Onderstepoort J. vet. Res., 53, 147–152 (1986)

INVESTIGATIONS INTO THE FUNCTION AND CHEMICAL COMPOSITIONS OF THE POROSE AREAS SECRETION OF *RHIPICEPHALUS EVERTSI EVERTSI* DURING OVIPOSITION

N. M. J. VERMEULEN[1], R. GOTHE[2], A. C. SENEKAL[1] and A. W. H. NEITZ[1]

ABSTRACT

VERMEULEN, N. M. J., GOTHE, R., SENEKAL, A. C. & NEITZ, A. W. H., 1986. Investigation into the function and chemical composition of the secretion of the porose areas of *Rhipicephalus evertsi evertsi* during oviposition. *Onderstepoort Journal of Veterinary Research*, 53, 147–152 (1986).

Major differences were observed in hexane, ethanol and butanol extracts of eggs obtained from *Rhipicephalus evertsi evertsi* females in which the porose areas functioned normally (AP^+ eggs) and from *R. evertsi evertsi* in which the porose areas were selectively destroyed by electrocautery (AP^- eggs).

Mass yields and UV spectra of the hexane extracts were similar for AP^+ and AP^- eggs. The UV spectra changed only slightly in the 294–320 nm range with respect to time and temperature. High performance liquid chromatography revealed 2 components which originate from the porose areas. Mass spectroscopy of these components indicated the presence of aliphatic and phenolic groups.

The ethanol and butanol extracts showed quantitative but no qualitative differences with respect to AP^+ and AP^- eggs. Electrophoretic fractionation of the butanol extracts revealed the presence of proteins in the secretion of the porose areas.

Apart from this information on the chemical composition of the secretion, no indication was obtained of their function during oviposition of *R. evertsi evertsi*.

INTRODUCTION

The porose areas and their associated tissues were originally described as sense organs (Williams, 1905; Bonnet, 1906, 1907; Nordenskiöld, 1909, 1911; Samson, 1909; Falke, 1931; Schulze, 1942; Douglas, 1943; Pomerantzew, 1950; Babos, 1964; Lagutenko, 1969; Woolley, 1972). More recently it was suspected (Feldman-Muhsam & Havivi, 1960), and subsequently proven experimentally, that blockage of the porose areas results in complete inhibition of Gené's organ (Feldman-Muhsam, 1963). It was therefore assumed that the main function of the porose areas, which are located on the dorsal side of the basis capituli, is the secretion of a lubricant essential for the evagination and invagination of Gené's organ (Feldman-Muhsam, 1963).

On the other hand, Atkinson & Binnington (1973) and Booth, Beadle & Hart (1984) have demonstrated that in replete female *Boophilus microplus* in which the porose areas were selectively destroyed Gené's organ continued to function normally. In addition, no significant differences in the size or hatchability of egg batches from treated and untreated females were noted. It was, however, shown that hexane extraction of the surface lipids of eggs exhibit UV absorption characteristic of $\Delta^{2,4,6}$ triene steriods. Furthermore, when eggs from cauterised *B. microplus* are incubated at 27 °C and 85 % RH the hatched egg shells contain little or no triene, in contrast to egg shells from untreated females.

Since the induction period for autoxidation of the triene steroids in the wax of eggs obtained from normal ticks was significantly longer irrespective of the abiotic environmental conditions, it was proposed that the secretion from the porose areas is incorporated in the waxy layer of the eggs where it functions to prevent the autoxidation of the unstable triene steroids (Atkinson & Binnington, 1973).

In *Rhipicephalus evertsi evertsi* with blocked porose areas, oviposition is likewise not impeded. Furthermore, contrary to expectations deduced from the results of Atkinson & Binnington (1973), the viability of eggs obtained from cauterised ticks is identical under various abiotic conditions with respect to temperature, RH and photoperiod, to that of eggs from untreated ticks (Gothe & Nadler, 1986).

In this study, the qualitative and quantitative differences in the chemical composition of the outer surface layer of eggs from *R. evertsi evertsi* with blocked and unblocked porose areas were investigated in an attempt to elucidate the possible function of the porose area secretion during oviposition.

MATERIALS AND METHODS

Origin and rearing of ticks

Laboratory colonies of *R. evertsi evertsi*, originally established from ticks collected in Nelspruit, Republic of South Africa in 1971, were maintained and reared in an incubator at 27 °C and 90–95 % RH.

Electrocautery of the porose areas of replete R. evertsi evertsi *and collection of eggs*

Female ticks were fed to repletion on black-headed sheep reared under tick-free conditions. The porose areas were then selectively blocked by cauterisation. Thereafter, the ticks were placed individually in glass vials and kept at 27 °C and 90–95 % RH in an incubator.

For each experiment eggs were collected from at least 10 females. These eggs were either pooled daily or in 3 batches collected during each third of the oviposition period. Alternatively, all the eggs laid by a single batch of females were pooled. Egg batches which were used as controls were likewise collected from analogous *R. evertsi evertsi* females in which the porose areas were not blocked.

Hexane, ethanol and butanol extraction of the outer chorion surface layer

Batches of eggs obtained from *R. evertsi evertsi* females in which the porose areas functioned normally (AP^+ eggs), and from *R. evertsi evertsi* in which the porose areas were destroyed by electrocautery (AP^- eggs) were extracted separately for 15 min at room temperature by gentle agitation with hexane followed by a 30 min extraction with ethanol. Fresh batches were also extracted in the same way for 15 min with n-butanol. All solvents were of analytical grade and glass-distilled before use. In each case 1 mℓ extractant was used per 10 mg egg mass. After filtration through a Whatman No. 40 filter paper under vacuum, the extract was dried in a

[1] Department of Biochemistry, University of Pretoria, Pretoria 0002, Republic of South Africa

[2] Institut für Vergleichende Tropenmedizin und Parasitologie, 8000 München, Federal Republic of Germany

Received 24 March 1986—Editor

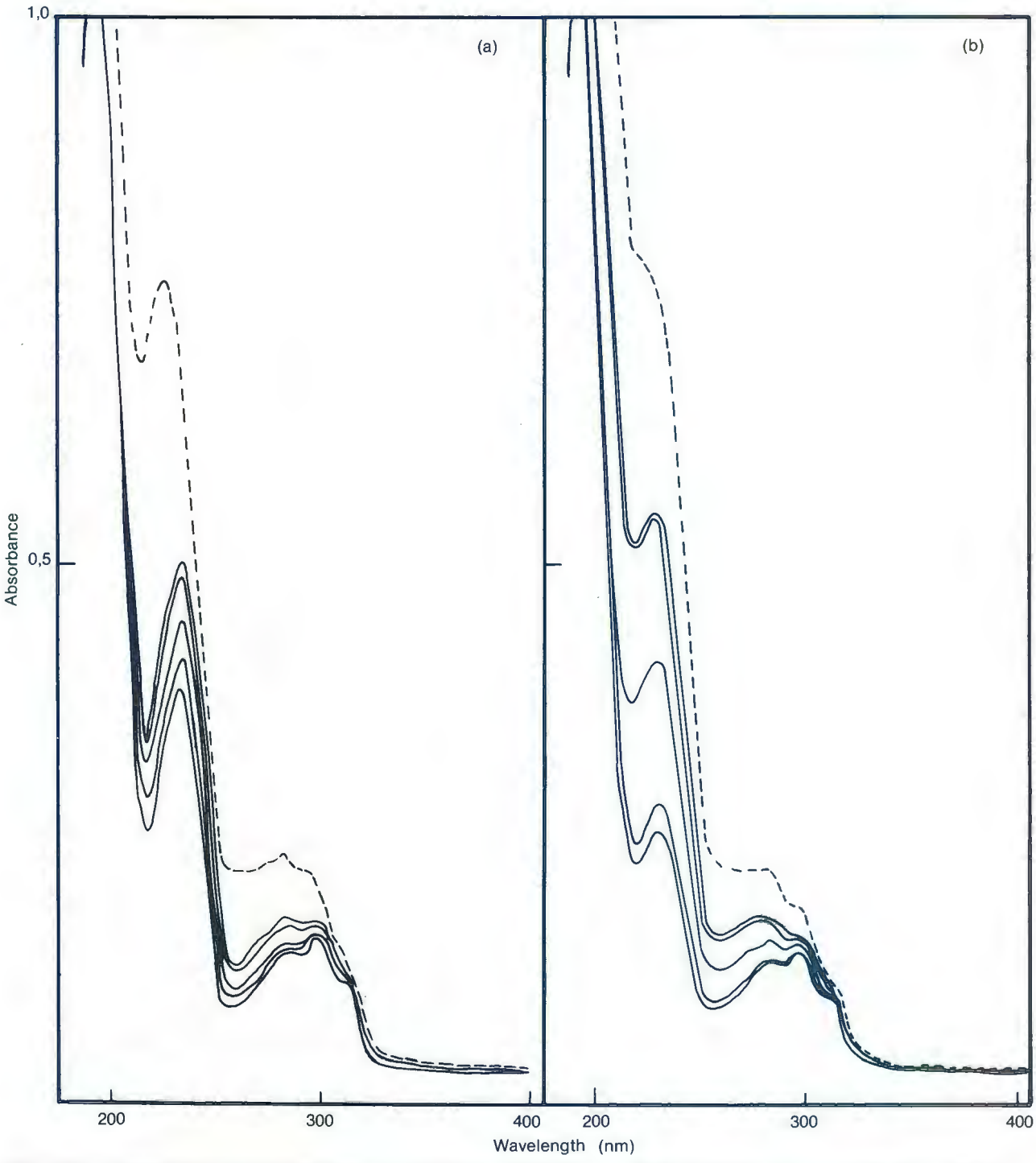

FIG. 1 a & b. Increase in absorbance of hexane extracts of AP^+ (1a) and AP^- (1b) eggs with time. Spectra are shown respectively immediately after extraction, after 36 hours, after 4 days and after 2 and 3 months. The changes after exposure to 70 °C for 3 hours are shown by the dotted lines

Buchi-rotary evaporator at 40 °C, the residue dissolved in 6 mℓ extractant and passed through a Millex-SR solvent resistant 0,5 μm filter. The filtered fractions were then dried with nitrogen and stored at −10 °C.

Initially, eggs collected on each individual day during oviposition were extracted separately. Since no differences were observed according to the analyses outlined below, the total egg mass obtained during the entire oviposition period was used.

Ultraviolet spectra

A Beckman Model 25 Spectrophotometer was used to determine whether differences existed in the components extracted by hexane and ethanol and to investigate if time-dependent changes occur. Spectra were obtained at various intervals up to 3 months after oviposition. Between measurements, samples were stored at 4 °C. The influence of temperature was also investigated by heating samples for 3 hours at 70 °C.

High performance liquid chromatography (HPLC)

HPLC was performed with a Model 322 Beckman Altex instrument equipped with a UV monitor. In all cases a 20 μℓ loop and flow rate of 1 mℓ/min was used. For chromatography of the hexane extracts, an Altex-silica gel column (particle size: 5 μm and column dimension of 25 × 0,46 cm) was employed. The eluent was hexane:isopropanol (95:5). The number of plates as determined with vitamin A standard was 4010 ± 8,7.

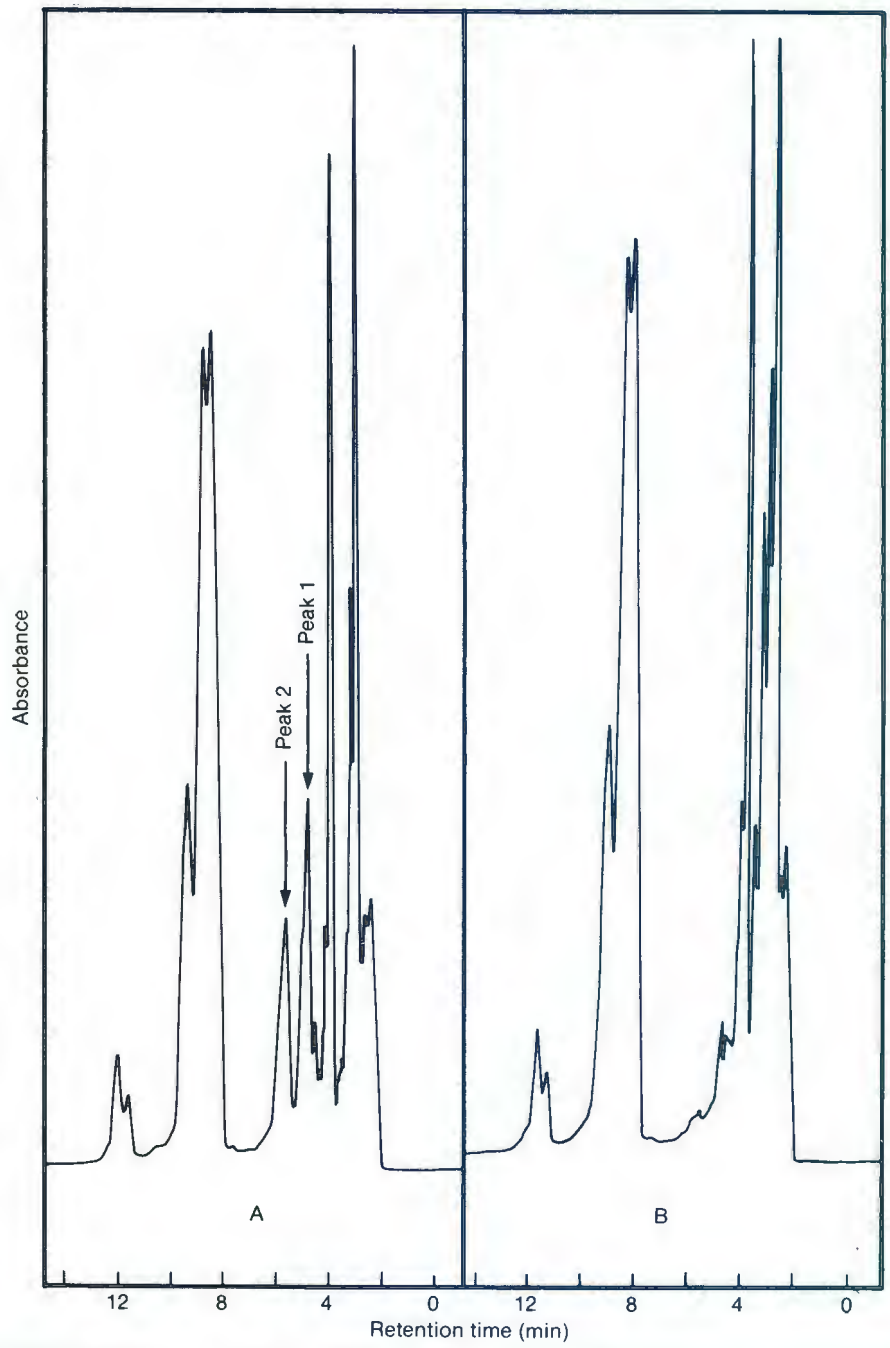

FIG. 2. Comparison of HPLC elution diagrams of AP^+ (profile A) and AP^- (profile B) egg hexane extracts. Arrows in profile A indicate additional peaks in AP^+ egg extracts

Ethanol extracts were chromatographed on a RP-8 Lichrosorb column (particle size: 10 μm and column dimension: 25 × 0,46 cm). The number of plates as determined with vitamin D was 1672 ± 5. The eluent was methanol:water (70:30) containing 1 % acetic acid. Absorbancies were monitored at 280 nm and 254 nm.

Thin layer chromatography

Silica plates (MQN Silgur-25, UV 254 with concentrating zone) were used. Spots were detected under UV light source at 254 nm and 366 nm or with a ninhydrin spray reagent (Famy, Niederwieser, Pataki & Brenner, 1961).

Protein determination

For the determination of protein, the modified Lowry method was used (Lowry, Rosenbrough, Farr & Randall, 1951). Colour development was monitored at 740 nm.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in a Pharmacia GE-2/4 electrophoresis apparatus using a 4–30 % gradient (Margolis & Kenrick, 1967). Samples were dissociated in the presence of SDS and dithiothreitol at 95 °C (Fairbanks, Steck & Wallach, 1971). Protein standards were used in order to estimate the molecular mass of unknowns (Weber & Osborn, 1969).

Mass spectroscopy

For mass spectroscopy, a Varian MAT 212 mass spectrometer was used.

RESULTS

Moisture content of eggs

The moisture content of AP^+ and AP^- eggs was found to be 65 % and 62 % respectively.

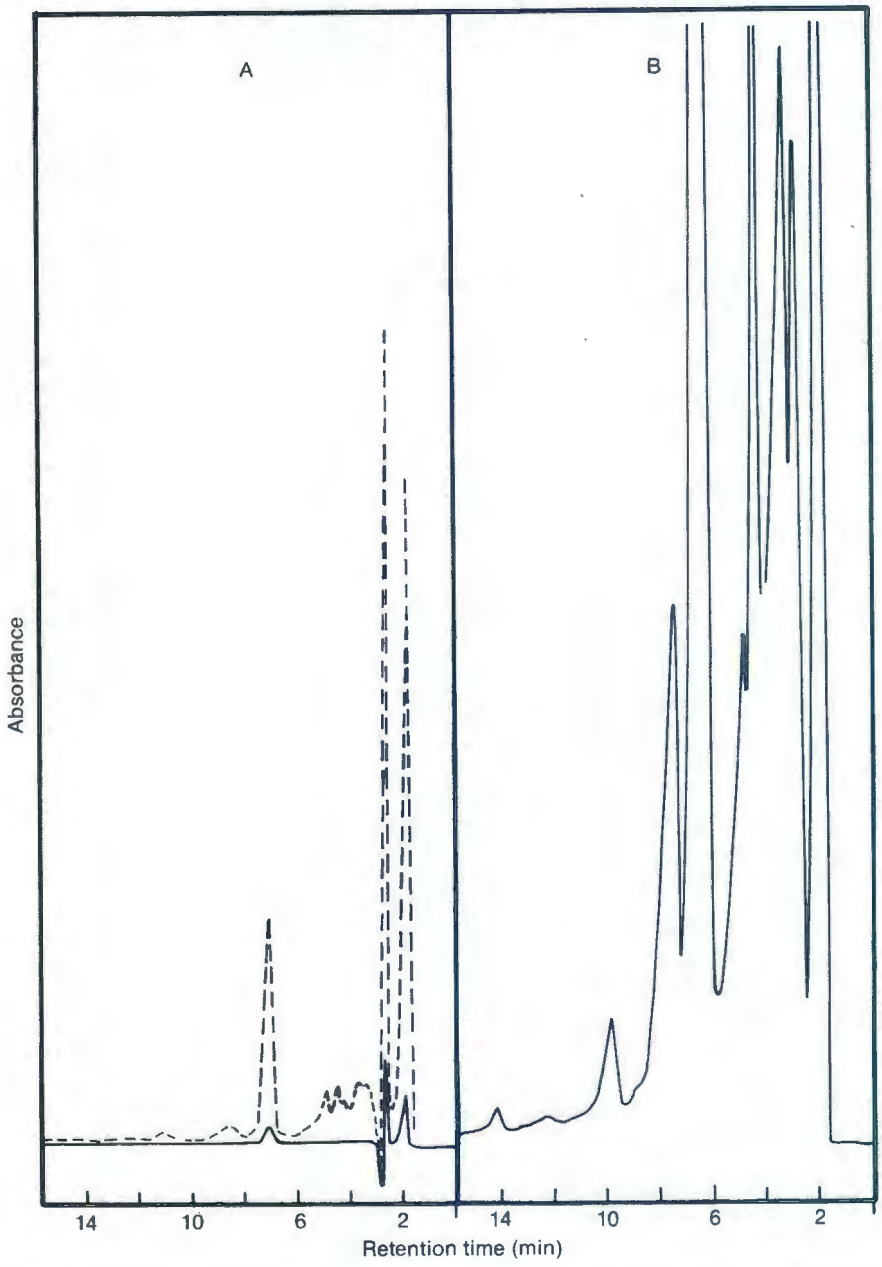

FIG. 3. Comparison of HPLC elution profiles of ethanol extracts obtained from AP^+ and AP^- eggs (profiles A & B respectively).—sensitivity 0,16 AUFS; --- sensitivity 0,02, AUFS

Hexane, ethanol and butanol extractions

A higher yield was obtained by hexane than by ethanol extraction. Furthermore, ethanol extracted more than 10 times as much material for AP^+ than from AP^- eggs. Ethanol extracts from AP^+ eggs were distinctly yellow in colour whereas extracts from AP^- eggs were colourless. A yellow precipitate was observed in hexane extracts from AP^+ eggs. This was absent in extracts made from AP^- eggs.

The UV spectra of the hexane extracts are shown in Fig. 1. Marked changes in the spectra occurred with respect to time in the AP^- eggs, especially at 230 nm. Smaller changes with respect to AP^+ eggs at this wavelength are evident. Only minor changes are noticed in the 290–320 nm region of both AP^+ and AP^- eggs. For extracts of AP^+ as well as AP^- eggs, the effect of high temperature is apparent at wavelengths below 300 nm.

The shapes of the UV spectra of ethanol extracts obtained from AP^+ and AP^- eggs were similar. Only slight changes occurred upon heat treatment. No changes were observed with respect to time.

High performance liquid chromatography

Slight quantitative differences were observed in the elution diagrams obtained from hexane extracts of AP^+ and AP^- eggs. However, qualitative differences are evident (Fig. 2); 2 additional peaks in the extracts of AP^+ eggs are apparent. The 2 peaks that were eluted at 5 and 6 min were collected and rechromatographed to estimate their purity. Single peaks were obtained. They were further investigated by TLC and MS.

Large quantitative differences were found in the ethanol extracts from AP^+ and AP^- eggs as shown by the HPLC elution diagrams (Fig. 3); up to a 230-fold higher content of the peaks was observed for AP^+ eggs. Peaks 6, 7, 8 and 9 reacted positively to ninhydrin. Peaks 4, 6 and 7 were isolated in pure form by rechromatography and analysed by TLC and MS.

The elution pattern of the yellow residue obtained in the hexane extract of AP^+ eggs was qualitatively identical to the ethanol extracts.

Thin layer chromatography

TLC patterns of the hexane extracts revealed 7 spots under UV light in the case of AP^+ eggs and 5 in the case of AP^- eggs. None of the spots reacted positively towards ninhydrin. The 2 additional components observed in the AP^+ eggs correspond to the 2 compounds isolated from the hexane extracts by means of HPLC (Fig. 2).

Large quantitative differences were observed in the TLC patterns of the ethanol extracts. A 30-fold concentration of extracts from AP^- eggs was necessary to obtain visible spots. Qualitative differences were also evident; AP^- eggs showed 4 fewer spots that AP^+ eggs. Of the 10 components seen in the latter eggs, 6 showed a positive ninhydrin reaction whereas only 1 of the 6 spots of AP^- eggs was ninhydrin positive.

Protein determination and SDS-polyacrylamide electrophoresis

Considerably more protein was shown to be present in both the ethanol and butanol extracts obtained from AP^+ eggs, compared with that from AP^- eggs.

Electrophoretic separation of proteins in the butanol extracts revealed 3 bands corresponding to molecular masses of 145 000, 66 000 and 59 000 in both the AP^+ and AP^- egg extracts. Quantitative differences were noted, however, as expected, from results of the protein determination.

Mass spectroscopy

Mass spectroscopy of the 2 isolated peaks obtained after HPLC of hexane extracts from AP^+ eggs showed results which are difficult to interpret, but there is an indication of the presence of aliphatic chains in both components. Phenolic rings are also evident. Peaks 4, 6 and 7, obtained after HPLC of ethanol extracts from AP^+ eggs, showed fragmentations characteristic of aliphatic chains. More detailed deductions concerning the structure of all these components cannot be made at this stage.

DISCUSSION

Our investigations have shown that distinct qualitative and quantitative differences exist with respect to components extracted by hexane, ethanol and butanol from the outer surface chorion layer of AP^+ and AP^- eggs. The UV spectra of hexane extracts obtained from AP^+ and AP^- eggs were found to be quite similar. The absorptions, occurring at 294, 306 and 320 nm, which are characteristic of steroid 2, 4, 6-trienes (Atkinson & Binnington, 1973; McCamish, Cannell & Cherry, 1977), changed only slightly with respect to time and high temperature.

An increase in absorbance, especially in the case of AP^- eggs, occurred at wavelengths in the 200–290 range after heat treatment. This is in clear contradiction to the findings of Atkinson & Binnington (1973), who reported a distinct decrease in absorbances at 294, 306 and 320 nm of hexane extracts obtained from eggs of *Boophilus microplus* females in which the porose areas were destroyed by electrocautery. These authors concluded that the secretion of the porose areas prevented the autoxidation of steroid trienes and other unstable compounds secreted by Gené's organ. This appears not to be the case for *R. evertsi evertsi* regarding compounds with absorbances between 294 nm and 320 nm since they were found to be stable in the absence of porose area secretion. Furthermore, the results of Atkinson & Binnington (1973) showed a decrease in absorption in the region of 220 nm–250 nm at high temperatures whereas the present study showed a marked increase in absorbances in this wavelength range.

Apart from quantitative differences, UV spectra of ethanol extracts from AP^+ and AP^- eggs were similar. Only minor changes were recorded following heat treatment in both egg samples.

Major differences were observed in the hexane, ethanol and butanol extracts obtained from AP^+ and AP^- eggs. Mass yields of the hexane extracts were similar for both and correspond to data obtained by McCamish *et al.* (1977) for *B. microplus* eggs. HPLC also revealed no quantitative differences. Two additional peaks were, however, observed which originate from porose area secretions. Apart from the indication by MS of aliphatic chains and phenolic rings present in both peaks, no details concerning their structure can be provided. The ethanol and butanol extracts revealed no qualitative differences. The conspicuous quantitative differences, however, clearly show that the porose area secretion contains, amongst other components, ninhydrin positive components as shown by TLC. The butanol extracts also confirm that at least some of these are proteinaceous in nature, as shown by Lowry protein determination and Coomassie staining after electrophoretic separations. The quantitative differences observed in these extracts cannot be explained by the moisture content of eggs since no major difference was observed for AP^+ and AP^- eggs. The moisture values are similar to those reported for *R. sanguineus* by Regendanz & Reichenow (1931).

In conclusion, it is evident that the porose area secretion of *R. evertsi evertsi* contains at least 2 hexane and several ethanol or butanol extractable components of which some are of proteinaceous and others of aliphatic or phenolic character. The function of these substances, and that of the porose area *per se*, during oviposition by *R. evertsi evertsi* remains unsolved, however. Contrary to the findings in previous investigations, Gené's organ continued to function normally in ticks with blocked porose areas and the viability of eggs was unchanged (Gothe & Nadler, 1986). No apparent autoxidation of the triene steroids occurred in eggs obtained from females in which the porose areas were blocked.

REFERENCES

ATKINSON, P. W. & BINNINGTON, K. C., 1973. New evidence on the function of the porose areas of ixodid ticks. *Experientia*, 29, 799–800.

BABOS, S., 1964. Die Zeckenfauna Mitteleuropas. Budapest: Akadémiai Kiadò.

BONNET, A., 1906. On the anatomy and histology of the Ixodidae. *Annals and Magazine of Natural History*, 27, 509–511.

BONNET, A., 1907. Recherches sur l'anatomie comparée et le développement des Ixodidés. *Annales de l'Université de Lyon*, 20, 1–185.

BOOTH, T. F., BEADLE, D. J. & HART, R. J., 1984. Ultrastructure of the accessory glands of Gené's organ in the cattle tick, *Boophilus*. *Tissue Cell*, 16, 589–599.

DOUGLAS, J. R., 1943. The internal anatomy of *Dermacentor andersoni* Stiles. *University of California Publications in Entomology*, 7, 207–271.

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

FALKE, 1931. Beiträge zur Lebensgeschichte und zur postembryonalen Entwicklung von *Ixodes ricinus* L. *Zeitschrift für Morphologie und Ökologie der Tiere*, 21, 567–607.

FAMY, R. A., NIEDERWIESER, A., PATAKI, G. & BRENNER, M., 1961. Dunnschicht-Chromatographie von Aminosauren auf Kieselgel G. *Helvetica Chimica Acta*, 44, 2022–2026.

FELDMAN-MUHSAM, B. & HAVIVI, Y., 1960. Accessory glands of Gené's organ in ticks. *Nature, London*, 187, p. 964.

FELDMAN-MUHSAM, B., 1963. Function of the areae porosae of ixodid ticks. *Nature, London*, 197, p. 100.

GOTHE, R. & NADLER, G., 1986. Zur Mechanik der Oviposition und funktionellen Bedeutung der Areae porosae bei *Rhipicephalus evertsi evertsi* Neumann, 1887. *Berliner und Münchener tierärztliche Wochenschrift*, (In press).

LAGUTENKO, Y. P., 1969. On morphology and topography of dermal sensory organs in ixodid ticks. I. *Hyalomma asiaticum*. (In Russian; English summary). *Zoologicheskii Zhurnal*, 48, 74–84.

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. & RANDALL, R. J., 1951. Protein measurement with Folin phenol reagent. *Journal of Biological Chemistry*, 193, 265–275.

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

McCAMISH, M., CANNELL, G. R. & CHERRY, L. M., 1977. The nonpolar egg wax lipids of cattle tick, *Boophilus microplus* (Canestrini). *Lipids*, 12, 182–187.

NORDENSKIÖLD, E., 1909. Zur Anatomie und Histologie von *Ixodes reduvius*. II. *Zoologischer Jahresbericht Abteilung Anatomie*, 27, 449–464.

NORDENSKIÖLD, E., 1911. Zur Anatomie und Histologie von *Ixodes reduvius*. III. *Zoologische Jahrbücher, Jena, Abteilung Anatomie*, 32, 77–106.

POMERANTZEW, B. I., 1950. Ixodid ticks (Ixodidae). *Fauna USSR Arachnida*, 4, 1–224.

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

SAMSON, K., 1909. Zur Anatomie und Biologie von *Ixodes ricinus* L. *Zeitschrift für wissentschaftliche Zoologie*, 93, 185–236.

SCHULZE, P., 1942. Über die Hautsinnesorgane der Zecken, besonders über eine bisher unbekannte Art von Arthropoden- Sinnesorganen, die Krobylophoren. *Zeitschrift für Morphologie und Ökologie der Tiere*, 38, 379–419.

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

WILLIAMS, S. R., 1905. The anatomy of *Boophilus annulatus* Say. *Proceedings of the Boston Society of Natural History*, 32, 313–334.

WOLLEY, T. A., 1972. Some sense organs of ticks as seen by scanning electron microscopy. *Transactions of the American Microscopical Society*, 91, 35–47.

Printed by the Government Printer, Private Bag X85, Pretoria, 0001