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

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ABSTRACT

VILJOEN, G. J., VAN WYNGAARDT, S., GOTHE, R., VISSER, L., BEZUIDENHOUT, J. D. & NEITZ, A. W. H., 1990. The detection and isolation of a paralysis toxin present in *Argas (Persicargas) walkerae*. *Onderstepoort Journal of Veterinary Research*, 57, 163–168 (1990).

One-day-old leghorn chickens were used in a laboratory assay to determine the toxicity of crude extracts of the tick *Argas (Persicargas) walkerae* and of fractions obtained during the isolation procedure. Extracts of unfed and engorged larvae, nymphae and females were tested using this *in vivo* test system. Only extracts of replete *A. (P.) walkerae* larvae produced paralysis.

A toxic fraction was isolated from replete larval extracts by gel-permeation and ion-exchange chromatography. This fraction with a pI of 4,5, showed 2 major bands corresponding to a M_r of 32 kDa and 60 kDa after SDS-polyacrylamide gel electrophoresis.

INTRODUCTION

Argas (Persicargas) walkerae causes paralysis in poultry during infestation. This tick, also called Walker's fowl tampan, is the commonest fowl tick in the Republic of South Africa (Howell, Walker & Nevill, 1978). It has been recorded in the Transvaal (Gothe & Schrecke, 1972; Gothe & Koop, 1974), the Cape Province at Fort Beaufort and Queenstown, Lesotho and Namibia (Kaiser & Hoogstraal, 1969), in Zimbabwe (Norval, Short & Chrisholm, 1985) and in Zambia (Colbo, 1973).

Larvae of *A. (P.) arboreus* (Gothe & Verhalen, 1975) and of *A. (P.) radiatus* (Gothe & Englert, 1978) have a greater paralysis-inducing capacity than those of *A. (P.) walkerae*, whereas the larval toxicity of *A. (P.) persicus* (Gothe & Verhalen, 1975; Gothe & Englert, 1978; Gothe, Buchheim & Schrecke, 1981), *A. (P.) sanchezi* (Gothe & Englert, 1978) and *A. (A.) africolumbae* is less pronounced.

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

From the earlier literature, conflicting opinions existed as to which stage or stages of the tick are capable of causing paralysis during feeding. According to Lounsbury (1904), nymphae as well as adult ticks are capable of causing paralysis. Emmel (1945) and Neitz (1962) were of the opinion that all postembryonal stages may cause this toxicosis, whereas Coles (1959), quoted by Stampa, S. 1959, reported that paralysis is caused by the simultaneous feeding of both larvae and adults. The confusion was eventually unravelled through intensive studies by Gothe *et al.* (1970). They unambiguously determined that only larvae caused paralysis after at least 4 days of feeding. Of interest was the finding that the larval body mass increased abruptly from approximately the 4th feeding day. It was concluded that the onset of symptoms may be correlated with increased rate of feeding. The interval between infestation and the onset of paralysis (at least 4 days) was found to be independent of the age of the host and of the number of ticks infesting it. The degree of paralysis, however, was dependent on these factors.

The inability of post larval ticks to cause paralysis during infestation is well estabished (Gothe *et al.*, 1970). It is not known, however, if this is due to the absence of toxin production or to an inability of these ticks to transfer the toxin to the host during their short feeding time. Larval ticks of this species imbibe blood during several days, whereas all the other stages are fully engorged within 1–2 h (Howell *et al.*, 1978).

To help solve this enigma, the paralysis-inducing capability of all post-larval stages of *A. (P.) walkerae* was investigated in the present study by inoculation of crude extracts into chickens. In addition, an attempt was made to isolate the implicated toxin from replete whole tick extracts.

MATERIALS AND METHODS

All glassware and equipment were sterilized with 70 % (v/v) ethanol and buffers by filtration through 0,22 µm filters (Millipore).

Origin and rearing of ticks

Laboratory colonies of *A. (P.) walkerae*, (Pretoria strain) were maintained and reared in an incubator at 27 °C and ≥ 80 % relative humidity. Three weeks after the hatching of eggs, the batch of larvae from 1 female was allowed to attach freely under the wings of white Leghorn chicken (approximately 7-week-old) as desribed by Alt (1971). The replete larvae were collected on the morning of the 5th, 6th, 7th and 8th day after attachment. To obtain fed nymphae, the larvae were allowed to moult, and the unfed nymphae were fed overnight on 7-month-old white leghorn hens, using approximately 100 ticks per animal. On the morning following the attachment, replete nymphae were collected. The same procedure was followed for feeding the subsequent stages.

Preparation of crude extracts from eggs, larvae, nymphae and adults for the detection of the paralysis toxin

A. (P.) walkerae eggs and fed as well as unfed larvae, nymphae and adult ticks were homogenized

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in distilled water with an Ultra Turrax[1] at low speed. Eggs and larvae were homogenized for 2 min and the other stages for 5 min at 4 °C. The homogenates were then sonified for 15 s with a Branson Model B-30 sonifier with the output set at 5 (continuous cycles). The sonified homogenates were centrifuged in a Beckman Model B microfuge for 10 min at 8 000 ×g and 4 °C. The resulting supernatants were freeze-dried and suspended in 0,154 M NaCl before use. Aliquots were injected sc in the neck region of one-day-old white Leghorn chickens (Table 1). At least 3 animals were used per assay and were kept under observation for at least 4 days. The degree of paralysis was evaluated according to the description provided by Gothe & Englert (1978) (See Table 2).

Preparation of crude larval extracts

Preliminary studies showed no differences in toxicity when extracts were prepared in the presence of either distilled water or 0,154 M NaCl.

For the preparation of a crude extract, 100 replete larvae were homogenized in 1 mℓ solvent. The homogenates were sonified and centrifuged for 10 min at 8 000 ×g and 4 °C. The supernatants were made up to 12 mℓ with solvent and centrifuged for 5 h at 80 700 ×g at 5 °C. The supernatants were freeze-dried. Salt containing samples were made up to 2,5 mℓ with distilled water, desalted on a Sephadex PD-10 column, and freeze-dried.

Gel-permeation chromatography

Sephadex G-100 gel was packed into a K26/40 column. A 2 mℓ crude larval extract, of 200 larvae in 3 mℓ of 0,154 M NaCl, was applied to the column and separated, using 0,154 M NaCl (pH 7,0) or H_2O (pH 7,0) as eluant by upward-flow elution at a flowrate of 30 mℓ/h. Pooled peak fractions were freeze-dried and stored at − 30 °C.

Pre-swollen Sephacryl S-200 was packed into a K26/40 column and calibrated with standard proteins. The crude larval extracts, obtained from 200 larvae in 2 mℓ of appropriate eluent, were separated by upward-flow elution with either distilled water, 0,154 M NaCl; 0,154 M NaCl, 0,05 % Tween 20; 0,154 M NaCl, 0,1 % SDS or 0,154 M NaCl, 0,1 % 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps). The detergent-containing eluents was included in an attempt to dissociate haemoglobin-toxin complexes. Fractions of 3,5 mℓ were collected and absorbancies read at 280 nm. When necessary, the fractions were desalted through a Sephadex PD-10 column and freeze-dried. For large scale isolation of toxin, a batch method was employed with 500 mℓ Sephacryl S-200 in water at pH 3,8. The crude larval extract, representing 1 600 larvae, was allowed to adsorb to the gel matrix for 1,5 h under mild stirring conditions at room temperature. The supernatant was separated from the gel by means of filtration through Whatman No. 1 filter paper. The supernatant was then freeze-dried.

Ion-exchange chromatography

The lyophilyzed filtrate obtained from the batch method was subjected to chromatography using DEAE-Sephacel. The gel in the chloride form was equilibrated with starting buffer, 0,05 M Phosphate, pH 6,7 and packed into a C16/20 column to obtain a 32 mℓ bed volume. The flow rate was 6 mℓ/h, and 1 mℓ fractions were collected. After 30 mℓ of buffer had passed through the column, the starting buffer was replaced by the 2nd buffer, 0,05 M sodium acetate, pH 4,0, and a further 90 mℓ fractions were collected. Fractions were monitored at 280 nm.

Chromatofocusing

A PBE 94 ion-exchanger was used for chromatofocusing (Sluyterman & Elgersma, 1978; Sluyterman & Wijdenes, 1978) in a K15/10 column at 10 °C and used over a pH range from pH 9,00 to pH 6,00. Fractions of 3,5 mℓ were collected and monitored at 280 nm, and peaks were freeze-dried.

Protein determinations

The protein content was determined by the heated biuret-Folin method described by Dorsey, McDonald & Roels (1977).

Toxicity determination of fractions obtained by chromatography

Fractions obtained by chromatography were tested for toxicity as described for crude extracts, using dilutions corresponding to the equivalent of 20–50 larvae. Fractions showing clinical symptoms of at least F11 or B11 (Table 2) in all animals were recorded as positive.

Electrophoresis

Analytical isoelectric focusing (Righetti, Gianzza & Bosisio, 1979) with polyacrylamide as supporting medium was performed with a Model FBE 3000 flat bed electrophoresis apparatus. Carrier ampholytes, pH range 3–10 were used. Coomassie Brilliant Blue R-250 (Fazekas de St. Groth, Webster & Datyner, 1963) or silver was used to stain the gel (Heukeshoven & Dernick, 1985). Sodium dodecyl sulphate gradient gel eletrophoresis (SDS-PAGE) was performed, according to the method of Laemmli (1970). Alternatively, electrophoresis was performed with the Pharmacia Phastsystem, according to the manufacturer's instructions.

RESULTS

Toxicity of crude tick extracts

Of all the tick stages tested, either fed or unfed, at extremely high dosages, only replete larvae induced paralysis in one-day-old chickens (Tables 1 and 2); extracts prepared from 2 000 unfed larvae failed to show paralysis symptoms

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

No difference in toxicity between the crude larval extracts in either distilled water or 0,154 M NaCl was observed. The addition of detergent at room temperature, whether Tween 20, SDS or Chaps, had no effect on the toxicity. Heating in the presence or absence of detergent, however, resulted in total loss in toxicity.

Gel-permeation chromatography

Elution of the crude *A. (P.) walkerae* larval extract on the Sephadex-G-100 column did not separate the toxic component from the haemoglobin fraction when using H_2O (pH 7,00) (fraction SN_1 in Fig. 1 or 0,154 M NaCl pH 7,00), with either 0,05 % Tween 20, 0,1 % SDS or 0,1 % Chaps, as eluting buffer (fraction SND_1 in Fig. 2).

[1] Janke & Kunkel, Kika-Werk, Fed. Rep. Germany

TABLE 1 Determination of the presence of paralysis toxin in crude extracts from various stages of *Argas (Persicargas) walkerae*

Developmental stage	Number used	Mean ± SD quantity of protein injected (mg/chicken)	Clinical symptoms[6,7]
Freshly laid eggs	2000	100,3 ± 12,8	N
Unfed larvae[1]	2000	87,7 ± 3,2	N
Unfed larvae[2]	2000	82,4 ± 8,1	N
Replete larvae[3]	300	32,3 ± 3,1	VP[5]
Unfed nymphae stage 1[4]	2000	217,4 ± 17,4	N
Replete nymphae stage 1[3]	400	123,1 ± 9,7	N
Unfed nymphae stage 2[4]	400	90,7 ± 10,5	N
Replete nymphae stage 2[3]	200	97,3 ± 14,9	N
Unfed nymphae stage 3[4]	200	82,6 ± 11,3	N
Replete nymphae stage 3[3]	200	101,5 ± 18,4	N
Replete adult male ticks[3]	200	89,9 ± 17,6	N
Replete adult female ticks[3]	200	203,1 ± 23,7	N

[1] Extracts prepared 3 days after hatching
[2] Extracts prepared 2 weeks after hatching
[3] Extracts prepared immediately after collection
[4] Extracts prepared 2 weeks after moulting
[5] Further investigations reported in Table 3
[6] See Table 2
[7] Result for at least 3 animals

TABLE 2 Description of the clinical symptoms in chickens from inoculation of crude extracts of *Argas (Persicargas) walkerae* larvae (Gothe & Englert, 1978)

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

TABLE 3 Toxicity determinations of crude extracts prepared from replete *Argas (Persicargas) walkerae* larvae

Number inoculated per chicken[1]	Mean ± SD inoculated (mg/chicken)	Clinical symptoms[2]
3 larvae	0,32 ± 0,05	F I
5 larvae	0,51 ± 0,07	F II
10 larvae	0,94 ± 0,06	F III/B I
15 larvae	1,39 ± 0,11	B I/B II
20 larvae	1,97 ± 0,15	B II
25 larvae	2,53 ± 0,13	B III
50 larvae	5,04 ± 0,17	VP

[1] At least 3 animals tested
[2] See Table 2

In all the cases, virtually 100 % of the protein as well as the toxicity was recovered.

The Sephacryl S-200 column gave reasonable resolution when the column was eluted with distilled water (pH 4,00) (Fig. 3). A colourless peak (SH_2) was obtained which represented 30 % of the original protein and 40 % of the original toxicity. This peak eluted at a volume corresponding to a molecular mass of 80 to 100 kDa. A red fraction (SH_4) adsorbed onto the column which could be eluted with 0,154 M NaCl. This fraction represented approximately 65 % of the sample protein and 70 % of the sample toxicity.

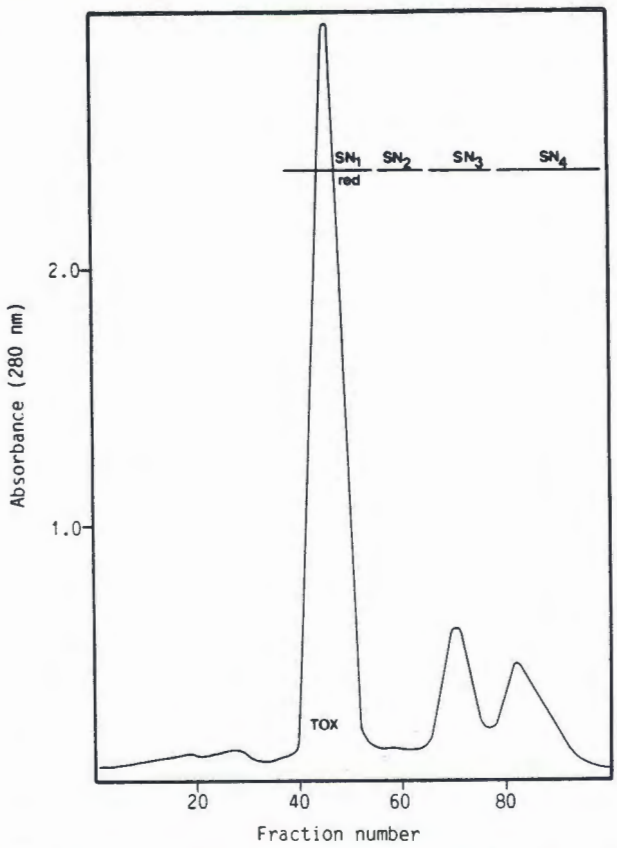

FIG. 1 Sephadex G-100 gel-permeation chromatogram of *Argas (Persicargas) walkerae* crude larval extract. The column was eluted with 0,154 M NaCl. Fractions of 3,5 mℓ were collected and monitored at 280 nm

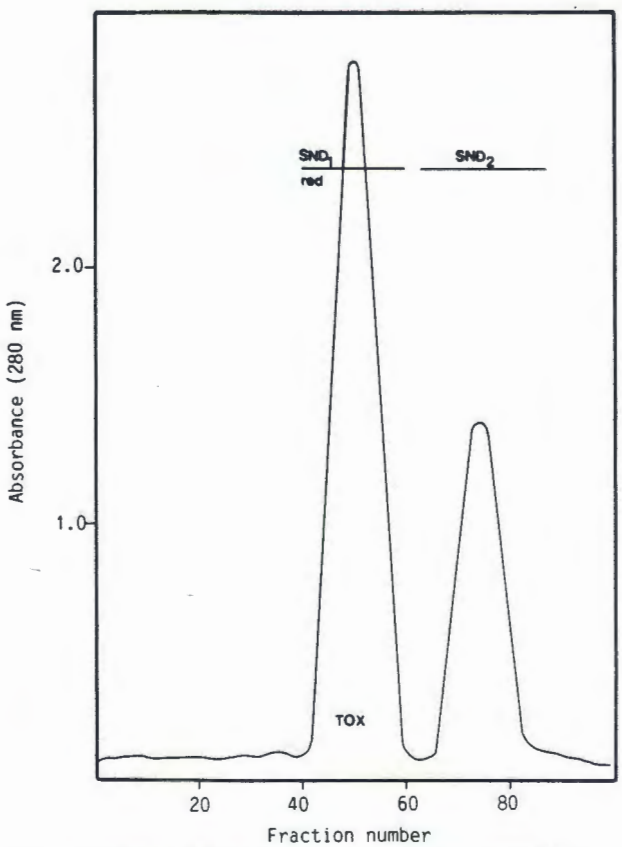

FIG. 2 Sephacryl S-200 gel-permeation chromatogram of *Argas (Persicargas) walkerae* crude larval extract. The column was eluted with 0,154 M NaCl containing either 0,05 % Tween 20 (v/v), 0,1 % SDS (w/v) or 0,1 % Chaps (w/v). Fractions of 3,5 mℓ were collected and monitored at 280 nm

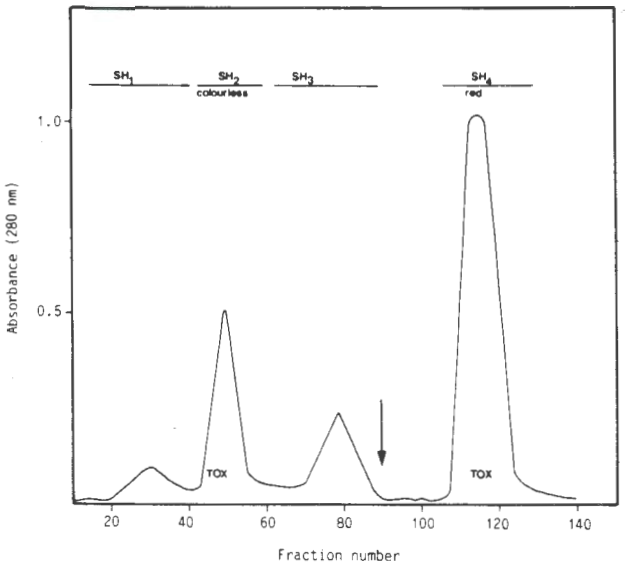

FIG. 3 Sephacryl S-200 gel-permeation chromatogram of *Argas (Persicargas) walkerae* crude larval extract. The column was eluted with acidified distilled water (pH 4,00). Fractions of 3,5 mℓ were collected and monitored at 280 nm. Arrow indicates an elution buffer change to 0,2 M NaCl

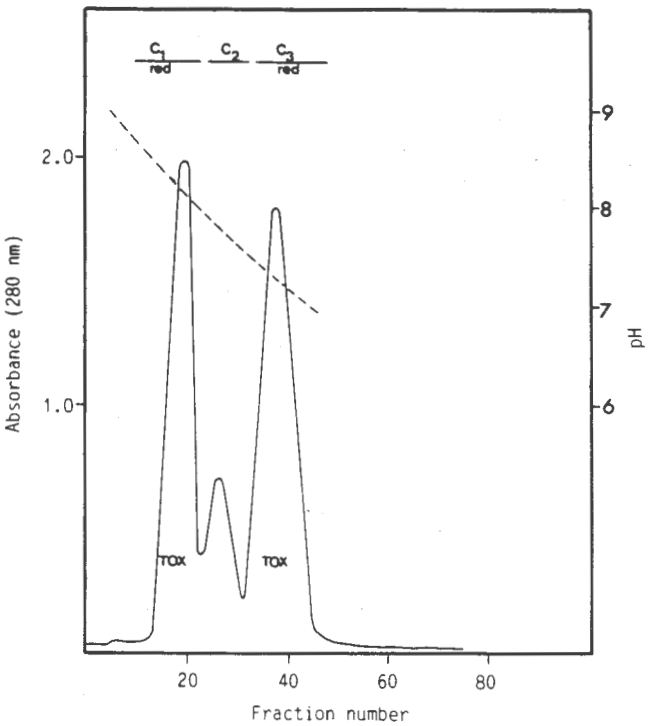

FIG. 4 Chromatofocusing chromatogram of *Argas (Persicargas) walkerae* crude extracts eluted with a pH gradient ranging from pH 9–7. Starting buffer was 0,025 M ethanolamine-HCl (pH 9,01) and eluent was polybuffer 96-HCl (pH 7,00). Fractions of 3,5 mℓ were collected and the pH (- - -) and 280 nm absorbance (———) were recorded

By means of the batch Sephacryl S-200 method, most of the haemoglobin could be separated from the sample. This toxic fraction (equivalent to SH_2) represented approximately 50 % of the original toxicity.

Chromatofocusing

This method gave good recoveries with respect to protein and toxicity (above 85 %). Two toxic fractions (C_1 and C_3), with pI of approximately 7,6 and 8,3, each associated with haemoglobin, were observed (Fig. 4).

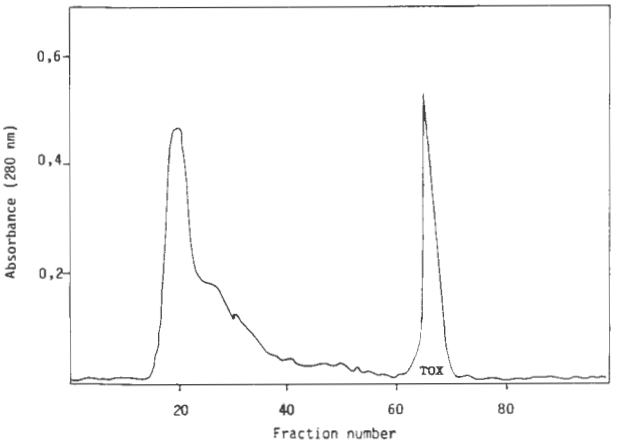

FIG. 5 DEAE-Sephacel chromatography of the toxic filtrate obtained from the Sephacryl S-200 batch method. Elution conditions are described in the text

Ion-exchange chromatography

DEAE-Sephacryl chromatography of the toxic filtrate obtained from the batch method resulted in 2 major peaks (Fig. 5), the 2nd of which showed high toxicity.

SDS-PAGE of this fraction revealed 2 bands with M_r of 32 kDa and 60 kDa. A faint band with M_r of 18 kDa was present when a high sample concentration was used. One band with a pI of 4,5 was visible after IEF. At high sample concentrations, additional faint bands with higher pI were visible.

DISCUSSION

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

These observations are in agreement with those of Gothe (1971a) and Gothe & Verhalen (1975) regarding the paralysis-inducing capabilities and the infestation rate of larval, nymphal and adult ticks during feeding. They showed that only larvae, after at least 4 days of infestation, caused paralysis, the intensity of which was directly proportional to the rate of infestation.

The present results also substantiate the finding of Gothe (1971a) that the paralysis is due to a toxin and does not develop as a result of blood loss in the host caused by feeding ticks, as suggested by Gulyàs (1952). Furthermore, it has been reported that the timely removal of ticks from paralyzed animals leads to a subsidence of symptoms and complete recovery (Gothe *et al.*, 1979). Likewise, with the toxicity tests of crude larval extracts, it was observed on many occasions that animals, paralysed by a single inoculation of a sub-optimal paralysis-inducing dosage, recovered within 1–2 days. It is evident that the noxious substance responsible for the paralysis is not cell-bound, but circulates humorally. The toxin possesses membranophilic properties, but its somatic linkage is labile (Kunze & Gothe, 1978).

The combined results of the present investigations and those of Gothe *et al.* (1970) thus show that toxin

production and transfer occur only after several days of feeding by larval ticks only. Post-larval stages do not produce toxin in amounts detectable by the present toxicity assays. The correlation between feeding behaviour and the developnment of pathophysiological syndromes has also been observed in other tick species that induce paralysis (Gothe, 1984).

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

Gel-permeation chromatography of crude larval extracts showed that elution in the presence of saline resulted in a toxic fraction which eluted at the void volume of Sephadex G-100 and Sephacryl S-200 columns. This fraction was reddish to brown in colour. Recovery of protein and toxicity were in excess of 90 %. The inclusion of 0,05 % Tween 20, 0,1 % SDS or 0,1 % Chaps to the sample and eluent had no effect on the elution pattern or on protein and toxicity recoveries. These results indicate the presence of large complexes between toxin and haemoglobin.

Elution of the Sephacryl S-200 column with distilled water (pH 4,00) resulted in the recovery of a colourless toxic fraction representing 40 % of the original toxicity at a volume corresponding to a M_r 80–100 kDa. Under these conditions, the haemoglobin adsorbed onto the column.

Elution of this fraction was achieved with 0,154 M NaCl. It contained approximately 70 % of the original toxicity. These results indicate the possibility that 2 toxins were present. More likely, however, is that the toxin is associated with haemoglobin through hydrophobic interaction. At low pH and low ionic strength, the toxin is partially liberated from a haemoglobin-toxin complex, with the result that some free toxin is present. The haemoglobin-toxin is adsorbed on the column, while the free toxin is eluted at a volume according to its molecular size and conformation.

Chromatofocusing was also tested as a means of toxin isolation starting with the crude larval extracts. Good resolution of peaks was obtained as well as high recoveries with respect to protein and toxicity. However, the same problem was experienced: toxicity was associated with haemoglobin. This indicates that several haemoglobin variants and probably their breakdown products are present in the crude extracts and that more than 1 of these may associate with the toxin.

SDS-PAGE showed that the toxic fraction obtained after ion-exchange chromatography consists of 2 bands with M_r of 32 and 60 kDa. This result indicates that the toxin may exist as an oligomer, since gel-permeation chromatography revealed a M_r in the region of 80–100 kDa. The M_r of 60 kDa is similar to that of the paralysis inducing toxins isolated from *Ixodes holocyclus* (Stone, Doube, Binnington & Goodger, 1979) and from *Rhipicephalus evertsi evertsi* (Viljoen, Bezuidenhout, Oberem, Vermeulen, Visser, Gothe & Neitz, 1986). The toxin from all 3 tick species have a pI in the acid range.

Although a homogeneous toxin was not obtained by these isolation attempts, useful information regarding the source, molecular mass, iso-electric point and the behaviour of the toxin with respect to ionic strength and pH has been obtained. This information is at present being exploited to further purify the toxin.

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