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PROTEINS AND FREE AMINO ACIDS IN THE SALIVARY SECRETION AND HAEMOLYMPH OF THE TICK *AMBLYOMMA HEBRAEUM*

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ABSTRACT

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 (1978).

Electrophoretic and chromatographic separations of the salivary secretion of *Amblyomma hebraeum* showed a less complex protein pattern than that of *Ornithodoros savignyi*. A hyaluronidase active component was isolated. The haemolymph protein pattern showed a major protein fraction with a mobility slightly faster than that of bovine serum albumin.

Résumé

PROTÉINES ET ACIDES AMINÉS LIBRES DANS LA SÉCRÉTION SALIVAIRE ET L'HÉMOLYMPHE DE LA TIQUE AMBLYOMMA HEBRAEUM

*Des séparations par électrophorése et chromatographie de la sécrètion salivaire d'*Amblyomma hebraeum *ont révélé un schéma protéinique moins complexe que celui d'*Ornithodoros savignyi. *On a isolé un composant hyaluronidase-actif. Le schéma protéinique de l'hémolymphe a révélé une fraction protéinique majeure dont la mobilité est légèrement supérieure à celle de la séralbumine bovine.*

INTRODUCTION

Numerous tick species being vectors of disease agents, it is important to investigate their body fluids, since the results of such investigations may explain the specificity of these parasites with respect to the biological activity of the fluids in the tissues of the host. In addition, the results may prove useful when suitable nutrient media for growing tick cell and *Rickettsia in vitro* are prepared (Barker & Lehner, 1976; Řeháček & Brzostowski, 1969).

A comparative study of both the chemical components and the biochemical activities of the salivary secretion of various species of ticks that transmit infectious diseases or cause tick toxicoses may form a basis for prophylactic and therapeutic research (Howell, 1966). Furthermore, the free amino acid pattern of the salivary secretion, like the free amino acid pattern of insect haemolymph, could serve as a taxonomic characteristic (Florkin, 1958).

A study of the haemolymph of ticks may yield valuable information on their intermediary metabolism, which is important in the investigation of the effects of acaricides. It is inevitable that an ultimate understanding of the resistance developed by some tick species to certain acaricides will have to be sought at a metabolic level. A knowledge of the metabolic fate of these compounds could aid in the development of alternative potent acaricides.

The protein pattern in the salivary secretion, salivary glands and haemolymph has been described for a number of tick species (Neitz, 1976; Howell, Neitz & Potgieter, 1975; Tatchell, 1971; Van Sande & Karcher, 1960). Van Sande & Karcher (1960) reported that the protein pattern in the haemolymph of ticks infected with pathogenic organisms was similar to that of normal individuals. Furthermore, the pattern was not influenced by the species of host on which the ticks had engorged. It is not known whether similar patterns may apply to the oral secretion proteins.

Several biological activities, including toxic cytolysin and haemolytic, anticoagulant and various enzymatic activities in salivary secretions of ticks, have also been reported (Kaire, 1966; Tatchell & Binnington, 1971; Neitz, Howell & Potgieter, 1969; Howell, Neitz & Potgieter, 1975; Neitz, 1976; Sauer, 1977). These activities may have an important role in the syndromes produced by the salivary secretions.

Amblyomma hebraeum is the transmitter of *Cowdria ruminantium* (syn. *Rickettsia ruminantium*) (Neitz, 1956, 1967), possibly by means of the salivary secretion. Alternatively, the secretion may aid in the distribution of the rickettsia in the tissues of the host. The secretion of uninfected ticks lacks toxic activity (C. J. Howell, unpublished data, 1970). This paper deals with the free amino acids and proteins of the salivary secretion and the haemolymph of uninfected specimens of *A. hebraeum*. The results of hyaluronidase activity determinations are also presented, together with a partial characterization of this active component.

It should be borne in mind that the results reported here were obtained from investigations on pilocarpine-stimulated salivary secretions. Barker, Burris, Sauer & Hair (1973) have shown that the electrolytic composition of the tick salivary secretions obtained by infra-red heat, pilocarpine injection and electrical stimulation varies considerably. These authors deduced that the highest concentrations of proteins and amino acids should be found after employing the infra-red heat method of stimulation. These findings are of considerable importance when the chemical composition and biological activity of tick salivary secretions are investigated. Future studies will necessarily entail investigations on secretions obtained by various stimulatory methods.

MATERIALS AND METHODS

Collection of salivary secretion and haemolymph

Semi-engorged *A. hebraeum* females fed on sheep at Onderstepoort were used for the collection of salivary secretions after injection with pilocarpine hydrochloride (Howell, 1966).

Haemolymph was collected by means of a capillary tube from a small incision made on the dorsum from ticks which had dropped from sheep 1–2 weeks previously.

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Gel permeation chromatography

The clear salivary secretion (2 mℓ) was subjected to gel permeation chromatography after centrifugation at 12 000 g for 20 min. Upward flow through a column (36,0×2,5 cm) of Sephadex G 100 (Pharmacia, Uppsala, Sweden) at a flow rate of 21 mℓ/h was used as the initial separation step. The eluent was distilled water. Fractions of 2,6 mℓ were collected and monitored at 280 nm with a Beckman DK2A spectrophotometer. Fraction GII (Fig. 1a) was subsequently subjected to Bio-Gel P10* gel filtration (Fig. 2). The column dimensions, rate of flow and the collection and monitoring of the fractions were similar to those described for the Sephadex G100 column.

Amino acid determinations

Determination of the amino acids in unhydrolysed fractions GIII (Fig. 1a) and PII (Fig. 2) was achieved with a Beckman Model 120B amino acid analyser using the procedure described by Neitz, Howell & Potgieter (1969). The sum of the individual amino acid concentrations in these 2 fractions was taken as the total of each free amino acid present in the salivary secretion.

To obtain the amino acid composition of fraction PI (Fig. 2), which has hyaluronidase activity, 0,3 mℓ 6N HCl was added to 0,53 mg freeze-dried fraction in a 16×0,75 cm glass tube. The tube was then inserted into a bath containing liquid nitrogen and, after the sample had frozen, it was evacuated with a water-jet pump and sealed under vacuum. Hydrolysis was performed at 110 °C for 24 h. The tube was subsequently cooled and opened, and the HCl evaporated with a stream of nitrogen. The dried sample was transferred to a 5 mℓ volumetric flask with 0,2 N sodium citrate buffer at pH 2,2 and made to volume. One-fifth aliquots were analysed for amino acids as described above.

The free amino acids in the haemolymph were determined with a Beckman Model 121M amino acid analyser after precipitation of the proteins with sulpho-salicylic acid**.

Microzone electrophoresis

The electrophoresis was carried out at pH 8,6 (sodium barbital buffer, ionic strength 0,075) in a Beckman microzone electrophoresis apparatus on cellulose acetate membrane, employing a constant voltage of 250V for 30 min. The strips were fixed and stained in Ponceau S solution and scanned with a Beckman densitometer.

Determination of hyaluronidase activity in fraction PI (Fig. 2)

The turbidity-reducing method was used (Dorfman, 1955). For activity measurements, 0,1; 0,15 and 0,2 mℓ aliquots of a solution containing 0,18 mg fraction PI per mℓ in 0,02M Tris, 0,08M NaCl at pH 7,9 were used. The substrate was human umbilical hyaluronic acid***. For each activity measurement, 0,05 mℓ of a stock solution containing 5,6 mg substrate per mℓ in 0,3M phosphate buffer (KH_2PO_4,Na_2HPO_4) at pH 5,3 was used. Turbidity was developed with egg albumen***, using 10 mℓ of a 0,1% albumen solution containing 0,33% sodium acetate and 0,46% glacial acetic acid at pH 3,75.

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Molecular mass determination of fraction PI (Fig. 2)

Conventional sedimentation equilibrium centrifugation was used employing a Spinco Model E ultracentrifuge equipped with an ultraviolet photoelectric scanner. Fraction PI was dissolved in 0,02M Tris, 0,08M NaCl at a pH of 7,9. The solution had an optical density of 0,18 at 280 nm determined in a 1 cm path length cell with a Beckman DK2A ratio-recording spectrophotometer. For the ultracentrifugal run, 0,12 mℓ of this solution was introduced into a charcoal double sector scanner cell. An An-D rotor was used and the temperature was 20 °C during the run. Overspeeding of the rotor was used to shorten the transient time to equilibrium. After the overspeeding period, the rotor was decelerated to 16 000 rpm and spun for 19 h before calculations of molecular mass were made. Additional calculations were made after 3 and 5 h at the same speed. To calculate the molecular mass, the optical density (A) was measured as a function of radial positions (r) at a series of points across the fluid column in the cell. A plot of log A *versus* r^2 was made and the slope calculated. The slope $d\log A/dr^2$ was then substituted in the equation, $M=(2,303)\ (2RT)\ (d \log A)/(1-\bar{v}p)\ W^2dr^2$ (Bowen, 1970).

Results

Gel permeation chromatography

The separation pattern of the salivary secretion of *A. hebraeum*, obtained after chromatography on Sephadex G100, is presented in Fig. 1a and that of *O. savignyi*, obtained on the same column, in Fig. 1b. Fraction G11 was subsequently fractionated on a Bio-Gel P10 column, with the result shown in Fig. 2. Fractions GIII and PII were analysed for free amino acids. The hyaluronidase activity of fraction PI was investigated as well as the amino acid composition and molecular mass.

Total free amino acids in the salivary secretion and haemolymph

The total free amino acids of the salivary secretion of *A. hebraeum* are summarized in Table 1, in which the total free amino acids of *O. savignyi* salivary secretion (Neitz *et al.*, 1969) and human saliva (Woldring, 1955) are also shown for comparison. The free amino acid composition of the haemolymph of *A. hebraeum* is presented in Table 2.

Microzone electrophoresis

Fig. 3a shows a scanned cellulose acetate membrane after electrophoresis of the salivary secretions of *A. hebraeum* and *O. savignyi*, and Fig. 3b of haemolymph of *A. hebraeum*. In both figures normal bovine blood serum is shown for comparison.

Hyaluronidase activity, amino acid composition and molecular mass of fraction PI

Fraction PI showed hyaluronidase (E.C. 3.2.1.35) activity. A reduction of 15% in turbidity of the hyaluronic acid-egg albumen complex resulted after 0,36 mg of fraction PI had acted on 0,28 mg substrate for 46 min at 37 °C. The amino acid composition of this fraction is compared with the composition of testicular hyaluronidase in Table 3 (Borders & Raftery, 1968; Brunish & Högberg, 1960, cited by Borders & Raftery, 1968). The molecular mass of fraction PI was calculated as 9 680, assuming a partial specific volume of 0,725 mℓ/g. The plot of log A *versus* r^2 is shown in Fig. 4.

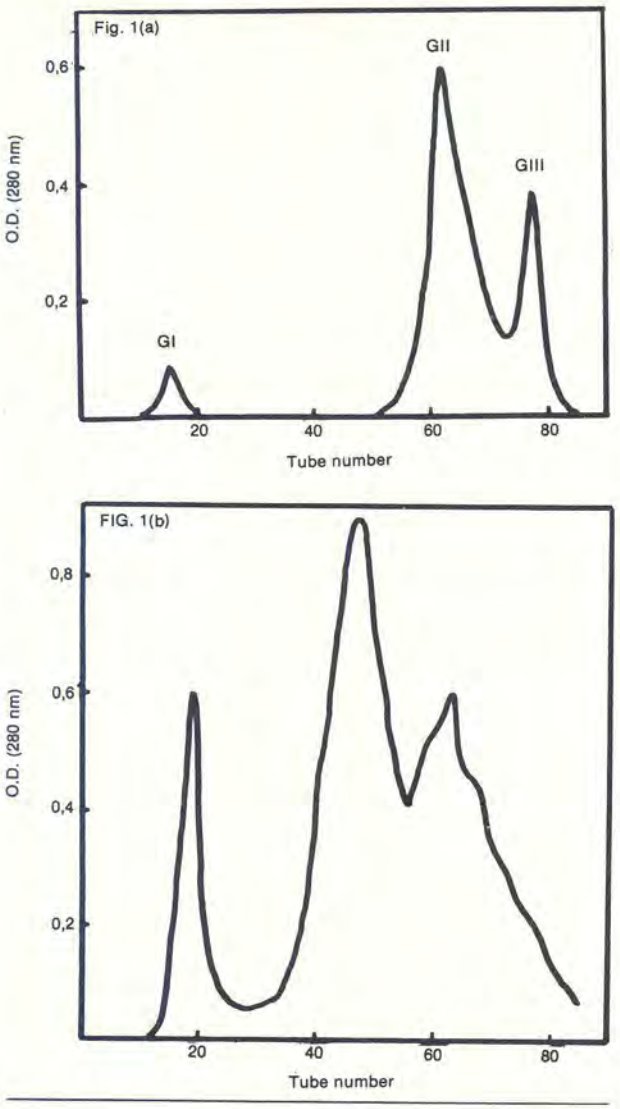

FIG. 1 Gel permeation chromatography of the salivary secretions of *A. hebraeum* (1a) and *O. savignyi* (1b) on a Sephadex G100 column (36×2,5 cm). Eluent: distilled water. Flow rate: 21 mℓ/h and fractions of 2,6 mℓ collected

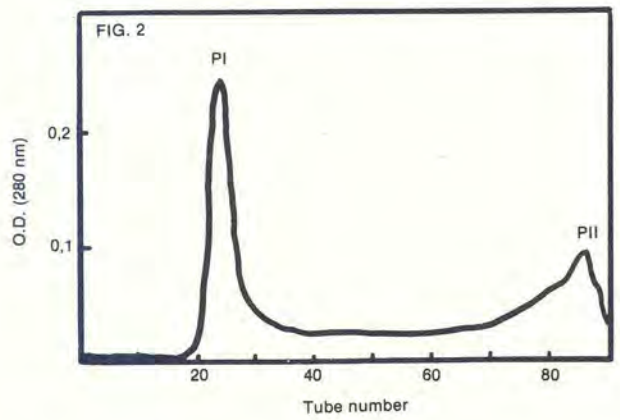

FIG. 2 Gel permeation chromatography of fraction GII on a Bio-Gel P10 column (36×2,5 cm). Eluent: distilled water. Flow rate: 21 mℓ/h and fractions of 2,6 mℓ collected.

TABLE 1 Free amino acids present in salivary secretions (μg/mℓ)

Amino acid	*A. hebraeum*	*O. savignyi*†	Human*
Lysine	36,7	98,9	7,7
Histidine	28,9	19,6	8,1
Arginine	70,2	0	1,9
Aspartic acid	13,3	trace	1,3
Threonine	0	42,6	2,6
Serine	731,6	4,6	2,6
Glutamic acid	32,7	trace	3,8
Proline	25,3	trace	5,1
Glysine	137,4	4,5	9,1
Alanine	81,7	17,6	4,6
Valine	139,1	68,3	1,6
Isoleucine	24,6	34,6	4,4
Leucine	52,2	77,9	3,4
Tyrosine	35,9	63,8	4,8
Phenylalanine	24,1	55,4	3,9
Taurine	119,6	44,5	4,1
Total	1 553,3	532,3	69,0

* Woldring, 1955

† Neitz, Howell & Potgieter, 1969

TABLE 2 Free amino acids present in the haemolymph of *A. hebraeum*

Amino acid	mg/100 mℓ	Amino acid	mg/100 mℓ
Lysine	4,79	Valine	6,74
Histidine	3,59	Isoleucine	0,17
Arginine	0,63	Leucine	2,96
Aspartic acid	0	Tyrosine	0,49
Methionine	0,21	Phenylalanine	1,21
Threonine	7,23	Citrulline	8,12
Serine	3,89	α-Aminobutyric acid	5,10
Glutamic acid	7,53	γ-Aminobutyric acid	0,06
Proline	7,52	Ethanolamine	1,17
Glycine	4,47	Ornithine	3,46
Alanine	15,42	3-Methylhistidine	0,52

TABLE 3 Amino acid composition of hyaluronidase (g residue/100 g enzyme)

Residue	*A. hebraeum* hycaluronidase	Testicular hyaluronidase†	Testicular hyaluronidase*
Lysine	4,68	4,77	4,21
Histidine	3,77	1,82	1,51
Arginine	3,59	4,37	3,39
Aspartic acid	7,19	7,83	7,78
Threonine	3,13	3,34	2,84
Serine	4,38	2,87	3,74
Glutamic acid	8,00	6,55	6,36
Proline	3,73	3,58	3,27
Glycine	2,54	2,13	2,09
Alanine	2,63	2,49	2,55
Valine	3,51	4,37	4,20
Isoleucine	2,26	2,87	2,73
Leucine	5,03	6,13	5,66
Tyrosine	3,43	3,98	3,22
Phenylalanine	3,61	3,61	3,29

* Brunish and Högberg, 1960, cited by Borders & Raftery, 1968

† Borders and Raftery, 1968

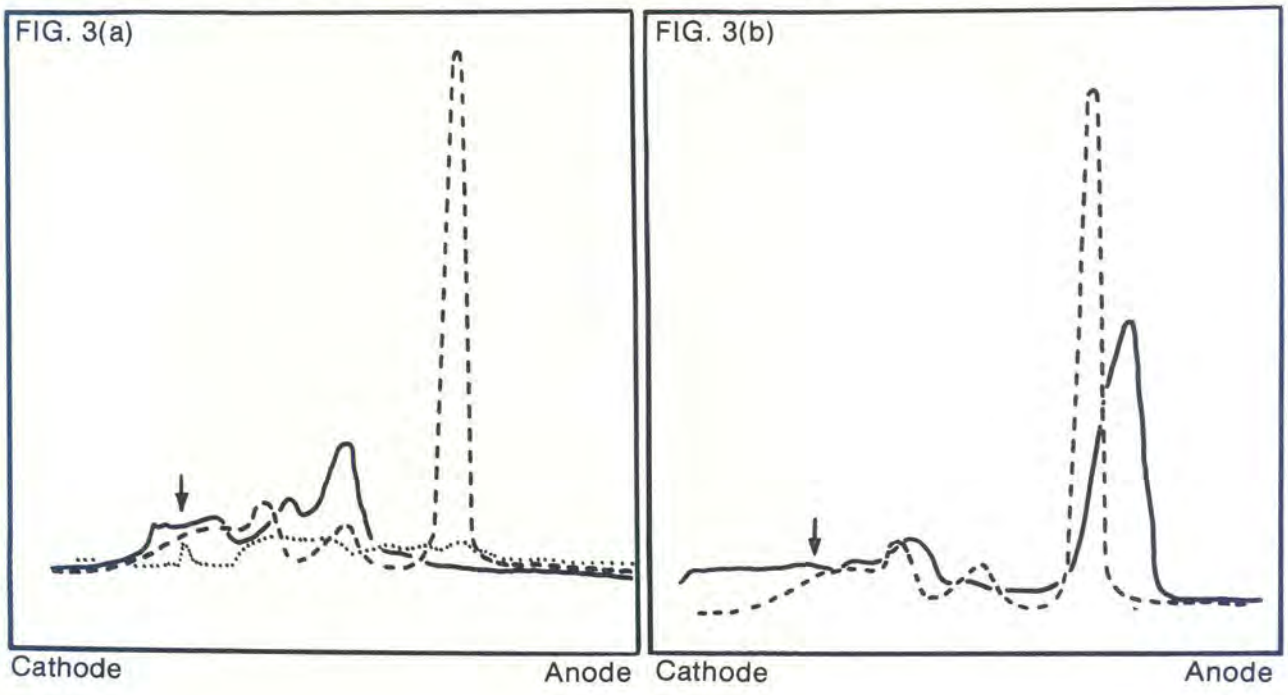

FIG. 3a Electrophoretic comparison of the oral secretions of *A. hebraeum* (. . .) and *O. savignyi* (solid line). The dashed line represents blood serum. The arrow indicates the origin
FIG. 3b Electrophoretic comparison of the haemolymph of *A. hebraeum* (solid line) with blood serum (dashed line). The arrow indicates the origin

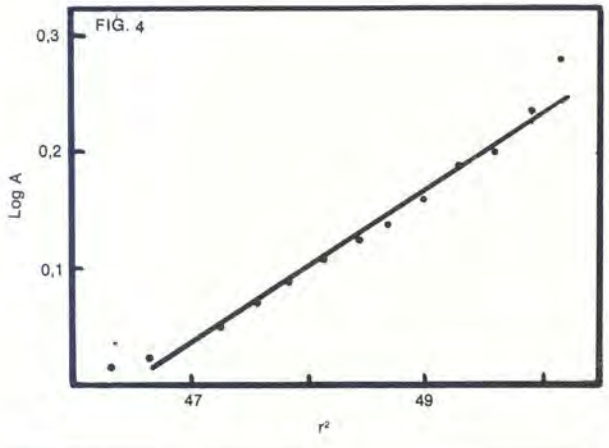

FIG. 4 Molecular mass determinations of hyaluronidase active fraction (PI) by sedimentation equilibrium centrifugation. Details of the determination are given in the text.

Discussion

This investigation has clearly shown that the salivary secretions, obtained by pilocarpine stimulation of *A. hebraeum* and *O. savignyi,* show definite variations with respect to the free amino acid content. Furthermore, when the free amino acids of human saliva are compared, it is evident that these 2 tick species have an appreciably larger amount of free amino acids in their salivary secretion.

It is a well-established fact that insect haemolymph contains extraordinarily high concentrations of free amino acids. Values of up to 2 000 mg/100 mℓ have been reported for the total free amino acids present in the haemolymph of some insect species (Gilmour, 1961; Candy & Kilby, 1975). This high free amino acid content may be considered as one of the main characteristic aspects of the biochemistry of insects (Florkin, 1958) and may apply to the free amino acids of the salivary secretions of ticks as well.

In the haemolymph, the free amino acid composition varies widely, not only from one species to another, but also within the same species. Temperature, diet and the stage of development affect the amino acid composition (Florkin, 1958). Nevertheless, certain species can be differentiated from others according to the concentration of some amino acids. In spite of the degree of variability within the same species, some attempts have been made to express the amino acidemia in terms of metabolism. For example, the free cysteine content of tick body fluids is of interest since this amino acid may participate in the metabolism of arsenical acaricides. Stocken & Thompson (1946) have clearly shown that one mode of action of arsenicals is their reaction with sulfhydryl compounds. Whitehead (1965) has shown that arsenic-resistant *B. decoloratus* ticks contain greater amounts of sulfhydryl compounds than susceptible ticks. The close relationship of tyrosine in the haemolymph to the O-Quinones in the cuticle is noteworthy (Hackman, 1958). A more complete knowledge of the cuticle composition and a detailed study of the biosynthesis of the components are of particular interest when penetrant carriers, which enhance acari-, cide penetration through the cuticle, are considered. Other significant correlations between free amino acids in the body fluids and metabolism will probably emerge in the future.

Comparison of the free amino acid content in the haemolymph of different tick species is difficult since it can be predicted that a large proportion of the amino acids present could have been obtained from the blood of the host. In addition, their concentration increases when the haemolymph volume is reduced by water loss as the bloodmeal is digested (Boctor &

Araman, 1971). In general, however, results show that the concentrations in tick haemolymph are much lower than in insect haemolymph (Florkin & Jeuniaux, 1964; Boctor, 1972). The presence of ornithine and citrulline in the haemolymph of *A. hebraeum* may indicate the operation of the urea cycle in tick tissues.

When the free amino acids of the salivary secretion of *A. hebraeum* are taken into account, it is evident that the serine content is particularly high and accounts for almost 50% of the total amino acid content. It is almost certain that the peak, emerging at the serine position during amino acid analysis, is contaminated with other amino acids, most likely glutamine and asparagine. These 2 amino acids are of particular interest in insect metabolism since their deaminated derivatives serve as effective trapping agents for the ammonia released after deamination (Winteringham, 1958). The deamination of amino acids to provide substrates for the tricarboxylic acid cycle may be an important function of the free amino acids in insect tissues. Winteringham, Harrison, McKay & Weatherley (1957) have shown that di-isopropyl phosphofluoridate causes an increase in the free glutamine concentration in the adult housefly. This glutamine accumulation may indicate fatal biochemical lesions in addition to that of cholinesterase inhibition.

Cellulose acetate electrophoresis of the haemolymph of *A. hebraeum* showed a major protein band with a higher mobility than serum albumin. Preliminary investigations have shown that the major protein components contain copper and resemble haemocyanin in many characteristics. It should be borne in mind that haemolymph proteins are influenced by the developmental stage, physiological state and sex of the ticks and possibly also by the host (Dolp & Hamdy, 1971). The importance of the effect of the developmental cycle of ticks on the biochemical composition of biological fluids has also been stressed by Hajjar (1971).

In contrast to the complex protein composition of the salivary secretion of *O. savignyi*, the secretion of *A. hebraeum* shows a relatively simple composition. The latter secretion apparently contains only one major protein component which possesses hyaluronidase activity. Hyaluronidase is widely distributed in nature and has been detected in snake venoms, in the salivary glands of the leech, sand tampan, mammalian testis and various micro-organisms (Meyer, Hoffman & Linker, 1960; Neitz, 1976). It is most probably involved in aiding diffusion by lowering tissue barriers, a function which may be particularly true for the enzyme in snake venoms and secretions of blood-sucking arthropods. The enzyme may also cause serious symptoms in the host since petechial hemorrhages caused by hyaluronidase have been reported by Chambers & Zweifach (1947). The mechanism by which the enzyme may affect capillary fragility has been described by Copley (1962). Hyaluronidases from different biological origins show marked differences with respect to their specific activity, substrate specificity, mechanism of action, chemical composition, physical characteristics and end-products produced (Meyer *et al.*, 1960). These differences may have a decisive effect on the symptoms produced by the various enzymes.

The hyaluronidase isolated from *A. hebraeum* shows some similarity to the testicular enzyme in respect of the amino acid composition. A comparison of the molecular mass is impossible, however, since values of between 11 000 and 61 000 have been reported for the bovine testicular enzyme (Borders & Raftery, 1968; Malmgren, 1953). The enzyme from *A. hebraeum* has a low activity, although it should be stressed that optimum conditions for activity measurements were not determined. The molecular mass was found to be 9 680 and it appears to be homogeneous according to the gel permeation chromatography eluent pattern. The sedimentation equilibrium results, however, show signs of slight heterogeneity.

A second minor protein component (Fraction GI, Fig. 1a) was not further investigated. It may prove to have acetyl cholinesterase activity since the salivary secretion of *O. savignyi* showed this activity in the fraction eluting in the same position.

This report has shown that the salivary secretions of *A. hebraeum* and *O. savignyi* show marked differences with respect to the free amino acid and protein composition. Undoubtedly many other differences exist, some of which may be characteristic of individual tick species. Furthermore, they may prove to be related to other characteristic features of the ticks such as host and pathogen specificity. An extensive comparative study of the salivary secretion and haemolymph composition of various ticks may ultimately prove essential for a true understanding of tick, host and pathogen inter-relationships, host immunization, chemotherapy and vector control.

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