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AN INVESTIGATION INTO THE TOXIC PRINCIPLE IN EGGS OF THE TICK *AMBLYOMMA HEBRAEUM*

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ABSTRACT

NEITZ, A. W. H., PROZESKY, L., BEZUIDENHOUT, J. D., PUTTERILL, J. F. and POTGIETER, D. J. J. 1981. An investigation into the toxic principle in eggs of the tick *Amblyomma hebraeum*. *Onderstepoort Journal of Veterinary Research*, 48, 109–117 (1981).

A purification procedure involving iso-electric focusing by means of which a toxic principle may be obtained in a pure form from crude egg extracts of *Amblyomma hebraeum* is described. The molecular mass of the toxin is approximately 10 000 according to sedimentation equilibrium sedimentation, Sodium dodecyl sulphate (SDS) gradient gel electrophoresis and calculations from the amino acid composition. Non-competitive proteinase inhibitory activity was found to be associated with the toxin. Histopathological lesions, observed in guinea-pigs inoculated with crude egg extracts or the purified toxin, included the following: focal areas of necrosis in the liver, with mineralization and oedema of the mucosa of the urinary bladder, and vacuolation of the lining epithelium. The genesis of the lesions in the various organs appears to be vascular.

Résumé

UNE INVESTIGATION DE L'AGENT TOXIQUE DES OEUFS DE LA TIQUE AMBLYOMMA HEBRAEUM

*Un procédé de purification comprenant une concentration iso-électrique, au moyen duquel un agent toxique peut être obtenu dans sa forme pure à partir d'extraits d'oeufs crus d'*Amblyomma hebraeum, *est decrit. Le poids moléculaire de la toxine est à peu près* 10 000 *suivant l'équilibre de sédimentation, l'électrophorèse à gradient de dodecyle sulphate de sodium (SDS) gélosé et les calculs de la composition d'acide aminés. Une activité inhibitrice d'une protéinase non-compétitive fut trouvée être associée avec la toxine. Des lésions microscopiques suivantes furent observées chez les cobayes inoculés avec des extraits d'oeufs crus ou de toxines purifiées: Foyers de nécrose hépatique, calsification et oedeme de la muqueuse de la vessie et vacuolation de l'épithélium. Les lésions dans les organes différents apparaissent être d'origine vasculaire.*

INTRODUCTION

Interest in the toxins present in the eggs of ticks stems largely from their possible bearing on toxins associated with tick toxicoses (Regendanz & Reichenow, 1931). Furthermore, the characterization of tick toxins is a prerequisite for the investigation into the possible symbiotic or commensal prokaryotic origin of the toxins and tick-microbial symbiotic associations in general (Koch, 1960; Houk & Griffiths, 1980). In addition, a chemical analysis of the composition of tick eggs and the associated biological activity of the individual components may lead to a better insight into the biochemistry of oogenesis, embryogenesis (Schwemmler, 1974), tick metabolism and selective antimicrobial defence mechanisms of ticks and tick eggs (Garibaldi, 1960; Lackie, 1980; Board & Fuller, 1974).

Toxins have been shown to be present in whole body extracts (Murnaghan, 1958) and in salivary (Howell, Neitz & Potgieter, 1975) and possibly in coxal secretions (Patton & Evans, 1929) of ticks. Furthermore, they have been shown to be present in the eggs of 17 species of ixodid ticks (Riek, 1957). Because few attempts have been made to isolate and characterize the toxins, a comparison of their structures and of the symptoms they produce has hitherto been impossible.

The present study was designed to investigate systematically the structures and biochemical activities of toxins and other components present in the eggs of different tick species.

Amblyomma hebraeum was selected as the first tick species to be investigated, since these ticks are relatively easy to breed and their eggs can be collected in fairly large numbers.

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This paper describes a procedure by means of which a pure toxic component in the eggs of *A. hebraeum* may be obtained as well as some characteristics regarding its structure. Several proteinaceous components present in the eggs have been found to have proteinase inhibitory activity. This activity was also found to be associated with the toxin. The kinetic properties have been studied, the results of which are also described in this paper. In addition, the prominent symptoms and microscopic pathological changes resulting from the introduction of crude egg extracts as well as the purified toxin and other egg components into test animals are reported.

MATERIALS AND METHODS

Tick eggs and preparation of crude egg extracts

Fifty female *A. hebraeum* ticks were allowed an uninterrupted period of 20 days for oviposition at 28 °C in 70–80% relative humidity. The eggs (60,3 g) were collected on the 20th day and ground up in a Tri-R Tissue Homogeniser in the presence of 0,9% saline so as to give a concentration of 2 g of eggs in 10 mℓ saline. The egg suspension was prefiltered under 80 kPa pressure through an asbestos pad on a Seitz filter. A 47 mm Millipore filter with a 0,22 μm disc was used for the final filtration. The filtrate (crude egg extract) was bottled in 10 mℓ batches and frozen at −20 °C.

Determination of toxicity

For all the toxicity determinations guinea-pigs (450–500 g body mass) were injected subcutaneously between the scapulas with 1 mℓ of crude egg extract, or various fractions obtained during the toxin isolation procedure. Lyophilized fractions were made up to the original volume of the crude extract with 0,9% saline. The animals were kept under observation for at least 48 h and the symptoms recorded.

Effect of age of eggs on toxicity

In the initial investigations female ticks were glued on their backs in a Petri dish and after the start of oviposition all the eggs were removed at 5-day intervals and incubated at 28 °C for various periods (Table 1).

Crude extracts of each batch were collected and the toxicity determined.

Effect of temperature on toxicity

The cold stability of the toxin was determined by sealing 1 mℓ batches of the crude egg extract in glass ampoules and storing them at −196 °C, −80 °C and −20 °C respectively. The toxicity of each was determined after 7 days.

The heat stability was determined by heating 2 mℓ aliquots of the crude extract in a water-bath for 10 min. Temperatures ranged from 45 °C–70 °C, with 5 °C intervals, and then with 10 °C intervals up to the boiling point (97 °C).

Purification of the toxic principle

Initially, the first isolation step involved gel permeation chromatography of the crude egg extract. The extract (4 mℓ) was centrifuged at 2 000 *g* for 20 min. and applied to a 36×2,5 cm column containing Sephadex G100*. The eluent was 0,1 M sodium phosphate buffer, the pH 7,4, and the flow rate 30 mℓ/h. Fractions of 3 mℓ were collected and monitored at 280 nm with a Beckman Model M25 spectrophotometer.

In subsequent investigations it was found that dialysis for 24 h of the crude extract against 4 changes of 3 ℓ of distilled water resulted in the formation of a heavy precipitate which was devoid of toxic activity and a supernatant containing the toxicity. The precipitate and supernatant were separated by centrifugation at 14 830 *g* for 90 min.

The supernatant obtained by the method described above was subjected to preparative iso-electric focusing (IEF) in a glycerol gradient. A LKB column of 440 mℓ capacity** was employed. Preparation of the pH and glycerol gradients (Moreno, Ochoa, Gascon & Villanueva, 1975) was performed as described in the LKB instruction manual. A pH gradient from 3–10 pH units was prepared with Pharmalyte carrier ampholytes***. The final ampholyte concentration was 2% and the column temperature 8 °C. The sample containing 12 mg of nitrogen was mixed with the light gradient solution before the column was filled. The anode electrolyte solution was placed at the bottom of the column and the cathode electrolyte solution at the top. Electrofocusing was performed for 48 h with a starting potential of 300V (12 mA) and a final potential of 600V (3 mA). The column was emptied by means of a peristaltic pump at a flow rate of 2 mℓ/min. Fractions of 3 mℓ were collected and monitored at 280 nm. The pH of each fraction was determined with an expanded scale pH meter. Pooled fractions were dialyzed for 48 h against several changes of distilled water at 5 °C. After lyophilization of the dialyzed fractions, chromatography on Sephadex G25 with water as eluent was used to remove remaining glycerol and carrier ampholytes.

* Pharmacia, Uppsala, Sweden
** LKB Instruments, Sweden
*** Pharmacia Fine Chemicals, Uppsala, Sweden

Analytical iso-electric focusing (IEF)

Analytical IEF with polyacrylamide or agarose as supporting medium was performed with a Pharmacia Model FBE 3000 flat bed electrophoresis apparatus and a Pharmacia Model ECPS 2000/300 power supply. Acrylamide and N, N'-methylene bisacrylamide were purified by means of Amberlite MB-1 ion exchanger resin. Polymerization was achieved under nitrogen in the presence of sodium sulfite, glycerol, carrier ampholytes (Pharmalytes, pH range 3–10) and potassium persulfate on silane-treated glass plates. For agarose IEF (Saravis, O'Brien & Zamcheck, 1979) plates were prepared by casting agarose* onto hydrophilic polyester sheets.**

IEF was performed at a constant power of 30 watt. The fixing of separated bands was achieved with 5% trichloro-acetic acid and 5% sulfosalicylic acid and staining with 0,2% coomassie brilliant blue R 250 in 45% methanol and 10% acetic acid (Fazekas de St. Groth, Webster & Datyner, 1963).

Sodium dodecyl sulphate (SDS) gradient gel electrophoresis

The electrophoresis was performed in a Pharmacia GE-2/4 electrophoresis apparatus at a voltage of 150V. Polyacrylamide gradient gel slabs (4-30%) were prepared by means of a Pharmacia GM-1 gradient mixer and peristaltic pump (Margolis & Kenrick, 1967). Potassium persulfate and 3-dimethylaminopropionitrile were used as catalysts and 0,09M Tris, 0,08M borate, 3 mM EDTA and 0,2% SDS, pH 8,3 was used as buffer (Neville, 1971). Samples to be investigated were suspended in the electrophoresis buffer and completely dissociated by heating at 95 °C for 5 min in the presence of 1% SDS and 1,25% dithiothreitol (Fairbanks, Steck & Wallach, 1971). For the determination of the molecular mass (Weber & Osborn, 1969) of the separated proteins, the following marker proteins*** were used: phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lactalbumin. Protein bands were stained with 0,2% coomassie brilliant blue R 250 in 45% methanol and 10% acetic acid. De-staining was achieved with 7% acetic acid in a Pharmacia GD 4II de-stainer and DPS Power Supply at 24 V for 60 min.

Nitrogen determination

Nitrogen determinations were performed with a micro-Kjeldahl apparatus**** according to the method described by McKenzie & Wallace (1954).

Analytical ultracentrifugation

Conventional sedimentation equilibrium centrifugation was performed in a Spinco Model E centrifuge equipped with an ultraviolet photo-electric scanner. Samples with absorbancies of approximately 0,5 as measured in a 1 cm light path cell at 280 nm were dialyzed for 16 h against 0,05 M Tris, 0,1 M KCl buffer, pH 8. For the ultracentrifugal run 0,12 mℓ of the sample solution was introduced into a charcoal double sector scanner cell. The rotor (Type An-D) was spun at 26 000 rpm for 4 h at 20 °C after which it was decelerated to 18 000 rpm and spun for 22 h before calculations of molecular mass were made.

* Pharmacia Fine Chemicals, Uppsala, Sweden
** Marine Colloids Division, FMC Corporation, Rockland, Maine, USA
*** Calibration Kit, Pharmacia Fine Chemicals, Uppsala, Sweden
**** Labconco Corporation, Kansas City, USA

The absorbancy (A) was measured as a function of radial distance (r) across the fluid column of the cell. A plot of log A versus r^2 was made and the slope calculated. The slope d log A/dr^2 was substituted in the equation, $M=(2,303)(2\,RT)(d \log A)/(1-\bar{v}p)w^2dr^2$, (Bowen, 1970). The partial specific volume was calculated from the amino acid composition (Freifelder, 1976).

Amino acid determinations

Amino acids were determined with a Beckman Model 121M amino acid analyser according to a method described previously (Neitz, Howell, Potgieter & Bezuidenhout, 1978).

Measurement of proteinase inhibitory activity

The inhibitory activity was analysed on pancreatic trypsin* and N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) as substrate. The method described by Fritz, Trautschold & Werle (1974) was used with a 0,1 M sodium phosphate buffer, pH 7,4. The dissociation constant Ki of the enzyme-inhibitor complex was determined according to the methods described by Segel (1975).

Histopathological examination

Formalin-fixed tissues from 17 guinea-pigs, 4 of which served as controls, were used for histopathological examination. The tissues examined were obtained from guinea-pigs which had been injected subcutaneously with crude egg extracts and with various fractions obtained during the purification of the egg toxin from *A. hebraeum*. Tissue blocks were embedded in paraffin wax, sectioned at 3–5 μm thickness and stained with haematoxylin and eosin (HE). The special staining techniques employed were Schmorl's technique (Pearse, 1961) and Dahl's Alizarin method (Anon. 1968).

RESULTS

Clinical symptoms

The symptoms in the guinea-pigs after administration of the crude extract, in order of appearance, were as follows: hyperaesthesia and anorexia, serous nasal and eye discharge, accompanied by conjunctivitis and rhinitis; hyperaemia of the exposed skin (ears, footpads and lips) and at the site of injection; disinclination to move when touched owing to an apparent paresis of the hindquarters or hyperaesthesia; watery diarrhoea within 24–36 h, which became haemorrhagic if left for a longer period with occasional prolapse of the rectum; ascending paresis evident when the animals were put on their sides and made no attempt to get back onto their feet; gradual loss of voice, this becoming progressively worse from about 15 h–36 h, after which time total loss of voice occurred.

Effect of age of eggs on toxicity

The crude extracts prepared from the various batches (Table 1) all produced the clinical symptoms as described above.

Effect of temperature on toxicity

Clinically, no differentiation could be made between the crude extracts stored at −196 °C, −80 °C and −20 °C. In the temperature range between 45 °C and 70 °C all the test animals reacted positively and showed the typical clinical symptoms described above. Above 70 °C no positive reaction was recorded.

* Boehringer, Mannheim, W. Germany

TABLE 1 Method used for egg collection to determine effect of age on toxicity

Day	Days of ovi-position	Batch No.	Incubation period at 28 °C (days)	Actual age of eggs (days)
5	5	1	0	0–5
10	5	2	5	5–10
15	5	3	10	15–20
20	5	4	15	20–25
25	5	5	20	25–30
30	5	6	25	30–35

Purification of the toxic principle

The initial attempt to isolate the toxin from the crude extract on Sephadex G 100 resulted in a toxic fraction that eluted at a position corresponding to a molecular mass of between 10 000 and 20 000 (Fig. 1). The nitrogen content of the 3 peaks was as follows: peak 1, 5,8 mg; peak 2, 6 mg and peak 3, 0,6 mg. The total recovery of the peaks from the column was 96%. An analytical iso-electric focusing separation of the crude toxic extract as well as of the 3 peaks obtained from the Sephadex column suggested that preparative IEF of the crude extract should yield a toxic fraction well separated from the non-toxic components. Preparative IEF of the crude extract resulted in the overloading of the column by some of the non-toxic components, this causing disturbance of the gradients. It was thus necessary to remove these components prior to the preparative IEF by extensive dialysis and centrifugation. The supernatant was toxic and contained 40% of the nitrogen, while the precipitate had no toxicity. Subsequent isolation procedures were performed on the supernatant.

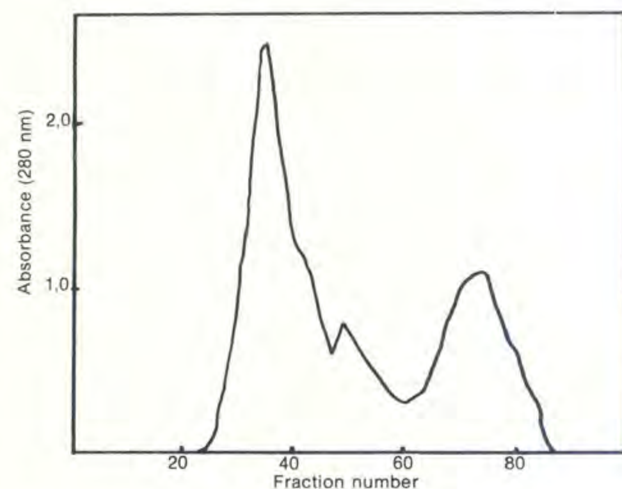


FIG. 1 Gel permeation chromatography of the crude egg extract of *A. hebraeum* on a Sephadex G100 column (36×2,5 cm). Eluent: 0,1 M sodium phosphate buffer, pH 7,4. Flow rate: 30 cm^3/h and fractions of 3 cm^3 collected

Several peaks were obtained after preparative IEF (Fig. 2). The fraction, focused between pH 7,5 and 8,5 (Fraction IEF 4), was found to be toxic. The clinical symptoms associated with the various fractions are summarized in Table 2. Analytical IEF patterns of fractions obtained by the isolation procedures are shown in Fig. 3.

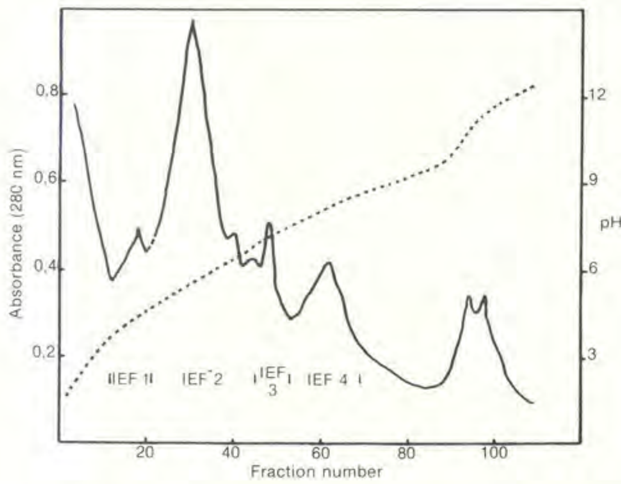


FIG 2 Iso-electric focusing of the supernatant obtained by dialysis and centrifugation of the crude egg extract. The dotted line represents the pH

FIG. 3 Separation of proteins present in the crude egg extract and Fractions IEF 1, IEF 2, IEF 3 and IEF 4 by analytical iso-electric focusing. D.S.—supernatant of dialyzed crude egg extract. (Sample before preparative IEF)

TABLE 2 Clinical symptoms in guinea-pigs after injection of fractions obtained from iso-electric focusing (IEF)

IEF Fraction	Symptoms
IEF 1............	No reaction during 24 h
IEF 2............	No reaction during 24 h
IEF 3............	Slight hypersensitivity after 24 h
IEF 4............	Symptoms as described for crude extract, prominent after 24 h

Homogeneity and molecular mass determination of Fraction IEF 4

The toxin was found to be homogeneous according to analytical IEF (Fig. 3). Sedimentation equilibrium centrifugation of the toxin showed a straight line relationship between log A and r^2 (Fig. 4). From the slope the molecular mass was calculated to be 10114.

SDS gradient gel electrophoresis showed a single band at a position corresponding to a molecular mass of 10 047 (Fig. 5). The minimum molecular mass calculated from the amino acid composition (see below) was 10 073.

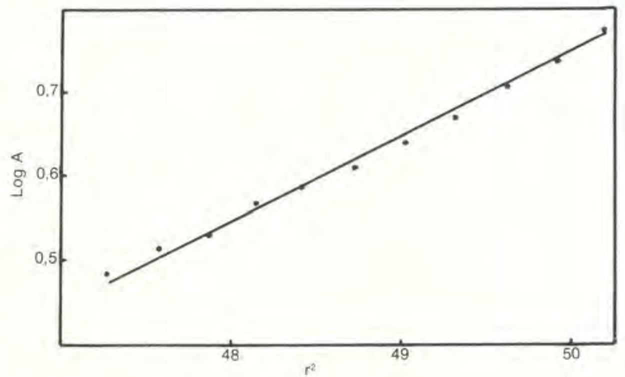


FIG. 4 Molecular mass determination of Fraction IEF 4 by sedimentation equilibrium centrifugation

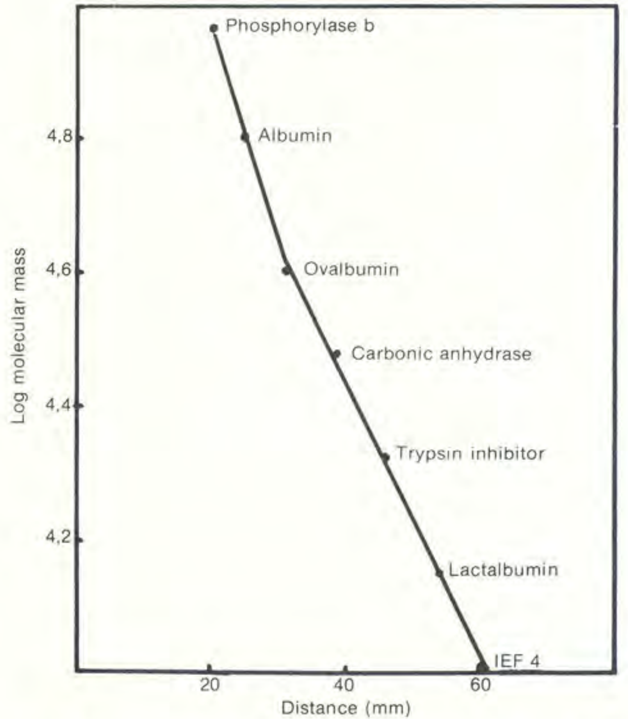


FIG. 5 Determination of the molecular mass of Fraction IEF 4 by SDS gradient gel electrophoresis

Amino acid composition of Fraction IEF 4

The amino acid composition expressed as residues per molecule is shown in Table 3. The amino acid composition of the toxin isolated from the salivary secretion of *Ornithodoros savignyi* is also shown for comparison (Neitz, 1976).

Ultraviolet absorption spectrum of Fraction IEF 4

The spectrum of Fraction IEF 4 is shown in Fig. 6. The spectrum of the toxin isolated from *O. savignyi* is also shown for comparison.

Antiproteinase activity of Fraction IEF 4

The inhibition curves of trypsin activity by Fraction IEF 4 at 2 substrate concentrations are shown in Fig.

7. Reciprocal plots at various Fraction IEF 4 concentrations (I) are shown in Fig. 8. The slope replot of data obtained from the reciprocal plot is presented in Fig. 9. The Dixon plot is shown in Fig. 10. From these data it is concluded that the inhibition is of the non-competitive type and that the Ki is $1,6 \times 10^{-6}$M.

TABLE 3 Amino acid composition of Fraction IEF 4 and the toxin isolated from the salivary secretion of *O. savignyi** (residues per molecule)

Amino acid	*A. hebraeum* IEF 4	*O. savignyi* salivary toxin
Lysine	4	4
Histidine	1	1
Arginine	5	1
Aspartic acid	7	5
Threonine	6	3
Serine	5	2
Glutamic acid	9	5
Proline	7	2
Glycine	9	5
Alanine	6	2
Valine	5	3
Isoleucine	8	1
Leucine	9	2
Tyrosine	5	2
Phenylalanine	4	1
Cysteine	3	2**
Methionine	1	1**
Tryptophan	nd	1***
Total	94	43

* (Neitz, 1976)

** Determined as cysteic acid and methionine sulfone respectively

*** Determined spectrophotometrically

nd not determined

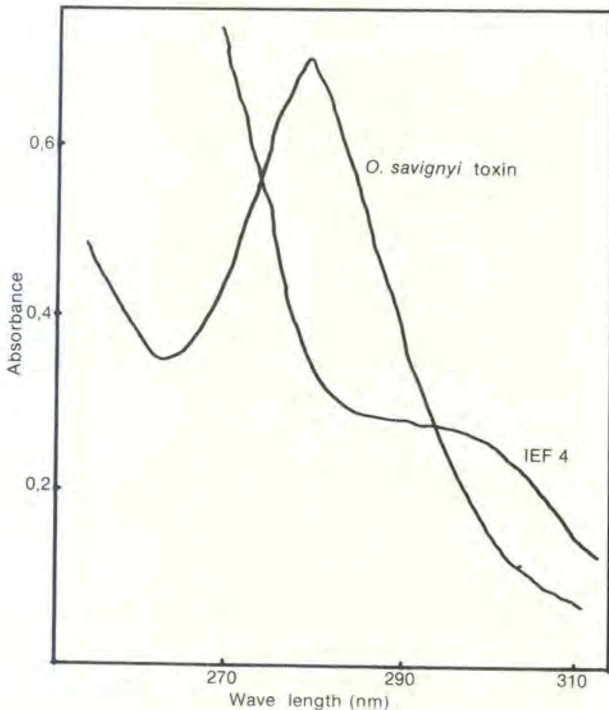


FIG. 6 Ultraviolet absorbance spectra of Fraction IEF 4 and the toxin isolated from the salivary secretion of *O. savignyi*

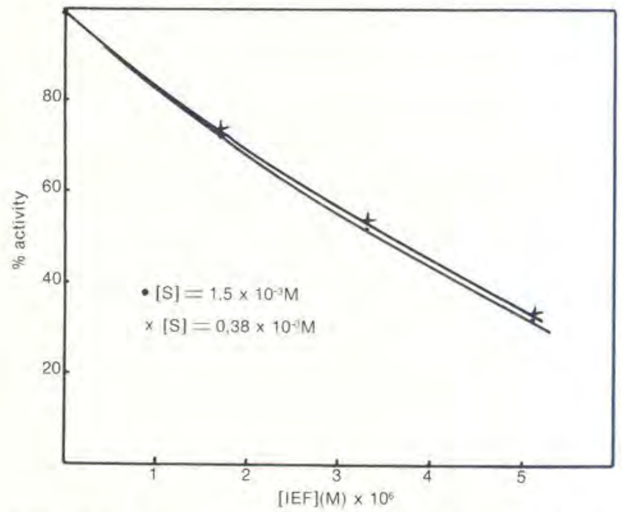


FIG. 7 Inhibition of trypsin by Fraction IEF 4 at 2 substrate concentrations (S). The trypsin concentration was 7×10^{-7}M

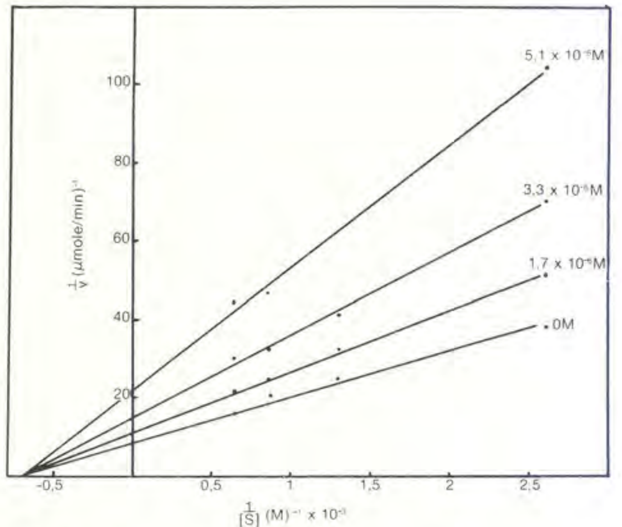


FIG. 8 Reciprocal plots (1/v *versus* 1/S) in the presence of different concentrations of Fraction IEF 4. The trypsin concentration was 7×10^{-7}M

Microscopic pathology

Liver

A prominent feature was the presence of large focal areas of coagulative necrosis comprising several lobules with a predilection for subcapsular tissue. In the subacute to chronic cases the lesions were characterized by a peripheral zone of mineralization which clearly demarcated viable and necrotic tissue (Fig. 11). In the HE sections, the cytoplasm of the necrotic cells, in which the outlines were still vaguely discernible, was either packed with small purplish-blue granules of varying size, had a purplish-blue homogeneous appearance, or a combination of both. The latter were regarded as indicative of mineralization and were positive with Dahl's Alizarin stain for calcium. The nuclei of the affected cells were indistinct or absent. During the acute phase the lesions were infiltrated by inflammatory cells, most of which were neutrophils. Subsequently, these were replaced by proliferating Kupffer cells, the cytoplasm of which contained phagocytozed material (Fig. 12).

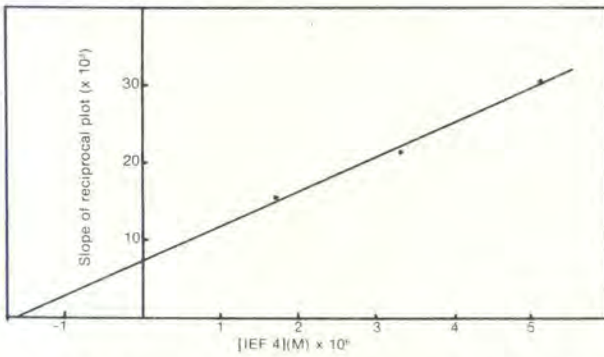


FIG. 9 Replots of the slopes taken from the reciprocal plots (Fig. 8) *versus* Fraction IEF 4 concentration

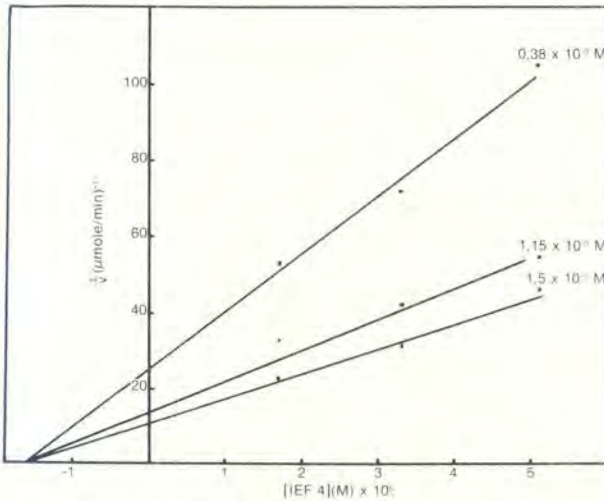


FIG. 10 Dixon plot for non-competitive inhibition: 1/v *versus* Fraction IEF 4 concentration in the presence of different substrate concentrations

Yellowish-brown pigment, which was positive with the Schmorl's reaction for lipofuscin, was observed in the cytoplasm of Kupffer cells and hepatocytes in the affected areas. Large spaces filled with red blood cells, fibrin and necrotic leucocytes were occasionally observed in the areas of necrosis. These areas of lytic necrosis were observed in only 3 animals. In the more chronic cases, bile duct proliferation, portal fibrosis and a slight infiltration of mono-nuclear cells and neutrophils were noted in all the portal tracts.

Throughout the liver, the walls of some portal vessels as well as those of several central veins appeared thickened. The affected vascular walls had a homogeneous eosinophilic appearance in the HE sections. The endothelial cells of these vessels were swollen and neutrophils could be seen adhering to these cells. In only one of the animals neutrophils were seen in the media and serosa of the vascular wall (Fig. 13). Dilatation of lymphatics, which were filled with eosinophilic fluid, was a prominent feature of the portal tracts confined to necrotic areas and was less conspicuous in others. There was also oedema of the interstitial tissue of the portal tracts within these necrotic areas.

Apart from the large necrotic areas described, small focal areas of necrosis (involving a few cells) with or without mineralization and/or neutrophil infiltration were a common feature. Single cell necrosis was present. These cells were recognizable in HE sections owing to their bright eosinophilic cytoplasm and pycnotic nuclei. Structures resembling Councilman bodies were observed in the cytoplasm of some of the hepatocytes. Some were also found lying free in the sinusoids or phagocytozed by Kupffer cells. Surviving parenchymal cells showed evidence of various forms of degeneration, including fatty degeneration, cloudy swelling, hydropic degeneration and, in one animal, hyalin droplet degeneration. Lipofuscin was observed in some of the cells showing degenerative changes.

Kidney

Lesions were confined mainly to the proximal and distal convoluted tubular epithelium. The epithelial cells were swollen with vacuolization of their cytoplasm. Desquamation of necrotic epithelial cells characterized by a homogeneous, bright pink, eosinophilic cytoplasm and pycnosis or karyorrhexis of nuclei resulted in the formation of cellular casts (Fig. 14 & 15). In severely affected cases some of the tubules were markedly dilated. Purplish-blue granules present in the vaguely discernible cytoplasm of necrotic cells stained positive for calcium with Dahl's Alizarin stain. Yellow-brown pigment seen in the cytoplasm of swollen and necrotic epithelial cells stained positive for lipofuscin with Schmorl's reaction. Leucostasis, which was observed as the accumulation of neutrophils in vascular lumens, was a constant feature. An interesting lesion observed in 6 of the animals was the presence of focal areas of tubular epithelial hyperplasia and hypertrophy in the kidney cortex. The affected cells had large vesicular nuclei with a bluish discoloration of their cytoplasm. Hypertrophic cells exhibited a high mitotic index. Occasionally these areas revealed necrosis and mineralization.

In several of the guinea-pigs, including 2 of the control animals, a chronic interstitial nephritis was observed. This was seen as the focal accumulation of round cells, most of which were plasma cells.

Urinary bladder

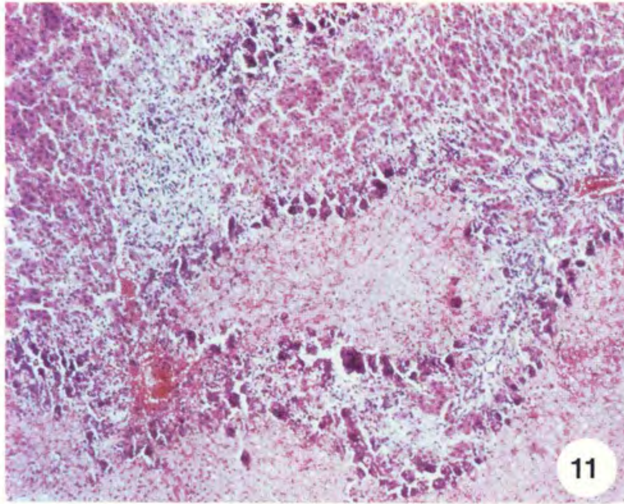
Oedema of the urinary bladder was a constant feature. This was seen as a thickening of the mucosa, dilatation of lymphatics filled with a pinkish fluid, infiltration of oedematous fluid into the interstitial tissue and congestion of blood vessels with haemorrhages. An infiltrate, composed mainly of neutrophils, was present in the mucosa. Lesions were seldom observed in the epithelium. When present, they were seen as a swelling of the epithelial cells with vacuolation of their cytoplasm (Fig. 16). There were focal areas of desquamated necrotic cells on the surface of the epithelium. In a few animals there was an increased mitotic index of the epithelium.

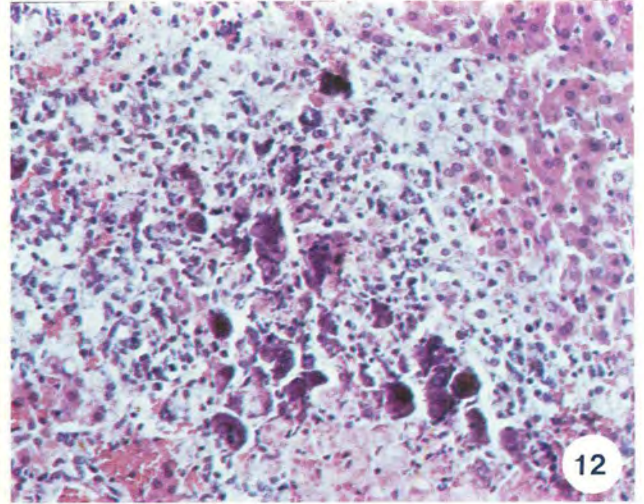
Digestive tract

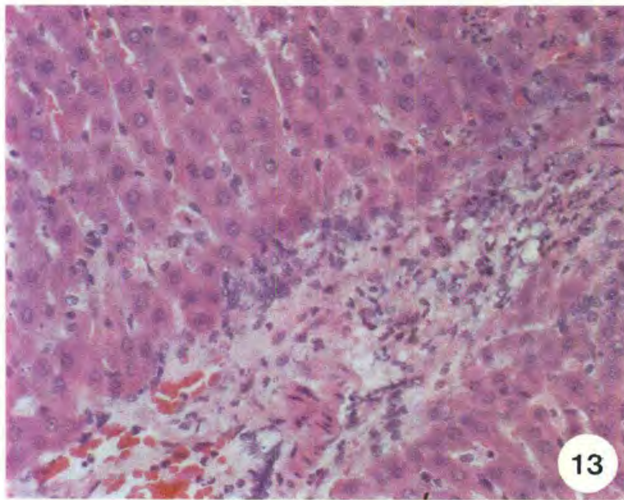
Lesions were mainly confined to the small intestine, although mild lesions were present in the large intestine. Pycnosis and karyorrhexis of mononuclear cells in the stroma of the distal villi with occasional necrosis of the epithelial cells were the most constant lesions observed. Lesions, observed in some but not in all cases, included dilatation of the central lacteal filled with eosinophilic fluid, infiltration of neutrophils into the mucosa and mucosal oedema.

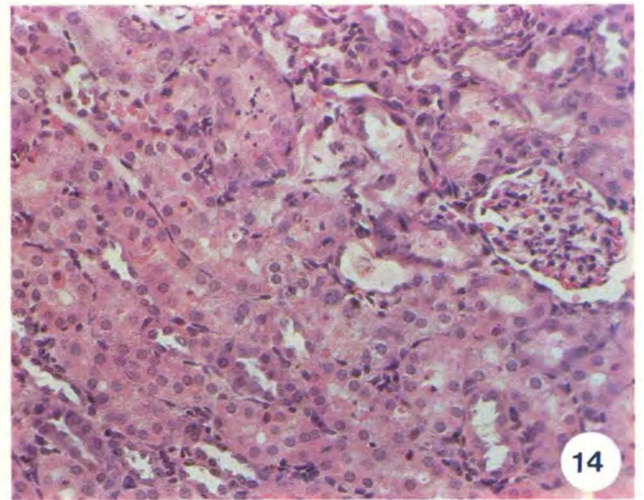
Spleen

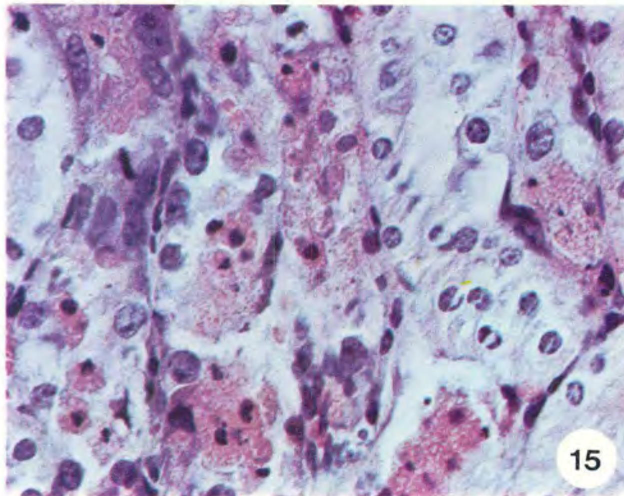
The accumulation of neutrophils in the red pulp was the only significant lesion observed.











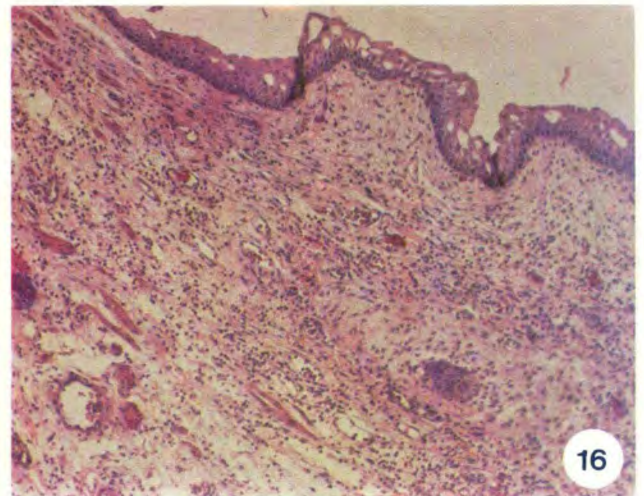


FIG. 11 A large focal area of coagulative necrosis in the liver; HE × 200
FIG. 12 Proliferating Kupffer cells in an area of necrosis; HE × 500
FIG. 13 Vasculitis of a hepatic vessel. Note the accumulation of neutrophils in the vascular wall; HE × 200
FIG. 14 Desquamation of necrotic epithelial cells resulting in the formation of cellular casts in the kidney; HE × 200
FIG. 15 A higher magnification of the lesions observed in Fig. 14; HE × 500
FIG. 16 Thickening of the urinary bladder mucosa with the infiltration of mainly neutrophils. Note the vacuolation of the epithelial cells; HE × 200

TABLE 4 Histopathological lesions observed in guinea-pigs inoculated with crude egg extract and various fractions of eggs from *Amblyomma hebraeum*

Guinea-pig No.	Fraction inoculated	Liver	Urinary bladder	Kidney	Small intestine	Skin	Lungs	Post inoculation day guinea-pigs autopsied
1	Crude egg extract	***	*	**	†	‡	*	3 days
2	Crude egg extract	**	**	**	‡	‡	*	3 days
3	Crude egg extract	**	***	**	**	‡	*	3 days[a]
4	Crude egg extract	**	**	**	*	‡	*	2 days
5	Crude egg extract	***	*	*	*	‡	*	3 days
6	Crude egg extract	*	**	*	‡	‡	*	3 days
7	Crude egg extract	**	*	†	*	‡	*	3 days
8	IEF 1	*	‡	*	*	‡	*	4 days
9	IEF 2	*	†	*	*	‡	*	4 days
10	IEF 3	*	*	†	†	‡	†	4 days
11	IEF 4	*	*	**	*	‡	*	4 days
12	IEF 4	***	**	**	*	**	***	4 days[a]
13	IEF 4	***	**	**	**	**	*	4 days

† = No lesions; * = Mild lesions; ** = Moderate lesions; *** = Pronounced lesions; ‡ = not examined; [a] = Guinea-pig died naturally

Lungs

Mild to moderate lung oedema, congestion, haemorrhages and leucostasis were present. In one of the animals, which died naturally, numerous fibrin thrombi were present in the lung capillaries, arterioles and venules.

Brain

Mild lesions consisting of congestion and petechial haemorrhages were observed in the meninges and brain substance.

Skin

Lesions present at the site of injection included the following: (i) congestion and oedema, (ii) steatitis characterized by focal groups of necrotic adipose cells, with pycnotic nuclei and the infiltration of macrophages and fibroblasts, (iii) fragmentation of collagen bundles, (iv) necrosis and degeneration of the underlying striated muscle, which appeared as swollen eosinophilic homogeneous fibres, some of which showed vacuolation of their cytoplasm. The necrotic muscle fibres were occasionally infiltrated by macrophages and some showed mineralization, (v) a few blood vessels showed fibrinoid changes with swelling of endothelial cells. Sections of skin distant from the site of injection were also examined. These revealed the above lesions but they were not so pronounced. There was no muscular tissue in these latter sections, thus it is not known whether necrosis was present.

Heart

Apart from congestion no lesions were observed.

The extent of the histopathological lesions observed in various organs is shown in Table 4.

DISCUSSION

The toxin isolation procedure described in this paper involves 2 main steps. One involves extensive dialysis against distilled water during which more than half the nitrogen content is removed, mainly by the precipitation of proteins at low ionic strength. The precipitated proteins have not been investigated in detail but preliminary work has shown that they contain the lipovittelins (Boctor & Kamel, 1976) according to their pyridine haemochromogen spectrum (Theorell & Åkeson, 1953; O'Hagan, 1974). In addition, a tight binding antiproteinase, several non-competitive trypsin inhibitors and proteinase and acetylcholinesterase activities have been found to be present in this fraction (to be published).

The second main isolation step involves preparative iso-electric focusing of the supernatant. The peak associated with the toxin showed tailing which is to be expected of a compound having a relatively low molecular mass. It is thus impossible to state the exact iso-electric point of the toxin. Homogeneity determinations by sensitive methods, including sedimentation equilibrium centrifugation, SDS gradient gel electrophoresis and analytical iso-electric focusing, all indicate that the isolated fraction is homogeneous with respect to molecular mass and charge.

The monitoring system used for determining toxicity is insensitive, because the clinical symptoms abate sharply upon dilution of the toxin. It is therefore impossible to report the yield, specific activity and enrichment of the toxin after each isolation step. The same problem is evident from the results reported by Riek (1957), who used mortality as an indication of toxicity for the crude egg extracts of various species of ixodid ticks.

The antiproteinase activity of Fraction IEF 4 was demonstrated with pancreatic trypsin. Most likely the activity is not specific for trypsin but for a wide variety of proteinases, the inhibition of which could cause a cascade of deleterious reactions in the host animal. Furthermore, the antiproteinase activity may not be the sole activity associated with the toxin. This possibility should be further explored.

Proteinase inhibitors have been found in the venom of certain snakes and insects (Strydom, 1973; Shkenderov, 1976) and in the salivary secretion of *Ornithodorus savignyi* (Neitz, 1976), where they apparently protect proteinaceous toxins from degradation by the victim's proteinases (Ryan, 1979).

The presence of antiproteinases in tick eggs is not surprising, since they have been found in the eggs of taxonomically distant avian species (Liu, Means & Feeney, 1971). The inhibition of microbial proteases may be the natural function of these ovoinhibitors and that this prevents the multiplication of some species of invading organisms (Board & Fuller, 1974). Inhibition

of animal proteinases is most probably only incidental, since they are similar in structure and function to some microbial proteinases (Davis, Zahnley & Donovan, 1969). Ovoinhibitors could well be partially responsible for specific symbiotic associations in tick eggs and act as a primitive humoral defence agent which does not depend on specific recognition of antigens (Lackie, 1980).

In the present study the histopathological lesions observed in guinea-pigs inoculated with crude egg extracts from various tick species, including *Amblyomma hebraeum, Boophilus decoloratus, Boophilus microplus* and *Rhipicephalus evertsi*, were comparable (unpublished data) and suggest that the toxins present in the eggs of these ticks are similar. When fractions derived from the crude egg extracts of *Amblyomma hebraeum* were injected into guinea-pigs, 2 out of the 3 animals inoculated with Fraction IEF 4 showed pronounced histopathological lesions (Table 4). This indicates that Fraction IEF 4 is the active toxic principle. The present study has shown that, in addition to other inhibitors and enzymes in the crude extract, non-competitive antiproteinase activity is associated with Fraction IEF 4. The significance of these observations with respect to the clinical symptoms and histopathological findings is not known at present.

Very little information is available about the histopathological lesions observed in laboratory animals inoculated with tick egg extracts. Riek (1957) mentions 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. No lesions were seen by De Meillon (1942) in guinea-pigs injected with egg extracts from *Rhipicephalus evertsi*.

Interesting features noticed in the present study include the adhesion of neutrophils to swollen endothelial cells in various organs and fibrinoid necrosis of hepatic vessels. Since a narrow rim of necrotic hepatocytes was noted around some of these vessels, the lesions described in the present study in the various organs appear to be of vascular origin. Lesions observed in the kidneys are more likely to be due to the direct effect of the toxin on the lining epithelium, as no prominent vascular lesions were observed.

In 1965 Reynolds reported the mineralization of hepatocytes in rats 16–22 h after being poisoned with carbon tetrachloride. Mineralization of hepatocytes was observed in lambs infected with Rift Valley fever by Coetzer (1977), and in an aborted bovine foetus due to infectious bovine rhinotracheitis by Kennedy & Richards (1964). According to Reynolds (1965), calcium appears to be primarily responsible for the mitochondrial lesions in carbon tetrachloride poisoning, although its entrance into the cell was due to altered permeability of the membrane. In the present study it is suggested that increased membrane permeability, associated with anoxia due to the vascular lesions and/or the direct deleterious effect of the toxic principle on the hepatocytes, resulted in acute necrosis with consequent rapid pH changes. The latter in turn resulted in a dystrophic type of mineralization.

It is tempting at this stage to make a comparison between the purified egg toxin from *A. hebraeum* and other tick toxins in respect of structure and biological activity. Few attempts, however, have been made regarding their isolation and characterization to an extent which makes comparisons feasible (De Meillon, 1942; Riek, 1957; Kaire, 1966; Neitz, 1976; Stone, Doube, Binnington & Goodger, 1979). Some comparisons are nevertheless possible. They all appear to be protein-like components and in many instances, where enough data are available, they show differences in respect of molecular mass, iso-electric point and amino acid composition.

Regendanz & Reichenow (1931), investigating various organs and different life stages of *R. sanguineus* to trace the origin and stage of paralysis toxin production, showed that salt extracts prepared from eggs are toxic to dogs and cause symptoms characteristic of tick paralysis. Furthermore, they showed that the imagines before engorgement and larvae possess very low concentrations of the toxin. They established that the toxin was produced during egg development and was present in the ovaries after completion of engorgement. The salivary glands, cephalic gland (ovipositing gland) and Malpighian tubules had no toxin, whereas the laid eggs contained high concentrations.

An important finding of Regendanz & Reichenow (1931) is that extracts made from whole female ticks after completion of oviposition contain very little toxin. This indicates that the toxin produced is virtually quantitatively incorporated into the eggs and, according to other findings, detoxified by developing larvae. The latter deduction is in agreement with the findings of Riek (1959). The authors hypothesized that in ticks producing tick paralysis the toxin is concentrated not only in the eggs but also in the salivary glands. The toxin, thus introduced by means of the salivary secretion into the host, produces paralysis, whereas in ticks that do not cause paralysis during engorgement, the toxin is concentrated in the eggs only.

It is of interest to mention an investigation into the origins of the toxins produced by other Arachnida. Buffkin, Russel & Deshmukh (1971), quote early papers by Kobert (1888; 1889) in which Kobert (1888; 1889) states that the toxins of *Latrodectus tredecimguttatus* are distributed throughout the body, including the legs and non-ripe eggs, and that the venom is only secreted but not formed in the venom gland. Kobert (1888; 1889) concluded that the poison was an albumin or toxic enzyme which is secreted by the genital organs of the female and is thus incorporated into the eggs, the spiderlings and adult spider. Buffkin *et al.*, (1971) investigated the relationship between toxins from black widow spider eggs and the spider's venom. They concluded from polyacrylamide gel electrophoretic separation data and the symptoms produced that the toxins differed markedly from one another.

The purification procedure described in this paper offers a means by which a toxic component from the eggs of *A. hebraeum* may be obtained in a pure form. Extensive structural, functional and comparative studies of egg toxins now become possible. Such studies would be of interest for their investigation into the origins of these toxins, their functions on a molecular level during the life cycle of ticks (Führer, 1980), their possible bearing on symbiotic or commensal prokaryotic associations (Martin, 1979), the transovarial passage of disease agents (Philip, 1963), invertebrate humoral immunity mechanisms (Lackie, 1980) and entomopathogens (Brader, 1980).

REFERENCES

ANON., 1968. Armed Forces Institute of Pathology, Washington. Manual of histologic staining methods. 3rd ed. New York, Toronto, London, Sydney: McGraw Hill.

BOARD, R. G. & FULLER, R., 1974. Non-specific antimicrobial defences of the avian egg, embryo and neonate. *Biological Reviews*, 49, 15–49.

BOCTOR, F. N. & KAMEL, M. Y., 1976. Purification and characterization of two lipovitellins from eggs of the tick, *Dermacentor andersoni*. *Insect Biochemistry*, 6, 233–240.

BOWEN, T. J., 1970. An introduction to ultracentrifugation. London: Wiley-Interscience.

BRADER, L., 1980. Advances in applied entomology. *Annals of Applied Biology*, 94, 349–365.

BUFFKIN, D. C., RUSSEL, F. E. & DESHMUKH, A., 1971. Preliminary studies on the toxicity of black widow spider eggs. *Toxicon*, 9, 393–402.

COETZER, J. A. W., 1977. The pathology of Rift Valley fever. Lesions occurring in natural cases in new-born lambs. *Onderstepoort Journal of Veterinary Research*, 44, 4, 205–212.

DAVIS, J. G., ZAHNLEY, J. C. & DONOVAN, J. W., 1969. Separation and characterization of the ovoinhibitors from chicken egg white. *Biochemistry*, 8, 2044–2053.

DE MEILLON, B., 1942. A toxin from the eggs of South African ticks. *South African Journal of Medical Science*, 7, 226–235.

FAIRBANKS, G., STECK, T. L. & WALLACH, D. F. H., 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*, 10, 2606–2617.

FAZEKAS DE ST. GROTH, S., WEBSTER, R. G. & DATYNER, A., 1963. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochemica Biophysica Acta*, 71, 377–391.

FREIFELDER, D., 1976. Physical biochemistry. San Francisco: W. H. Freeman and Company.

FRITZ, H., TRAUTSCHOLD, I. & WERLE, E., 1974. Protease inhibitors. *In:* BERGMEYER, H-U (ed.). Methods of enzymatic analysis, 2, 1064–1080. Weinheim: Verlag Chemie.

FÜHRER, E., 1980. Physiologische Wechselbeziehungen zwischen *Pieris brassicae* und dem endoparasiten *Apanteles glomeratus*. II. Der einfluss der parasitierung auf die Ernährung des Wirtis. *Entomologia Experimentalis et Applicata*, 27, 179–187.

GARIBALDI, J. A., 1960. Factors in egg white which control growth of bacteria. *Food Research*, 25, 337–344.

HOUK, E. J. & GRIFFITHS, G. W., 1980. Intracellular symbiotes of the Homoptera. *Annual Review of Entomology*, 25, 161–187.

HOWELL, C. J., NEITZ, A. W. H. & POTGIETER, D. J. J., 1975. Some toxic, physical and chemical properties of the oral secretion of the sand tampan, *Ornithodoros savignyi* (1827). *Onderstepoort Journal of Veterinary Research*, 42, 99–102.

KAIRE, G. H., 1966. Isolation of tick paralysis toxin from *Ixodes holocyclus*. *Toxicon*, 4, 91–97.

KENNEDY, P. C. & RICHARDS, W. P. C., 1964. The pathology of abortion caused by the virus of infectious bovine rhinotracheitis. *Pathologia Veterinaria*, 1, 7–17.

KOBERT, R., 1888. Ueber Spinnengift. *Zeitschrift für Naturforschung*, 61, 441.

KOBERT, R., 1889. Lehrbuch der Anatomie der Spinnesorgane. *Biologisches Zentralblatt*, 8, 287.

KOCH, A., 1960. Intracellular symbiosis in insects. *Annual Review of Microbiology*, 14, 121–140.

LACKIE, A. M., 1980. Invertebrate immunity. *Parasitology*, 80, 393–412.

LIU, W. H., MEANS, G. E. & FEENEY, R. E., 1971. The inhibitory properties of avian ovoinhibitors against proteolytic enzymes. *Biochimica et Biophysica Acta*, 22, 176–185.

MARGOLIS, J. & KENRICK, K. G., 1967. Polyacrylamide gel-electrophoresis across a molecular sieve gradient. *Nature* (London), 214, 1334–1336.

MARTIN, M. M., 1979. Biochemical implications of insect mycophagy. *Biological Reviews*, 54, 1–21.

McKENZIE, H. A. & WALLACE, H. S., 1954. The kjeldahl determination of nitrogen: a critical study of digestion conditions, temperature, catalyst, and oxidizing agent. *Australian Journal of Chemistry*, 7, 55–70.

MORENO, F., OCHOA, A. G., GASCON, S. & VILLANUEVA, J. R., 1975. Molecular forms of yeast invertase. *European Journal of Biochemistry*, 50, 571–579.

MURNAGHAN, M. F., 1958. Neuroanatomical site in tick paralysis. *Nature* (London), 181, 131.

NEITZ, A. W. H., 1976. Biochemical investigation into the toxic salivary secretion of the tick, *Ornithodoros savignyi* Audouin (1827). D.Sc. (Agric.) Thesis, University of Pretoria.

NEITZ, A. W. H., HOWELL, C. J., POTGIETER, D. J. J. & BEZUIDENHOUT, J. D., 1978. Proteins and free amino acids in the salivary secretion and haemolymph of the tick *Amblyomma hebraeum*. *Onderstepoort Journal of Veterinary Research*, 45, 235–240.

NEVILLE, D. M., 1971. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous Buffer System. *Journal of Biological Chemistry*, 246, 6328–6334.

O'HAGAN, J. E., 1974. Boophilus microplus: digestion of hemoglobins by the engorged female tick. *Experimental Parasitology*, 35, 110–118.

PATTON, W. S. & EVANS, A. M., 1929. Insects, ticks, mites and venomous animals Croydon: H. R. Grubb.

PEARSE, A. G. E., 1961. Histochemistry theoretical and applied. 2nd ed. London: J. & A. Churchill.

PHILIP, C. B., 1963. Ticks as purveyors of animal ailments: A review of pertinent data and of recent contributions. *In:* NAEGELE, J. A. (ed.). *Advances in Acarology*, 1, 285–325.

REGENDANZ, P. & REICHENOW, E., 1931. Über zeckengift und Zeckenparalyse. *Archiv für Schiffs- und Tropen-Hygiene*, 35, 255–273.

REYNOLDS, E., 1965. Liver parenchymal injury. III. The nature of calcium-associated electron-opaque masses in rat liver mitochondria following poisoning with carbon tetrachloride. *Journal of Cell Biology*, 25, 53–75.

RIEK, R. F., 1957. Studies on the reactions of animals to infestation with ticks. *Australian Journal of Agricultural Research*, 8, 215–223.

RIEK, R. F., 1959. Studies on the reactions of animals to infestation with ticks. *Australian Journal of Agricultural Research*, 10, 604–613.

RYAN, C. A., 1979. Proteinase inhibitors. *In:* ROSENTHAL, G. A. & JANZEN, D. H. (eds). Herbivores. Their interaction with secondary plant metabolites, 599–618. London: Academic Press.

SARAVIS, C. A., O'BRIEN, M. & ZAMCHECK, N., 1979. Direct tissue iso-electric focusing in agarose. *Journal of Immunological Methods*, 29, 97–100.

SCHWEMMLER, W., 1974. Endosymbionts: Factors of egg pattern formation. *Journal of Insect Physiology*, 20, 1467–1474.

SEGEL, I. H., 1975. Enzyme kinetics. London: John Wiley & Sons.

SHKENDEROV, S., 1976. Further purification, inhibitory spectrum and some kinetic properties of the protease inhibitor in bee venom. *In:* OHSAKA, A., HAYASHI, K. & SAWAI, Y. (eds.). Animal, plant and microbial Toxins, 1, 263–272. London: Plenum Press.

STONE, B. F., DOUBE, B. M., BINNINGTON, K. C. & GOODGER, B. V., 1979. Toxins of the Australian paralysis tick (*Ixodes holocyclus*). *In:* RODRIQUES, J. C. (ed.). Recent advances in acarology, 1, 347–356. Academic Press.

STRYDOM, D. J., 1973. Protease inhibitors as snake venom toxins. *Nature New Biology*, 243, 88–89.

THEORELL, K. G. P. H. & ÅKESON, Å., 1953. The molar light absorption of pyridine ferroprotoporphyrin (pyridine haemochromogen). *Acta Chemica Scandinavica*, 7, 1284–1287.

WEBER, K. & OSBORN, M., 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry*, 244, 4406–4412.