PURIFICATION AND CHARACTERISATION OF TICK TOXINS

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LIST OF SYMBOLS AND ABBREVIATIONS

A	area under schlieren peaks (ultracentrifugation)
A.heb.	*Amblyomma hebraeum*
BAPNA	N-benzoyl-D-L-arginine 4-nitroanilide-HCl
B.dec.	*Boophilus decoloratus*
B.mic.	*Boophilus microplus*
ca.	*circa*
C	percentage concentration of the cross-linker relative to the total concentration T.
CF	chromatofocusing
C.rum.	*Cowdria ruminantium*
E	enzyme
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immuno sorbent assay
g_{av}	centrifugal force calculated at a distance from axis of rotation to mid point of tube
g	gram
h	hours
H.trun.	*Hyalomma truncatum*
I	inhibitor
In	initial
IEF	isoelectric focusing
KDal	kilo Daltons
mA	milliampere
mg	milligram
min	minutes
MLD	minimum lethal dose for 100% of the experimental animals
MM	molecular mass
NSS	sweating sickness negative
O.sav.	*Ornithodoros savignyi*
OD	Absorbance
P	Product
ρ	solution density
PBE	Polybuffer exchanger
pbs	phospho buffered saline
pI	isoelectric point
r	radial distance in cm, corrected for camera lens magnification (ultracentrifugation)

R	universal gas constant
rbs	red blood cells
R.app.	*Rhipicephalus appendiculatus*
R.ee.	*Phipicephalus evertsi evertsi*
rpm	revolutions per minute
S	substrate
sa	specific activity
SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec.	seconds
SIP	iso-osmotic stock solution of Percoll
SPNA	N-succinyl-L-phenylalanine p-nitroanilide
SS	sweating sickness positive
T	total percentage concentration of gel
Tox	Toxin
Tris	tris (hydroxymethyl) aminomethane
uv	ultraviolet
V	volts
v	velocity
$\bar{v}$	partial specific volume
Ve	eluting volume
Vi	internal volume
Vo	void volume
Vt	bed volume
W	mass
ω	angular velocity
λ	wavelength

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CHAPTER 1

INTRODUCTION

1.1 Scope of the tick problem

Ticks are the most important exoparasites of domestic animals in the Republic of South Africa. They cause direct and indirect damage resulting in severe economic reductions in livestock production (1).

The direct damage results from a decrease in the condition of their hosts because of exsanguination, tick burden, tick toxicoses (Table 1) and secondary infection of organisms at the attachment sites. Indirectly they cause damage since they may serve as vectors of numerous pathogenic organisms (Table 1).

Reliable estimates of the cost due to tick infestation is not known. This is due to uncertainties of the numerous factors which have to be considered in the estimation of monatory losses involved (2). Data is required concerning mortality rates and control costs, including labour, equipment and chemicals. In addition the costs involved in the research and development of acarisides, 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, concervative estimates have shown that the annual losses add up to several hundred million Rand (2,3).

1.2 General characteristics of ticks

Ticks are arthropods that belong to the class Arachnida, which are characterised by possessing four pairs of walking legs and no antennae (4). All but a single specie of ticks, *Nuttallielidae*, fall into two families; the

the Ixodidae (hard ticks) and the Argasidae (soft ticks). There are 644 known species of Ixodid ticks and 149 of Argasidae ticks (4).

Ticks transmit some pathogens transstadially. In many cases pathogens may migrate through the ovary into the egg (Table 1). Hereby the larvae become infested and transmit the pathogen while feeding (transovarial transmission). The pathogens under consideration are of six principal types: Spirochetes, Rickettsiaceae, Babesidae, bacteria *(Pasteurella tularensis)*, Anaplasmataceae and viruses (5).

Ticks are highly adaptable and are capable of filling a wide range of ecological niches. Generally, however, they favour warm moist climates. Consequently they are particularly hazardous in many parts of Africa, Australia and South America (6). Their adaptability and effectivity as pathogen transmitters is due to their longevity, extreme resistance to starvation, overwintering or interepizootic survival and ability to attack more than one host within one generation. Furthermore they are capable of transstadial as well as transovarial transmission of pathogens (7).

1.3 Tick-borne diseases and tick toxicoses

1.3.1 Tick-borne diseases

Smith and Kilbourne's discovery in 1893 that the pathogen of bovine piroplasmosis is transmitted by a tick, is the first recorded instance in history in which the transmission of a protozoan parasite by an arthropod was observed (8).

Subsequently all the economical important diseases were described by various workers. In all the diseases

TABLE 1 IMPORTANT SOUTH AFRICAN TICKS AND TICK-BORNE DISEASES. (ADAPTED FROM HOWELL (1) AND NEITZ (10))

TICK	COMMON NAME	DISEASE TRANSMITTED	TRANSMISSION*	AGENT
Rhipicephalus evertsi evertsi	The red-legged tick	Spring lamb paralysis		
		Biliary fever		*Babesia equi*
		Spirochaetosis	a,b	*Borrelia theileri*
Rhipicephalus appendiculatus	The brown ear tick	East Coast fever	b	*Theileria parva*
		Redwater	a,b	*Babesia bigemina*
		Corridor disease	b	*Theileria Lawrencei*
		Louping ill	b	Virus of louping ill
Boophilus decoloratus	The common blue tick	Redwater	a,b	*Babesia bigemina*
		Gallsickness	a,b	*Anaplasma marginale*
		Spirochaetosis	a,b	*Borrelia theileri*
Boophilus microplus	The pantropical blue tick	Redwater	a,b	*Babesia bigemina*
		Gallsickness	a,b	*Anaplasma marginale*
		Spirochaetosis	a,b	*Borrelia theileri*
Hyalomma truncatum	The small smooth bont-legged tick	Sweating sickness		
Amblyomma hebraeum	The South African bont tick	Heartwater	b	*Cowdria ruminantium*
Ixodes rubicundus	The Karoo paralysis tick	Tick paralysis		
Ornithodoros savignyi	The sand tampan	Sand tampan toxicosis		
Rhipicephalus simus	The glossy brown tick	Tick paralysis		
		East Coast fever	b	*Theileria parva*
		Gallsickness	a,b	*Anaplasma marginale*

a: Transovarial (18)
b: Transtadial (18)
*: Known pathogenic organisms only

of Africa, and *A.(P.) radiatus* of North America (13).

"Karoo" paralysis of sheep caused by *Ixodes rubicundus* and "Spring" lamb paralysis of sheep caused by *R. evertsi evertsi* are common in South Africa. Paralysis is also caused by certain strains of *Hyalomma truncatum* ticks (10) (Table 2).

The first symptoms are clinically perceptible as early as 5 days (10) after infestation and a single female tick suffices to completely paralyse and kill an adult human; thus there arises the potential for serious consequences owing to erroneous diagnosis by inexperienced physicians. Treatment, however, is simple: timely removal of the tick, which usually attaches to feed on the hairy parts of the body.

A major enigma concerning paralysis is the origin of the toxin (16). There are three possibilities:

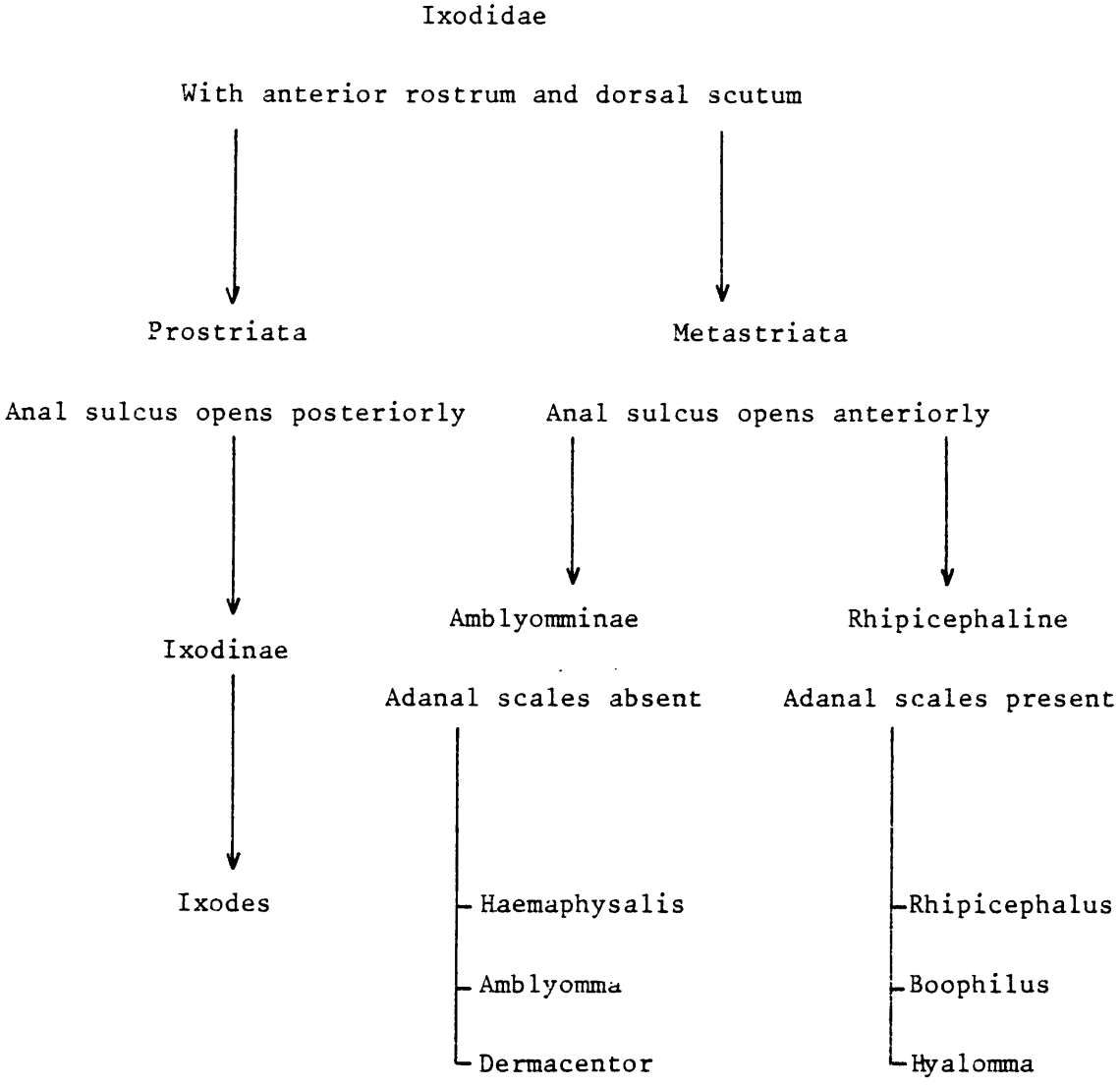
1) It is a product of the tick tissues *per se*,
2) It is a product of metabolic breakdown of host tissue, and
3) the toxin is produced by an organism within the tick.

The possibility that the toxin is a product of the host tissue can be divided into two different mechanisms:

1) Ingested host materials are made toxic by digestion in the gut of the tick and then regurgitated, and
2) Nontoxic saliva is introduced into the host where toxin is produced by enzymatic action.

With this as a general basis, various possibilities as to the origin of toxins causing tick toxicoses may be envisaged (Fig 1)(17).

TABLE 2 CLASSIFICATION OF IXODIDAE, LISTING ONLY THE GENERA KNOWN TO CAUSE TICK PARALYSIS (4)



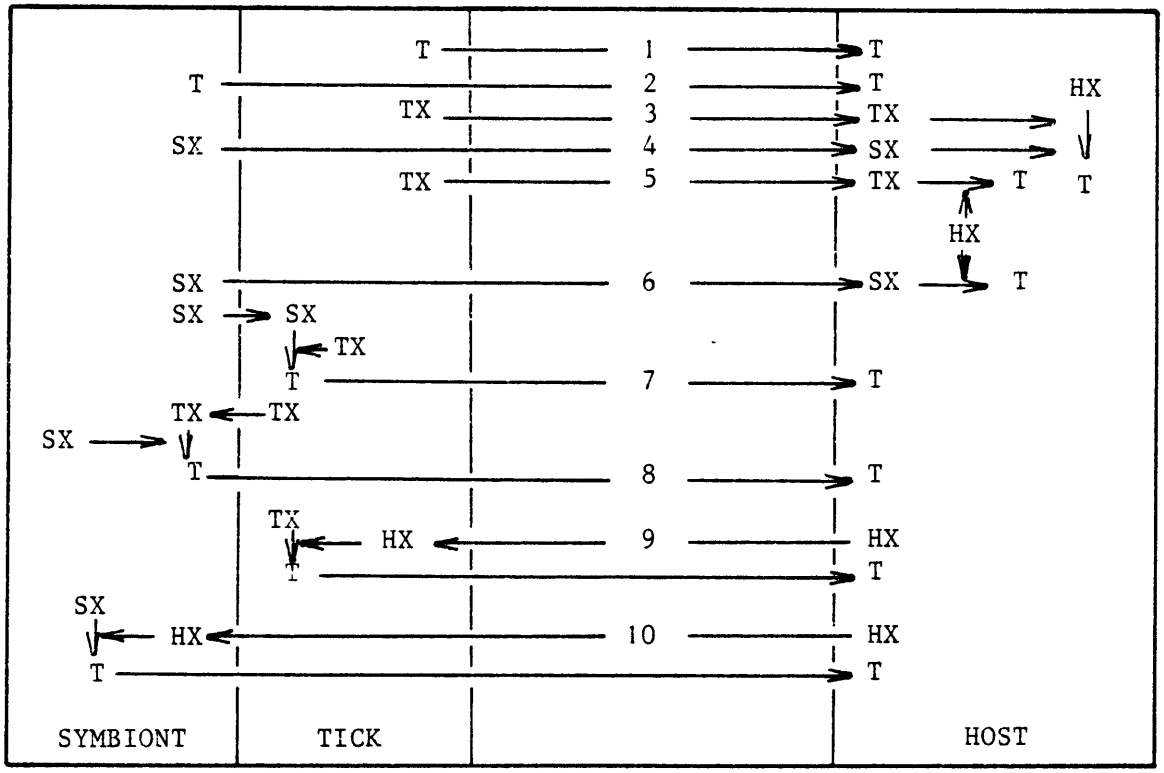


Fig 1 Some possible origins of tick toxins

Toxin is a product of tick 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.

No convincing evidence has yet been deduced for any of the alternatives, and both the sources and the chemical natures of the tick toxins are enigmatic.

1.4 Egg toxins

Toxins have been shown to be present in whole body extracts, in salivary and coxal secretions and in the eggs of ticks (18). Egg toxins have been shown to be present in the eggs of 17 species of ixodid ticks (19). Studies carried out by Neitz (18) on the toxic principle in eggs of the tick *Amblyomma hebraeum* showed that the toxin, causes paresis. Accordingly, we isolated and studied the egg toxins of different strains of *Rhipicephalus evertsi evertsi*, *Boophilus microplus*, *Boophilus decoloratus* and *Hyalomma truncatum*.

The reason why the egg toxins are being studied is to determine whether there is any resemblance between them and the toxins associated with tick toxicosis. The characterization of the tick toxins (18) is a prerequisite for the investigation into the possible symbiotic or commensal prokaryotic origin of the toxins. The chemical analysis of the composition of the egg toxins and their biological activity may lead to a better insight into the biochemistry of tick metabolism and selective antimicrobial defence mechanisms of ticks and tick eggs (18). Neitz (20) reported that no toxic component could be found in the eggs of the soft tick, *Ornithodoros savignyi*.

1.5 Tick-symbiont relationship

Symbiosis can be defined as the living together of two or more organisms in close association. The association may be beneficial to both (mutualism), beneficial to one

without effect on the other (commensalism), beneficial to one and detrimental to the other (parasitism), detrimental to one without effect on the other (amensalism) or detrimental to both (synnecrosis)(21).

Smith and Kilbourne (1893) proved the cattle tick *(Boophilus annulatus)* to be the intermediate host of *Babesia bigemina* (8). Similar discoveries followed and today a great variety of causal agents (protozoa, rickettsiae, anaplasms, viruses, spirochaetes, bacteria, etc.) are known to be transmitted by members of the families Ixodidae Murray and Argasidae Canestrini (10). These causal agents can be pathogenic (22). Their function in ticks is still unknown.

The study presented in this thesis was conducted on rickettsiae and the heartwater agent *C. ruminantium*.

1.5.1 Rickettsiae

Rickettsiae are except for *Rickettsia quintana* obligate intracellular parasites, which separates them from all the bacteria. They are distinguished from the Chlamydia, by virtue of their mode of reproduction and by fundamental differences in metabolism (23). Rickettsiae possess a extraordinarily wide natural host range which includes insects, ticks, mites, birds and mammals (1).

Characteristically, rickettsiae are transmitted from vertebrate to vertebrate, in which they usually cause disease, by an arthropod. They are transmitted transovarially from arthropod to arthropod, in which they do not cause disease (16). Exceptions are *R. prowazekii* and *R. typhi* which are not transmitted transovarial. Rickettsiae are also transmitted transstadially from

one stage to another.

Studies by Anacker *et al* (24) and Weiss (25,26) showed overwhelming evidence that their fine structure and chemical composition is quite similar to that of Gram-negative bacteria. The typical cell is rod-shaped, 0,3 to 0,7 µm by 1,5 to 2 µm in dimension. Rickettsiae have not been grown in the absence of host cells (27), despite several elaborate attempts, but are most commonly cultivated in fertile chicken eggs (26). They have been most useful for the propagation of large quantities of rickettsiae.

1.5.2 *Cowdria ruminantium*: the causal organism of heartwater (10)

Cowdria ruminantium was described morphologicaly in 1926 as pleomorphic, Gram-negative organisms present in the endothelial cells of the renal glomeruli, cerebral cortex and other organs of the host animal. Cowdry (27) also described these organisms in the intestinal epithelial cells of the tick *Amblyomma hebraeum*. Cowdry was the only person to identify the agent of heartwater in tick tissue to present. Pienaar (27) and Du Plessis (28,29) have identified the organisms in brain smears of host animals and with the indirect fluorescent antibody test on many occasions.

Electron microscopical studies on host tissue (30) showed *C. ruminantium* as pleomorphic, varying in size from 0,49 µm to 2,7 µm in diameter. They occur as dense masses, varying from a few individuals up to several hundred in the cytoplasm of an infected cell.

In South Africa, *C. ruminantium* is transmitted exclusively by ticks belonging to the genus *Amblyomma*. Four *Amblyomma* species proved to be vectors of heartwater

(10). Stage to stage transmission occurs only within the same generation but transovarial transmission does not take place. Preliminary studies on the immunity of heartwater indicate that serum antibodies play little or no role in the resistance against the disease (9). Isolation of *C. ruminantium* could lead to its characterisation and culture which may possibly contribute to improve methods of control of the disease (9).

1.6 Abatement strategies for the tick problem

Possible methods available for dealing with the tick problem are summarised below (adapted from Philip (7)).

1. Environmental control

1.1 Area and premises management.
1.1.1 Sanitation to reduce breeding of vectors.
1.1.2 Pasture spelling (31).
1.2 Physical manipulation.
1.2.1 Use of traps and lures.

2. Chemical control

2.1 Acaricides.
2.1.1 Soil treatment.
2.1.2 Treatment of host body (dipping).
2.1.3 Systemic treatment of host.
2.1.3.1 Resulting in tick resistance.
2.1.3.2 Treatment of affected hosts (antibiotics).
2.1.3.3 Prophylactic treatment (avermectins)(32).
2.2. Use of repellents.
2.3 Use of baits (eg. carbon dioxide)(33).

3. Biological control

3.1 Induced destruction by natural enemies.

3.1.1 Promoted predation (eg. red-and-yellow billed oxpeckers)(34).

3.1.2 Environmental contamination with pathogenic agents of diseases of vectors.

3.2 Interference in biotic potential (Sterilisation of insect sexes).

3.3 Biological baits (pheromones)(35)

3.4 Reduction of disease donors.

3.4.1 By vaccination of hosts against tick toxins, pathogenic agents or against tick tissue constituents (eg. tick hormones, enzymes etc.)(36).

3.5 Breeding of tick-resistant hosts (37).

3.6 Culling and supplementary feeding of hosts (32).

3.7 Pasture plants detrimental to ticks (38).

In spite of the numerous available control measures, there are at present no realistic prospects for the eradication of ticks and tick-borne diseases. In fact some control measures have led to serious problems. For example, the wide-spread use of chemicals has resulted in environmental pollution, development of resistance to pesticides in tick population, chemical residues in meat and milk products. The use of pesticides may also lead to the reduction of useful nontarget species populations such as predators and parasites (2).

Furthermore, many of the control measures are ineffective. For example, environmental control and control programs based on tick resistant cattle and strategic chemical applications can only be successful if exact ecological data on tick species is available (39). Unfortunately this data is inadequate at present. The effective application of biological control, in particular the use of vaccines, which have tremendous potential as a control measure will ultimately depend

upon our understanding of tick, host and symbiont associations. This in turn will only be possible if investigations on the molecular level are extended. Far too little is known at present concerning the biochemistry of tick tissues and the pathogenic organisms they accommodate and transmit (40).

With this in mind the present study was undertaken. The isolation and characterisation of several tick toxins from various species is described as well as the isolation of symbionts. The aim being to eventually describe the aetiology of tick-borne diseases and toxicoses on a molecular level. These findings could hopefully be exploited advantageously for the abatement of the tick problem.

CHAPTER 2

ISOLATION AND CHARACTERISATION OF EGG TOXINS

2.1 Materials and Methods

2.1.1 Ticks and tick eggs

The eggs of the following tick species were investigated:

1.	*Rhipicephalus evertsi evertsi*:	Boshoff strain
		Duncan strain
		Warrenton strain
		Sweetwater strain
2.	*Hyalomma truncatum:*	Uitenhage strain
		SWA strain
		Kaalplaas strain
		SWA x Kaalplaas strain
3.	*Boophilus decoloratus:*	Van Dyk strain
		Wessels strain
4.	*Boophilus microplus:*	Onderstepoort strain

Female ticks that engorged to repletion on rabbits or sheep were collected and placed in sterile petri trays at 28°C and 70-90% relative humidity. After an oviposition period of approximately 5 days, the eggs were collected and used to prepare crude egg extracts.

2.1.2 Preparation of crude egg extracts

Analytical reagent quality reagents were used in all the experiments. The eggs were homogenised at 5°C in 2 g batches (wet mass) with an ultra turrax

(Janke & Kunkel; Kika-werk) in 10 cm^3 0,9% NaCl for 8-10 min at low speed. The egg suspension was centrifuged for 5 h at 5°C in a Rotor 40 at 80 700 g_{av} in a Beckman L5-65 centrifuge. Half maximum acceleration and braking was used. The supernatant (crude egg extract) was stored at -10°C.

2.1.3 Toxicity determinations

Guinea-pigs (200-250g) of either sex were injected subcutaneously between the scapulas in 1 cm^3 quantities. Fractions obtained during the toxin isolation procedures were made up to the original volume of the crude extract before injection. The animals were kept under observation for 7 days and the symptoms recorded. The tests were done in duplicate.

2.1.4 Histopathological investigations

The tissues used for the investigation were obtained from guinea-pigs which had been injected subcutaneously with crude egg extracts or with purified egg toxins. The investigations were performed by Dr. L. Prozesky of the Veterinary Research Institute, Onderstepoort, as described in the paper of Neitz (18).

2.1.5 Isolation procedures

2.1.5.1 Gel permeation chromatography

Upward-flow elution was used in all the gel chromatographic separations. The gel was prepared as follows: Sephadex G 100 gel (Pharmacia Fine Chemicals) (12,5g) 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: Vt, 210 cm^3;

Vo, 74 cm^3; Vi, 125 cm^3.

The crude egg extracts (ca.10 cm^3) were applied on the columns and separated using a LKB peristaltic pump, Type 4192 A, and distilled water as eluent (flow rate 30 cm^3/h). Fractions of 3 cm^3 were collected with a LKB fraction collector Type 7000 and monitored at 280 nm, with a Beckman Model 25 spectrophotometer.

The peaks were freeze-dried in a Virtus Model 10-146 MR-BA freeze-drier for 16-21 hours.

The Sephadex G100 column was calibrated with standard proteins (Pharmacia Fine Chemicals calibration kit) under the same conditions as for the sample. The proteins, 5 mg ribonuclease A (MM 13700), 5 mg chymotrypsinogen A (MM 25000), 5 mg ovalbumin (MM 43000) and 5 mg bovine serum albumin (MM 67000), were dissolved in 1 cm^3 distilled water.

2.1.5.2 Chromatofocusing

Chromatofocusing is a technique which gives exceptional resolution. The main advantages of this technique compared to conventional ion exchange chromatography are due to the focusing effect, to the fact that the sample is not exposed to extremes of pH and the separation time is much shorter (41,42).

Depending on the pI of the toxins either a PBE 118 or PBE 94 ion exchanger (Pharmacia Fine Chemicals) was used. They are supplied pre-swollen as a suspension in 24% ethanol. PBE 118 gel was degassed under vacuum and packed into a column (K15/10, Pharmacia Fine Chemicals) and regenerated with 3 bed volumes of 1 M NaCl and equilibrated with 0,025 M triethylamine (Merck), pH 11 (starting buffer).

A flow rate of 30 cm^3/h was used. The eluting buffer was prepared as follows: 4,5 cm^3 Pharmalyte, pH 8-10,5 (Pharmacia Fine Chemicals), was made up with distilled water to a volume of 200 cm^3. The pH was adjusted to pH 7 with 1 M HCl. The buffers were degassed for 1 h under vacuum before use.

The PBE 94 column was packed and regenerated as described for the PBE 118 column. Equilibration was achieved with 0,025 M imidazole-HCl, pH 7,4. The eluent was prepared as follows: Polybuffer 74 (Pharmacia Fine Chemicals), 25 cm^3 was diluted to a final volume of 200 cm^3 with distilled water and adjusted with 1 M HCl to pH 4. The buffers were degassed for 1 h under vacuum before use.

The freeze-dried sample, dissolved in 2 cm^3 starting buffer, was applied after 1 cm^3 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 and the eluent was collected with a LKB fraction collector Type 2112 and monitored at 280 nm with a Beckman Model 25 spectrophotometer.

2.1.5.3 Removal of Polybuffer 74 and Pharmalyte from sample fractions

Polybuffer 74 was separated from sample components by means of a 19,5 x 1 cm Sephadex G 10 column, using distilled water as eluent.

Pharmalyte was removed with a Pharmacia Fine Chemicals PD-10 column (containing Sephadex G 25 gel) and subsequent dialysis using Spectrapor tubing (Spectrum Medical Industries Inc.) with a cutoff of 10 KDal. The tubing was washed extensively with

distilled water, followed by washing in deionized water.

2.1.6 Characterisation procedures for tick egg toxins

2.1.6.1 Analytical isoelectric focusing

The method as described in the isoelectric focusing Manual of Pharmacia Fine Chemicals was used. A stock polyacrylamide solution containing 15% (w/v) acrylamide (BDH) and 0,5% (w/v) N,N'-methylene bisacrylamide (BDH) was used. The charged species were removed by mixing the solution with an Amberlite MB-1 (Serva) ion exchanger resin. The Amberlite was filtered off with Whatman nr. 1 filterpaper. The stabilising medium for isoelectric focusing was prepared by mixing the following solution:

10 cm^3 stock polyacrylamide
12 cm^3 distilled water
2 cm^3 Pharmalyte pH 3-10 (Pharmacia Fine Chemicals)
6 cm^3 50% (v/v) Glycerol (Merck).

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%.

The solution was degassed under vacuum for ca. 1 h while the glass cassette (225 x 100 x 1 mm) was flushed with nitrogen gas for ca. 1 h. Immediately before casting the gel into the cassette 0,1 cm^3 of a 5% (w/v) Sodiumsulphite (Protea Laboratory Services) and of a 2,28% (w/v) ammonium persulphate (BDH) solution was added to the solution.

The samples were applied onto the gel with a mask (ca. 0,005 cm^3) or by using a filterpaper (3 x 6 mm) containing ca. 0,025 cm^3 sample. Standard proteins with known isoelectric points (Pharmacia Fine Chemicals) were always run simultaneously with samples.

Isoelectric focusing was carried out with a Pharmacia electrophoresis constant power supply ECPS 2000/300 and flat bed apparatus FBE-3000, at a constant power of 30 Watt. The gel was fixed with 5% trichloroacetic acid (Merck) and 5% sulphosalicylic acid (Merck). The protein bands were stained with 0,2% (w/v) Coomassie brilliant blue R 250 (Merck) in 45% methanol and 10% acetic acid (43).

In some cases the direct colouring method of Blakesley (44) was used. Coomassie brilliant blue G 250 (Merck), 0,2% (w/v) solution was thoroughly mixed with 60 cm^3 2 N H_2SO_4 and left for 4 h. The precipitate was filtered off with Whatman nr 2 filterpaper. To the filtrate, one-ninth volume of 10 M KOH was added followed by 100% trichloroacetic acid to a final concentration of 12% (w/v).

The isoelectric points of the separated sample components were determined from a standard curve of pI *versus* distance of the zones, measured from the cathode.

2.1.6.2 Sodium dodecyl sulphate (SDS) gradient gel electrophoresis

Acrylamide (BDH) and N,N'-methylene bisacrylamide (BDH) were used to prepare a 4 to 30% polyacrylamide gradient gel. The gel concentrations in the solutions were: T = 4%, C = 4% and T = 30%, C = 4%. The gradient was formed with a Pharmacia Fine Chemicals GM-1 gradient mixer and a Pharmacia Fine Chemicals peristaltic pump P-1 (45). The gradient was pumped into a Pharmacia Fine Chemicals gel slab casting apparatus GSC-8 containing 4 glass cassettes (80x80x3 mm). The method used is described in the

polyacrylamide gel electrophoresis in SDS Manual of Pharmacia Fine Chemicals. Ammonium persulphate was used as catalyst and 0,09 M Tris, 0,08 M borate, 3 mM EDTA and 0,2% SDS (Merck) as electrophoretic buffer. Samples were dissolved in the electrophoretic buffer and heated for 5 min at 95°C on a warm-bath in the presence of 1% SDS and 1,25% dithiothreitol. After dissociation of the sample an equal volume of 60% sucrose was added. Electrophoresis was carried out in a Pharmacia Fine Chemicals GE-4 apparatus with a voltage of 150V. Standard proteins with known molecular mass (Pharmacia Fine Chemicals) were always run simultaneously with samples. Fixing of the gradient gel was performed with 25% *iso*-propanol and 10% acetic acid for 30 min at 24 V with a Pharmacia Fine Chemicals GD-4 destainer apparatus and DPS power supply. The protein bands were stained with 0,2% Coomassie brilliant blue R250 in 45% methanol and 10% acetic acid for ca. 12 h and destained with a Pharmacia Fine Chemicals GD-4 destainer apparatus and a DPS power supply at 24 V for 60 min.

2.1.6.3 Determination of direct haemagglutination

Haemagglutination was carried out using the microtiter technique in U-shaped microtiter plates (46, 47,48). Sheep-rbs, Chicken rbs and Human-rbs were used. The rbs were washed 4 times with ca. 8 cm^3 0,075 M NaCl, 0,075 M phosphate buffer, pH 7,2. The cells were sedimented by centrifugation with a Piccolo bench top centrifuge for 5 min at 270 g_{av} and 25°C. The sedimented rbs (ca. 2 cm^3 were made up to 10 cm^3 with the NaCl-phosphate buffer. The 10 ml represented a 100% cell suspension from which a 10% cell suspension was prepared for haemagglutination.

2.1.6.4 Molecular mass determination by sedimentation equilibrium centrifugation

Conventional sedimentation equilibrium centrifugation was performed in a Spinco Model E ultracentrifuge equipped with an ultraviolet photo-electric scanner. Samples were dissolved in 0,05 M Tris, 0,1 M KCl pH 8 so as to give a solution with absorbance of 0,35-0,4 at 280 nm, in a Beckman Model 25 spectrophotometer. A charcoal double sector cell and an An-D rotor was employed. The sample volume in the cell was 0,12 cm^3. Overspeeding of the rotor was used to shorten the transient time to equilibrium. Absorbance (A) was measured as a function of radial distance (r) across the fluid column of the cell. After sedimentation equilibrium, maximum overspeed was used for 3 h to determine the absorbance base line. The slope of log A *versus* r^2 plot was calculated and the slope $d\log A/dr^2$ was substituted in the equation, $MM = (2,303)(2RT)(d\log A)/1-\bar{v}\rho)\omega^2 dr^2$ (49) with $\bar{v}$ as 0,725 $cm^3 g^{-1}$.

2.1.6.5 Amino acid determinations

Samples (1 mg) were weighed on a Cahn-RG electrobalance and hydrolysed with 1 cm^3 constant boiling HCl, for 24 h at 110°C (50). The hydrolisate was dried under nitrogen at room temperature and made up to 1 cm^3 with a 0,2 N lithium citrate buffer, pH 2,2. A Beckman Model 121M amino acid analyser was employed for the separation and quantitative estimation of the amino acids. A program based on the Beckman program (Beckman amino acid instruction manual for the Model 121M (121M-TB-003)) for the analysis of amino acids in physiological fluids was used.

2.1.6.6 Anti-protease activity determinations

2.1.6.6.1 Fast binding inhibition

Inhibition studies of trypsin and chymotrypsin by isolated egg toxins was performed according to the methods of Frits (51) and Erlanger (52) respectively. The reactions involved are shown in Scheme 1 and 2.

The following stock solutions were used: for Trypsin inhibition studies: $6,9x10^{-8}$M trypsin, $7,6x10^{-4}$M BAPNA, $8,4x10^{-7}$M inhibitor (*R.ee.* egg toxin) and 0,1 M sodium phosphate buffer pH 7,4; for chymotrypsin inhibition studies: $1x10^{-4}$M chymotrypsin, $5,2x10^{-5}$M SPNA, $5,1x10^{-4}$M inhibitor (*B.dec.* egg toxin) and 0,1 M sodium phosphate buffer pH 7,4.

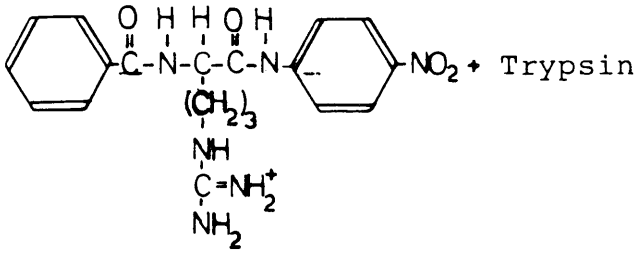
Typical concentrations and volumes for trypsin and chymotrypsin assays are shown in Tables 3 and 4.

The formation of p-nitroaniline was monitored at 405 nm with a Beckman Acta M VI spectrophotometer, at 25^{o}C.

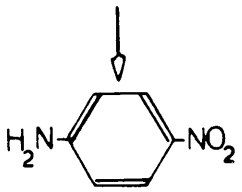
2.1.6.6.2 Slow-binding inhibition

Due to the complexicity of the kinetics involving slow-binding inhibitors a short general description of proposed models is outlined in 2.1.6.6.2.1, 2.1.6.6.2.2 and 2.1.6.6.2.3.

2.1.6.6.2.1 Kinetic model for reversible inhibition

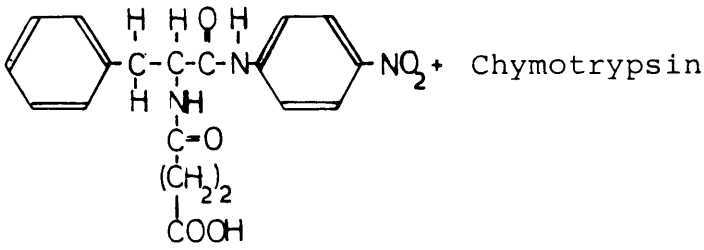


BAPNA

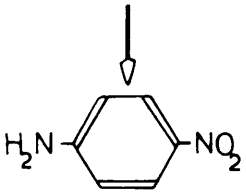


p-nitroaniline

Scheme 1 Trypsin assay with BAPNA as substrate



SPNA



p-nitroaniline

Scheme 2 Chymotrypsin assay with SPNA as substrate

TABLE 3 TYPICAL CONCENTRATIONS AND VOLUMES USED IN A TRYPSIN ASSAY

	VOLUME (cm^3)[a]	VOLUME (cm^3)[b]	CONCENTRATION IN CUVETTE (M)
Buffer	0,99	0,98	0,033
BAPNA	2	2	$5,1x10^{-4}$
Trypsin	0,01	0,01	$2,3x10^{-10}$
Inhibitor[c]	0	0,01	$2,8x10^{-9}$
Total volume in cuvette	3	3	

a: Activity determined of trypsin

b: Activity determined of trypsin in the presence of inhibitor

c: *R.ee.* egg toxin used as example.

TABLE 4 TYPICAL CONCENTRATIONS AND VOLUMES USED IN A CHYMOTRYPSIN ASSAY

	VOLUME (cm^3)[a]	VOLUME (cm^3)[b]	CONCENTRATION IN CUVETTE (M)
Buffer	0,99	0,98	0,033
SPNA	2	2	$3,5x10^{-5}$
Chymotrypsin	0,01	0,01	$3,3x10^{-7}$
Inhibitor[c]	0	0,01	$1,7x10^{-6}$
Total volume in cuvette	3	3	

a: Activity determined of Chymotrypsin

b: Activity determined of chymotrypsin in the presence of inhibitor

c: *B.dec.* egg toxin used as example.

The slow establishment (53) of the inhibition of enzymes has long been known. When a reaction involving a reversible slow-binding inhibitor, is started by the addition of enzyme, the relatively rapid initial velocity decreases to a slower steady-state rate (Fig 2). If the enzyme is pre-incubated with inhibitor and the reaction started with substrate, there will be a slow decrease of inhibition and ultimately a steady-state rate will be attained. With no significant enzyme inactivation, substrate depletion or product inhibition, the two steady-state rates will be identical (Fig 2).

Reversible inhibitors can be grouped into four categories (Table 5) on the basis of inhibitor and enzyme concentrations and whether the equilibria are established rapidly or slowly.

TABLE 5 CLASSIFICATION OF REVERSIBLE ENZYME INHIBITORS

	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-binding	$I_t > E_t$	slow
Slow, tight-binding	$I_t \simeq E_t$	slow

I_t: Total inhibitor concentration.

E_t: Total enzyme concentration.

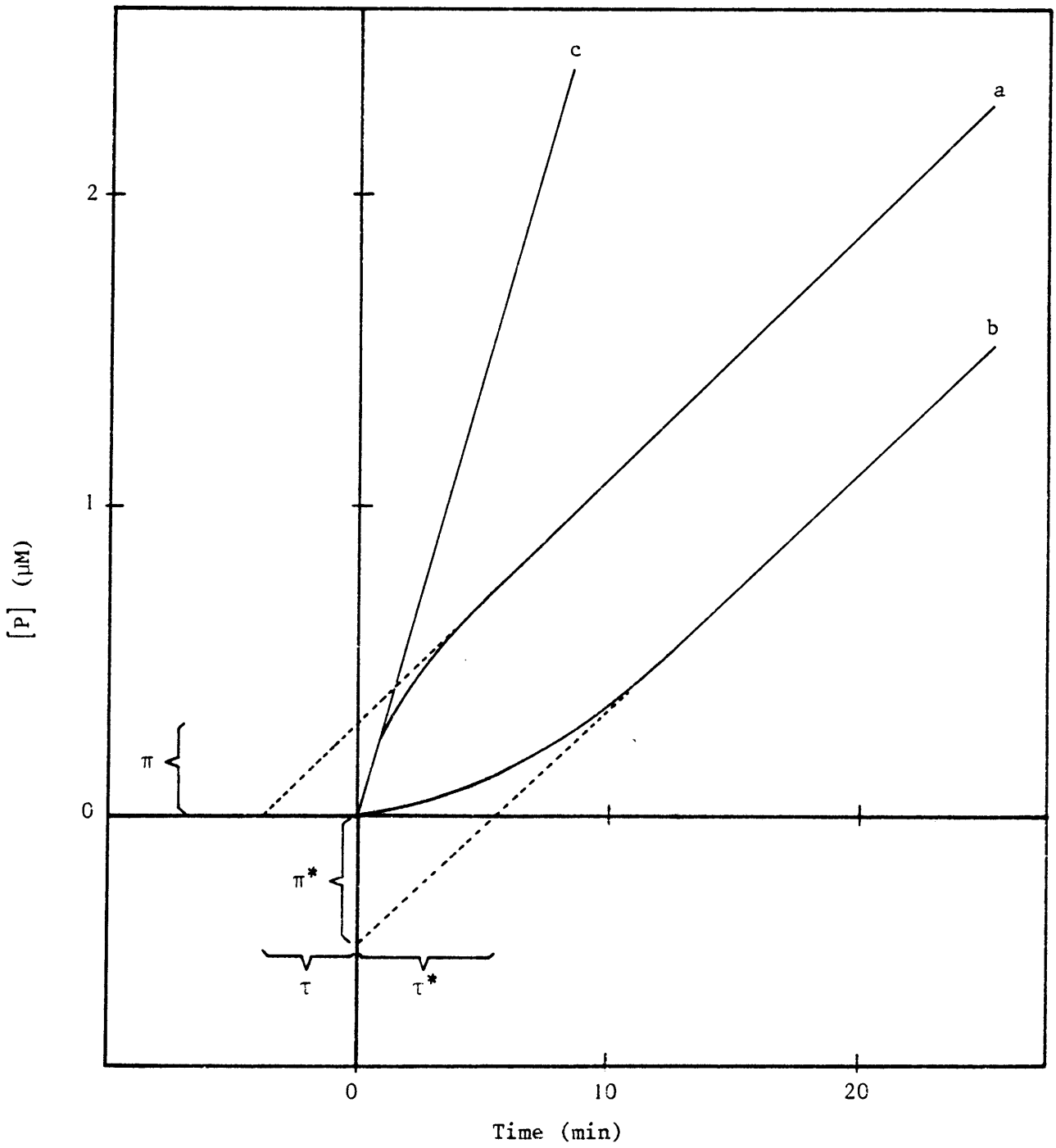


Fig 2 Theoretical curves for reversible slow-binding inhibition when a reaction is started (a) with enzyme, (b) with substrate after pre-incubation of enzyme and inhibitor and (c) no inhibitor

When the concentration of the inhibitor is more than ten times greater than that of the enzyme (53) the theoretical curves (Fig 2) can be described by the integrated equation:

$$P = v_s t + (v_o - v_s)(1 - e^{-kt})/k \qquad \text{Eqn (1)}$$

in which P is the product concentration, t is the time, v_o the initial velocity, v_s the steady-state velocity and k the apparent first-order rate constant. v_o, v_s and k will depend on the mechanism followed (Table 6).

TABLE 6 RELATIONSHIPS FOR THE APPARENT FIRST ORDER RATE CONSTANTS (k) ASSOCIATED WITH MECHANISMS A AND B[a]

MECHANISM	k	INHIBITION CONSTANT
A	$k_4\left[1 + \frac{1}{Ki(1 + S/Ka)}\right]$	$Ki = \frac{k_4}{k_3}$
B	$k_6\left[\frac{1 + \frac{1}{Ki^*(1 + S/Ka)}}{1 + \frac{1}{Ki(1 + S/Ka)}}\right]$	$Ki = \frac{k_4}{k_3}$ $Ki^* = Ki\left[\frac{k_6}{k_5 + k_6}\right]$

[a] It is assumed that the inhibitor (I) gives rise to linear competitive inhibition with respect to substrate (S). Ka is the Michaelis constant for S and Ki^* is the overall inhibition constant. The steps associated with the rate constants are shown in Fig 3.

The values obtained for v_o and v_s at different concentrations of inhibitor (53) can be fitted to the equation:

$$v = v_o/(1+I/Ki_{app}) \qquad \text{Eqn(2)}$$

and the true Ki values calculated by using the relationship:

$$Ki = Ki_{app}/(1+S/K_a) \qquad \text{Eqn(3)}$$

The two mechanisms described in Fig 3 assume that slow-binding inhibition is not due to the slow isomerisation of the inhibitor to a more potent specie.

The two mechanisms (Fig 3 and Table 6) exhibit different characteristics. The initial velocity is independent of the inhibitor concentration [I] for mechanism A, but varies as a function of [I] for mechanism B.
Mechanisms A and B although considered as being distinct, could vary. If the value of Ki >> Ki* (for mechanism B) and the inhibitor is varied in the region of its Ki* value, then the steady-state concentration of EI would not be kinetically significant. Mechanism B degenerates into mechanism A under the above circumstances.

It means then theoretically, that the slow, tight-binding inhibition could occur under different enzyme and inhibitor conditions as shown in Tables 6 and 7 (53,54).

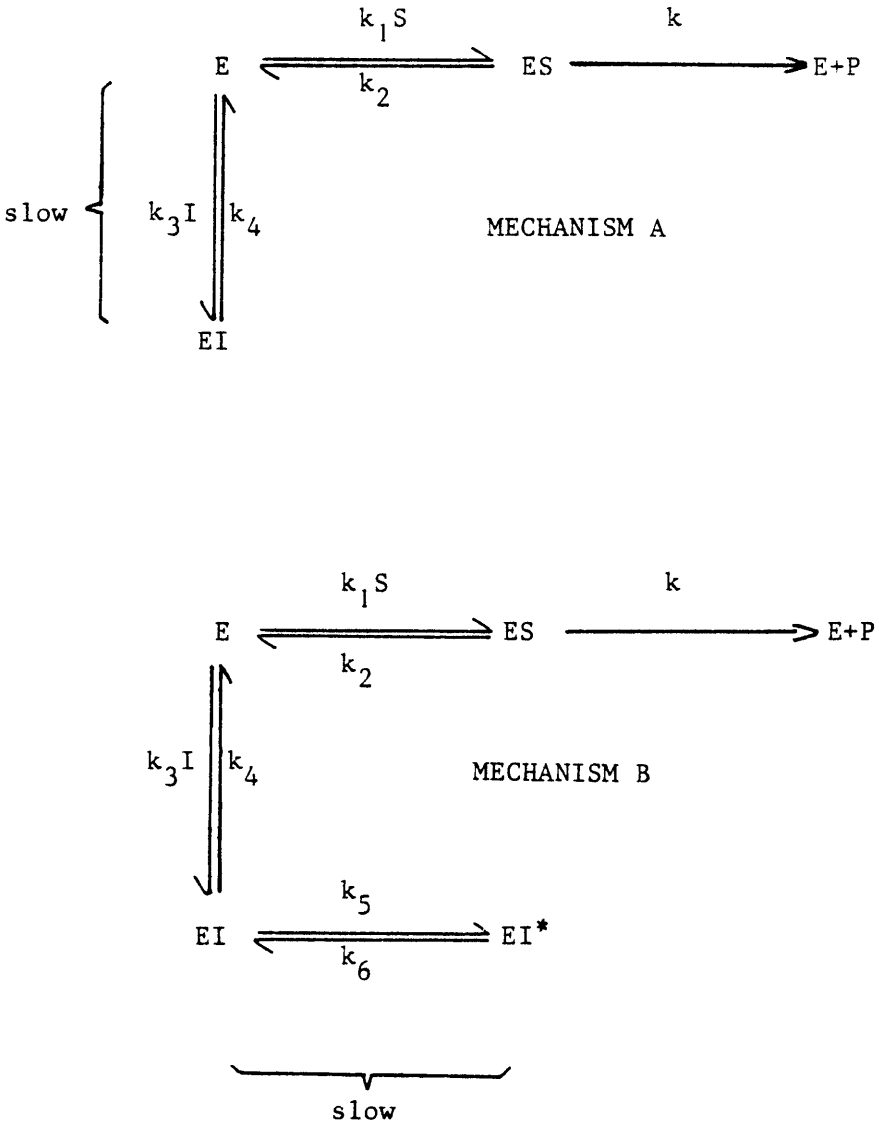


Fig 3 Mechanisms that describe reversible slow-binding and slow, tight-binding inhibition

TABLE 7 DIFFERENT CONDITIONS FOR SLOW AND SLOW, TIGHT-BINDING INHIBITION

MECHANISM	INHIBITOR/ENZYME	DETERMINATION OF CONSTANTS
A	$[I]_t > [E]_t$	Graphically and Computor
A	$[I]_t \simeq [E]_t$	Computor
B	$[I]_t > [E]_t$	Graphically and Computor
B	$[I]_t \simeq [E]_t$	Computor

The determination of Ki from the progress curve when $[I]_t > [E]_t$ is relatively simple for both meganisms A and B (55). This is discussed in 2.1.6.6.2.3.

However if slow-binding inhibition occurs at inhibitor concentrations which are comparable to that of the enzyme (53), mechanisms A and B (Fig 2) can still apply, but additional complexities are introduced. An integrated rate equation has been derived for mechanism A and used for analysis of progress curve data, but it has not been possible to derive such an equation for mechanism B. Instead, numerical integration procedures have been used for data analysis (54).

2.1.6.6.2.2 Kinetic model for irreversible inhibition

The kinetic model for irreversible inhibition has been described by a number of workers (56, 57,58,59). The time dependent inhibition can be described by the scheme below (60).

$$E + I \rightleftharpoons EI \xrightarrow{k_p} EI^*$$

$$\text{and } Ki = [E][I]/[EI]$$

Scheme 3 Model for irreversible inhibition where EI^* is the newly formed complex.

If the inhibitor concentration is much larger ($[I]/[E] > 10$) than that of the enzyme, it can be shown that the decrease in the $[E] + [EI]$ concentration in the incubation mixture follows pseudo-first order kinetics, at any fixed value of I. The pseudo-first order rate constant (k) can be measured by drawing aliquots periodically and assaying any residual activity. Dilution of the assay mixture is likely to lead to the disappearance of EI, so that the enzyme concentration will represent the total enzyme (E + EI) present in the aliquot (59). A semilog plot of enzyme activity against time gives k directly. It can be shown that k_{app} is related to Ki and k_p by Eqn(4) (56).

$$k_{app} = \frac{k_p[I]}{Ki+[I]} \qquad \text{Eqn(4)}$$

$$\frac{1}{k_{app}} = \frac{Ki}{k_p[I]} + \frac{1}{k_p} \qquad \text{Eqn(5)}$$

If data are available over a range of inhibitor concentrations, Ki, the dissociation constant of the EI complex, the limiting rate of inactivation,

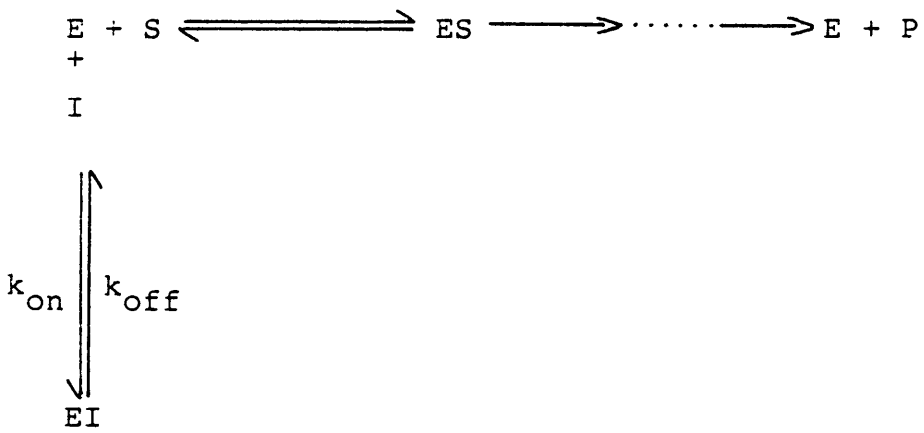
may be determined from a plot of $1/k_{app.}$ against $1/[I]$ and Eqn(5).

Rich (61) assumed that in the case where Ki of the inhibitor is in the region of 10^{-10}M and $[I]_t > [E]_t$ that "pseudo" irreversible inhibition occurs, therefore these types of tight-binding inhibitors could possibly also be studied as irreversible inhibitors. This argument would especially be valid with transition state analogs. Since preliminary inhibition data suggested potent inhibition of trypsin by the egg toxin from *H. trun.*, in a similar fashion than that of pepstatin on pepsin (61), it was decided to use this method to study the egg toxin of *H. trun.*

2.1.6.6.2.3 Graphical determination of dissociation constants

Slow-binding kinetics can be studied according to the computer programs developed by various workers (53,54), or by the method described by Baici *et al* (55), as was shown in Fig 2, an enzyme-catalysed reaction in the presence of a reversible slow-binding inhibitor, a concentration-dependent transient state lasting several minutes, preceeds the attainment of steady-state rate and is characterised by a concave upward or a concave downward lag phase depending on whether the enzyme had been pre-incubated with the inhibitor or not.

A model suggested by Biaci *et al* for this type of inhibition is shown in Scheme 4.



Scheme 4 A model for fully competitive, slow-binding inhibition

The following conditions are assumed:

a) k_{off} and k_{on} are smaller than all rate constants;
b) the reversible reaction (catalytic step) is negligibly slow;
c) $[E]_o << [I]_o$, which means also that $[I] \simeq [I]_o$;
d) $[I]_o >> Ki$
e) $Ki = k_{off}/k_{on}$

The general equation for the appearance of product contains an $[EI]_o$ term, i.e. the concentration of the enzyme-inhibitor complex at the beginning of the reaction. Depending on whether the enzyme has been pre-incubated with the inhibitor or not this value is equal to the total enzyme concentration or zero, respectively. Under these two experimental conditions, the formation of the reaction product P as a function of time is:

$$[P] = \frac{v_o k_{off}}{\lambda} t + \frac{v_o k_{on} [I]}{(1+\sigma)\lambda^2} (1-e^{-\lambda t}) \qquad \text{Eqn(6)}$$

$$[P]^* = \frac{v_o k_{off}}{\lambda} t - \frac{v_o k_{off}}{\lambda^2}(1-e^{-\lambda t}) \qquad \text{Eqn(7)}$$

In the equations; $[P]^*$ denotes that the enzyme has been pre-incubated with the inhibitor, and $[P]$ denotes no pre-incubation, v_o the initial velocity in the absence of inhibitor, $\sigma = S/Km$ and λ is an apparent pseudo-first-order rate constant (s^{-1}) which describes the exponential approach to the steady state:

$$\lambda = \frac{k_{on}}{1+\sigma}[I] + k_{off} \qquad \text{Eqn(8)}$$

After the attainment of the steady state the exponential terms in Eqns (6) and (7) become negligibly small and the above equations can be rewritten as follows:

$$[P] = \frac{v_o k_{off}}{\lambda} t + \frac{v_o k_{on}[I]}{(1+\sigma)\lambda^2} \qquad \text{Eqn(9)}$$

$$[P]^* \quad \frac{v_o k_{off}}{\lambda} t - \frac{v_o k_{off}}{\lambda^2} \qquad \text{Eqn(10)}$$

The plots of $[P]$ and $[P]^*$ *versus* time are now straight lines with the same slope = $(v_o k_{off})/\lambda$ (Fig 2). The value of the slope is equal to v_i, the inhibited reaction velocity in the steady state, for fully competitive inhibition:

$$\text{Slope} = v_i = \frac{v_o k_{off}}{\lambda} = \left[\frac{V \frac{[s]}{Km}}{1 + \frac{[s]}{Km} + \frac{[I]}{Ki}} \right] \qquad \text{Eqn(11)}$$

The intercepts of the linear portions with the $|P|$ axis are given by:

$$\pi = \frac{v_o k_{on}[I]}{(1+\sigma)\ \lambda^2} \qquad \text{Eqn(12)}$$

$$\pi^* = -\frac{v_o k_{off}}{\lambda^2} \qquad \text{Eqn(13)}$$

The intercepts of the linear portions with the time axis are given by:

$$\tau = -\frac{k_{on}[I]}{k_{off}(1+\sigma)\lambda} \qquad \text{Eqn(14)}$$

$$\tau^* = 1/\lambda \qquad \text{Eqn(15)}$$

From the above equations k_{on}, k_{off} and Ki can be calculated.

From Eqns (14) and (15):

$$k_{off} = \frac{1}{[\tau] + \tau^*} \qquad \text{Eqn(16)}$$

This is verified with the introduction of the explicit formula for λ(Eqn(8) into Eqns(14) and (15).

From Eqns (12),(13) and (16):

$$k_{on} = \frac{\pi(1+\sigma)}{[\pi^*]\ (\tau^* + [\tau])\ [I]} \qquad \text{Eqn(17)}$$

From Eqns (9),(10),(14) and (15) it can be verified that:

$$k_{off} = \frac{vo - vi}{vo} \quad \frac{1}{[\tau]} \qquad \text{Eqn(18)}$$

It is found that the experiments in which the reaction is started by the addition of the enzyme (with no pre-incubation) give more accurate and reproducible results than experiments in which the enzyme is pre-incubated with inhibitor. Therefore π^* and τ^* can not be determined with the necessary accuracy and this is particularly valid for high inhibitor concentrations, in which cases both π^* and τ^* become very small (see Eqns (13) and (15) and Fig 2). Only when the substrate and inhibitor concentration are appropriately chosen can the values of π^* and τ^* be determined with accuracy. Eqn(14) allows the correct evaluation of both k_{off} and Ki, since the value of τ can be measured precisely. Rearranging Eqn(14) into the reciprocal form we obtain:

$$\frac{1}{[\tau]} = Ki\,k_{off}(1+\sigma)\,\frac{1}{[I]} + k_{off} \qquad \text{Eqn(19)}$$

Thus, a plot of $1/[\tau]$ versus $1/[I]$ gives a straight line with intercept on the ordinate axis corresponding to k_{off} and intercept on the abscissa equal to

$$-1(1+\sigma)Ki \qquad \text{Eqn(20)}$$

Baici's model can be used generally for cases where $[I]_t >> [E]_t$ but not for cases where $[E]_t \simeq [I]_t$.

In cases $[E]_t \simeq [I]_t$ it becomes very complicated, since the equations above are no longer valid and therefore the computor programs developed by Morrison *et al* should be used (Table 7).

2.1.6.6.3 Toxicity determinations of anti-proteases

The toxicity of soybean trypsin-inhibitor (Boehringer Mannheim GmbH) and hen egg white trypsin-inhibitor (Boehringer Mannheim GmbH) was tested at dosage rates of 0,15 mg and 1,5 mg dissolved in 1 cm^3 0,9% NaCl respectively and subcutaneously injected into guinea-pigs, body mass of 150 to 200 g.

2.2 Results

2.2.1 Isolation of the egg toxins

Guinea-pigs were injected with crude egg extracts, obtained in 2.1.2. They showed symptoms on the 3rd day after injection and died on the 5th or 6th day. The guinea-pigs injected with the sediments showed very little or no symptoms. Sick guinea-pigs which survived recovered rapidly.

Chromatography of the crude egg extracts on a Sephadex G 100 column, resulted in a toxic fraction for each of the four tick species (Fig 4, 5, 6 and 7). The toxic fractions were RG II, HG II, MG I and DG I. According to the standard curve (Fig 8) the molecular mass ranges for these fractions are given in Table 8.

Each fraction was monitored with flat bed isoelectric focusing, pH range 3 to 10,5 (Fig 18, Table 12). The isoelectric focusing results showed that a separation depending on charge differences by chromatofocusing was feasable after gel chromatography. Toxicity tests done on the fractions eluted from the chromatofocusing columns (Fig 9, 10, 11 and 12) showed that fractions RCF II, HCF IV, MCF II and DCF II were toxic. According to flat bed isoelectric focusing at a concentration

TABLE 8 MOLECULAR MASS RANGES OF THE TOXIC FRACTIONS OBTAINED FROM A SEPHADEX G 100 COLUMN

FRACTION	MOLECULAR MASS RANGE* (KDal)
RG II	13,5 to 45
HG II	13,5 to 48
MG I	56 to 150
DG I	48 to 150

* Standard calibration curve was used (Fig 8)

of 1 mg freeze-dried mass in 0,1 cm^3 distilled water, the respective peaks were pure except for *B. dec.* (DCF II) which showed two bands. DCF II was subjected to rechromatofocusing on a PBE 118 column (Fig 13). At a concentration of 1 mg/0,1 cm^3 distilled water, the flat bed isoelectric focusing showed that fraction DRCF I (Fig 13) was homogeneous.

The removal of Polybuffer 74 with a Sephadex G10 column (Fig 14 and 15) and the removal of Pharmalyte with a Pharmacia Fine Chemicals PD-10 column followed with dialysis (Fig 16 and 17) were very satisfactory.

Fractions 6, 7 and 8 were combined (Fig 14) and an absorbance spectrum was recorded from 300 nm to 250 nm on a Beckman Model 25 spectrophotometer (Fig 15).

Polybuffer 74 shows a high absorbance below 265 nm (Chromatofocusing Manual of Pharmacia Fine Chemicals). A trough in the uv spectrum of the toxic samples thus served as a good indication that polybuffer was removed (Fig 15). The *R.ee.* egg toxin was freeze-dried

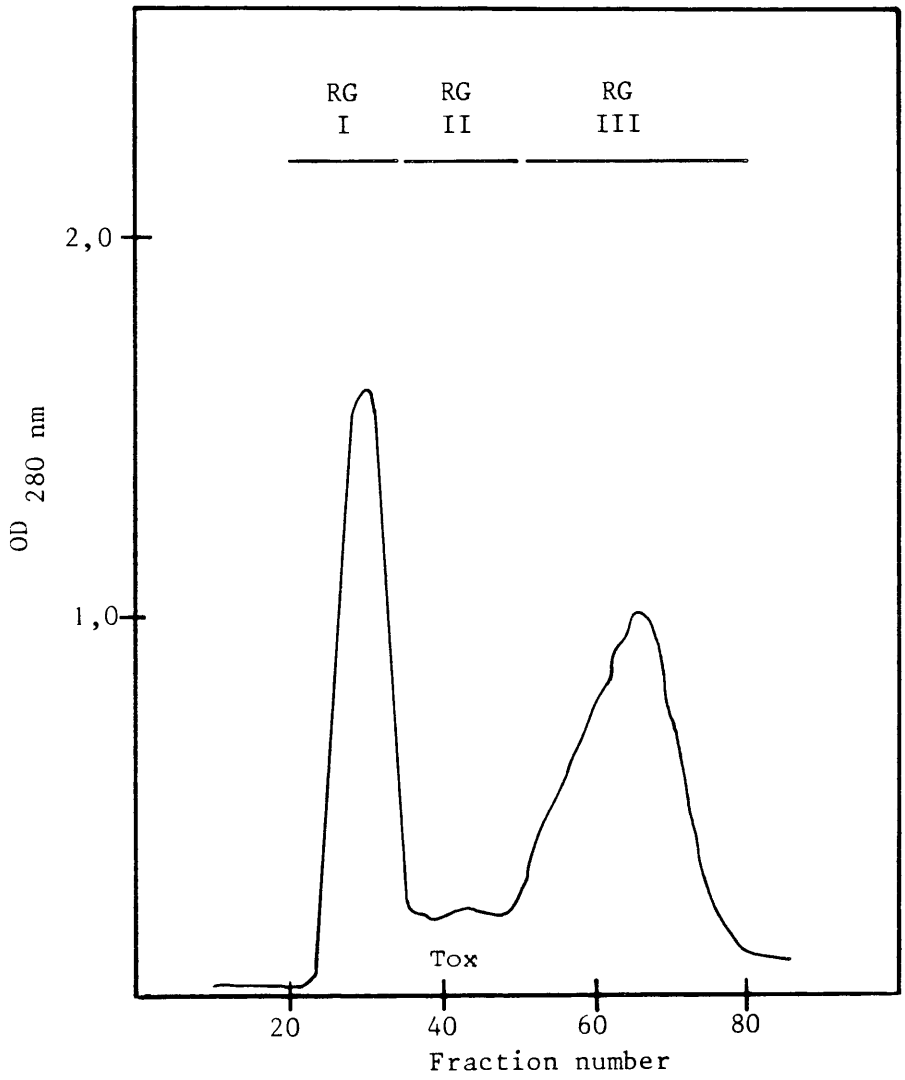


Fig 4 Gel permeation chromatography of the *R.ee.* crude egg extract on a Sephadex G 100 column (40x2,6 cm). Flow rate: 30 cm^3/h and volume/fraction: 3 cm^3. Eluent: distilled water.

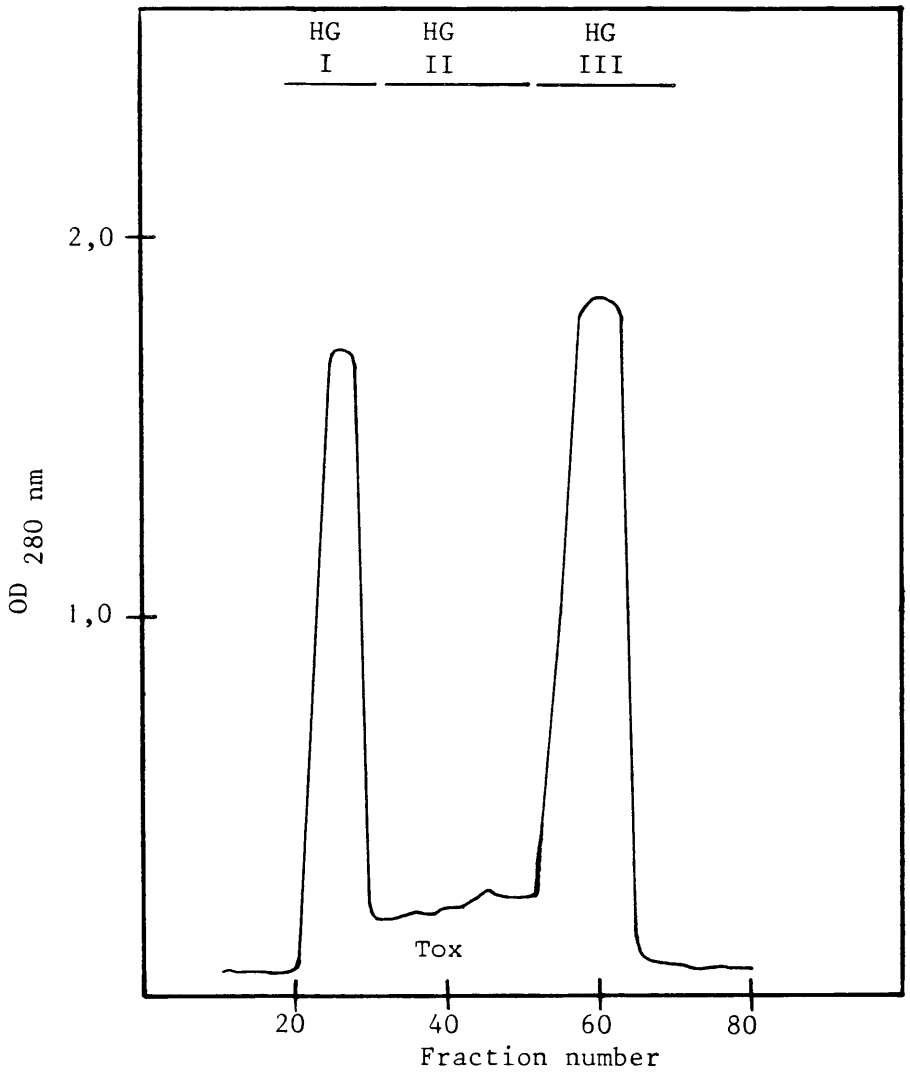


Fig 5 Gel permeation chromatography of the *H.trun.* crude egg extract on a Sephadex G 100 column (40x2,6 cm). Flow rate: 30 cm^3/h and volume/fraction: 3 cm^3. Eluent: distilled water.

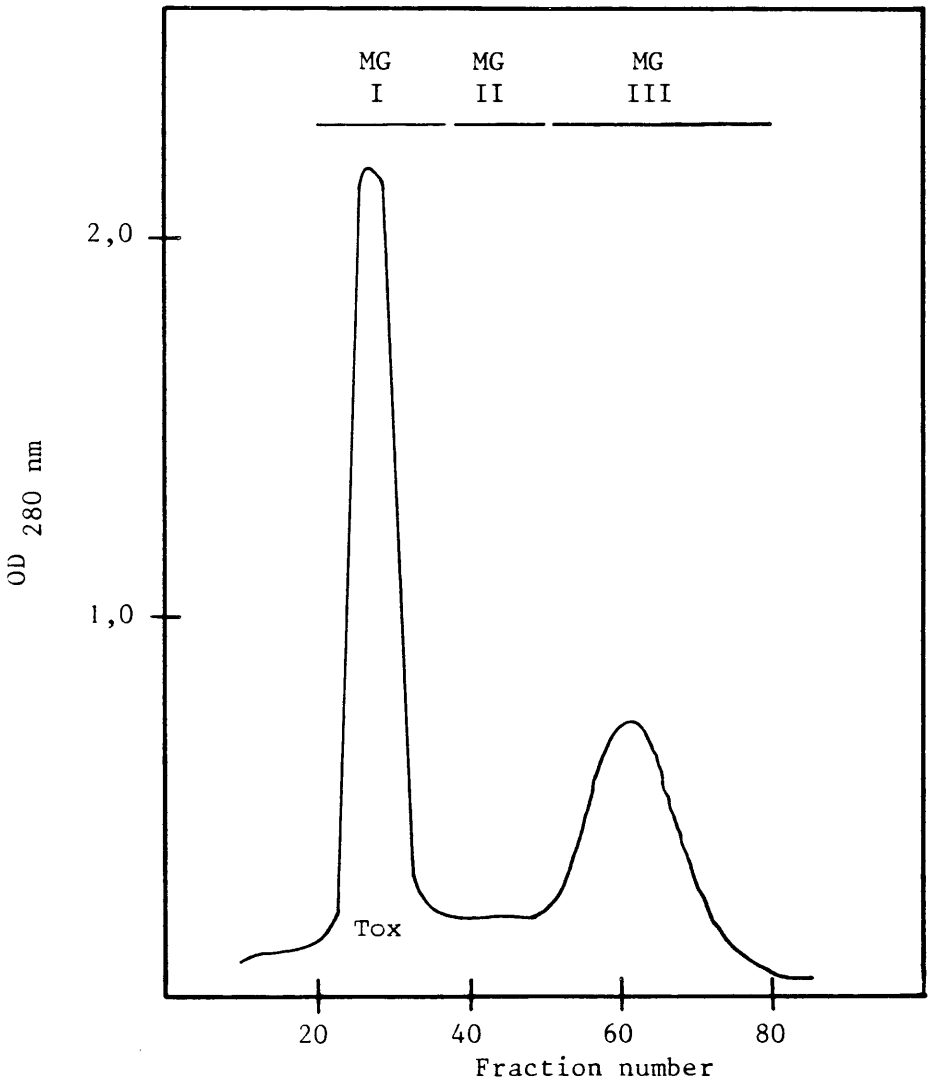


Fig 6 Gel permeation chromatography of the *B.mic.* crude egg extract on a Sephadex G 100 column (40x2,6 cm). Flow rate: 30 cm^3/h and volume/fraction: 3 cm^3. Eluent: distilled water.

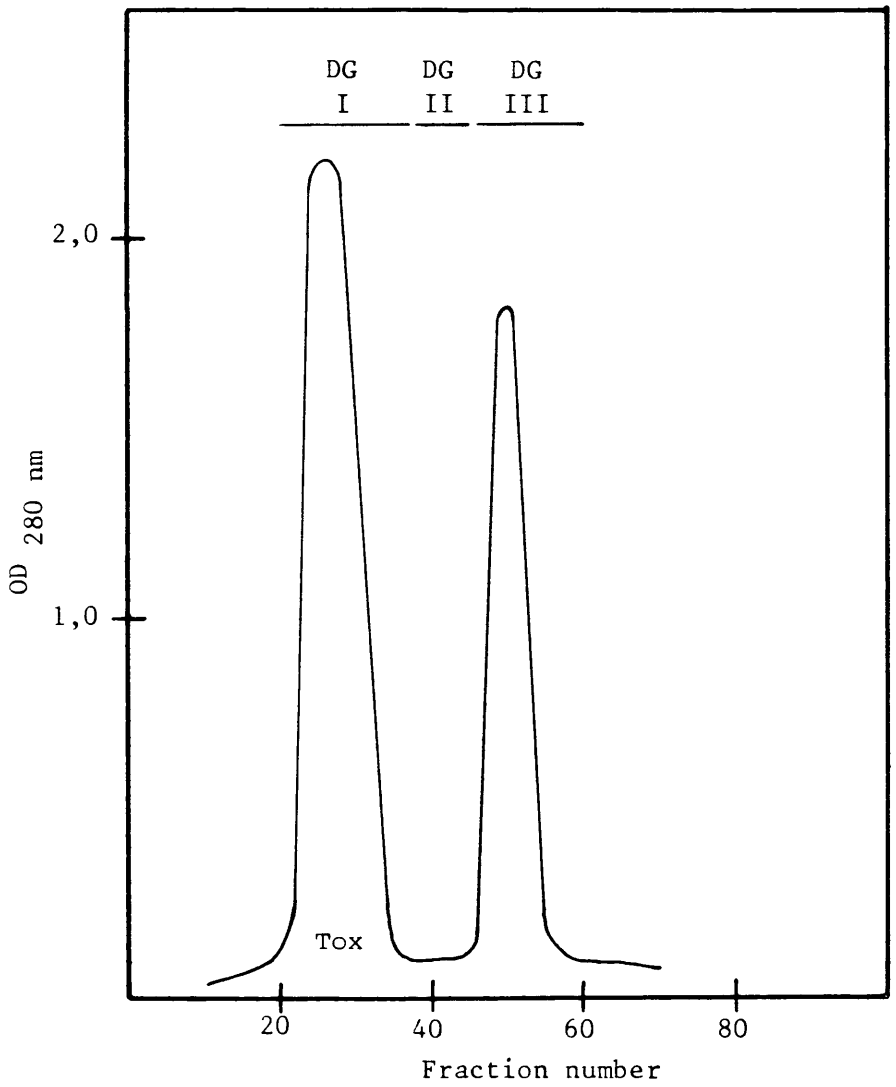


Fig 7 Gel permeation chromatography of the *B.dec.* crude egg extract on a Sephadex G 100 column (40x2,6 cm). Flow rate: 30 cm^3/h and volume/fraction: 3 cm^3. Eluent: distilled water.

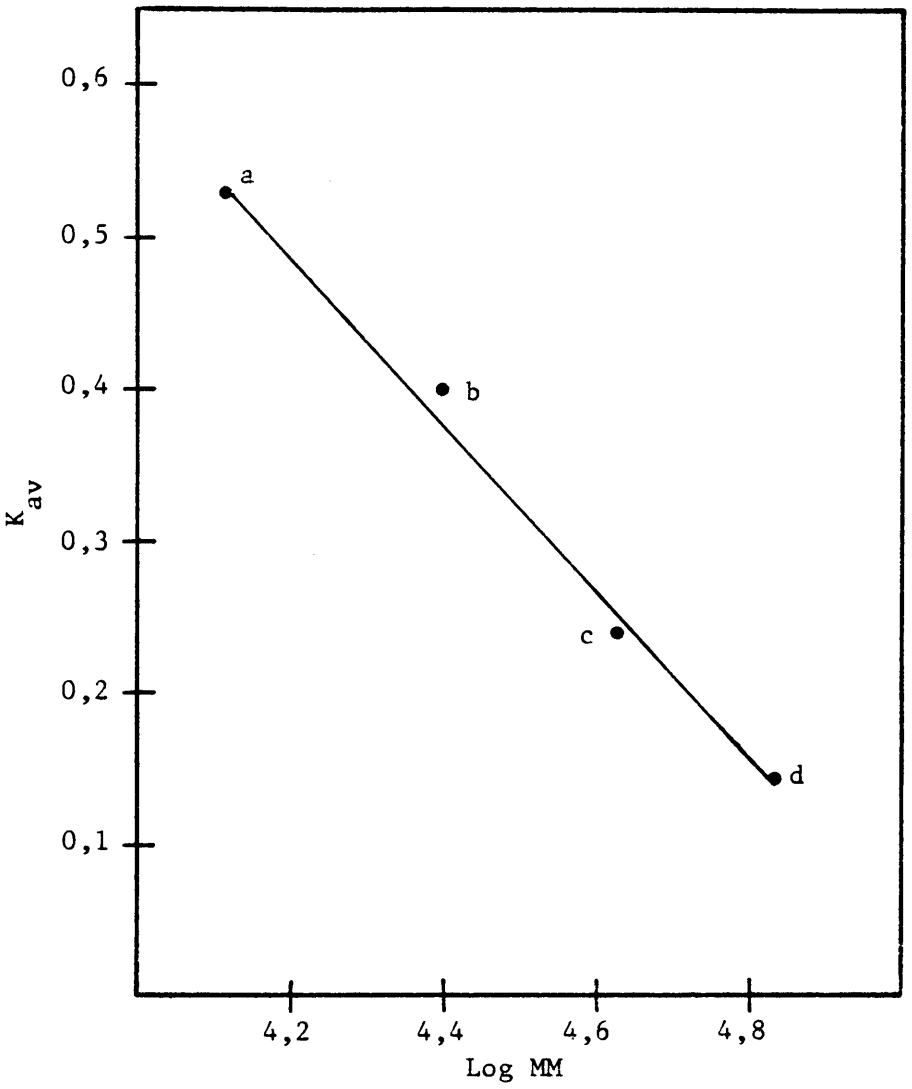


Fig 8 Calibration curve for the Sephadex G 100 column. a: ribonuclease A, b: chymotrypsinogen A, c: ovalbumin and d: bovine serum albumin. $K_{av} = (V_e-V_o)/(V_t-V_o)$

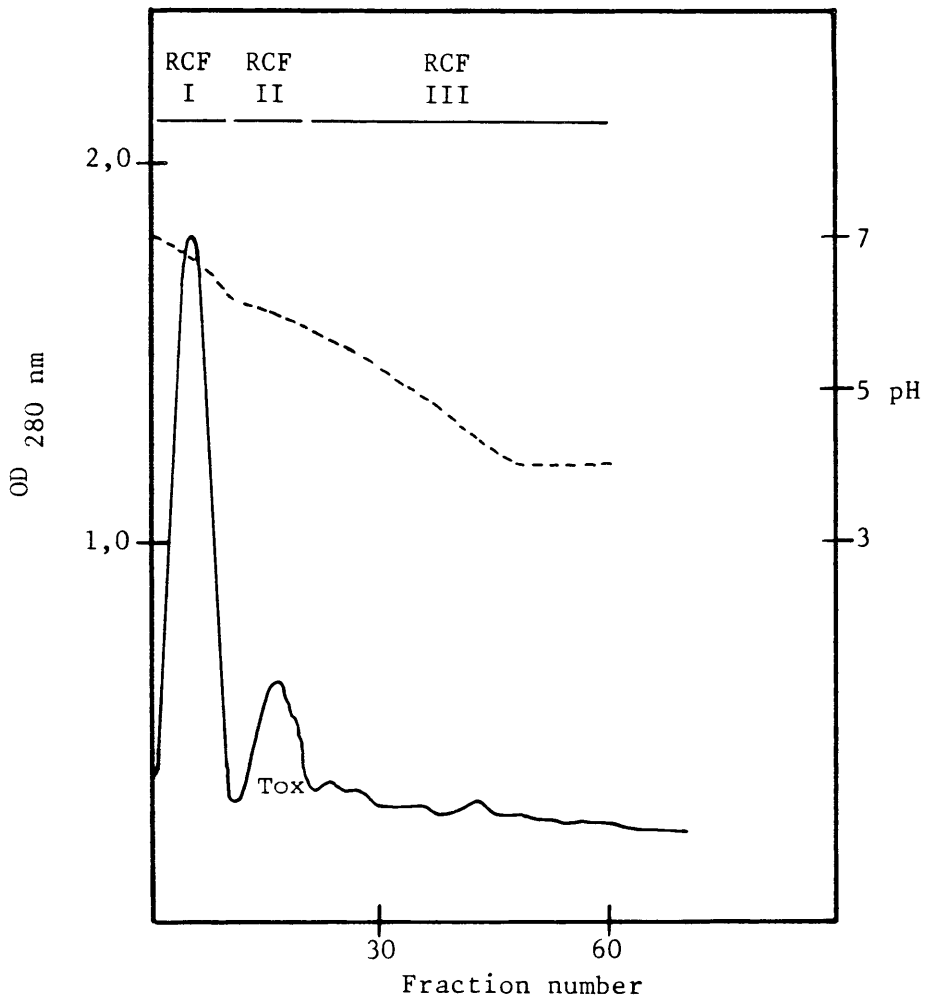


Fig 9 Eluting diagram of *R.ee.* (RG II) after chromatofocusing. Flow rate: 30 cm^3/h, volume/fraction: 3 cm^3. OD: (——); pH gradient: (---)

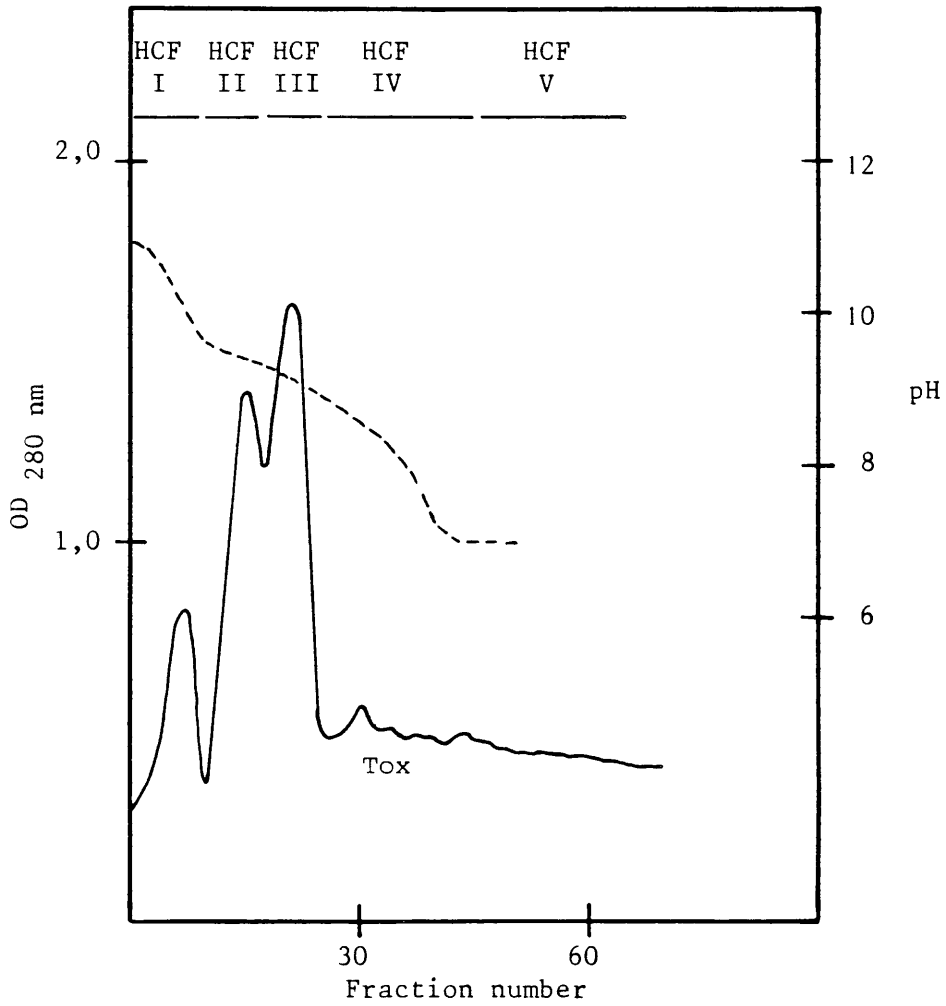


Fig 10 Eluting diagram of *H.trun.* (HG II) after chromatofocusing. Flow rate: 30 cm^3/h, volume/fraction: 3 cm^3. OD: (——); pH gradient: (---)

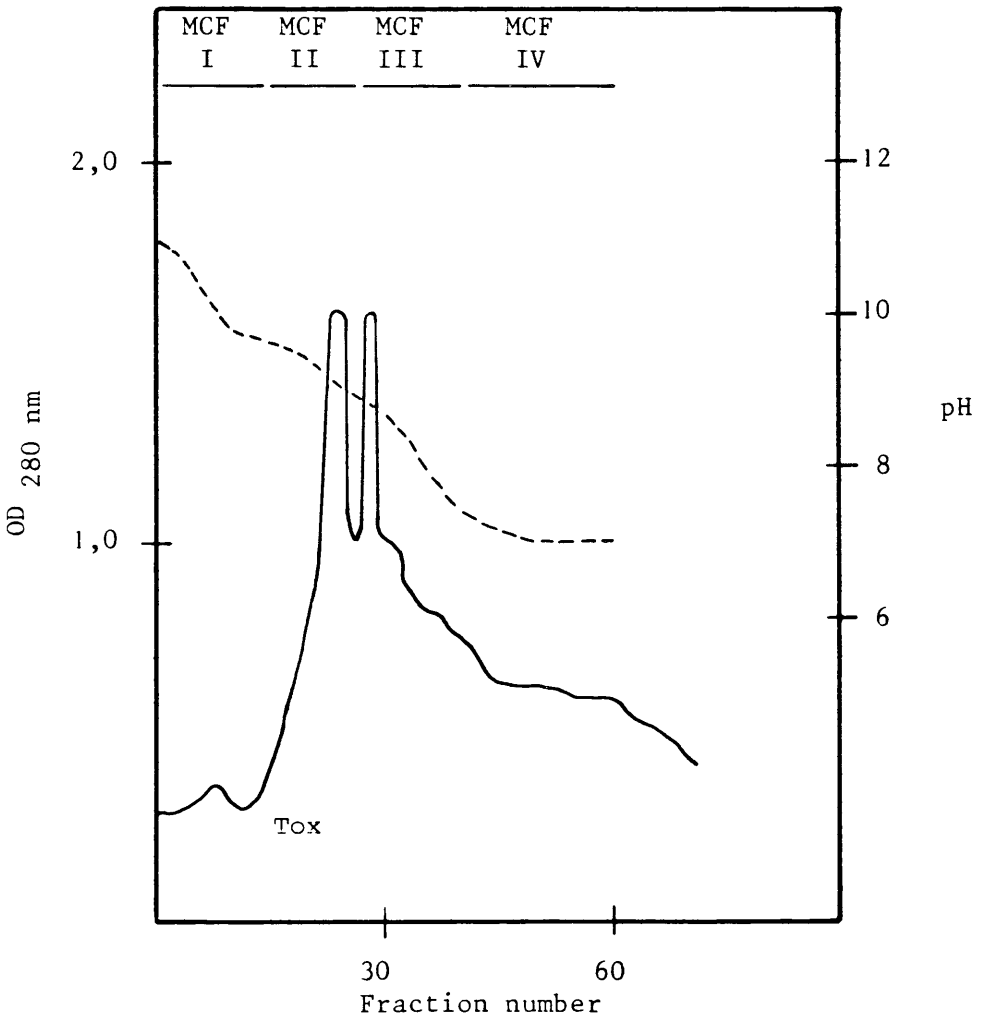


Fig 11 Eluting diagram of *B.mic.* (MG I) after chromatofocusing. Flow rate: 30 cm^3/h, volume/fraction: 3 cm^3. OD: (——); pH gradient: (---)

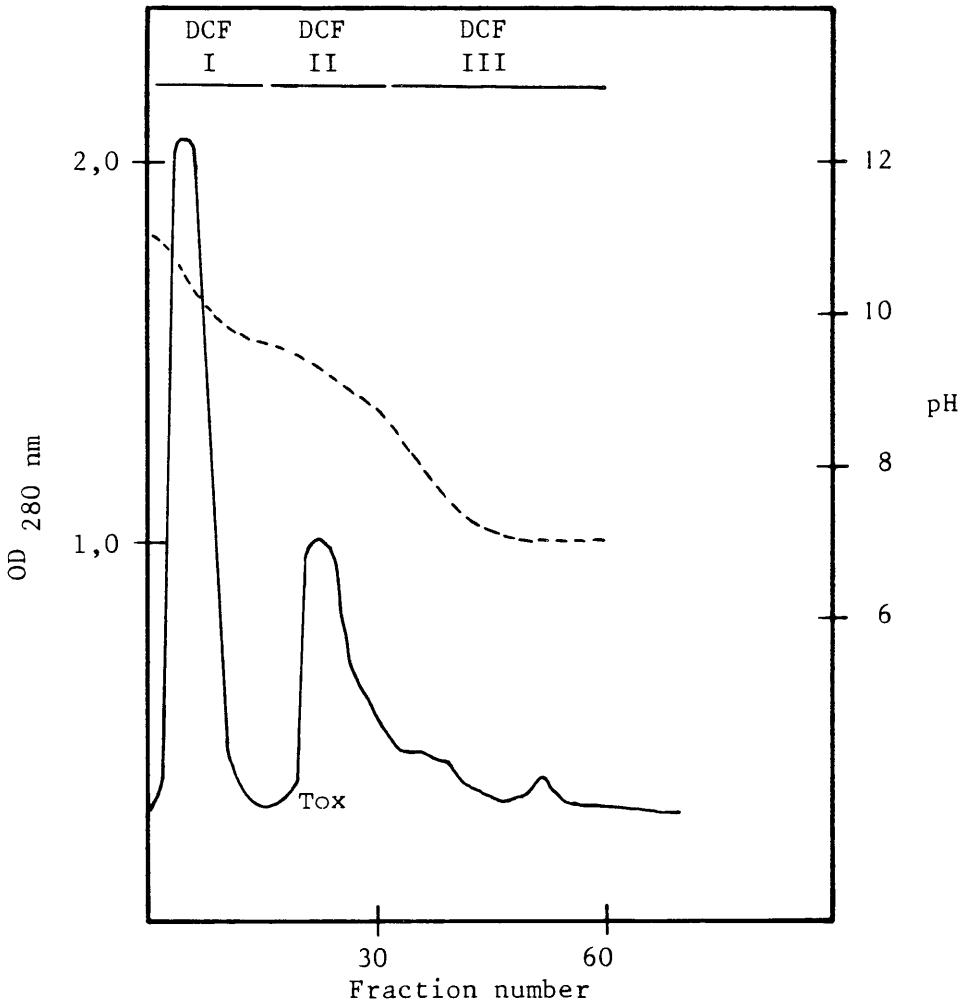


Fig 12 Eluting diagram of *B.dec.* (DG I) after chromatofocusing. Flow rate: 30 cm^3/h, volume/fraction: 3 cm^3. OD: (——); pH gradient: (---)

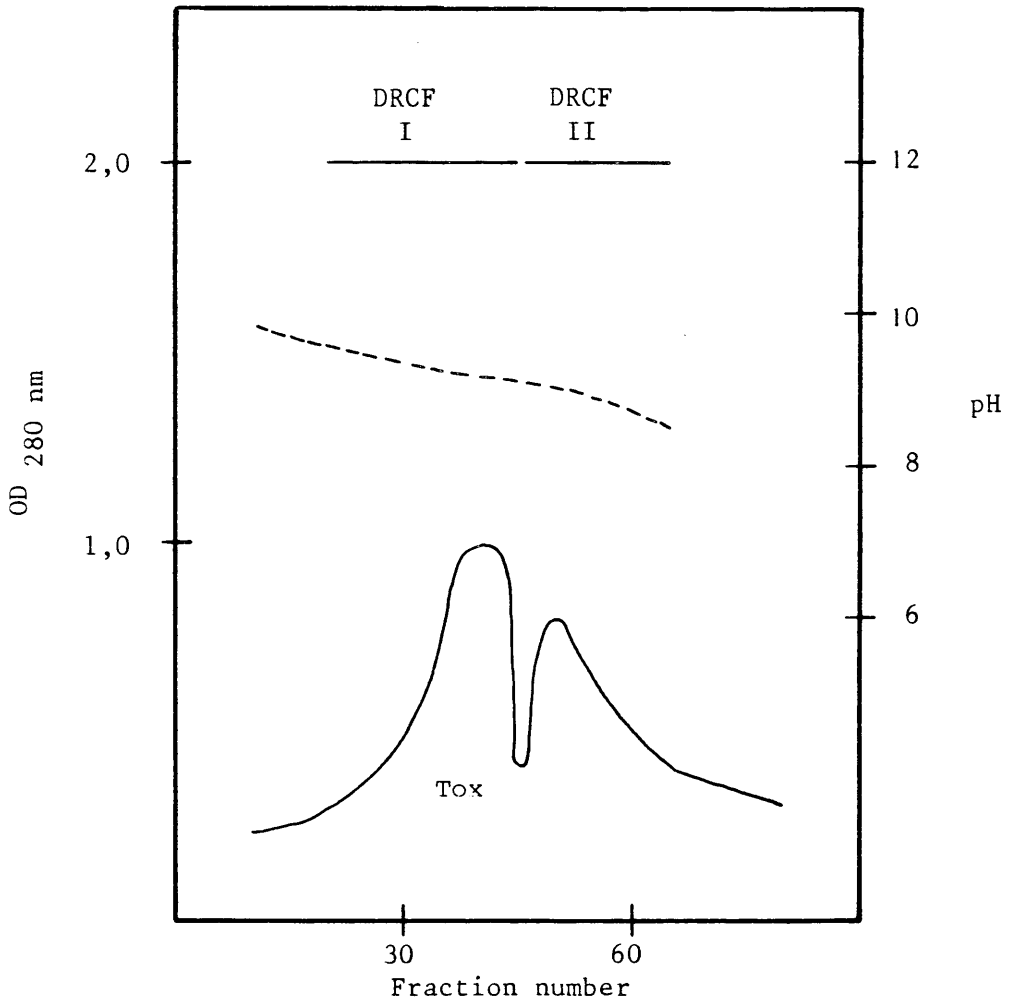


Fig 13 Rechromatofocusing of *B.dec.* (DCF II) on a PBE 118 column. Flow rate: 30 cm^3/h, volume/fraction: 1 cm^3. OD: (——); pH gradient (---)

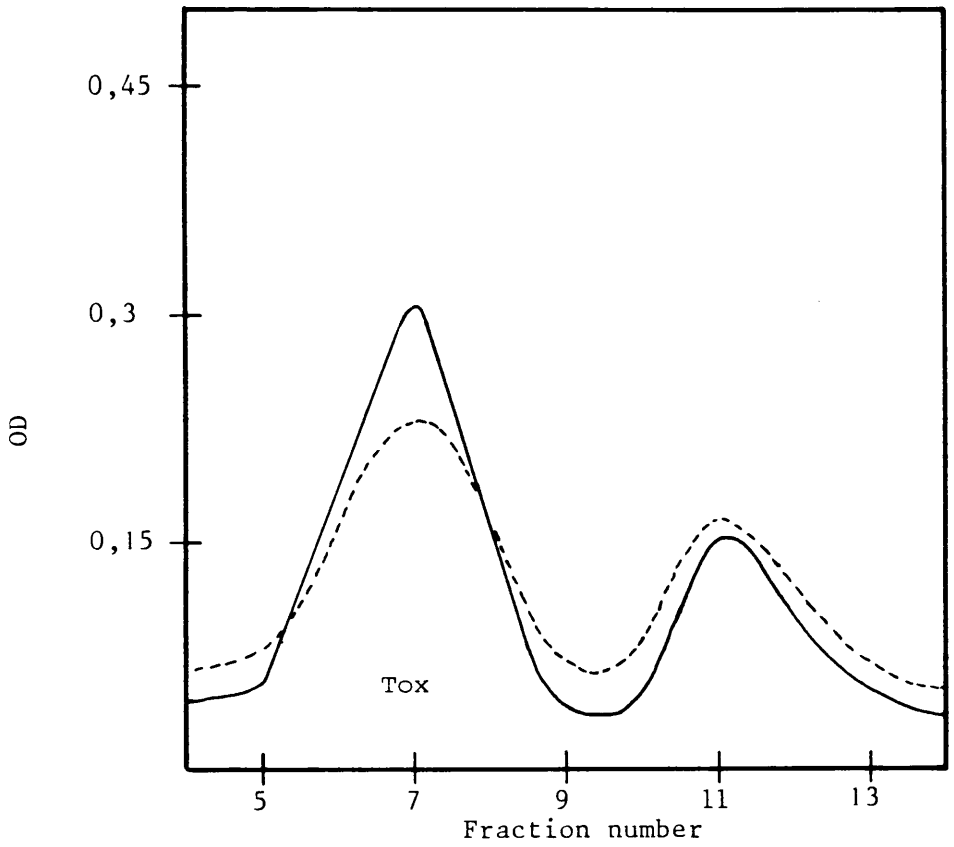


Fig 14 Separation of Polybuffer 74 from the *R.ee.* egg toxin by means of a Sephadex G 10 column. Flow rate: 30 cm^3/h, volume/fraction: 1 cm^3, OD: 280 nm (——), 265 nm (---)

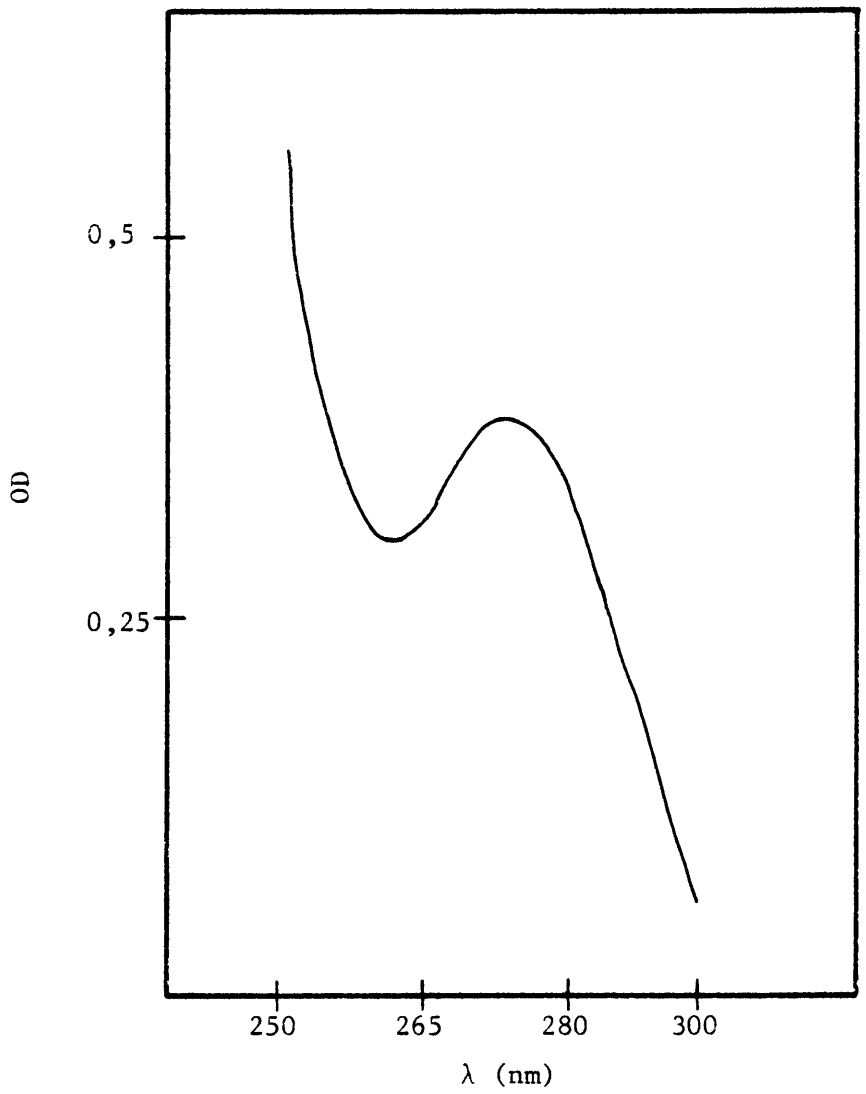


Fig 15 Ultraviolet absorption spectra of the combined fractions 6, 7 and 8 of the Sephadex G 10 column. Chartspeed: 2,5 cm/min, wavelength/min: 50 nm/min and the span: 1A

for 2 h at and stored at -10°C.

Fractions 6, 7 and 8 (Fig 16) were combined and dialysed for 24 h in Spectrapor membranes with cutoff margin of molecular mass 10 000. Dialysis was carried out against 5 dm^3 distilled water at 4°C. The ultraviolet absorption spectrum (Fig 17) of the retentate was recorded. Comparison of the spectrum (Fig 17) with that of pharmalyte, which shows a high absorbance below 265 nm (Chromatofocusing Manual of Pharmacia Fine Chemicals), served as a good indication of pharmalyte removal.
Summaries of the isolation procedures for the egg toxins are shown in Tables 9, 10, 11 and 12.

TABLE 9 PURIFICATION OF *R.ee.* EGG TOXIN

STEP	TOTAL MASS mg	MLD* mg	TOTAL MLD	MLD/mg≠	TOXICITY ENRICHMENT	% YIELD IN TOXICITY
Crude egg extract	2 000	400	5	$2,5x10^{-3}$	-	100
After: Sephadex G 100 column	28	5,6	5	$1,8x10^{-1}$	72	100
After: CF, PBE 94 Sephadex G 10	6,2	1,7	3,6	$5,8x10^{-1}$	232	72

* expressed as 1 000 g body mass; guinea-pigs as experimental animals.

≠ expressed as mg material after each step; nitrogen determinations could not be determined accurately after each step because of the low yield. Only freeze-dried material was used.

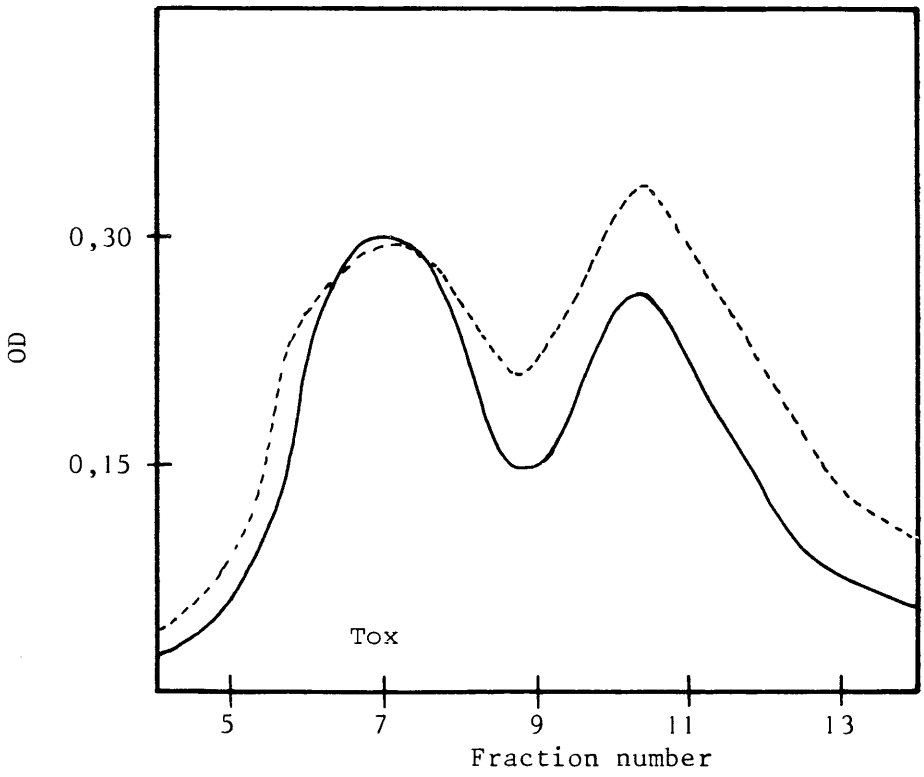


Fig 16 Typical eluation diagram of *H.trun.*, *B.mic.* and *B.dec.* egg toxins after chromatography on a Sephadex G 25 column. Flow rate: 30 cm^3/h, OD: 280 nm (——), 265 nm (---)

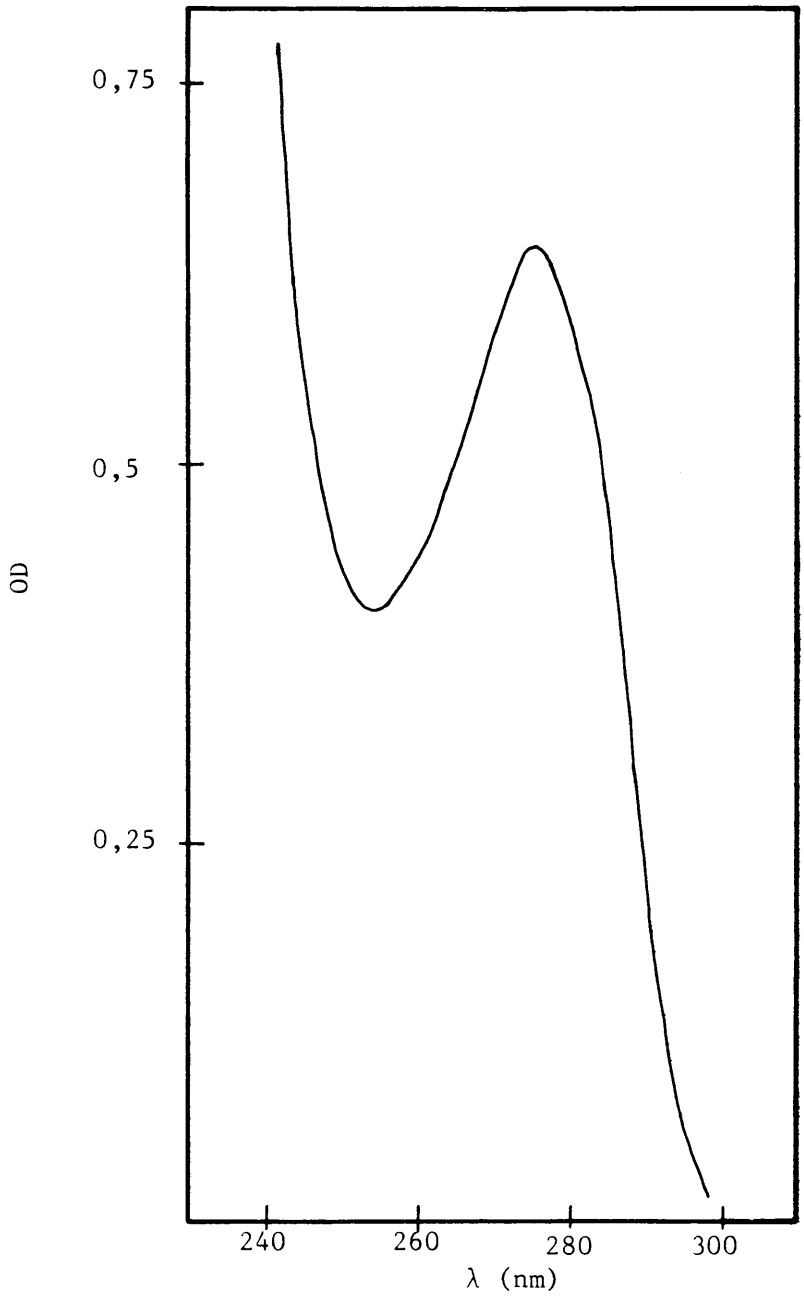


Fig 17 Ultraviolet absorbance spectrum from 300 to 240 nm of the retentate (p.51). Chartspeed: 2,5 cm/min, span: 1A, wavelength/-min: 50 nm/min

TABLE 10 PURIFICATION OF *H. trun.* EGG TOXIN

STEP	TOTAL MASS mg	MLD* mg	TOTAL MLD	MLD/mg≠	TOXICITY ENRICHMENT	% YIELD IN TOXICITY
Crude egg extract	2 000	250	8	4×10^{-3}	-	100
After: Sephadex G 100 column	105	13,1	8	$7{,}6 \times 10^{-2}$	19	100
After: CF, PBE 118, Sephadex G 25 24 h dialysis	3,2	0,43	7,4	2,5	625	93

* expressed as 1 000 g body mass; guinea-pigs as experimental animals

≠ expressed as mg material after each step; nitrogen determinations could not be determined accurately after each step because of the low yield. Only freeze-dried material was used

TABLE 11 PURIFICATION OF *B.mic.* EGG TOXIN

STEP	TOTAL MASS mg	MLD* mg	TOTAL MLD	MLD/mg≠	TOXICITY ENRICHMENT	% YIELD IN TOXICITY
Crude egg extract	2 000	250	8	4×10^{-3}	-	100
After: Sephadex G 100 column	400	50	8	2×10^{-2}	5	100
After: CF, PBE 118, Sephadex G 25 24 h dialysis	6,2	0,82	7,6	1,27	318	95

* expressed as 1 000 g body mass; guinea-pigs as experimental animals

≠ expressed as mg material after each step; nitrogen determinations could not be determined accurately after each step because of the low yield. Only freeze dried material was used

TABLE 12 PURIFICATION OF *B.dec.* EGG TOXIN

STEP	TOTAL MASS mg	MLD* mg	TOTAL MLD	MLD/mg≠	TOXICITY ENRICHMENT	% YIELD IN TOXICITY
Crude egg extract	2 000	380	5,3	$2,6 \times 10^{-3}$	-	100
After: Sephadex G 100 column	325	61	5,3	$1,6 \times 10^{-2}$	6,1	100
After: CF, PBE 118, Sephadex G 25 24 h dialysis	3,6	0,75	4,8	1,33	513	91

* expressed as 1 000 g body mass; guinea-pigs as experimental animals

≠ expressed as mg material after each step; nitrogen determinations could not be determined accurately after each step because of the low yield. Only freeze-dried material was used.

2.2.2 Characterisation of the egg toxins

2.2.2.1 Electrophoretic characterisation

According to the isoelectric points of the toxins, no differences between the strains of each specie was found. Consequently only the isoelectric focusing patterns of the crude egg extracts and toxins of the species are shown in Fig 18.

The isoelectric points of the egg toxins (Table 13) were determined by using a plot of pI against distance measured from cathode after analytical isoelectric focusing (Fig 19).

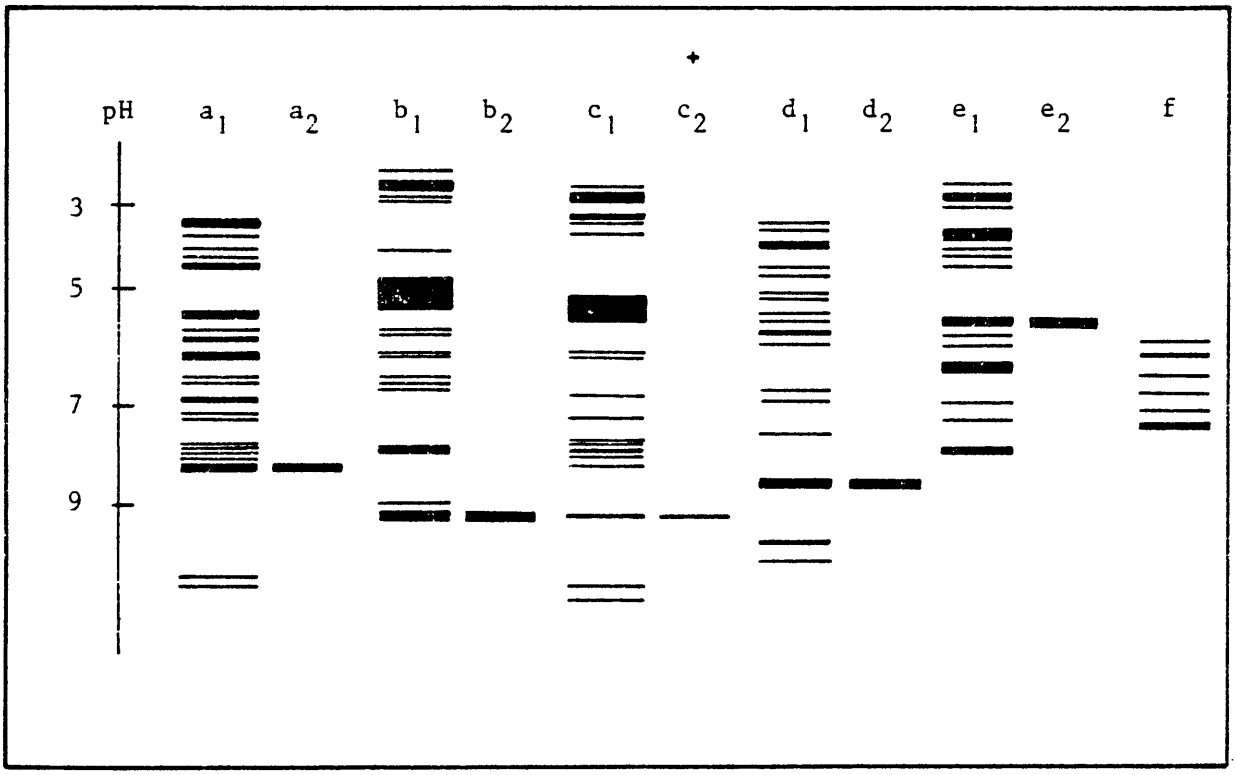


Fig 18 Analytical isoelectric focusing of the crude egg extracts and the isolated toxins.

a_1: Crude *A.heb.* extract (18); a_2: *A.heb.* toxin (18)

b_1: Crude *B.dec.* extract; b_2: *B.dec.* toxin

c_1: Crude *B.mic.* extract; c_2: *B.mic.* toxin

d_1: Crude *H.trun.* extract; d_2: *H.trun.* toxin

e_1: Crude *R.ee.* extract; e_1: *R.ee.* toxin

f_1: Crude *O.sav.* extract (20) - non toxic

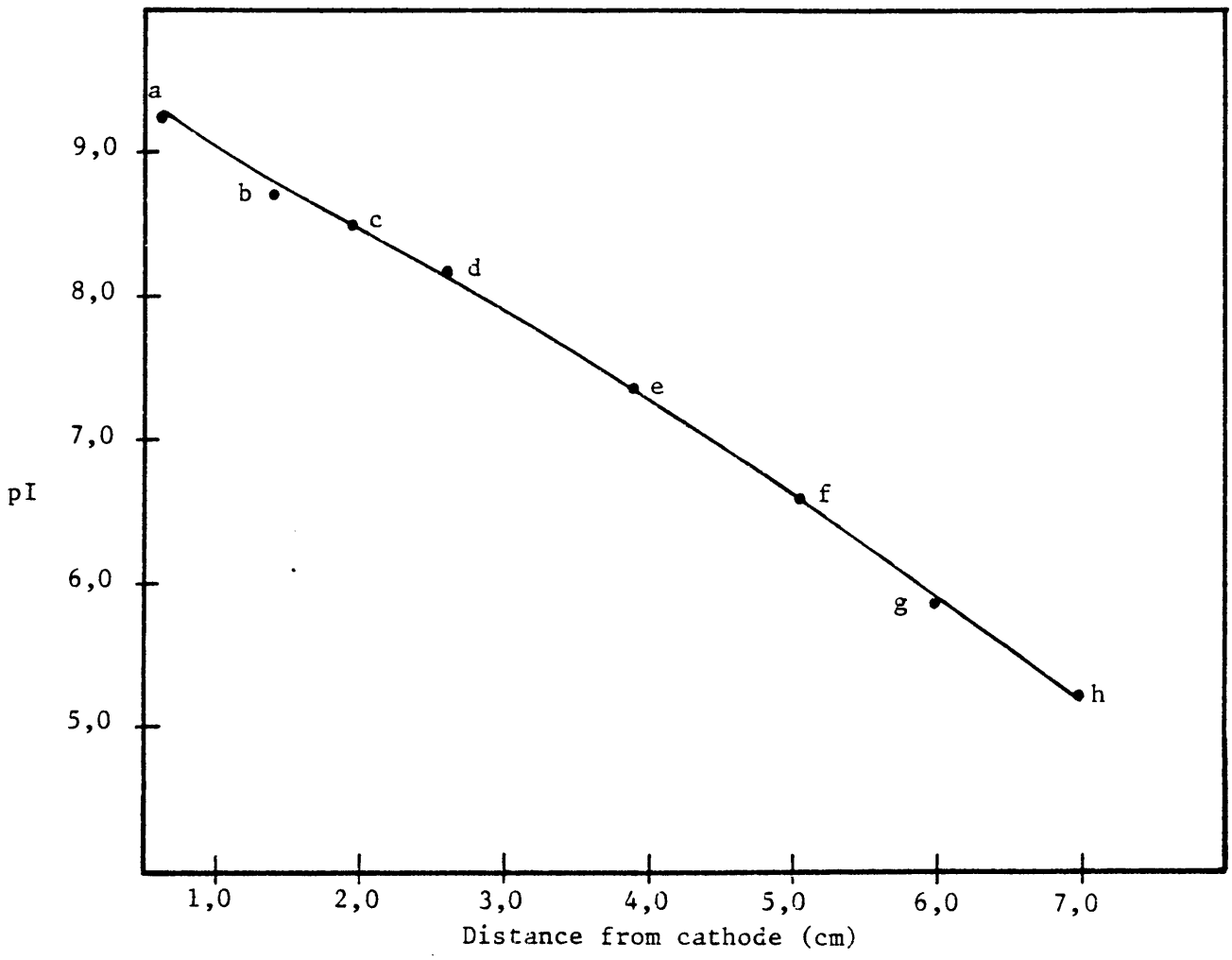


Fig 19 Determination of isoelectric focusing gradient profile using the broad isoelectric calibration kit (Pharmacia Fine Chemicals)
a: trypsinogen, b: lentil lectin-basic, c: lentil lectin-middle, d: lentil lectin-acidic, e: myoglobin-basic, f: human carbonic anhydrase, g: bovine carbonic anhydrase, h: β-lactoglobulin A

TABLE 13 ISOELECTRIC POINTS OF THE TICK EGG TOXINS

SPECIES	pI
R.ee.	6,0
H.trun.	8,3
B.mic.	9,1
B.dec.	9,2
A.heb (18)	8,0

The molecular mass of each egg toxin was determined from a calibration curve (Fig 20), after SDS-polyacrylamide gradient gel electrophoresis (Fig 21).

The different strains of each specie showed the same molecular mass on the SDS-polyacrylamide gradient gel.

2.2.2.2 Determination of direct haemagglutination

Throughout the series of U shaped holes, each hole was represented by a twofold dilution of the preceding hole. No haemagglutination was obtained with 0,050 cm^3 of crude extract and 0,050 cm^3 of 1 mg egg toxin/0,1 cm^3 0,9% NaCl after 2 h. The experiments were performed at both 25°C and 4°C.

2.2.2.3 Determination of the molecular mass of egg toxins with the analytical ultracentrifuge

The molecular mass of the egg toxins are shown in Table 14.

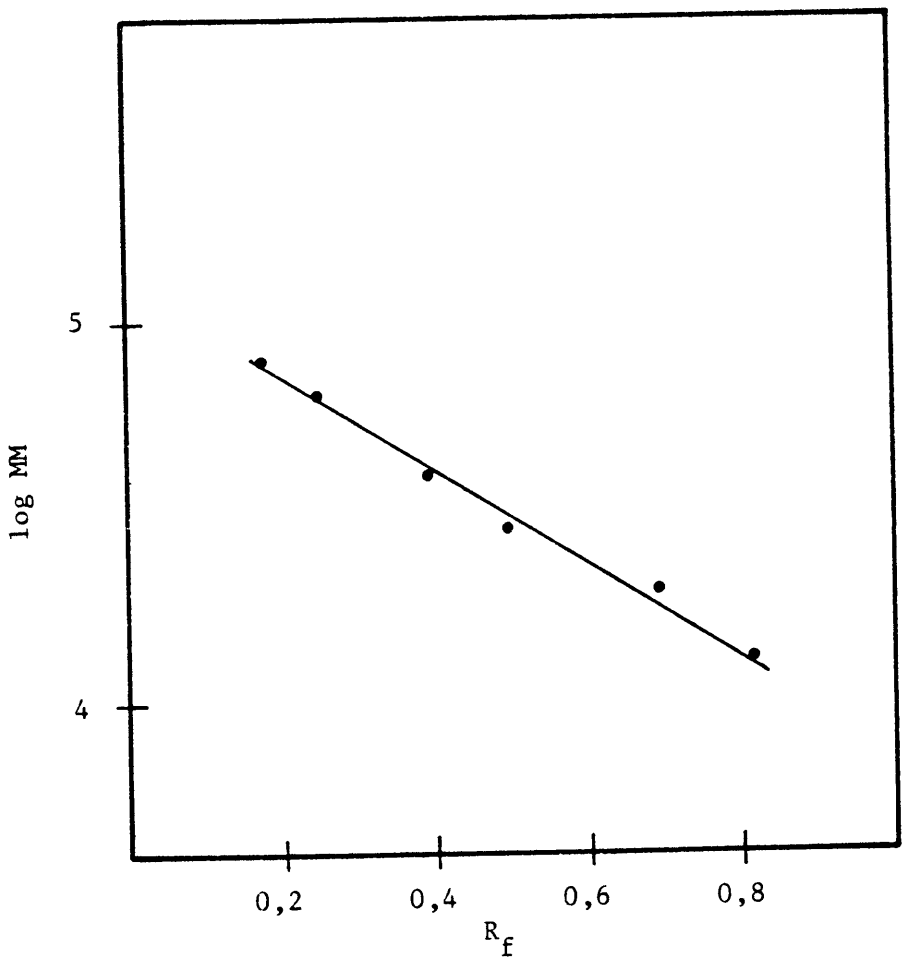


Fig 20 Calibration curve for the determination of molecular mass employing SDS-polyacrylamide gradient gel electrophoresis

TABLE 14 MOLECULAR MASS OF THE EGG TOXINS (BUFFER: 0,05 M TRIS, 0,1 M KCl, pH 8)

STRAIN		MOLECULAR MASS (KDAL)
R.ee:	Boshoff	5
	Warrenton	2,8
	Sweetwater	5
H.trun:	SWA	30
	SWA & Kaalplaas	26
	Kaalplaas	25
B.mic:	Onderstepoort	31
B.dec:	Ethel	40
A.heb:	(18)	10

No change in molecular mass was observed at pH 5.

2.2.2.4 Amino acid composition of the egg toxins

The amino acid composition of each toxin from 2.2.1 is shown in Table 15.

2.2.2.5 Clinical symptoms

The crude extracts of all the tick species investigated, caused symptoms on the 3rd to 4th day. The animals died on the 5th to 6th day. Purified egg toxins caused symptoms on the 2nd to 3rd day and death on the 3rd to 4th day. There was no difference between the symptoms caused by the crude egg extracts, the isolated egg toxins and the different species. The symptoms were: hyperaesthesia and anorexia folfowed by nasal and eye discharge. Hyperaemia of the ears, feet pads and lips. The watery diarrhea became

TABLE 15 AMINO ACID COMPOSITION OF THE EGG TOXINS (MOLE RATIOS WITH RESPECT TO HISTIDINE)

AMINO ACID	*R.ee.*(B)	*H.trun.*(SWA)	*H.trun.*(KT)	*B.mic.*(Y)[a]	*B.mic.*(O)	*B.dec.*(V)	*A.heb.*[a]
ASP	2	7	7*	5	5	8	7
THR	2	7	7*	5	5	6	6
SER	2	4	4	7	7	4	5
GLU	4	9	10	9	9	10	9
PRO	2	6	5	4	3	7	7
GLY	4	8	7	9	9✓	9	9
ALA	2	5	5	4	4	5	6
VAL	1	6	6	5	5	6	5
ILE	1	3	3	3	3	4	8
LEU	2	7	5	4	4	6	9
TYR	1	4	3	1	2	4	5
PHE	1	3	3	2	2✓	4	4
LYS	1	9	9	6	6	9	4
HIS	1	1	1	1	1	1	1
ARG	1	4	4	2	2	4	5

B: Boshoff strain, KT: SWA x Kaalplaas strain, Y: Y Pilly strain

O: Onderstepoort strain, V: Van Dyk strain

*: Average of two determinations

✓: Average of two determinations

a: *B.mic.* Y and *A.heb.* according to Neitz (18)

haemorrhagic, apparent paralysis of the hind legs which spread to the front legs.

2.2.2.6 Histopathology

The pathological symptoms caused by the egg toxins of the different tick species appeared to be the same. The lesions in the kidney were characterised by a peripheral zone of mineralisation and necrotic tubular epithelial cells. The liver showed focal areas of coagulative necrosis with mineralisation. Furthermore degenerative changes in the hepatocytes were observed. Oedema of the urinary bladder was present and infiltration of neutrophils in the mucosa. Oedema of the white matter of the brain was also present.

2.2.2.7 Anti-protease activity

2.2.2.7.1 *R.ee.* egg toxin inhibition of trypsin

Inhibitor activities of *R.ee.* (Sweetwater strain) egg toxin showed fast-binding inhibition with bovine pancreas trypsin and BAPNA as substrate. The plot of % activity against inhibitor concentration is shown in Fig 22.

A decrease of velocity with an increase of *R.ee.* egg toxin concentration is shown in Fig 23.

The Lineweaver-Burk plot (61) showed non-competitive inhibition for the toxin with trypsin (Fig 24). The 1/v-axis intercept showed that:

$$1/v_{max(toxin)} = (1+ \frac{[I]}{Ki})\ 1/v_{max(trypsin)} \quad \text{Eqn(21)}$$

$$Ki = 1{,}6 \times 10^{-8}\ M$$

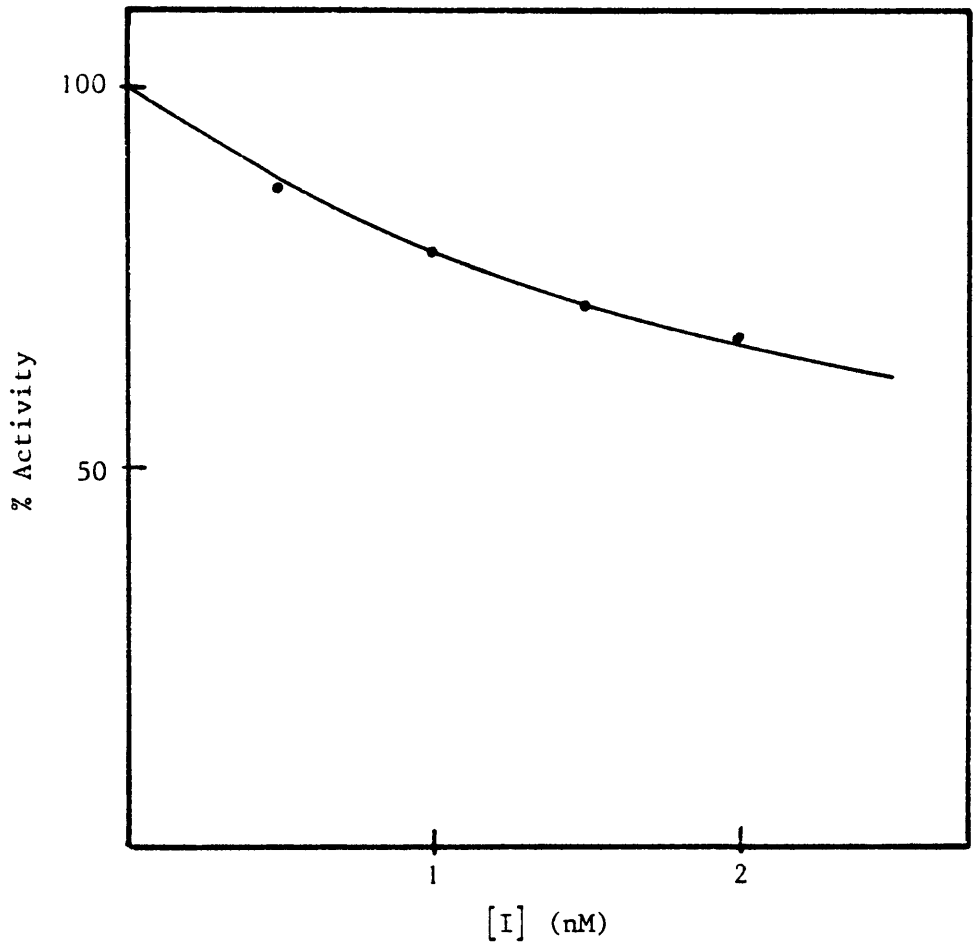


Fig 22 Anti-trypsin activity of *R.ee.* (Sweetwater strain) egg toxin. [BAPNA] = $5,1 \times 10^{-4}$ M, [Trypsin] = $2,3 \times 10^{-10}$ M

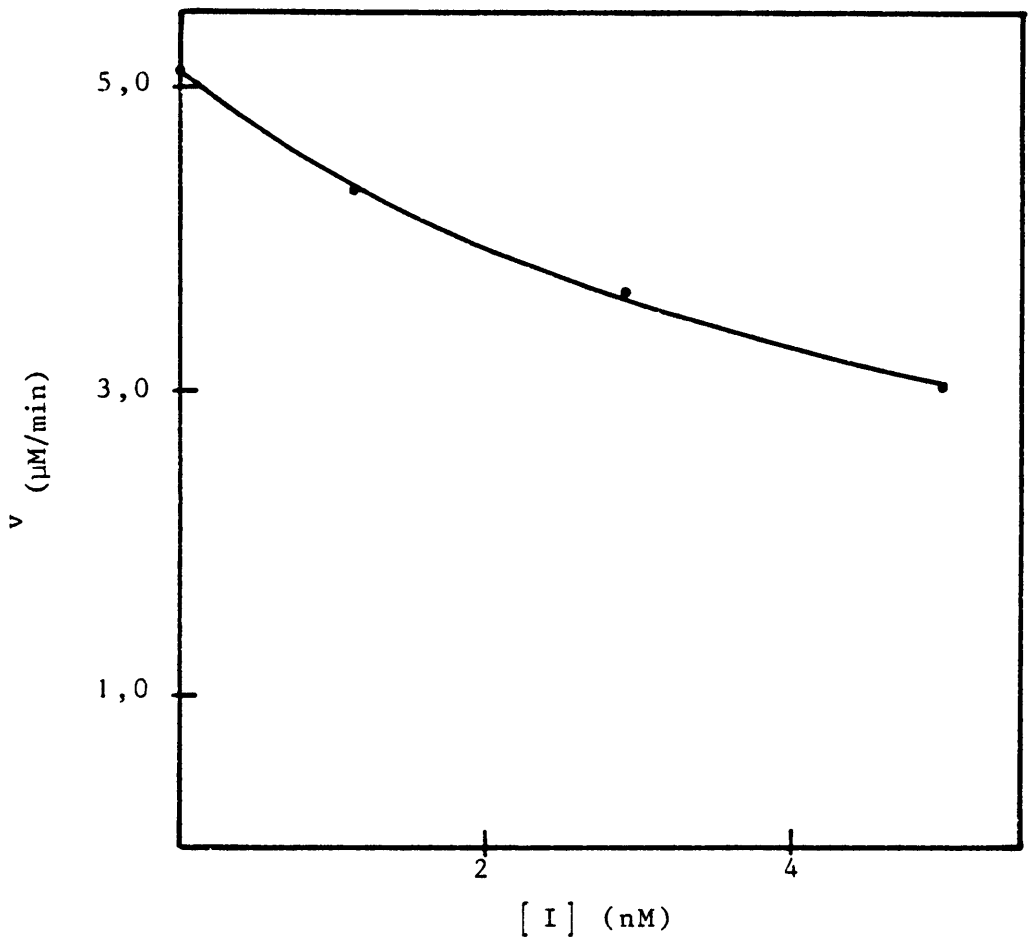


Fig 23 The velocity *versus* inhibitor plot for the *R.ee.* (Sweetwater strain) egg toxin. [BAPNA] = $5,1 \times 10^{-4}$ M, [Trypsin] = $2,3 \times 10^{-10}$ M

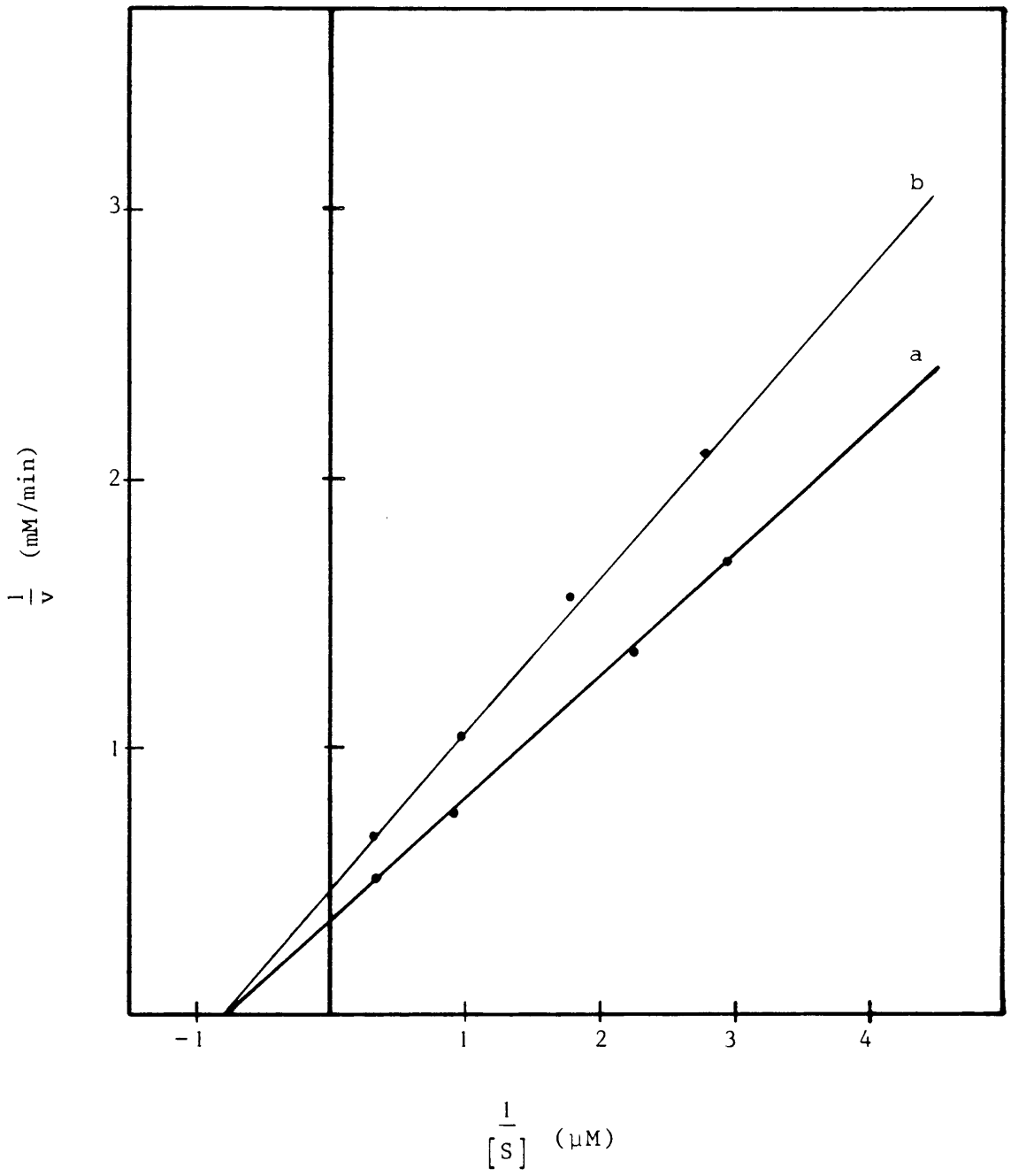


Fig 24 The Lineweaver-Burk plot of *R.ee.*(Sweetwater) egg toxin inhibition of bovine pancreas trypsin. [Trypsin] = $2,3 \times 10^{-10}$ M, [*R.ee.* egg toxin] = $2,8 \times 10^{-9}$ M, a: No inhibitor b: with inhibitor, BAPNA as substrate

2.2.2.7.2 *R.ee.* (Sweetwater strain) egg toxin inhibition of chymotrypsin

At concentrations of *R.ee.* egg toxin $1,5x10^{-5}$ M and chymotrypsin $3,3x10^{-7}$ M, no inhibition of the enzyme could be detected. No inhibition could also be detected when the inhibitor and enzyme were pre-incubated for 30 min.

2.2.2.7.3 *B.mic.* (Onderstepoort strain) egg toxin inhibition of trypsin

The toxin showed slow, tight-binding inhibition, when studied according to the method of Morrison (Fig 25)(53). The % activity against *B.mic.* egg toxin concentration is shown in Fig 26. The velocity of the reaction *versus* *B.mic.* egg toxin concentration is shown in Fig 27. At concentrations $[I] > 10[E]$ the inhibition could not be determined.

2.2.2.7.4 *B.mic.* (Onderstepoort strain) egg toxin inhibition of chymotrypsin

At [*B.mic.* egg toxin] = $4,2x10^{-6}$ M and [chymotrypsin] = $3,3x10^{-7}$ M, no inhibition was observed. No inhibition could also be detected when the inhibitor and enzyme were pre-incubated for 30 min at the same concentrations.

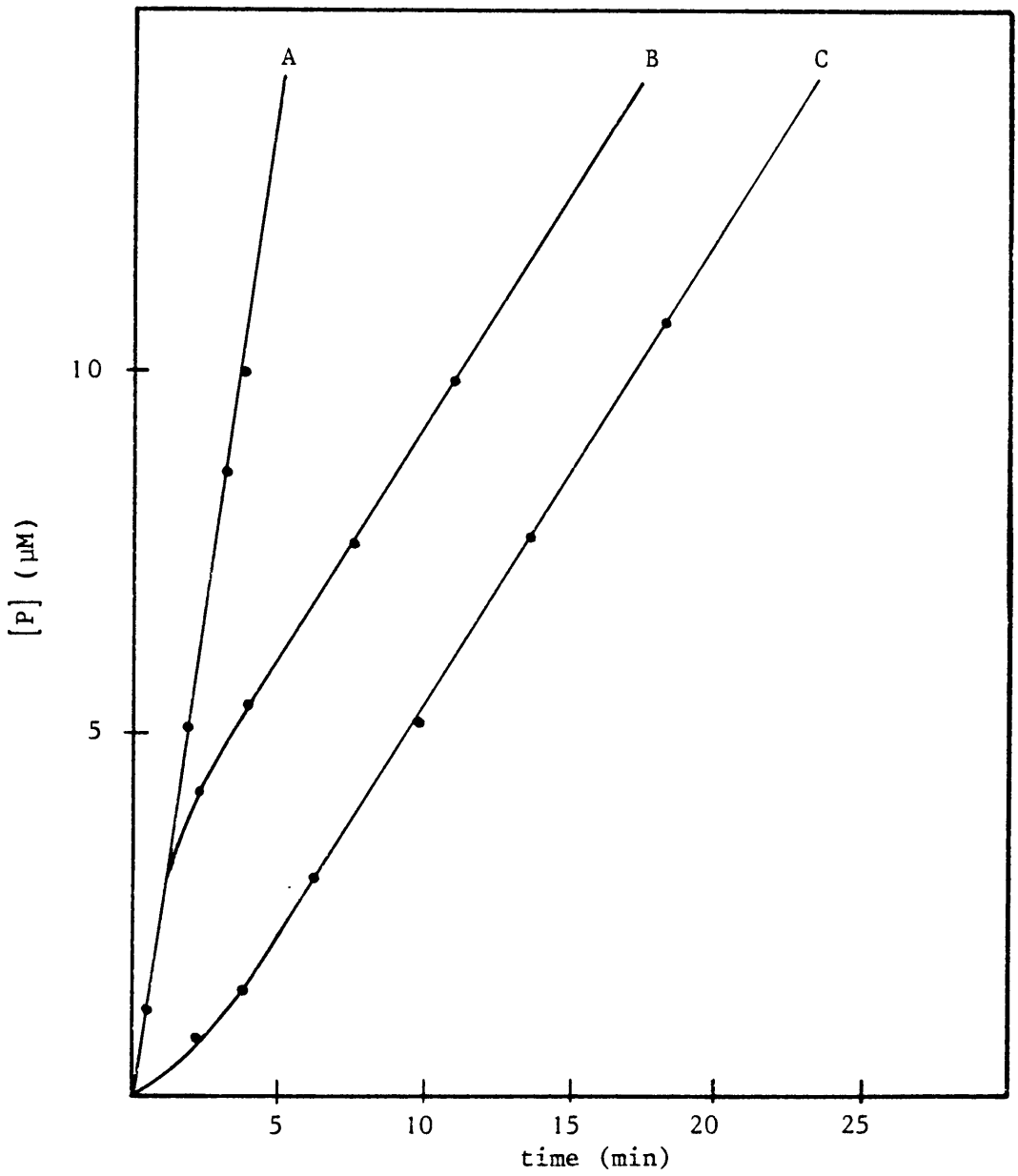


Fig 25 Lag phases and steady-state conditions for *B.mic.* (Onderstepoort strain) egg toxin. A: No inhibitor, B: inhibitor not pre-incubated with enzyme, C: enzyme and inhibitor pre-incubated for 30 min. [BAPNA] = $5,1 \times 10^{-4}$ M, [*B.mic.* egg toxin] = $6,5 \times 10^{-10}$ M, [Trypsin] = $2,3 \times 10^{-10}$ M

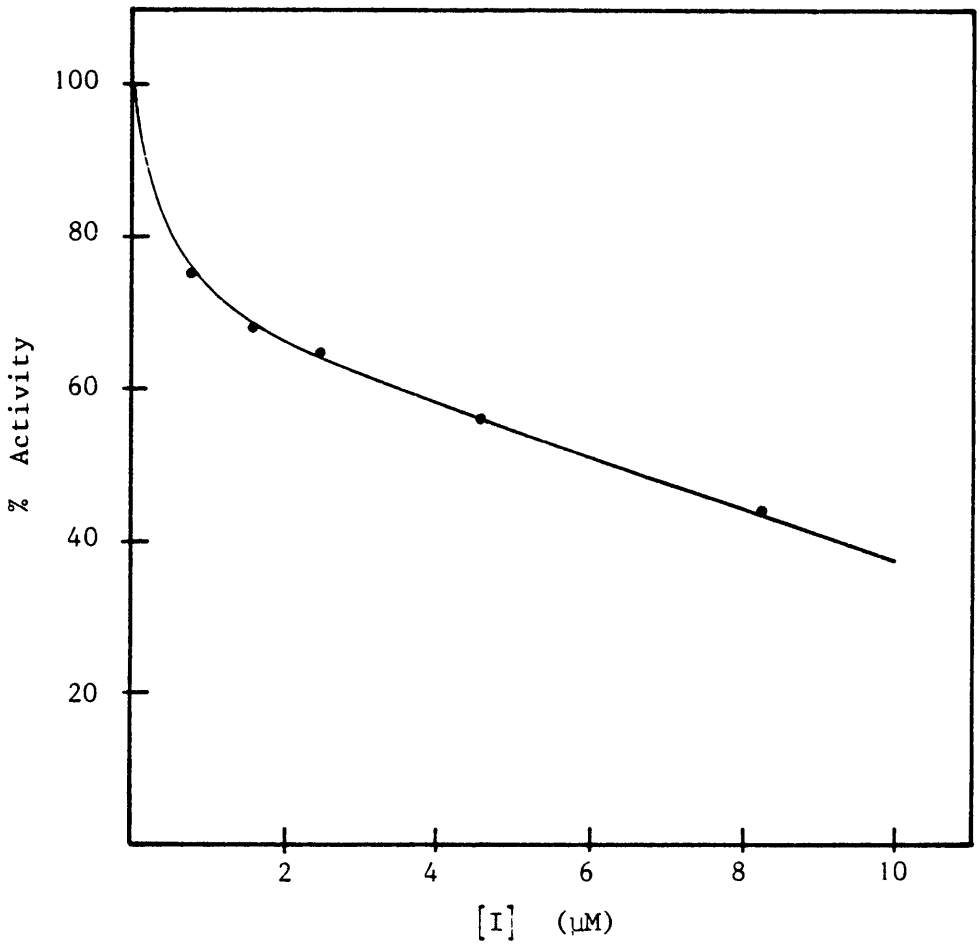


Fig 26 Anti-trypsin activity of *B.mic.* (Onderstepoort strain) egg toxin. $[BAPNA] = 5,1 \times 10^{-4}$ M, $[Trypsin] = 2,3 \times 10^{-10}$ M

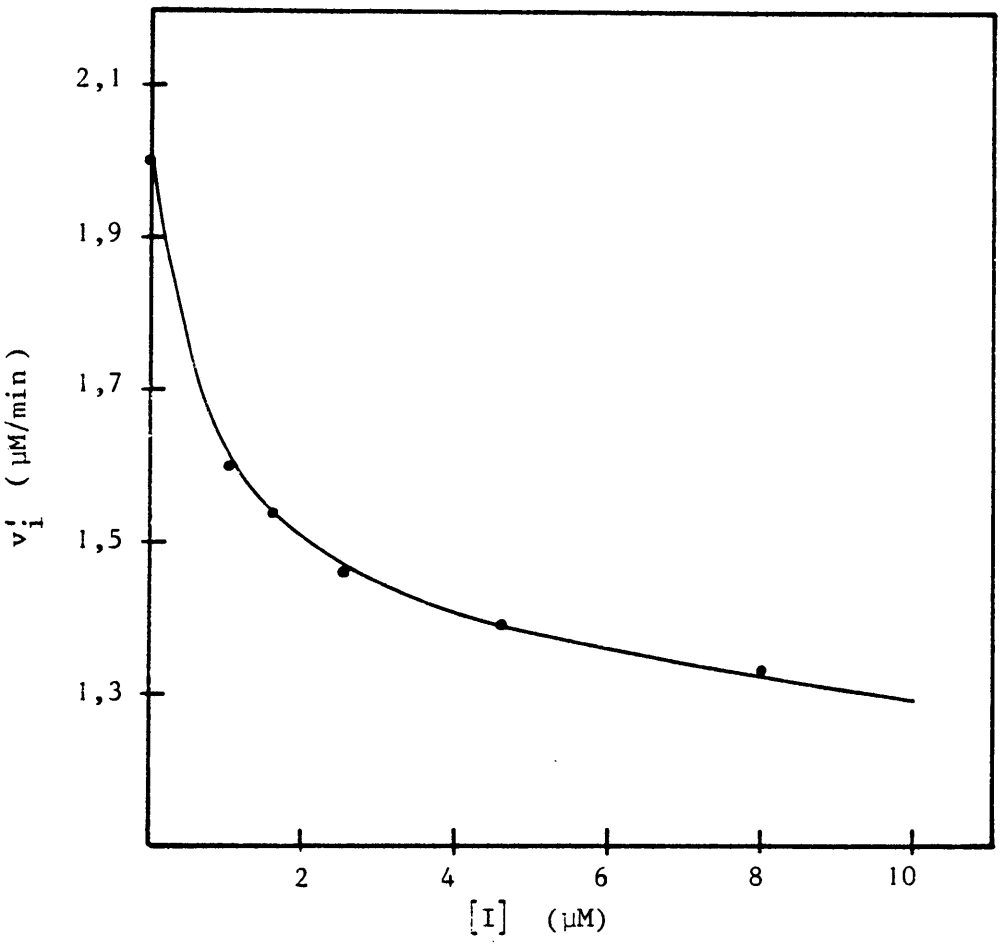


Fig 27 Velocity of reaction against *B.mic.*(Onderstepoort strain) egg toxin concentration. [BAPNA] = $5,1 \times 10^{-4}$ M, [Trypsin] = $2,3 \times 10^{-10}$ M. v_i' was estimated from the tangent at t=0.

2.2.2.7.5 *B.dec.* (Ethel strain) egg toxin inhibition of trypsin

The toxin showed slow, tight-binding inhibition when determined by the method of Morrison (Fig 28) (53). The % activity of trypsin against *B.dec.* egg toxin concentration and the decrease of the velocity of the reaction with increase of *B.dec.* egg toxin concentration is shown in Fig 29 and Fig 30 respectively.

2.2.2.7.6 *B.dec.* (Ethel strain) egg toxin inhibition of chymotrypsin

The egg toxin of *B.dec.* showed fast-binding inhibition of chymotrypsin. The method of Dixon (62), was employed to determine the type of inhibition and the Ki (Fig 32). The anti-chymotrypsin activity of *B.dec.* (Ethel) egg toxin is shown in Fig 31.

With different SPNA concentrations, the Dixon plot of velocity of the reaction against *B.dec.* egg toxin concentration, was the same (Fig 32). A SPNA concentration of $3,5x10^{-5}$ M is plotted in Fig 32. The inhibitor showed non-competitive inhibition. The distance between successive intercepts on the $[I]_t$-axis gives Ki directly (62). It can also be determined from:

$$[I]_n = nKi + [E]_t \qquad \text{Eqn(22)}$$

The Ki of *B.dec.* (Ethel strain) egg toxin with chymotrypsin according to Fig 32 is:

$$Ki = 3,6x10^{-8} \text{ M}$$

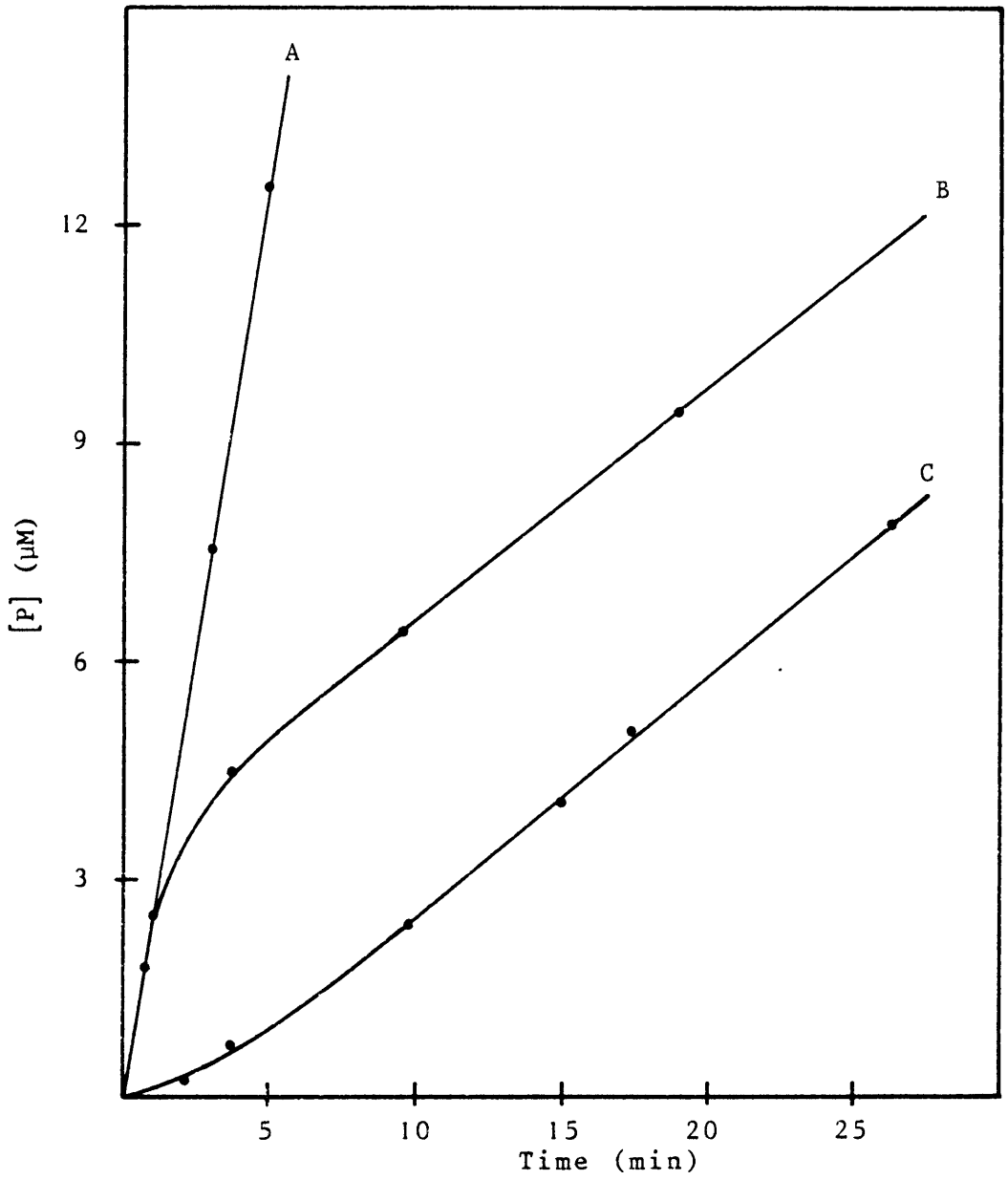


Fig 28 Lag phases and steady-state conditions of *B.dec.* (Ethel strain) egg toxin inhibition. A: No inhibitor, B: inhibitor not pre-incubated with enzyme, C: enzyme and inhibitor pre-incubated for 30 min. [BAPNA] = $5,1 \times 10^{-4}$ M, [*B.dec.* egg toxin] = 4×10^{-10} M, [Trypsin] = $2,3 \times 10^{-10}$ M.

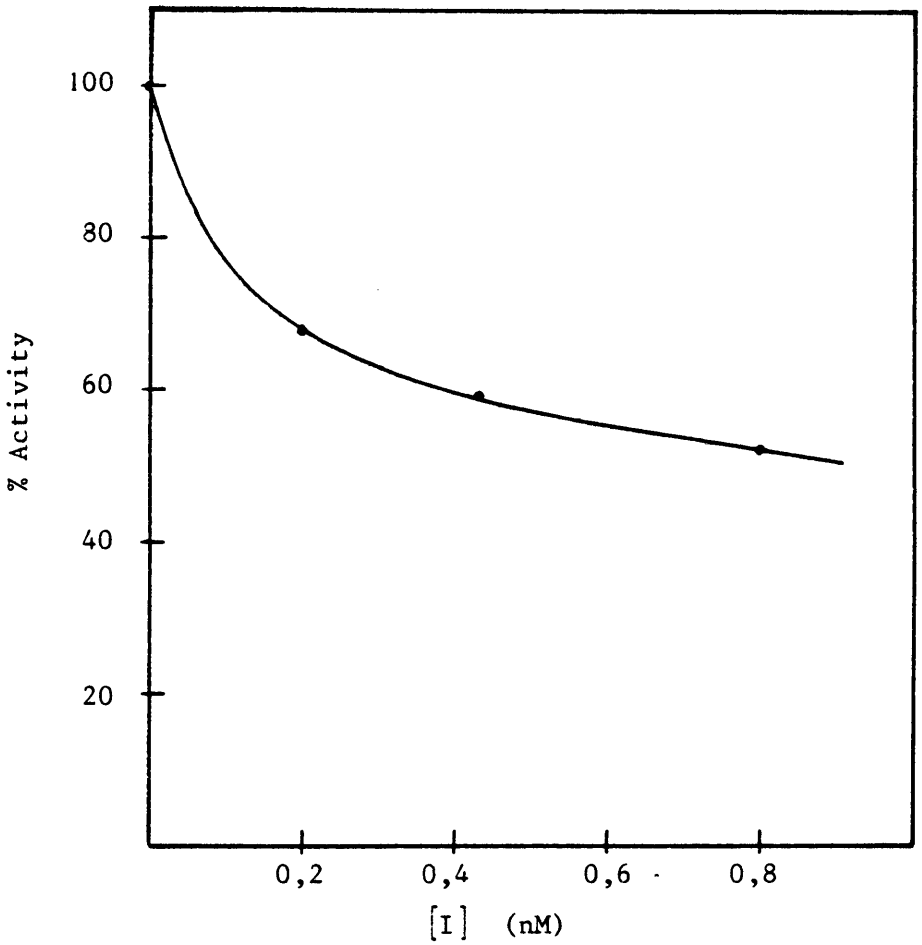


Fig 29 Anti-trypsin activity of *B.dec.* (Ethel strain) egg toxin. [BAPNA] = $5,1 \times 10^{-4}$ M, [Trypsin] = $2,3 \times 10^{-10}$ M.

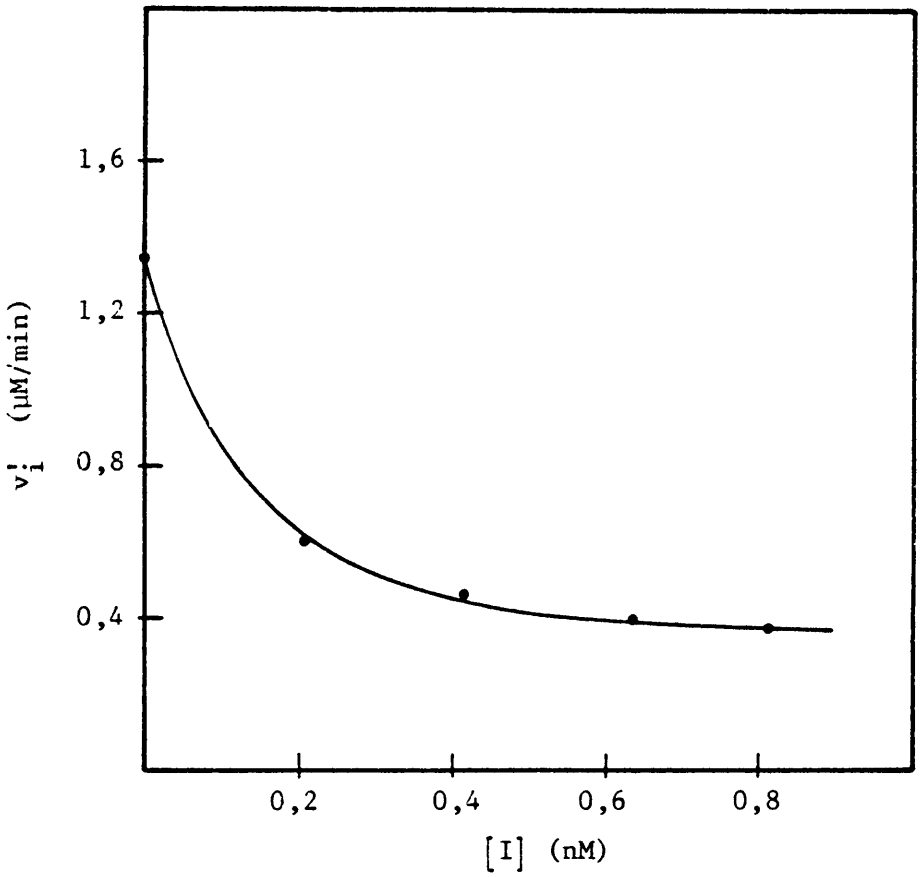


Fig 30 Relationship between the velocity of the reaction and an increase in *B.dec.* (Ethel strain) egg toxin concentration. $[BAPNA] = 5,1x10^{-4}$ M, $[Trypsin] = 2,3x10^{-10}$ M, v_i' was estimated from the tangent at t = 0.

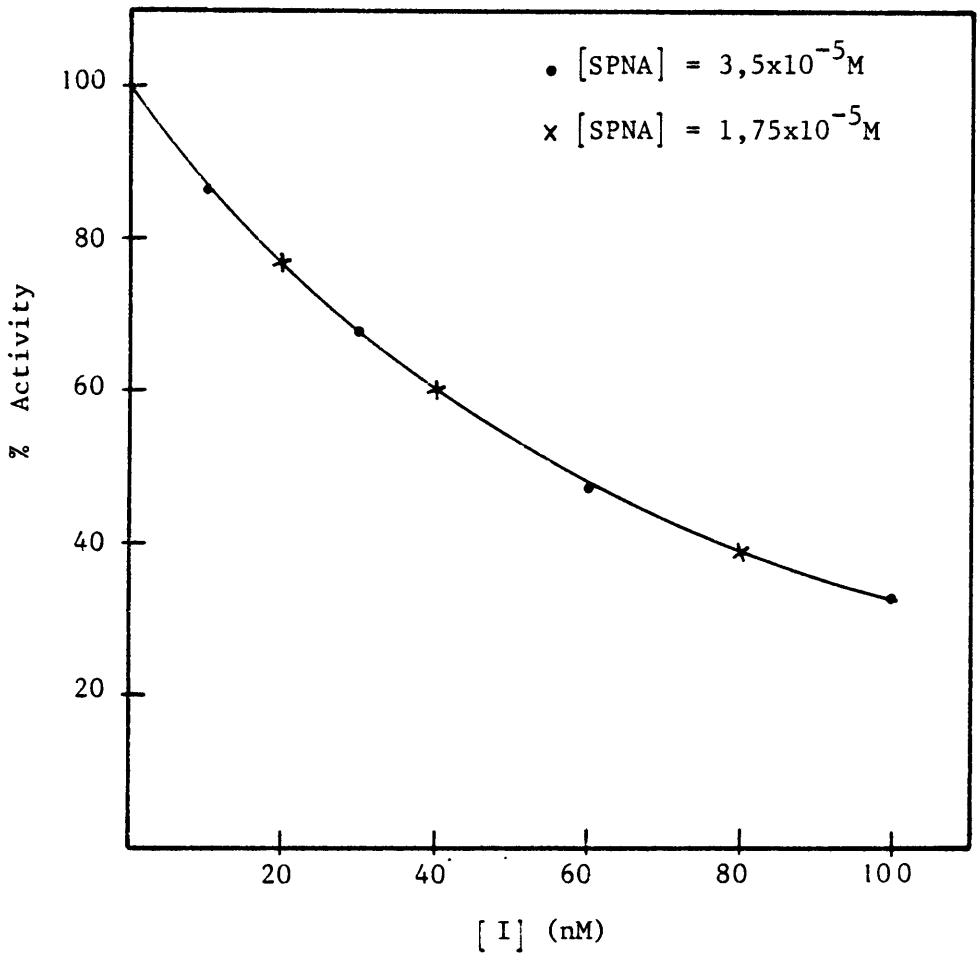


Fig 31 Anti-chymotrypsin activity of *B.dec.* (Ethel strain) egg toxin. [Chymotrypsin] = 3,3x10^{-8} M.

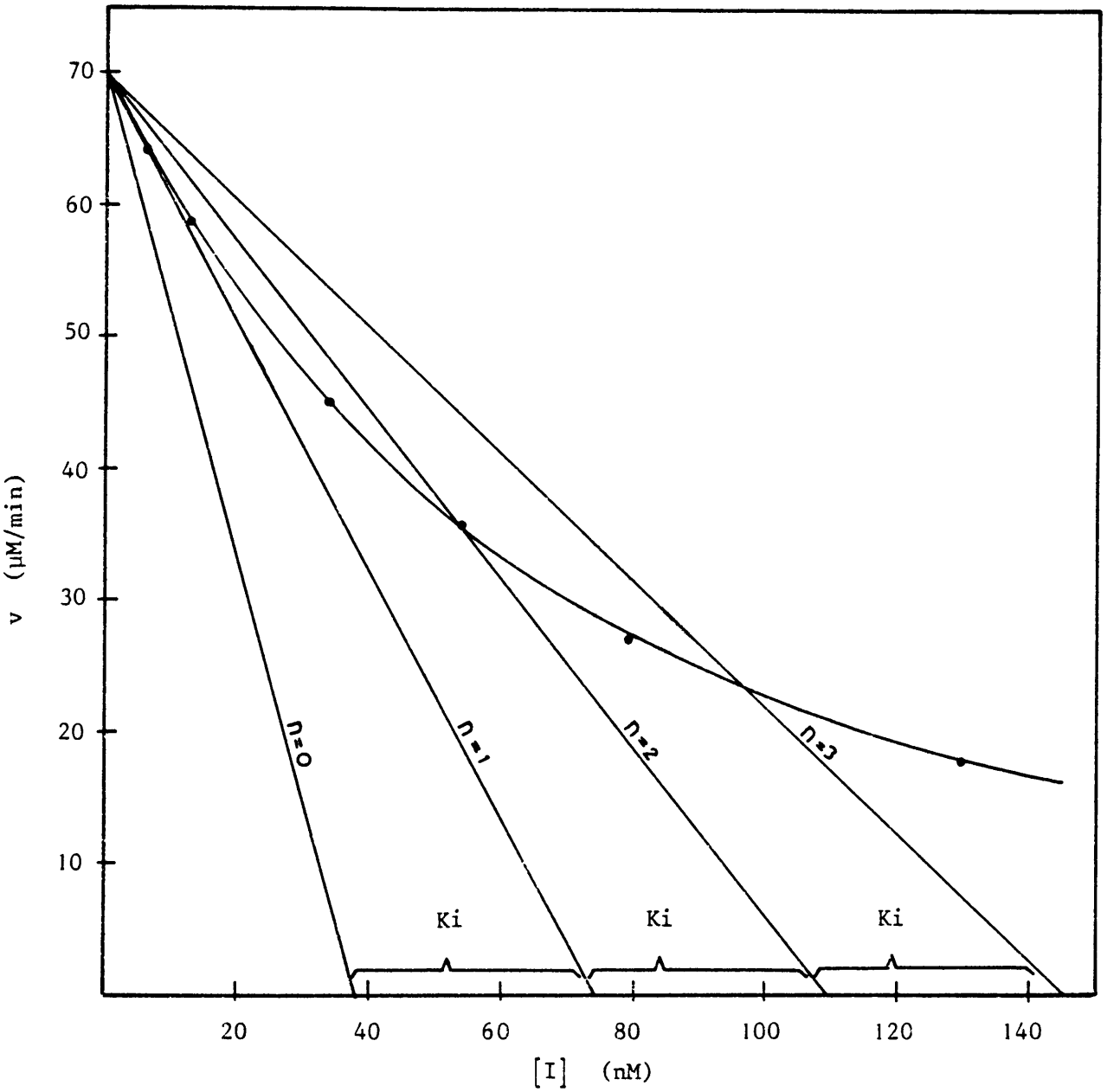


Fig 32 The relationship of v *versus* I, of *B.dec.* (Ethel strain) egg toxin. [SPNA] = $3,5 \times 10^{-5}$ M; [Chymotrypsin] = $3,3 \times 10^{-8}$ M.

2.2.2.7.7 *H.trun.* (SWA x Kaalplaas strain) egg toxin inhibition of trypsin

H.trun. (SWA x Kaalplaas strain) egg toxin showed slow-binding inhibition with trypsin. The anti-trypsin activity of the toxin is shown in Fig 33 and the decrease in velocity of the reaction with an increase in the toxin concentration in Fig 34.

The inhibition was determined according to the method described by Rich *et al* (61).

The reciprocal of the k_{app} (Fig 35) is plotted against the reciprocal of *H.trun.* egg toxin concentration in Fig 36.

$$E + I \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} EI \xrightarrow{k_3} EI^* \qquad (6) \qquad \text{Eqn(23)}$$

$$\text{and } \frac{1}{k_{app}} = \frac{Ki}{k_3[I]} + \frac{1}{k_3} \qquad \text{Eqn(24)}$$

$$\therefore \quad Ki = 9{,}6 \times 10^{-10} \text{ M}$$

2.2.2.7.8 *H.trun.* (SWA x Kaalplaas strain) egg toxin inhibition of chymotrypsin

Anti-chymotrypsin activity of *H.trun.* egg toxin is shown in Fig 37.

Dixon's method (62) was employed since the toxin showed fast-binding inhibition with chymotrypsin (Fig 38). The curve was essentially the same for $[SPNA] = 3{,}5 \times 10^{-5}$ M and $[SPNA] = 1{,}75 \times 10^{-5}$ M.

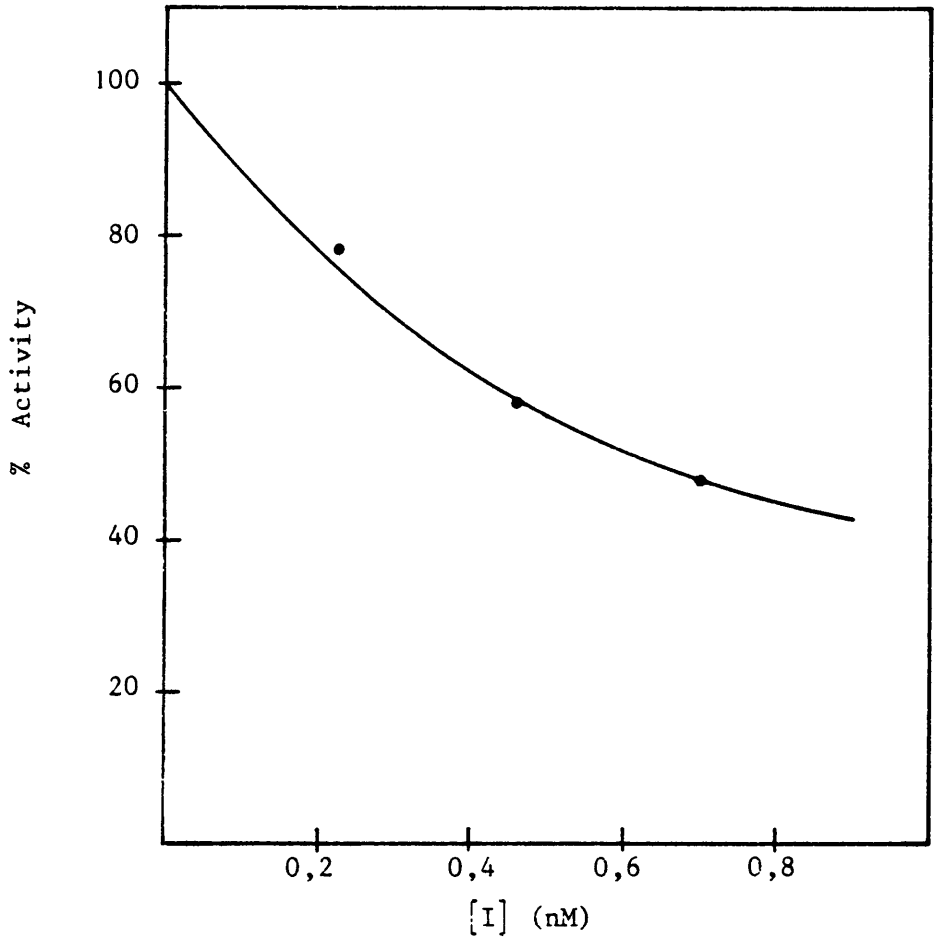


Fig 33 Anti-trypsin activity of *H.trun.* (SWA x Kaalplaas strain) egg toxin. [BAPNA] = $7,6 \times 10^{-4}$ M and [Trypsin] = $2,1 \times 10^{-10}$ M.

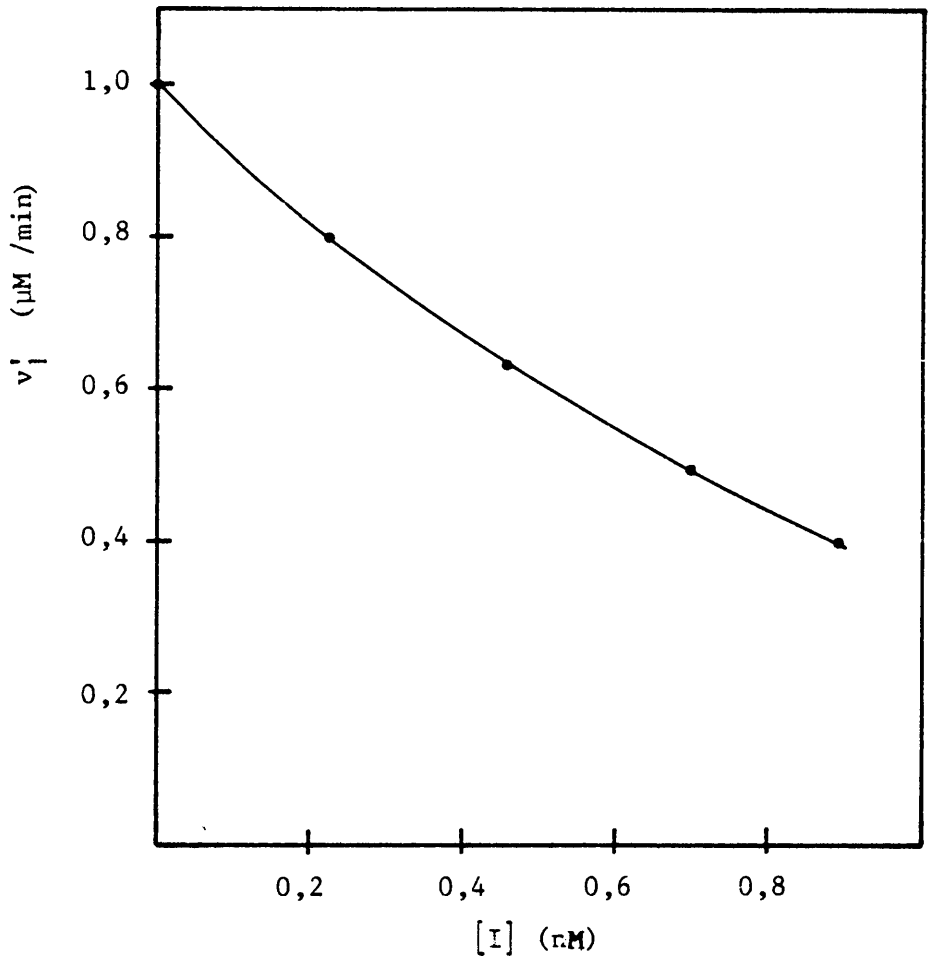


Fig 34 Relationship between the velocity of the reaction and an increase in *H.trun.* (SWA x Kaalplaas strain) egg toxin concentration. [BAPNA] = $7,6 \times 10^{-4}$ M, [Trypsin] = $2,1 \times 10^{-10}$ M, v_1' was estimated from the tangent at t = 0.

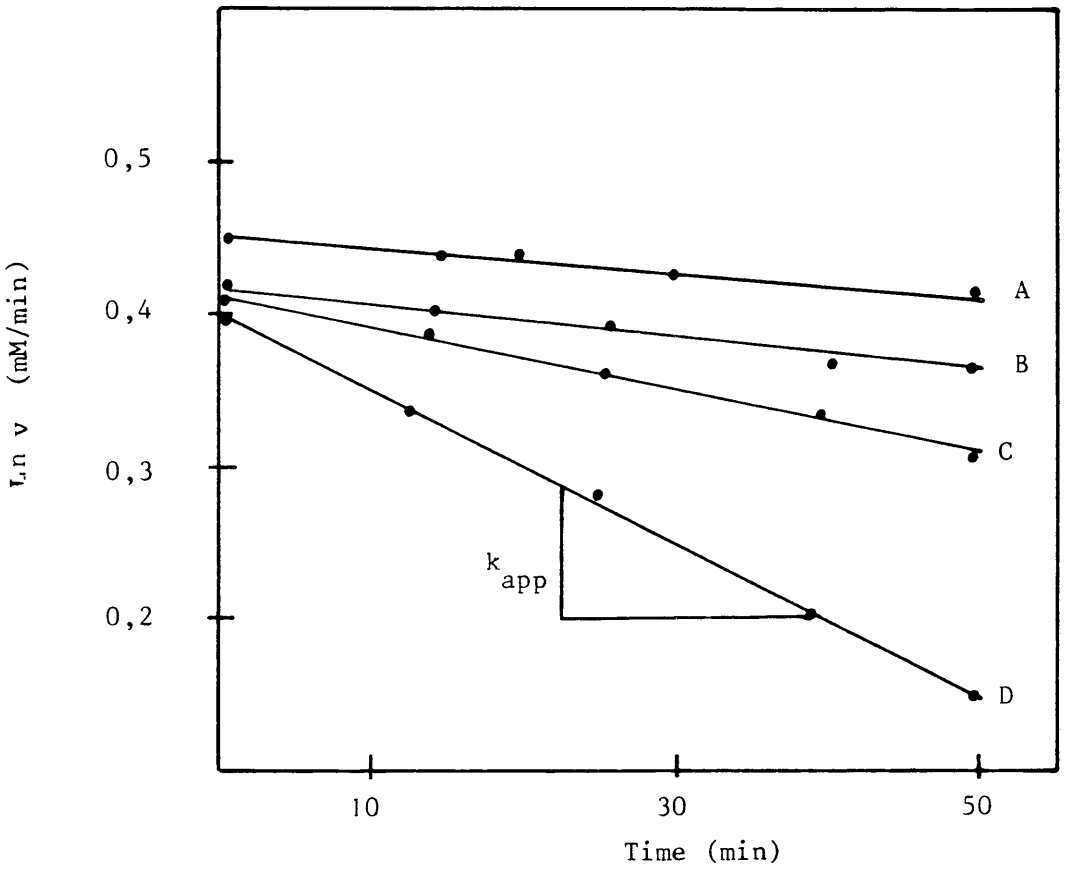


FIG 35 Determination of the slope (k_{app}) from the plot of In v against time, of *H.trun.* egg toxin. [Trypsin] = $2,1 \times 10^{-10}$ M, [BAPNA] = $7,6 \times 10^{-4}$ M, A: [*H.trun.* egg toxin] = $2,3 \times 10^{-10}$ M, B: [*H.trun.* egg toxin] = $4,6 \times 10^{-10}$ M, C: [*H.trun.* egg toxin] = 7×10^{-10} M, D: [*H.trun.* egg toxin] = 18×10^{-10} M.

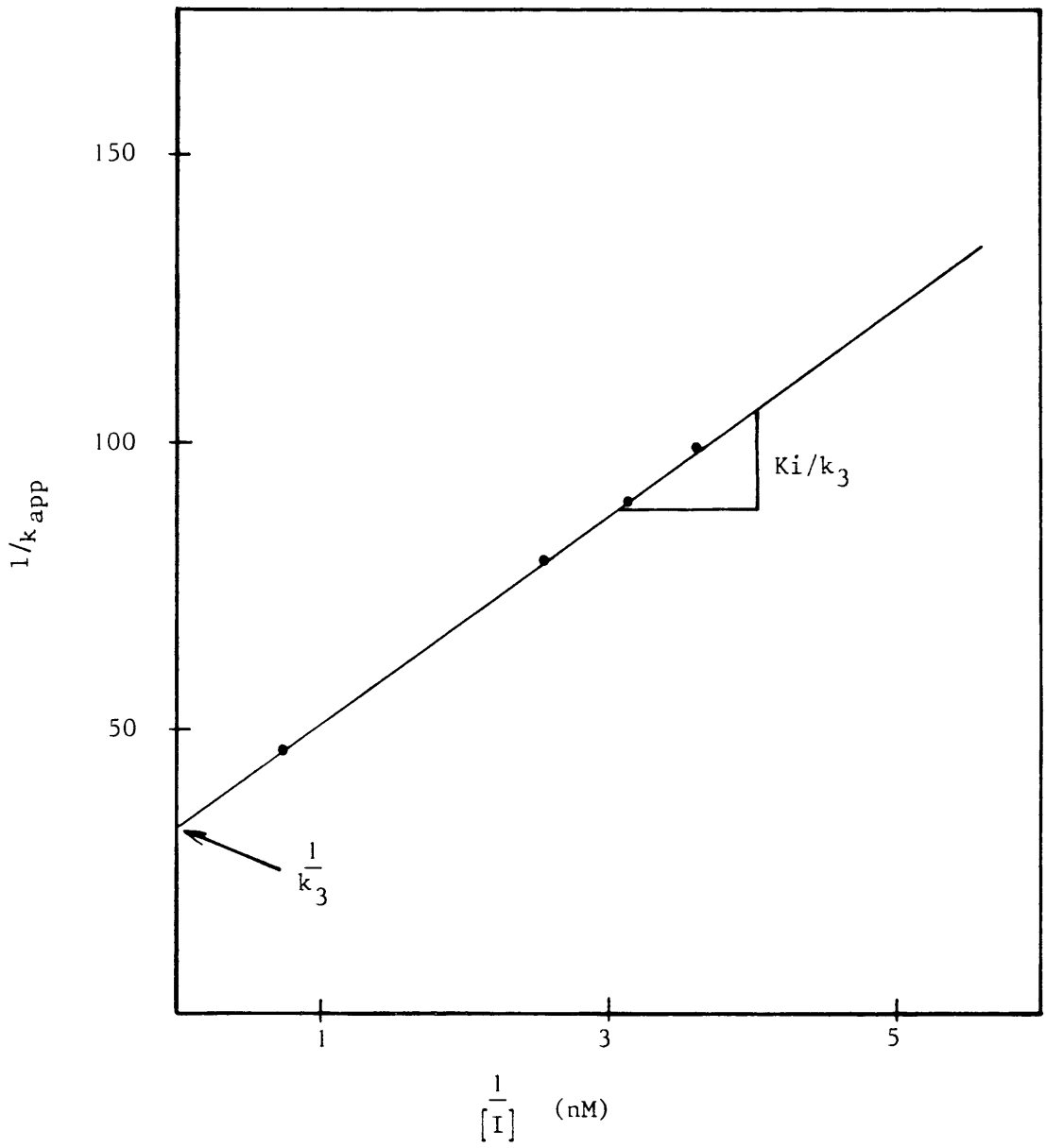


Fig 36 Reciprocal plot of k_{app} against *H.trun.* (SWA x Kaalplaas strain) egg toxin concentration
$|Trypsin| = 2,1x10^{-10}M$

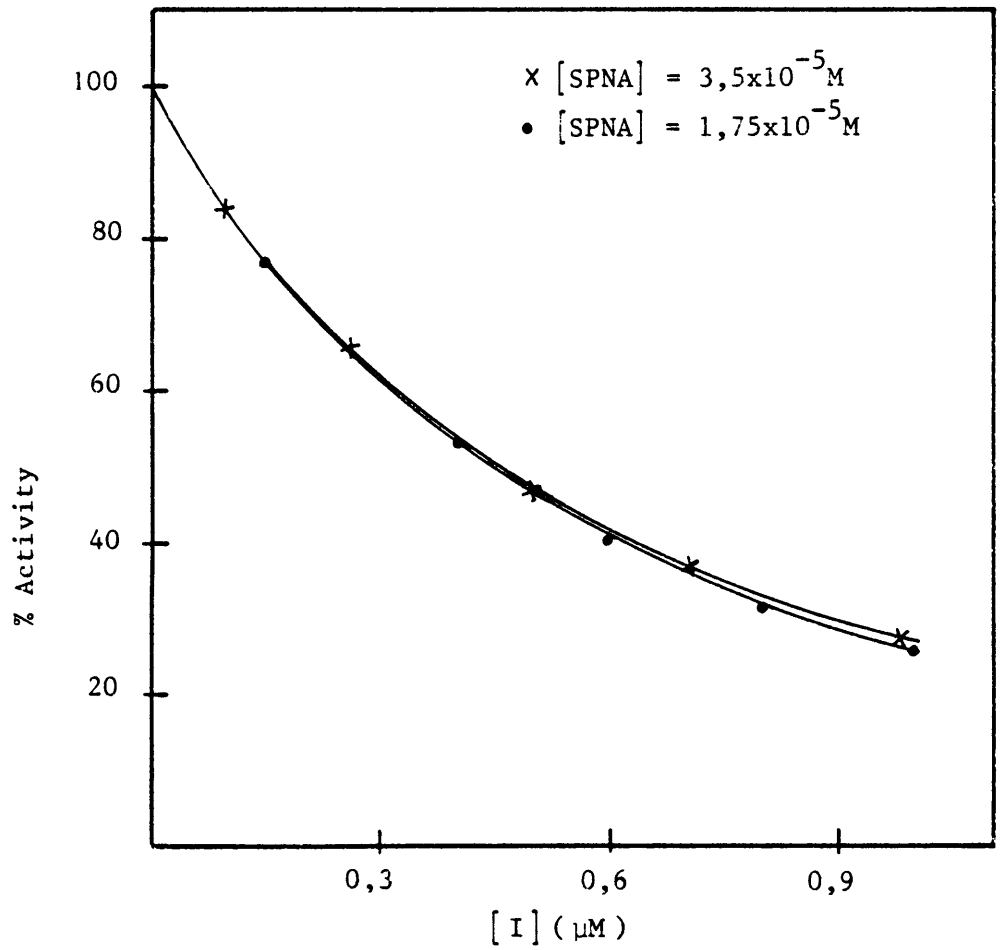


Fig 37 Anti-chymotrypsin activity of *H.trun.* (SWA x Kaalplaas strain) egg toxin, [Chymotrypsin] = 4,8x10^{-7} M.

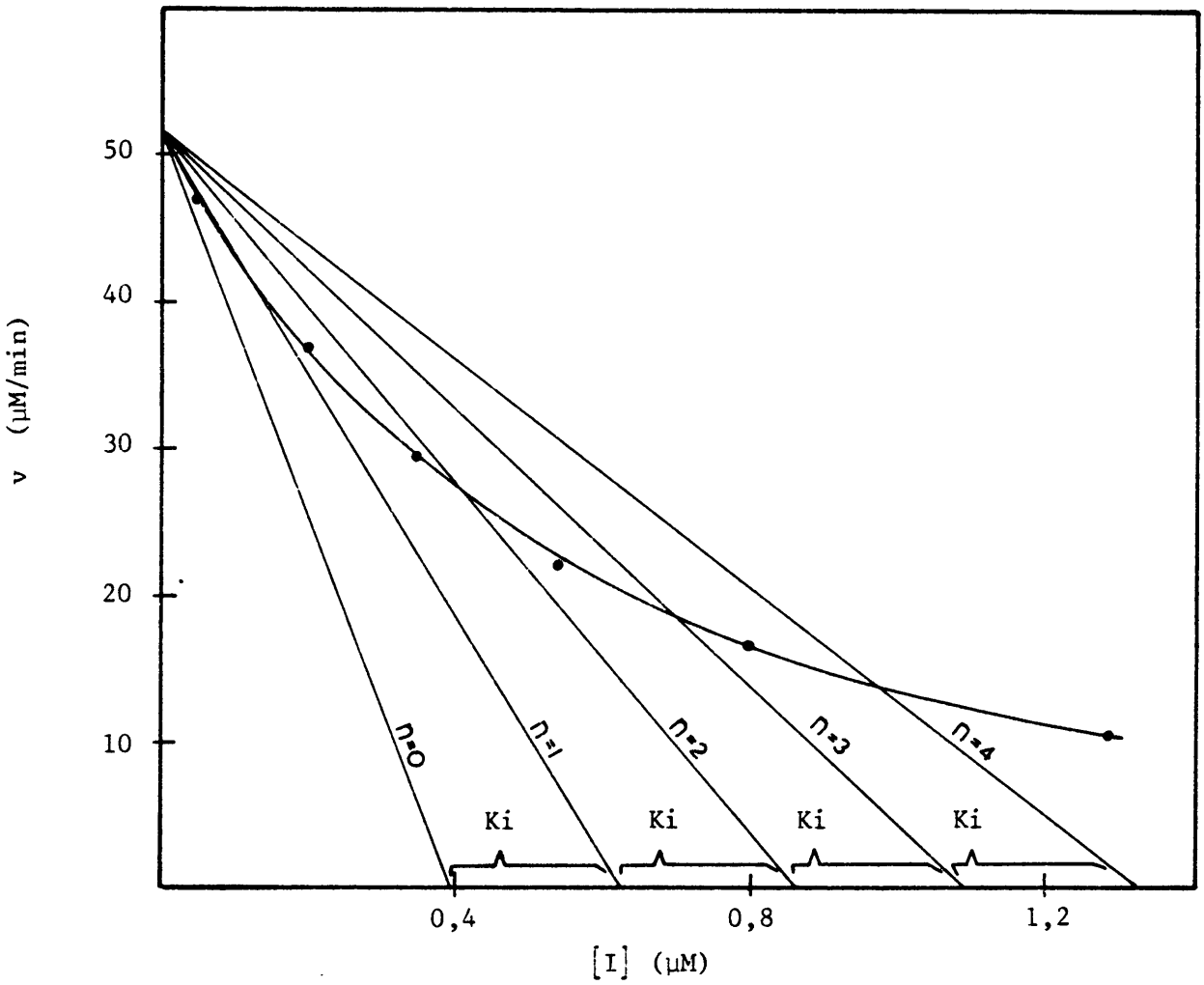


Fig 38 Relationship of velocity against *H.trun.* (SWA x Kaalplaas strain) egg toxin concentration with chymotrypsin.
[SPNA] = $3,5 \times 10^{-5}$ M, [Chymotrypsin] = $4,8 \times 10^{-7}$ M.

According to Fig 38, the inhibition was non-competitive and the distance between successive intercepts on the $|I|_t$-axis gives the Ki directly (62).

$$Ki = 2,3x10^{-7} M$$

2.2.2.8 Toxicity determinations of anti-proteases

The guinea-pigs injected subcutaneously with soybean-and-hen egg white trypsin-inhibitor were observed for 14 days and showed no symptoms.

CHAPTER 3

PARTIAL PURIFICATION OF ADULT, LARVAL AND NYMPHAL TOXINS

3.1 Materials and Methods

3.1.1 Preparation of crude tick extracts

Adult male and female *R. ee.* (Boshoff) ticks were synchronously infested on sheep. The female ticks were collected on day 5 after the infestation started. The toxic phase of female ticks, confined to the initial active sucking phase, is confined to the weight range of 20 to 129 mg or day 1 to 6 with highest toxin transmittion on days 4 and 5 (63).

The replete female ticks were homogenized in phospho buffered saline (64) at a ratio of ten ticks in 5 ml pbs, in a Virtus Model 60K homogeniser for 5 min at 15 000 rpm. The final volume was ca. 10 cm^3 and ice was packed round the special Virtus conical flask.

The tick suspension was centrifuged with a Piccolo bench top centrifuge at 270 g_{av} and 5°C in glass centrifuge tubes to separate the soluble from the cuticle and other non-soluble materials (65). The supernatant was withdrawn with a pasteur pipette and stored at -10°C.

The larvae and nymphs of *R. evertsi evertsi* (Mukuzi strain), *R. appendiculatus* (Grembéeck strain) and *R. simus* (Zululand strain) were used. The larvae and nymphs were frozen in liquid nitrogen and weighed off in 2 g batches and homogenised with an ultra-turrax (Janke & Kunkel; Kika-werk) in 10 cm^3 0,9% NaCl for

8 to 10 min at low speed. The homogenisation was done at 5°C with ice packed round the conical flask used for homogenisation.

The suspension was centrifuged with a Beckman Model L5-65 ultracentrifuge in a Rotor 40 at 80 700 g_{av} for 5 h at 5°C. The clear supernatant was siphoned off with a pasteur pipette and stored at -10°C.

3.1.2 Toxicity determination

Toxicity of the crude extracts obtained in 3.1.1 was tested as described in 2.1.3.

3.1.3 Electrophoretic characterisation of the crude extracts

Analytical isoelectric focusing was done as described previously (2.1.6.1).

3.2 Results

3.2.1 Toxicity determinations

The larval and nymphal crude extracts were all toxic. The guinea-pigs with larvae of *R. ee.*, *R. app.* and *R. simus* showed symptoms on day 5 after injection with the crude extracts and died on days 6 to 7. The guinea-pigs showed symptoms on days 5 to 6 and died on days 7 to 8 after being injected with the nymphal crude extracts. The symptoms arising from the nymphal, larval and crude egg extracts were essentially identical. The guinea-pigs injected with the adult crude extracts showed no symptoms.

3.2.2 Electrophoretic characterisation of the crude extracts with analytical isoelectric focusing

The isoelectric focusing pattern of *R.ee.*, larval and nymphal extracts showed no correlation with the corresponding band position of the egg toxin (Fig 39).

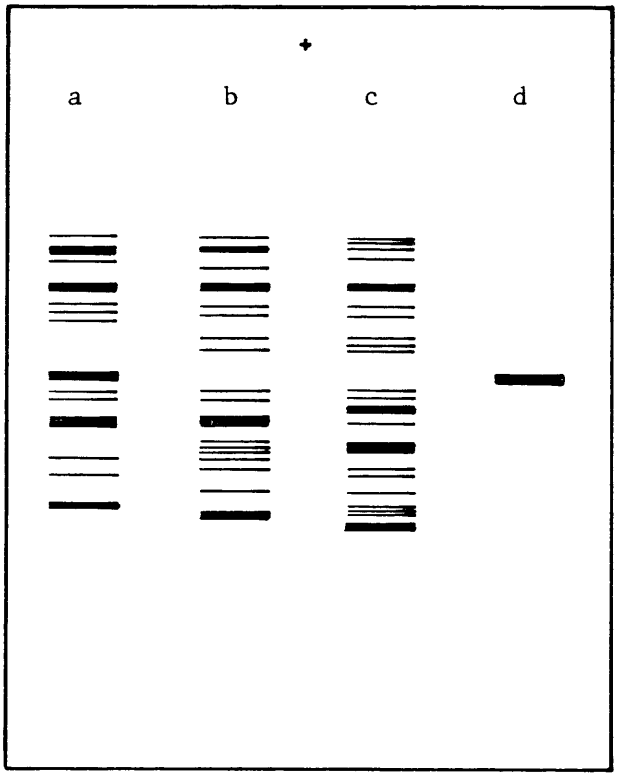


Fig 39 Isoelectric focusing patterns of *R.ee.* larval, nymphal and egg crude extracts. a: Crude egg extract, b: crude larval extract, c: crude nymphal extract, d: egg toxin.

CHAPTER 4

PURIFICATION OF SYMBIONTS IN TICK TISSUE

4.1 Materials and Methods

4.1.1 Rickettsiae and ticks

Pure, freeze-dried rickettsiae species; *Rickettsia typhi*, *R. prowazekii* (both belonging to the typhus group) and *R. conorii* (from the spotted fever group) were obtained from the Medical Research Institute, Johannesburg. These species, which represent typical rickettsiae found in ticks (66) were used to standardise cultivation and isolation methods. These rickettsiae species were the only available at the time.

For the isolation of symbionts in *H. trun.* ticks, unengorged adult sweating sickness positive (SS) and sweating sickness negative (NSS) ticks were used. Symbionts were also isolated from *H. trun.* (Uitenhage strain) (SS) and *H. trun.* (Warrenton strain) (NSS) tick eggs. The eggs were cultivated as described in 2.1.1.

Well fed *A. heb.* nymphs (Wessels or Spesbona strains) infected with *Cowdria ruminantium* Ball 3 strain were used for the isolation of the organism. The unfed nymphs were fed for approximately 7 days on a heartwater infected sheep. They were then collected and used directly.

4.1.2 Cultivation of rickettsiae

Under sterile conditions, 1 g each of the freeze-dried *R. prowazekii*, *R. typhi* and *R. conorii* organisms were suspended in 24 cm^3 of 0,9% NaCl and 1 cm^3 Ruscosulf (ICI) (an anti-microbic agent). The 25 cm^3 rickettsial suspensions were divided into 5 equal parts. The shells of fertile 7 day old chicken eggs were broken at the blunt pole with a small punch. Each of the 5 cm^3 fractions were injected into the embryo's with a 10 cm^3 syringe. The egg shells were sealed with candle wax. Control eggs were injected with 4,8 cm^3 0,9% NaCl and 0,2 cm^3 Ruscosulf. About 5% of the inoculated eggs were discarded on the second day after inoculation, because of mechanical damage.

The chicken eggs, inoculated with the rickettsiae, were incubated at 33°C. At 30°C the chicken embryo's showed optimal growth, while the rickettsiae showed optimal growth at 35°C (67,68). The eggs were harvested after 7 days. Embryo's which died before 7 days, were harvested as follows: the spotted fever group rickettsial infected embryo's, 24-48 h after death. The typhus group rickettsial infected embryo's on the day they died (65,68). In all cases embryo's were harvested under sterile conditions and frozen with pieces of broken glass (4.1.3) in sterile bottles. They were then stored in liquid nitrogen.

Some yolk sacs were used for further cultivation. The embryo's were gently shaken during thawing until a homogeneous suspension was obtained. Each 5 cm^3 homogeneous yolk sac suspension was diluted with 20 cm^3 0,9% NaCl and 1 cm^3 Ruscosulf and chicken egg embryo's were inoculated with this suspension as described previously.

4.1.3 Preparation of crude chicken yolk extracts

Rickettsial infected yolk sacs were homogenised while thawing, by gentle shaking. Each yolk sac was suspended in 15 cm^3 0,9% NaCl. The suspension was shaken until homogeneous and then centrifuged in glass centrifuge tubes for 15 min with a Piccolo bench top centrifuge at 270 g_{av} and 25°C. The supernatant was siphoned off with a pasteur pipette. The pieces of glass were removed with the aid of a tweezer and the precipitate suspended in 10 cm^3 0,9% NaCl. Hereafter the suspension was sonified (69) with a Sonifier Cell Disruptor B-30 (Branson Sonic Power Co.) for 8 sec at minimum power. The suspension was then centrifuged at room temperature in a Piccolo bench top centrifuge for 15 min at 270 g_{av}. The supernatant was siphoned off with a pasteur pipette and combined with the previously mentioned supernatant.

4.1.4 Preparation of crude *H.trun.* and *A. heb.* tick extracts

Thirty sweating sickness positive (SS) and thirty sweating sickness negative (NSS) unengorged adult female *H. trun.* ticks and three hundred replete heartwater infected nymphs of *A. heb.* were sterilised with 70% ethanol and thoroughly rinsed (4x) with sterilised water. The ticks were homogenised in a conical flask containing 20 cm^3 0,9% NaCl with an ultra-turrax (Janke & Kunkel, Kika-werk) at low power for 6 min. Ice was packed round the conical flask during homogenisation.

The tick-NaCl suspension was centrifuged with a Beckman Model L5-65 ultracentrifuge for 25 min at 2200 g_{av} and 5°C to remove all the insoluble material.

4.1.5 Preparation of crude *H.trun.* tick egg extracts

Eggs of *H.trun.* (SS) and *H.trun.* (NSS) were used. The eggs (2 g wet mass) were frozen in the presence of pieces of glass in liquid nitrogen. While they thawed, they were gently handshaken. After 10 min of shaking the solution appeared homogeneous and was gently mixed with 2 cm^3 0,9% NaCl. The solution was centrifuged with a Piccolo bench top centrifuge for 25 min at 270 g_{av} and 5°C. The supernatant was siphoned off with a pasteur pipette.

4.1.6 Isolation of symbionts

Further purification of rickettsiae was achieved by means of Percoll (Pharmacia Fine Chemicals) density gradient centrifugation (70). An iso-osmotic Percoll stock solution (SIP) was prepared by adding 9 cm^3 of Percoll to 1 cm^3 of 0,9% NaCl.

The crude extracts prepared from chicken yolk (4.1.3), *H.trun.* ticks (4.1.4) and *H.trun.* eggs (4.1.5), were centrifuged with a Beckman Model L5-65 ultracentrifuge for 25 min in a Rotor 30 at 19 800 g_{av} and 5°C to sediment the rickettsiae (65). The crude extract of *A.heb.* ticks (4.1.4) were centrifuged with a Beckman Model L5-65 ultracentrifuge for 25 min in a Rotor 30 at 30 000 g_{av} and 5°C to sediment the organisms.

The supernatant was siphoned off with a pasteur pipette and stored at -4°C. The sediment was suspended in 15 cm^3 NaCl and then mixed with 15 cm^3 SIP to form a 50% SIP solution. As reference a tube containing 50% SIP solution and density marker beads (Pharmacia Fine Chemicals) was used.

The tubes were centrifuged in a Beckman Model L5-65 ultracentrifuge for 15 min at 30 000 g_{av} in a Rotor 30 at 5°C. At maximal acceleration the rotor took 4,5 min

to reach a speed of 18 750 rpm. The braking time was 7,5 min at half maximal braking rate. The gradient was pumped out from the bottom of the tube with a Beckman fraction recovery system. Ten fractions of 3 cm^3 each were collected, 2,5 cm^3 of each of the ten fractions were immediately taken to Onderstepoort for toxicity determinations while the other 0,5 cm^3 of each fraction were used for electron microscopic and light microscopic studies.

4.1.7 Light microscopic studies

Smears of the fractions obtained during the symbiont isolations were stained by the Gimènez (71) method and investigated under a light microscope (Wild-Heerbrugg Model 20) to determine whether rickettsiae were present. The Gimènez reagent was prepared as follows:

Basic fuchsin (Merck)(2,5 g) was dissolved in 50 cm 95% ethanol. Crystallised phenol (5 g) was dissolved in 450 cm^3 distilled water. The basic fuchsin and phenol solutions were combined and incubated in a dark bottle for 48 h, at 37°C. After incubation, 4 parts of this solution was mixed with 5 parts of 0,1 M phosphate buffer, pH 7. The solution was filtered through a Whatman nr 1 filterpaper before use. A new solution was prepared after every 3 days. Malachite green (Merck) (1 g) was dissolved in 125 cm^3 distilled water and filtered with a Whatman nr 1 filterpaper before use.

Staining was achieved as follows:

Smears were heat fixed and stained in the basic fuchsin-phenol-phosphate solution for 5 min at 25°C. The smears were rinsed with tap water and counter stained with malachite green for 30 sec. The smears were then rinsed with tap water and

allowed to dry. The dried smears were investigated under the light microscope.

4.1.8 Infectivity of isolated organisms

Fractions obtained from the Percoll density gradients (4.1.6) which contained *R. prowazekii*, *R. typhi* and *R. conorii* organisms were diluted to 25 cm^3 with 0,9% NaCl and 1 cm^3 Ruscosulf. The suspensions were inoculated in chicken egg embryo's as described in 4.1.2.

Density gradient fractions obtained from *A. heb.* ticks (4.1.6) were each made up to 6 cm^3 with 0,9% NaCl. The fractions were injected intravenously into 10 sheep at a dose rate of 5,5 cm^3 per animal. The injection needle was dipped prior to the injection into a adrenalin solution (34 g adrenalin in 100 cm^3 water). This reduced the initial shock of the injection. The remaining 0,5 cm^3 of each fraction was used for electron microscopic studies (4.1.9).

4.1.9 Electron microscopic studies of organisms isolated from *A. heb.* ticks

Organisms isolated from the *A. heb.* ticks were prepared for electron microscopic investigation in the following way: to 0,5 cm^3 of each fraction obtained from the density gradient (4.1.6) was added 0,9% NaCl. The suspension was centrifuged with a Beckman Model L5-65 ultracentrifuge, for 25 min at 30 000 g_{av} and 4°C, in a Rotor 40 to sediment the organisms. The supernatants were siphoned off and the sediment of each fraction, was mixed with ca. 0,1 cm^3 2% agar at 45°C and siphoned into a glass capillary tube. The contents of the tubes were blown out onto filter-

paper and cut into thin sections with a scalpel (0,1 to 0,3 cm^3). The sections were fixed in 3% glutaraldehyde for 3 h, followed by fixing in 2% osmium tetroxide (72,73) in a fume cupboard for 3 h. Dehydration was achieved with 90% and 100% ethanol for 15 min. Hereafter the sections were embedded in a 100% spurr (Polaron Equipment Ltd) solution (74) and left at 70°C for 20 h for maximum penetration of the spurr solution into the sample.

4.1.10 Discontinuous electrophoresis

The method described by Laemmli (75), was employed. The gel concentrations were as follows:

Stacking gel; T = 5%, C = 2,6%

Separating gel; T = 15,4%, C = 5,2%.

The samples (2 mg) were made up in 0,1 cm^3 distilled water and applied onto the vertically mounted gel (150x160x3 mm). Electrophoresis was performed with a deluxe regulater power supply of Gelman Instruments Comp. at 200 V (constant) for 16 h, 10°C. Starting current was 52 mA and end current, 12 mA.

4.2 Results

4.2.1 Purification of rickettsiae *(R. prowazekii, R. typhi* and *R. conorii)* cultivated in yolk sacs of chicken eggs

Fractions obtained from the density gradient (4.1.6) were stained (4.1.7) and investigated under the light microscope (X1500) for the presence of rickettsiae. The results are shown in Table 16.

TABLE 16 PRESENCE OF RICKETTSIAE IN A PERCOLL DENSITY GRADIENT

FRACTION	VOLUME (cm^3)	DENSITY*($g\ cm^{-3}$)	PRESENCE OF ≠ *R. prowazekii*	PRESENCE OF ≠ *R. typhi*	PRESENCE OF ≠ *R. conorii*
1	0 - 3	1,126	0	1+	0
2	4 - 6	1,091	3+ v	3+ v	1+ v
3	7 - 9	1,065	2+	1+	2+
4	10 - 12	1,059	0	0	0
5	13 - 15	1,057	0	0	0
6	16 - 18	1,055	1+	1+ v	1+
7	19 - 21	1,052	0 v	0	0
8	22 - 24	1,049	2+	0	2+ v
9	25 - 27	1,041	2+	1+ v	3+ v
10	28 - 30	1,026	0 v	2+ v	0

≠: Stained with Giménez (X1500)(71). Each rickettsiae sighted at X1500 per field accounts for 10^6 rickettsiae per gram dried mass of the suspension investigated, 1 to 5, 6 to 15 and more than than 15 rickettsiae per field are given as 1+, 2+ and 3+ respectively (65,89).

v: Visual bands.

*: Average density of each fraction.

Microscopic investigations showed that fraction 2 of *R. typhi*, fraction3 of *R. conorii* and fraction 2 of *R. prowazekii*, were the fractions containing the highest concentration of rickettsiae and the least contaminating particles. The Laemmli method of disc-electrophoreses showed that the above fractions had no chicken egg proteins present.

A typical distribution of density marker beads in a 50% SIP gradient is shown in Fig 40.

4.2.2 Purification of rickettsiae from *H. trun.* ticks and *H. trun.* eggs

The distribution of rickettsiae is shown in Table 17.

Fractions that were positive for rickettsiae of the *H. trun.* (SS) and (NSS) ticks (Table 17) were inoculated in chicken eggs in a similar way as described in 4.1.2. The growth of rickettsiae were dominated by the growth of *Citrobacterium* (a general agent in water and the air) and no success was obtained with the cultivation of rickettsiae in the embryo's of chicken eggs.

4.2.3 Purification of *A.heb.* symbionts

Four experiments were performed. The distribution of heartwater organisms are shown in Table 18.

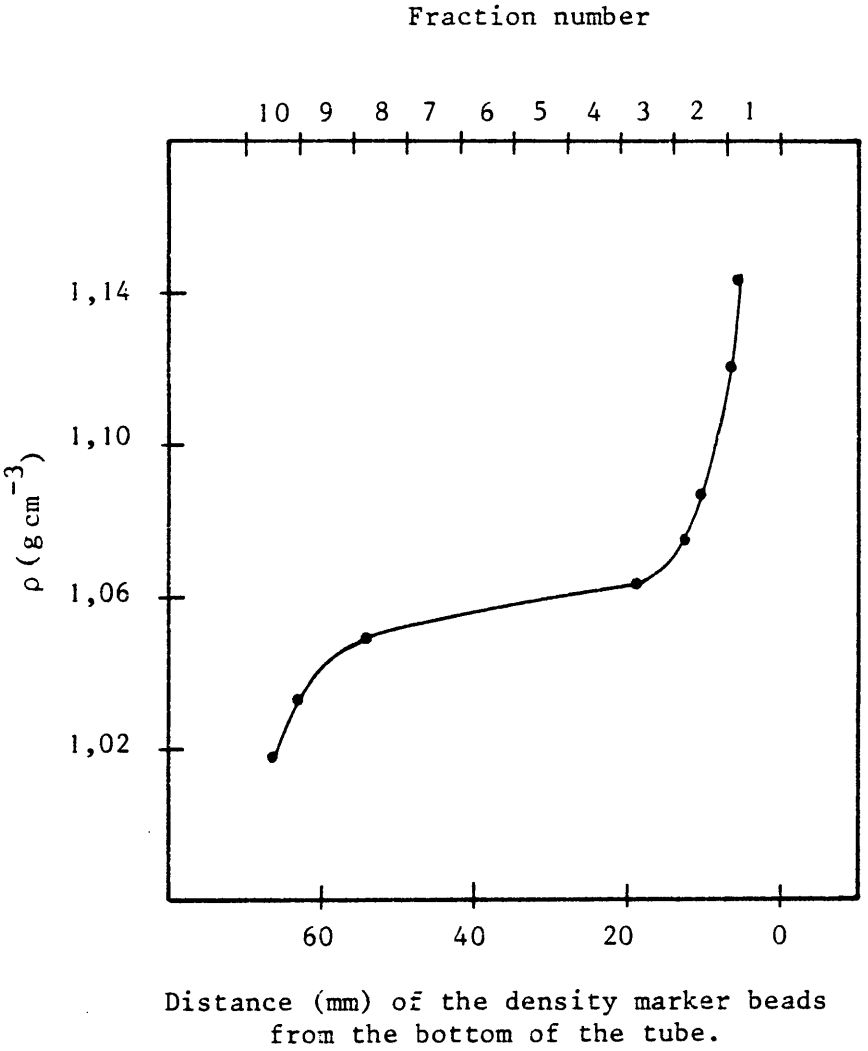


Fig 40 Typical distribution of density marker beads in a 50% Pecoll gradient. Marker bead distribution (•—•), Rotor 30, 50% SIP, 30 000g_{av}, 15 min.

TABLE 17 PRESENCE OF RICKETTSIAE IN *H. trun.* TICKS AND *H. trun.* EGGS

FRACTION	VOLUME (cm^3)	DENSITY* ($g\ cm^{-3}$)	SS TICKS ≠	NSS TICKS ≠	SS EGGS ≠	NSS EGGS ≠
1	0 - 3	1,126	0	1+	1+ v	1+ v
2	4 - 6	1,091	1+ v	2+ v	2+	1+
3	7 - 9	1,065	1+	1+	1+	1+
4	10 - 12	1,059	1+	0	0	0
5	13 - 15	1,057	0	1+	0	0
6	16 - 18	1,055	1+	0	1+	1+
7	19 - 21	1,052	2+	0	1+	1+
8	22 - 24	1,049	1+	1+	0	0
9	25 - 27	1,041	1+ v	2+ v	1+ v	0 v
10	28 - 30	1,026	0	1+	1+ v	1+ v

≠: Stained with Gimènez (X1500)(71). Each rickettsiae sighted at X1500 per field account for 10^6 rickettsiae per gram dried mass of the suspension investigated, 1 to 5, 6 to 15 and more than 15 rickettsiae per field are given as 1+, 2+ and 3+ respectively (65,89).

v: Visible bands.

*: Average density of each fraction.

TABLE 18 DISTRIBUTION OF HEARTWATER ORGANISMS IN A PERCOLL DENSITY GRADIENT

FRACTION* SHEEP NUMBER	DENSITY (g cm^{-3})	EXPERIMENT I	II	III	IV
		≠	≠		≠
1	1,126 v	✓ (11)	✓ (18)	X	✓ (10)
2	1,091 v	✓ (8)	✓ (18)	X	✓ (8)
3	1,065	✓ (8)	✓ (17)	X	✓ (7)
4	1,059	✓ (10)	X	X	✓ (7)
5	1,057	✓ (10)	X	X	✓ (8)
6	1,055 v	✓ (8)	X	X	✓ (9)
7	1,052	✓ (8)	X	X	✓ (8)
8	1,049	✓ (11)	X	X	✓ (9)
9	1,041 v	0	✓ (17)	X	✓ (8)
10	1,026	0	X	X	✓ (8)

≠ Day after injection on which first heartwater symptoms (constant high body temperature) occurred.

✓ Reaction positive.

X No reaction.

0 Sheep died ca. 15 min. after injection probably as a result of anaphylactic shock.

v Visible bands.

I Spesbona strain.

II Spesbona strain.

III Alldays strain.

IV Spesbona strain.

* Each fraction from gradient (4.1.6) numbered 1 to 10 was injected into the corresponding numbered sheep.

CHAPTER 5

DISCUSSION

The egg toxin isolation procedures described in this thesis involve three main steps. The first involves the preparation of a crude extract. The crude extract was obtained by centrifuging the homogeneous egg-NaCl suspension for 5h at 80 700 g_{av}. The sediment contained very little or no toxin and a 100% toxicity yield was obtained.

The second isolation step involves gel permeation chromatography. This separation, although not giving a high toxicity enrichment, gave a high (100%) yield in toxicity. It was however a suitable method to separate most of the contaminating coloured components.

The third step in the isolation of the toxins involves chromatofocusing. The toxicity enrichments were high as well as the percentage yield in toxicity which varied from 72 to 95%. Nitrogen determinations could not be determined accurately after each isolation step because of the low yield with respect to mass.

The monitoring system used for determining toxicity may be considered as insensitive, since a limited number of test animals were used. Furthermore, the clinical symptoms abate sharply upon dilution of the toxin. The same tendency was observed by Neitz *et al* (18) and Riek (19) for crude egg extracts of various species of ticks.

According to gel permeation chromatography the toxins were relatively large. SDS-gradient gel electrophoresis and sedimentation equilibrium centrifugation however showed that the toxins have lower molecular masses. The reason for this discrepancy could be that the toxins are associated with

other components under the conditions prevailing during the isolation step involving gel permeation chromatography. On the other hand the toxins may not conform to the behavior expected of smaller molecules as the result of a greater exclusion from the gel because of their structural features. These possibilities may be tested by subjecting the purified toxin to gel permeation chromatography. Log A *versus* r^2 plots for all the toxins showed a slight upward curvature near the bottom of the cell. This could indicate association-dissociation of the toxins during centrifugation (49). To test if the proposed dissociation-association was pH dependent, the pH was lowered from 8 to 5. No change in the curves were observed. Another possibility could be that the samples were heterogeneous. However, because the samples showed single bands with analitical isoelectric focusing and SDS-gradient polyacrylamide gel electrophoresis, even at high concentrations, it can be concluded that the curvature is likely due to thermodynamic non-ideality (76).

Isoelectric focusing of the crude larval and nymphal extracts of *R.ee.* showed no correlation with the respective egg toxin band position (Fig 39). This suggests that the toxin found in larval and nymphal crude extracts differ from that present in tick eggs. The clinical symptoms however are similar, although it appears that the specific activity of the larval and nymphal toxins is lower. The findings suggest that the toxin undergoes structural changes during the life cycle of the tick.

The amino acid composition of *B. mic.*, *B. dec.* and *H. trun.* toxins (Table 15) show an acidic character, whereas the isoelectric points are relatively high (Table 13). The discrepency is most probably due to the fact that many of the acidic residues determined by amino acid analysis exist as the amides in the intact toxins.

The clinical symptoms found in the experimental animals injected with egg toxins differ from those resulting from tick paralysis (4). The symptoms in animals as a result of tick paralysis are similar to that of an ascending flaccid paralysis due to involvement of the lower motor neurons. Test animals show a lack of coordination in the hind limbs. This is followed by paralysis which spreads to the forelimbs and chest and neck muscles. The egg toxins cause a partial paralysis (paresis) only.

In the present study the histopathological lesions observed in guinea-pigs inoculated with the egg toxins of *R. ee.*, *B. mic.*, *B. dec.* and *H. trun.* were comparable. This suggests that the toxins, although different in structure, have the same mode of action. The histopathological findings indicate that the toxins exert their effect on cell membranes (18). Neitz *et al* (18) reported similar histopathological observations as described in this thesis. Riek (19) mentioned degeneration and necrosis of hepatocytes as well as degenerative changes in the kidney tubular epithelial cells of guinea-pigs inoculated with egg or tick extracts of various Ixodidae. The haemagglutination activity of the crude egg extracts as well as of the toxins was determined in order to gain an insite into their mode of action on cell membranes. No such activity could be detected however under the conditions employed in this project. This investigation should be repeated using other conditions, for example pre-sensitisation of red blood cells.

Trypsin inhibitors and protease inhibitors have long been known to be present in plants (78), animals (79) and microorganisms (79). More recently they have been shown to be present in ticks (18), where they may be responsible for specific symbiont associations and act as primitive humoral defence agents (90). Protease inhibitors in animal and plant cells or in blood plasma are proteins and are involved

in the control of a variety of cell functions, inflammation and blood coagulation. The inhibitors obtained by extraction from microbial cells are also macromolecular peptides, but many protease inhibitors released extracellularly are low-molecular-weight peptides of unusual structures (80).

All the tick egg toxins described in this thesis showed anti-protease activity when tested against trypsin. In addition some showed inhibitory activity against chymotrypsin. The toxicity of the tick egg toxins is probably not dependent on the anti-protease activity alone but to other activities as is shown by the following observations. The trypsin inhibitors from soybean and chicken egg white (2.2.2.8) showed no toxicity when injected subcutaneously into guinea-pigs. Furthermore non-toxic fractions obtained from the Sephadex G 100 column showed anti-protease activity with trypsin (Fig 4) and Neitz, *et al* (81) showed that numerous non-toxic anti-proteases were present in the eggs of the tick, *Amblyomma hebraeum*. It is also of interest that Rosenberg, *et al* (82) showed that in snake venom phospholipase A_2, there are at least two distinct but perhaps overlapping active sites; one, being primarily responsible for enzymatic activity and the other, being associated with lethal and pharmacological effects.

Inhibitors are classified as single-headed if they have only one reactive site, double-headed if they have two reactive sites and when they have more than two, multi-headed (83). Results reported in this thesis indicate that the egg toxins of *B. mic.*, *B. dec.*, *H. trun.* and *R. ee.* are at least double-headed, since they all showed different types of inhibition with trypsin and chymotrypsin. It is possible for a single-headed inhibitor to inhibit both trypsin and chymotrypsin, but usually the inhibition of the enzymes occurs by identical meganisms (84). During the present study of the various protease inhibitors an attempt was made to determine the type

of inhibition and the dissociation constants. The results showed distinct differences between toxins which are indicated by the following findings: *R. ee.* egg toxin showed non-competitive fast-binding inhibition of trypsin, according to the Lineweaver-Burk method, with a Ki of $1,6x10^{-8}$ M. The egg toxins of *B. dec.* and *H. trun.* on the other hand showed non-competitive fast-binding inhibition of chymotrypsin. The Dixon method (62) was employed and the Ki values were found to be $3,6x10^{-8}$ M and $2,3x10^{-7}$ M respectively.

Furthermore the egg toxins of *R. ee.* and *B. mic.* showed no inhibition with chymotrypsin. At this stage it must be mentioned that protease inhibitors have been defined as being strictly competitive (85,86). However when studied with the Lineweaver-Burk method this type of inhibitor show non-competitive inhibition. It has been shown that when the Ki is much smaller than the Km of the enzyme used in the assay, the substrate does not substantially perturb the enzyme inhibitor equilibrium. Furthermore whatever the value of Ki, if the half-time for dissociation of the enzyme inhibitor complex (since substrate is usually added to the previously prepared complex) is long compared to the assay time, the three components do not equilibrate. In both cases, Lineweaver-Burk plots will indicate non-competitive inhibition.

A number of experiments have been performed to show that the protease inhibitors are indeed of the competitive type (85). Dixon (62) derived a method to study tightly bound inhibitors, which is able to differenciate between competitive and non-competitive inhibitors. This method used, was successfully in a number of inhibition studies with compounds of this type (85,87,88). It was found however that the inhibitors from *B. dec.* and *H. trun.* showed non-competitive inhibition with both the Lineweaver-Burk and Dixon methods. These results should be confirmed by studies of the type described by

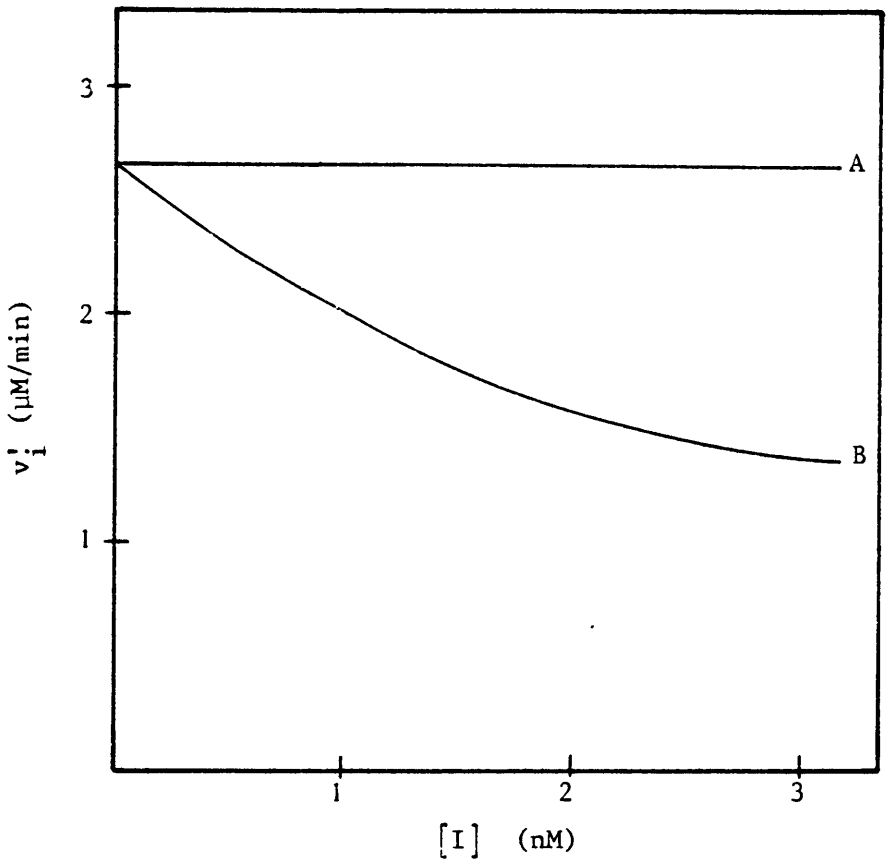


Fig 41 Relationship between the velocity of the reaction and the inhibitor concentration. v_i' is the tangent at t = o. A follows mechanism A and B follows mechanism B.

other described methods.

The isolation procedure of rickettsiae involves centrifugation in two main steps. The first involves high and low speed centrifugation. The second step involves density gradient centrifugation with Percoll (70) as density forming medium. The reasons why Percoll was used was the following: It can be prepared to have physiological ionic strength and pH. Furthermore it is iso-osmotic throughout the gradient, it is non-toxic, unable to penetrate biological membranes, and capable of forming self-generated gradients by centrifugation at moderate centrifugal field strengths. The only problem encountered was that samples containing Percoll could not, because of its colloidal nature, be studied directly under the electron microscope with positive staining. Negative staining however, was not effected.

Sonification of the yolk sacs was necessary because the gentle breaking of the yolk sac cells with glass was inadequate to release the organisms quantitatively from the egg cells.

The cultivation of the rickettsiae present in *H. trun.* ticks was unsuccessful since the growth of *Citrobacterium* dominated that of the rickettsiae in the yolk sacs. This occurred in spite of the presence of an anti-bacterial agent (Ruscosulf) during the cultivation. The presence of rickettsiae at the same densities in Percoll gradients in both SS and NSS strains of the *H. trun.* ticks is important. It indicates that sweating sickness is indeed a tick toxicoses. On the other hand if sweating sickness is a rickettsial disease, either different species of rickettsiae are present in NSS and SS ticks or the NSS ticks contain the rickettsiae in a avirulent form.

The distribution of *Cowdria ruminantium* through almost all

the density gradient is noteworthy. This substantiates the pleomorphic nature of this organism as seen by electron microscopic investigations (27). In addition it stresses the resolving power of Percoll density gradients.

The advantage of the isolation procedure developed during this study, is that the time for the isolation procedure is short (ca. 4 h) and can be performed under mild conditions. Hereby viable and infective organisms may be obtained. A test procedure to determine the purity of the isolated organisms has not been developed. A sensitive method involving ELISA techniques is envisaged.

CHAPTER 6

CONCLUSIONS

The purification procedure described in this thesis offers a means by which the toxic components from the eggs of *B. mic.*, *B. dec.*, *R. ee.* and *H. trun.* may be obtained in a pure form.

There is no difference between the strains of each specie but the toxins differ with respect to molecular mass, iso-electric point and anti-protease activity from specie to specie. The anti-protease activity of the toxic principle of all the tick eggs must be studied with different methods to confirm the type of inhibition described in this thesis.

The toxin found in the eggs of the tick *R. ee.* is different from the toxin causing tick paralysis. The main problem in isolating the tick paralysis toxin, is to find a suitable *in vitro* test procedure, since the tick paralysis toxin is host specific and small laboratory animals are not susceptible.

The isolation and cultivation procedure described for various rickettsial organisms, is favourable to obtain viable and infective organisms.

Much concerning the tick problem is still unknown and it is hoped that this study will serve to foster further investigations into the origins of the toxins, their mode of action, vector-parasite relationships and the tick problem in general.

SUMMARY

Ectoparasites can serve as vectors of numerous disease agents i.e. they are involved in maintaining and transmitting for example viral and rickettsial organisms pathogenic to man and animals (S1). An understanding of these diseases inevitably must involve a study of the vector, pathogen, possible toxins involved and the host.

The present study was designed to investigate the origin and characteristics of the toxins. Tick eggs were selected as starting material since they are known to be toxic (S2) and they can be collected in fairly large numbers. Furthermore, these toxins may have a bearing on toxins associated with tick paralysis and tick-symbiont association.

Rhipicephalus evertsi evertsi, *Hyalomma truncatum*, *Boophilus decoloratus* and *Boophilus microplus* were used to prepare crude egg extracts. Toxin purification procedures involved centrifugation, gel permeation chromatography and chromatofocusing. They were shown to be pure by isoelectric focusing and SDS-PAGE. Protease inhibitory activity was analysed on pancreatic trypsin with N-benzoyl-D-L-arginine 4-nitroanilide-HCl as substrate, and pancreatic chymotrypsin with N-succinyl-L-phenylalanine-p-nitroanilide as substrate. The toxins differed with respect to amino acid composition, isoelectric point, molecular mass and inhibitory action on trypsin and chymotrypsin. Molecular mass was determined with SDS-gradient polyacrylamide gel electrophoresis and sedimentation equilibrium centrifugation and the isoelectric points with analytical isoelectric focusing. Some characteristics of the toxins are summarised in Table S1.

The relatively well studied organisms, *Rickettsia prowazekii*, *R. typhi* and *R. conorii* were selected as test organisms. These studies were later extended to *Cowdria ruminantium* and orga-

TABLE S1 COMPARISON OF EGG TOXINS

TICK SPECIES	SPECIFIC ACTIVITY sa	MOLECULAR MASS KDal	YIELD[Y] mg	KI M	pI[f]
Amblyomma Hebraeum (18)	nd	$10^{uc,sds,aa}$	nd	$1,6x10^{-6}nct$	8
Rhipicephalus evertsi evertsi					
Strain: Boshoff		5^{uc}; 3^{aa}			6
Duncan		$<14^{sds}$			6
Warrenton		28^{aa}			6
Sweetwater	0,3	5^{uc}; $<14^{sds}$	3	$1,6x10^{-8}nct$	6
Hyalomma Truncatum					
Strain: SWA (SS)		30^{uc}; 26^{sds}			8,3
Kaalplaas (NSS)		25^{uc}; 24^{sds}			8,3
SWA X Kaalplaas (SS)	2,3	26^{uc}; 27^{sds}	2	$9,6x10^{-10}sbt$, $2,3x10^{-7}ncc$	8,3
Uitenhage (SS)	2,2	25^{sds}			8,3
Boophilus Microplus					
Strain: Onderstepoort	1,2	31^{uc}; 36^{sds}	2		9,1
Boophilus Decoloratus					
Strain: Ethel	1,4	$40^{sds,uc}$	2	$3,6x10^{-8}ncc$	9,2
Ornithodoros Savignyi (20)	non-toxic				

nd; not determined, SS; virulent strain, NSS; avirulent strain, uc, aa and sds; determined by sedimentation equilibrium centrifugation, amino acid analysis and SDS-PAGE respectively, Y; mg toxin/gram wet eggs, f; determined by analytical IEF, nct; non-competitive trypsin inhibitor, sbt; irreversible slow-binding trypsin inhibitor, ncc; non-competitive chymotrypsin inhibitor. sa;MLD/mg toxin (dose expressed/kg body mass)

nisms from ticks producing sweating sickness. The motivation was to investigate possible toxins produced by *C. ruminantium* and to investigate the possibility that organisms from sweating sickness producing ticks are involved as agents of the disease.

The isolation of rickettsia-like organisms involved high and low speed centrifugation as well as Percoll density gradient centrifugation. Electron microscopic studies of the organisms isolated from heartwater infected *Amblyomma hebraeum* ticks, showed morphological similarities to those found in the choroid plexus of an infected animal. The rickettsia-like organisms isolated from sweating sickness positive and negative *H. truncatum* ticks and eggs showed a similar distribution of organisms for both strains in Percoll density gradients.

The above results show that rickettsia-like organisms from ticks may be harvested in a pure, infective form and in large numbers.

SAMEVATTING

Ektoparasiete kan dien as vektore van talryke agente wat siektes veroorsaak. Hulle is betrokke by die onderhoud en oordraging van byvoorbeeld virale en rickettsiale organismes wat patogenies is vir mens en dier (S1). Begrip van hierdie siektes sluit onvermydelik 'n studie van die vektor, patogeen, moontlike toksiene en die gasheer in.

Hierdie studie is ontwerp om die oorsprong en eienskappe van die toksiene te ondersoek. Bosluiseiers is as begin materiaal gekies omdat dit bekend is dat hulle toksies is (S2) en in relatief groot hoeveelhede verkrygbaar is. Verder mag die eier toksiene betrekking hê op toksiene geassosieerd met bosluisparalise en bosluis-simbiont assosiasies.

Rhipicephalus evertsi evertsi, *Hyalomma truncatum*, *Boophilus decoloratus* en *Boophilus microplus* is vir die bereiding van ru-eier ekstrakte gebruik. Die toksien isolasie prosedure sluit sentrifugasie, jel permeasie chromatografie en chromatofokussering in. Volgens iso-elektriese fokussering en SDS-gradiënt jel elektroforese was die geïsoleerde toksiene suiwer. Anti-protease aktiwiteite is op pankreas tripsien met N-bensoïel-D-L-arginien 4-nitroanilied-HCl as substraat en pankreas chimotripsien met N-suksiniel-L-fenielalinien-p-nitroanilied as substraat bestudeer. Die toksiene verskil ten opsigte van hulle aminosuur samestelling, iso-elektriese fokussering, molekulêre massa en protease inhibitor aktiwiteite ten opsigte van tripsien en chimotripsien. Die molekulêre massas van die toksiene is bepaal met SDS-gradiënt jel elektroforese en sedimentasie ewewig sentrifugasie en hulle iso-elektriese punte met analitiese iso-elektriese fokussering. Sommige karakteristieke van die toksiene is in Table S1 opgesom. Die relatief goed bestudeerde organismes, *Rickettsia prowazekii*, *R. typhi* en *R. conorii* is as toets organismes gebruik. Hierdie studies is later na *Cowdria ruminantium* en na organis-

TABEL S1 VERGELYKING VAN BOSLUISEIER TOKSIENE

BOSLUIS SPESIE	SPESIFIEKE AKTIWITEIT sa	MOLEKULêRE MASSA KDal	OPBRENGS[Y] mg	KI M	pI[f]
Amblyomma Herbraeum (18)	nb	$10^{uc,sds,aa}$	nb	$1,6x10^{-6nct}$	8
Rhipicephalus evertsi evertsi					
Stam: Boshoff		5^{uc}; 3^{aa}			6
Duncan		$<14^{sds}$			6
Warrenton		28^{aa}			6
Sweetwater	0,3	5^{uc}; $<14^{sds}$	3	$1,6x10^{-8nct}$	6
Hyalomma Truncatum					
Stam: SWA (SS)		30^{uc}; 26^{sds}			8,3
Kaalplaas (NSS)		25^{uc}; 24^{sds}			8,3
SWA X Kaalplaas (SS)	2,3	26^{uc}; 27^{sds}	2	$9,6x10^{-10sbt}$, $2,3x10^{-7ncc}$	8,3
Uitenhage (SS)	2,2	25^{sds}			8,3
Boophilus Microplus					
Stam: Onderstepoort	1,2	31^{uc}; 36^{sds}	2		9,1
Boophilus Decoloratus					
Stam: Ethel	1,4	$40^{sds,uc}$	2	$3,6x10^{-8ncc}$	9,2
Ornithodoros Savignyi (20)	nie toksies				

nb; nie bepaal, SS; virulente stam, NSS; avirulente stam, uc; aa en sds; bepaal deur sediment equilibrium sentrifugasie, aminosuur analise en SDS-gradiënt poliakrielamied jel eletroforese respektiewelik, Y; mg toksien/gram nat eiers, f; bepaal deur analitiese iso-elektriese fokussering, nct; nie-kompeterende tripsien inhibitor, sbt; onomkeerbare stadig bindende tripsien inhibitor, ncc; nie-kompeterende chimotripsien inhibitor, sa; MLD/mg toksien (dosis uitgedruk/kg Liggaamsmassa)

mes van sweetsiekte veroorsakende bosluise uitgebrei. Die motivering vir hierdie studie was om die moontlike toksiene wat deur *Cowdria ruminantium* geproduseer word te ondersoek en die moontlikheid dat organismes van sweetsiekte veroorsakende bosluise as die agente van die siekte optree.

Die isolasie van rickettsiale organismes behels hoë en lae spoed sentrifugasie sowel as Percoll dightheidsgradiënt sentrifugasie. Elektron mikroskoop studies van die organismes geïsoleer vanaf hartwater geïnfekteerde *Amblyomma hebraeum* bosluise toon morfologiese ooreenkomste met die wat in die choroïedpleksus van geïnfekteerde diere gevind word. Die rickettsiale organismes geïsoleer uit sweetsiekte positiewe en negatiewe *H. truncatum* bosluise en eiers toon dieselfde distribusie van organismes in Percoll digtheidsgradiënte.

Bogenoemde resultate toon, dat rickettsiale organismes van bosluise, in 'n suiwer, infektiewe toestand en in groot hoeveelhede geïsoleer kan word.

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