolate a paralysis toxin from whole *A. (P.) walkerae* fully engorged larvae. Problems were encountered due to the

very high concentrations of host hemoglobin in these ticks. No homogeneous toxin could be obtained. Toxicity was assayed with 1 day old chickens.

The egg toxins of *R. evertsi evertsi*, *Boophilus decoloratus*, *B. microplus* and *Hyalomma truncatum* were found to be fast-binding or slow-binding inhibitors of trypsin and chymotrypsin. Fast-binding inhibition of trypsin by *R. evertsi evertsi* (K_i = 16 nM) and of chymotrypsin by *B. decoloratus* (K_i = 36 nM) and by *H. truncatum* (K_i = 23nM) was observed. Slow-binding inhibition of trypsin by *B. microplus* (K_i = 4.8 nM), by *B. decoloratus* (K_i = 4.1 nM) and by *H. truncatum* (K_i = 0.9 nM) was demonstrated. Immunological cross reaction was observed between the toxins from *B. microplus* and *B. decoloratus*. The *in vivo* functions of the toxins need to be further investigated.

C. ruminantium organisms were isolated by lectin affinity chromatography and Percoll density gradient centrifugation. Isolated fractions were analyzed by intravenous inoculation into sheep, protein determination, electron-microscopy and enzyme-linked immunosorbent assay. The purification procedures could be completed in a few hours using either infected sheep brain or nymphae as starting material. The density gradient centrifugation method permitted the recovery of viable populations of the organism possessing different densities.

ELISA methods for the detection of *C. ruminantium* antibodies during the course of heartwater disease in sheep as well as antigen in blood and ticks were developed. By using appropriate controls the assays were rendered specific for *C. ruminantium*.

The antibody assay showed that IgM antibodies reached an maximum on the 4 th day after infection and disappeared on the 7 th day. IgG

antibodies first appeared on the 8 th day and continued to increase up to at least the 28 th day.

The earliest day of *C. ruminantium* antigen detection in the host was in plasma and serum on day 4 after inoculation. Erythrocytes showed the highest concentration of antigen which reached a maximum two days after the febrile reaction.

C. ruminantium antigen was detected in various tick tissue and hemolymph. In females, the gut and synganglion and in males the salivary glands and gut showed the highest levels.

ELISA also showed that the infective mouse strain (Kumm strain) and infective sheep strain (Ball 3 strain) of *C. ruminantium* possess common antigens.

SAMEVATTING

Die belangrikste ektoparasiete van diere in die Republiek van Suid-Afrika is bosluise. Jaarlikse verliese te wyte aan bosluise beloop etlike honderd miljoen Rand. Siektes word veroorsaak omrede bosluise as vektore van patogene kan dien. Sekere bosluisspesies veroorsaak toksikose wat algemeen aanvaar word as synde te ontwikkel as gevolg van die oordra van toksiene vanaf die bosluis na die gasheer.

Met die huidige studie is daar gepoog om toksiene van *Rhipicephalus evertsi evertsi* en *Argas (Persicargas) walkerae* bosluise te isoleer en te karakteriseer. Hierdie bosluise veroorsaak verlamming in skape (Lente lamverlamming) en in pluimvee respektiewelik. Bosluis eiertoksiene is ook bestudeer omdat hulle moontlik verband hou met die verlammingstoksiene. Metodes vir die isolasie en bepaling van *C. ruminantium* is ondersoek as 'n eerste stap in die studie van die moontlike toksien wat deur hierdie patogeen geproduseer word. *C. ruminantium* is die patogeen verantwoordelik vir die ontstaan van hartwater. Die organisme word deur *Amblyomma* bosluise oorgedra.

'n Toksien is uit speekselkliere van vroulike *R. evertsi evertsi* bosluise in die toksiese voedingsfase deur chromatofokusering geisoleer. Die toksien bereik gedurende hierdie fase sy maksimum konsentrasie. Die toksien is homogeen met behulp van iso-elektriese fokusering, natriumlaurielsulfaat poliakrielamied gradient jel-elektroforese en jelpermeasie chromatografie bevind. *In vitro* toksisiteitstoetse is met behulp van 'n *Xenopus laevis* senu-spierpreparaat uitgevoer. Die toksien het 'n omkeerbare inhibisie van spiersametrekking veroorsaak wanneer dit direk op die senuwee toegedien is. Die molekulêre gewig van die toksien, soos bepaal deur jelpermeasie chromatografie is 68 kdal en die pI = 6.

Die isolasie van 'n verlammingstoksien uit volgesuigde heel *A. (P.) walkerae* larves was minder suksesvol. Toksisiteit is met behulp van 1 dag oue kuikens bepaal. Probleme is ondervind as gevolg van die geweldige hoë konsentrasie van gasheer hemoglobien in hierdie bosluise. Geen homogene toksien kon geïsoleer word nie.

Die eiertoksiene van *R. evertsi evertsi*, *Boophilus decoloratus*, *B. microplus* and *Hyalomma truncatum* het inhibisie van tripsien en/of chimotripsien getoon. Vinnige bindingsinhibisie van tripsien deur *R. evertsi evertsi* (K_i = 16 nM) en van chimotripsien deur *B. decoloratus* (K_i = 36 nM) en deur *H. truncatum* (K_i = 23 nM) is waargeneem. Stadige bindingsinhibisie van tripsien deur *B. microplus* (K_i = 4.8 nM) sowel as deur *B. decoloratus* (K_i = 4.1 nM) en deur *H. truncatum* (K_i = 0.9 nM) is aangetoon. Immunologiese kruisreaksie is tussen die toksiene van *B. microplus* en *B. decoloratus* waargeneem. Die *in vivo* funksies van die toksiene behoort verder ondersoek te word.

C. ruminantium organismes is geisoleer met behulp van koringkiem lektien-affiniteitschromatografie en Percoll digtheidsgradient-sentrifugasie. Geïsoleerde fraksies is geanaliseer deur intraveneuse inokulasie in skape, proteïenbepalings, elektronmikroskopie en ensiengekoppelde immuno-adsorbent bepaling. Die suiweringsprosedures kon binne 'n paar uur afgehandel word met of geïnfekteerde skaapbrein of muis lewer en milt of nimfe as uitgangsmateriaal. Die digtheidsgradiënt sentrifugasie metode het dit moontlik gemaak om lewensvatbare populasies van die organisme met verskillende digthede te herwin.

ELISA metodes vir die bepaling van *C. ruminantium* teenliggaampies gedurende die verloop van hartwater in skape sowel as antigeen in bloed en bosluise is ontwikkel. Deur gebruik te maak van geskikte kontroles is hierdie metode spesifiek gemaak vir *C. ruminantium*.

Teenliggaampiebepalings het getoon dat immunoglobulien M teenliggaampies 'n maksimum bereik op die 4 de dag na infeksie en dat hulle op die 7 de dag verdwyn. Immunoglobulien G teenliggaampies was op die 8 ste dag waarneembaar en het toegeneem tot ten minste die 28 ste dag.

Die vroegste dag van *C. ruminantium* antigeen verskyning in die gasheer was in plasma en serum en wel op die 4de dag na inokulasie. Eritrosiete het die hoogste konsentrasie van antigeen getoon en het 'n maksimum bereik 2 dae na die verhoogde temperatuurreaksie.

C. ruminantium antigeen is in verskeie bosluisweefsels en hemolimf waargeneem. In vroulike bosluise was die hoogste vlakke *C. ruminantium* in die brein en ingewande en in manlike individue in die speekselkliere en ingewande.

Die ELISA metode het ook getoon dat die infektiewe muis stam (Kumm stam) en die infektiewe skaap stam (Ball 3 stam) gemeenskaplike antigene van *C. ruminantium* besit.

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