PART 2

Evolution of the tick lipocalin family

The next three chapters concern the evolution of the tick lipocalin superfamily and their diverse role in host pathogenesis, hemostasis and salivary gland granule biogenesis.

Chapter 5 investigates the localization of savignygrin and apyrase to specific salivary gland granules. While not part of the tick lipocalin family, this chapter serves as a introduction to salivary gland biology and ultrastructure. It shows that anti-hemostatic factors are synthesized in specific cell types where they are localized to granules. These results indicate that there are more than the previously described three cell types of argasid ticks present.

Chapter 6 describes the identification of putative proteins (TSGPs) involved in granule biogenesis based on their abundance in salivary glands and granules. These proteins are characterized in terms of their molecular properties and their possible involvement in granule biogenesis is considered. Two of these proteins are also identified as being toxins that affect the cardiac system of the host rather than being causative agents of tick paralysis. The absence of both toxins in *O. moubata* has interesting implications for the origins of tick toxicoses.

Chapter 7 indicate that the proteins described in the previous chapter are all part of the tick lipocalin family. Their relationship to hard tick lipocalins is investigated and the implications of the lipocalin fold for structure and function is considered. A platelet aggregation inhibitor (savignygen), a homologue of moubatin, a platelet aggregation inhibitor from the soft tick *O. moubata*, was partially characterized. This inhibitor shows close homology to the TSGPs.

Chapter 5: Localization of anti-hemostatic factors to specific salivary gland granules

5.1.1 Introduction: Tick salivary glands

During tick feeding, host fluid is imbibed and saliva is secreted in alternate directions, through a common buccal canal. Saliva originates from paired, alveolar salivary glands lying anterolaterally and extending posteriorly from both sides of the body, resembling bunches of grapes (Fig. 5.1) (Sauer *et al.* 1995). General salivary gland structure and biology have been described in several reviews (Kemp, Stone and Binnington, 1982; Fawcett, Binnington and Voight, 1986; Sauer *et al.* 1986; Sonenshine, 1991; Sauer *et al.* 1995), while salivary gland ultrastructure has been described for the soft ticks *A. arboreus*, *A. persicus* and *O. moubata*, (Roshdy and Coons, 1975; El Shoura, 1985). Several gross differences can be observed between hard and soft tick salivary glands. (1) Soft tick salivary glands are surrounded by a myo-epithelial sheath, that is absent in hard tick salivary glands (L.B. Coons, personal communication). (2) During the extended feeding period observed for hard ticks, the ultrastructure of their granular cells undergoes a great change in structure, size and protein expression levels. The granular cells of soft ticks, which feed more than once for short periods do not undergo such changes.

Fig. 5.1: Salivary glands from the soft tick *O. savignyi*. Note the pair of large (5 mm X 1 mm X 1 mm), white salivary glands lying in an oblique position (Photograph, B.J. Mans, 1996).

5.1.2 Agranular acini

Salivary glands are composed of agranular (type I) and granular acini. Type I acini are found in the anteromesal or anterior portion of the salivary glands of soft and hard ticks, respectively. Type I acini is multi-cellular and include a single large lamellate cell as well as a constrictor cell and a variable number of peripheral lamellate and peritubular cells. The morphology of these acini is similar to that of the salt glands from marine birds and it has been proposed that they function in the regulation of tick water balance. These acini might function by active water sorption from the atmosphere through secretion of hygroscopic substances onto the hypostome and subsequent swallowing of the diluted salt solution (Needham and Teel, 1986).

5.1.3 Granular acini

Morphological studies have revealed significant differences between the granular acini of hard and soft ticks. Argasid salivary glands consists of a single granular acini, with three granular cell types while salivary glands of hard ticks are more complex with at least three granular acini. Type II acini consist of six granular cell types (a, b, c1-c4) and type III acini of three granular cell types (d, e, f). Type IV acini only occur in males (Sauer *et al.* 1995).

5.1.4 Argasid granular acini structure

The classical histological and histochemical studies of the salivary glands of *A. persicus*, provided a detailed view of argasid salivary gland structure. It was shown that at least three distinct granular cell types existed (Roshdy, 1972). This was confirmed during ultrastructural studies of the salivary glands of *A. arboreus* (Roshdy and Coons, 1975). The granules of these cell types differed in electron density and submicroscopic appearance. Cell types a and b contain granules of varying electron density, while cell type c contain granules with an electron dense core (Fig. 5.2). Studies of the ultra-structure of *O. moubata* showed similar granule types (El Shoura, 1985). The "a" cell granules contains an electron dense core similar to "c" cell granules of *A. persicus* and are secreted within 5 minutes of attachment to the host. The "b" cells granules are large and electron dense, while the "c" cells are small (1 μm diameter) with both electron-

lucent and dense zones. For practical purposes, the descriptions of El Shoura (1985) will be used in this discussion as *O. moubata* is the closest to *O. savignyi* and no equivalent for the "c" cell granules have been found in Argas ticks.

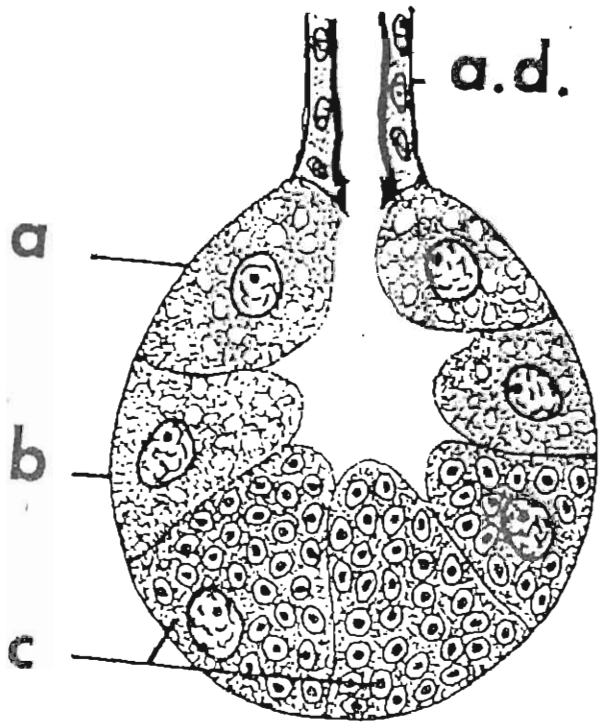

Fig. 5.2: A schematic representation of the granular acini of the argasid tick, *A. persicus*. Adapted from Roshdy (1972). In a toluidine blue stain, "a" and "b" cell type granules appear unstained, while "c" cell type granules show dense core granules. Adlumenal cells (a.d.) line the salivary gland duct. Note the lumen leading into the salivary gland duct, into which granules are secreted during feeding.

5.1.5 Biological significance of salivary gland proteins during feeding

The use of pharmacological stimulants such as pilocarpine (Howell, 1966b) or dopamine (Kaufman, 1977) that induces salivation of ticks, are normally used to infer physiological secretion of a compound by the tick. Proof of secretion is an essential requirement in the characterization of such bio-active compounds, as this is the only way to ascribe possible biological function in tick-host interactions (Law, Ribeiro and Wells, 1992). Whole salivary glands have also been used as a source of bio-active compounds, the rationale being that any bio-active components derived from salivary glands are liable to be

secreted during feeding. This is a rather naïve way to characterize secretory components and require more specific proof that they are indeed secreted. A specific indication of secretion can be targeting to the secretory pathway, as exemplified by the ER-signal peptide or specific localization to secretory granules, which are secreted during feeding.

5.1.6 Evolutionary significance of tick salivary glands

A study into the evolution of secretory components cannot fail to consider the biology of the salivary glands and their possible influence on tick evolution. For example, the number of granular cells differs in hard and soft ticks and can be distinguished based on morphological, histochemical and immunochemical means. These differences must obviously be accounted for by the expression of different proteins or synthesis of other chemical entities, such as carbohydrates by the different cell types. In evolutionary terms the issue is raised whether these different cells all evolved from a single ancestral cell type and if so what effect this had on tick adaptation to a blood-feeding environment. This could be investigated by comparative ultrastructural studies of both hard and soft tick salivary glands and salivary glands from *Nuttalliella namaqua*, as well as glands from their closest sister-group, the holothyrids. If holothyrids have less complex glands this could indicate that tick salivary glands evolved in complexity only after or during their adaptation to a blood-feeding environment. This hypothesis is supported by the fact that hard and soft tick salivary glands differ in complexity and number of granular cells.

In hard ticks, in which extensive changes occur in the glands during feeding, it may be argued that different granular types function at different times during feeding. However, the reason why soft ticks need more than one granular cell type is more problematic. They feed fast, so that granule contents are probably secreted at the same time or very shortly after one another, making more than one granular cell almost redundant. It can so far only be assumed that the different granular cell types perform specific functions.

5.1.7 Salivary glands of other arthropods

The presence of salivary glands and the secretion of salivary gland components is by no means a unique property of ticks, but can be found throughout the arthropoda. As such,

the question of how many processes are shared in salivary gland biology between ticks and other arthropoda and how many processes are unique to ticks are raised. Examples of processes include mechanisms of granule secretion, targeting to the secretory granules and granule biogenesis. Investigations into these properties can indicate whether salivary glands evolved unique ways to accommodate a blood-feeding environment.

5.1.8 Localization of components to granule types

Elucidation of the issues raised is a daunting task at the least and one could expect them to be answered over a number of years of hard and painstaking work. As such, future progress can only be expected with the development of techniques able to distinguish individual granular cell types on an analytical level. Until then, individual localization of different bio-active compounds to one or more granular cell types can contribute to an understanding of how these cells differentiated into such a complex organ as the modern tick salivary gland. It would also allow an initial, although slow step, in the cataloging of granule composition and will give an indication of the similarities and differences that exist between different granule types. This chapter explores the biological significance of savignygrin through specific localization to salivary gland granules.

5.2 Materials and Methods

5.2.1 Assay for apyrase activity

As a marker of secretory activity, the enzyme apyrase was used during this study, as it is known that this enzyme is secreted during pilocarpine stimulation (Mans, 1997). Apyrase activity was assayed as described previously (Mans, 1997; Mans *et al.* 1998a). Fractions tested (10 μl) were added to 90 μl of reaction medium (20mM Tris-HCl, 0.15M NaCl, 5mM $MgCl_2$ and 2mM nucleotide, pH 7.6) per well of a microtitre plate, covered with plastic wrap and incubated at 37 °C for 30 min. The enzyme reaction was stopped by the addition of the molybdic acid mixture (reducing solution) and free phosphate was determined as described below. The amount of phosphate released was determined from a standard orthophosphate standard curve. One enzyme unit (U) is defined as 1 μmole of inorganic phosphate released/min. A mixture (25 ml) of 2.5% molybdate (w/v) and 13.3% concentrated sulphuric acid (v/v) was added to 8 ml of a 1% ascorbic acid solution

and mixed thoroughly. Thirty-three microlitres of this reducing reagent were then added to a standard orthophosphate or sample solution (100 µl) in a microtitre well and shaken for 10 minutes after which the absorbance was read at 620 nm with a SLT 340 ATC scanner (SLT Labinstruments).

5.2.2 Purification of apyrase by HPLC

Apyrase was purified by SEHPLC and AEHPLC (Mans *et al.* 1998b). A size-exclusion column (G3000SWxl, 7.8 mmx30 cm, TosoHaas) and step gradient conditions were employed in the first purification step. SGE was applied in 20mM Tris-HCl, pH 7.6, followed by elution with 20 mM Tris–HCl, 0.15 M NaCl, pH 7.6. A flow speed of 1 ml/min was maintained for 20 min, while the effluent was monitored at 280 nm. Apyrase activity was then eluted with higher ionic strength buffer (20 mM Tris–HCl, 0.5 M NaCl, pH 7.6) from the column. Fractions from the size-exclusion column were dialyzed against 0.1 M NaCl (Slide-A-Lyzer cassettes, Pierce), before application to the AEHPLC as described in Chapter 2.

5.2.3 Preparation of anti-sera

Purified apyrase and savignygrin were prepared for immunization by tricine SDS-PAGE and Coomassie Brilliant Blue staining. Bands (~10 µg) were cut out, homogenized and suspended in 400 µl of 0.1M phosphate buffered saline, pH 7.2 (PBS). The adjuvant was prepared as described (Vulliet, 1996). New Zealand White rabbits were first bled to obtain naïve sera and then immunized with antigen emulsified in Freund's complete adjuvant. After 6 weeks the rabbits were boosted with antigen prepared in Freund's incomplete adjuvant and this schedule repeated until a specific response could be seen at x1000 dilution (~18 weeks) with western blotting. Rabbits were bled ten days after each immunization to gauge the immune response.

5.2.4 Western blot analysis of SGE

SGE (~0.5 glands) was fractionated with tricine SDS-PAGE and blotted onto Hybond-P membranes (Amersham Pharmacia Biotech) using a Trans-Blot SD Semi-Dry Transfer

Cell (Bio-Rad) blotting apparatus (20V, 45 min). Membranes were blocked with 1% non-fat milk powder in PBS for 1 hour before rotary incubation overnight in their respective anti-sera (x500 dilution of sera in 0.1% non-fat milk powder). Blots were washed in PBS (x3) and incubated with peroxidase conjugated goat anti-rabbit IgG antibody (Sigma Co., USA) for 3 hours. After washing in PBS (x3), blots were developed in 20% methanol, 0.06g chloronaphtol (Sigma Co., USA) and 60μl peroxide (SigmaCo., USA) in 100 ml PBS.

5.2.5 Secretion of salivary gland components by various stimulants

To investigate the secretion of salivary gland components, several procedures were investigated. (A) Ticks were allowed to feed to complete engorgement on rabbits, by confining them to the ears. Salivary glands were then dissected at various time intervals for EM analysis. The salivary glands of two ticks were analyzed for each time interval taken. (B) Female ticks were also injected with a 1% pilocarpine hydrocloride or dopamine solution into the genital pore with a 24G syringe (Howell, 1966b). Salivation was allowed to proceed until completion, before glands were dissected for EM analysis. (C) A backless-tick explant (Bell, 1980) was prepared by removing the dorsal cuticle and gut before bathing the glands in a 1% pilocarpine or dopamine solution. Ticks were monitored for saliva secretion and glands were removed for EM analysis. (D) Salivary glands were dissected and bathed in solutions of 1 μM pilocarpine or dopamine dissolved in salivary gland solution (20 mM TrisHCl, 0.15M NaCl, 2 mM KCl, 50 mM D-glucose, pH 7.6). After 5 minutes of incubation, the supernatant was tested for secretion of salivary gland components by assaying for apyrase. (E) Salivary glands from ten female ticks were dissected in 9% saline. Salivary glands were then transferred to 1 ml salivary gland solution. Salivary glands were sheared by repetitive trituration using a 100μl pipette. Acini were allowed to settle by gravitation and were washed 3X with 1 ml TBS. Acini were suspended in 1 ml TBS and 100 μl were added to 900μl TBS with pilocarpine or dopamine to give a final concentration of 1 μM. After incubation for 10 minutes, aliquots (10μl) were taken of the supernatant and tested for apyrase activity. Acini were also processed for SEM and TEM analysis. (F) Twenty unfed female ticks with similar masses were compared to assess the efficiency of salivary gland secretion. Ten unfed

female ticks were allowed to feed to complete engorgement on mice before dissection of their salivary glands. Salivary glands from ten unfed ticks were dissected as controls and apyrase activity as well as protein concentration were determined as described previously.

5.2.6 Preparation of tick salivary glands for immuno-localization

Salivary glands from female ticks that had not fed for at least two months were dissected in PBS and immediately fixed in 1% formaldehyde and 0.1% gluteraldehyde for 3 hours. For SEM analysis, glands were additionally fixed in 0.1% osmium tetra-oxide. Glands were then dehydrated in a sequential series of 30, 50, 70 and 100% ethanol (15 min x3) and critical point dried in CO_2, sputter-coated with gold and analyzed with a JEOL 840 SEM (5 or 30 kV). For immuno-localization and TEM analysis, glands were sequentially incubated in 30, 50, 70 and 100% LR White resin, for 15 minutes each. After 3 hours incubation in 100% LR White resin, the glands were embedded in 100% LR White and polymerized at 60 °C for 48 hours. Sections of 0.5 μm were prepared using an ultramicrotome for light-microscopy studies. Sections were either stained with a solution of 0.1% toluidine blue or were further processed for immuno-cytochemistry at the light microscope level. Sections were blocked with 1% antibody dilution solution (ADS, 1% fish skin gelatin, 10 min) and stained with primary anti-sera or naïve sera (200 X dilution) for 1 hour. Sections were washed 3 X with ADS before incubation with biotinylated goat α-rabbit IgG (1:200) for 1 hour. Sections were washed 3 X with PBS (0.15M phosphate buffer, pH 7.4) before incubation with an avidin-biotinylated horse-radish macromolecular complex (VECTASTAIN Elite ABC Reagent, Vector Laboratories, Inc., CA, USA). Sections were washed 3 X with PBS before incubation with DAB. Color development was observed until an adequate level of marking was reached.

5.2.7 Immuno-cytochemical labeling of thin sections

Thin sections (0.1 μm) were prepared with an ultramicrotome and mounted on nickel grids for immuno-cytochemical labeling. To remove excess aldehyde groups, thin sections were blocked in 50 mM glycine for 5 minutes. Non-specific binding of

antibodies was prevented by blocking in 10% fetal calf serum. Sections were washed in PBS (x3) and then incubated in prepared anti-serum (x500 dilution) for 60 minutes. Anti-sera were changed every 30 minutes. After washing in PBS (x3) the sections were incubated with gold conjugated (10 nm) goat anti-rabbit serum (Sigma) at x500 dilution for 60 minutes and then washed in PBS (x3) before fixation in 0.1% glutaraldehyde for 5 min. The sections were washed in PBS (x5) followed by water (x5) before contrasting with uranyl acetate. It was found that with additional contrasting in lead citrate, no morphological differences could be detected for different granules. Samples were analyzed with a Philips 301 TEM.

5.2.8 Immuno-fluorescent localization of savignygrin, using confocal microscopy

Fixed glands were permeabilized in a graded series of methanol (0-100%) before rehydration in PBS. To minimize auto-fluorescence from glutaraldehyde fixation, glands were incubated overnight at 4 °C in 100 mM $NaBH_4$ with the Eppendorf caps open. Glands were washed 3 X with TBSN buffer (10 mM Tris-HCl, pH 7.4, 155 mM NaCl, 0.1% Triton X-100) for 90 minutes with constant rotation before blocking with 2% BSA in TBSN. Glands were incubated with 50 X dilution of primary anti-sera or naïve sera in PBS and 2% BSA for 8 hours. Glands were washed with TBSN for a further 48 hours with buffer washes every 8-12 hours before incubation with fluorescein-goat-α-rabbit secondary antibody in TBSN (2% BSA) for 8 hours at 4 °C in the dark. Glands were washed in TBSN for 48 hours before dehydration in a graded series of methanol (0-100%) over 90 minutes. Glands were mounted on microscope slides in clearing solution. Glands were viewed with an Olympus BX50 microscope and Bio-Rad MRC-1000 confocal laser scanning imaging system and COMOS imaging software (BioRad, Hercules, CA, USA). For low magnification a series of 2 μm optical sections (Z focus series) spanning 32 μm were merged to form a single image. For high magnification a series of nineteen, 1.34 μm optical sections (Z focus series) were merged to form a single image. Naïve sera were used to assess both specificity as well as auto-fluorescence.

5.2.9 Immuno-fluorescent localization of microtubules using confocal microscopy

Using the same procedures as for savignygrin, α-tubulin was also localized with polyclonal rabbit anti-tubulin antibodies (a kind gift from Prof. Charles Lessman, University of Memphis, Tennessee, USA).

5.2.10 Carbohydrate and membrane staining of salivary glands using the Thièry test

Thin sections (0.1 μm), embedded in LR White resin, were stained with the periodic acid, thiocarbohydrazide (TCH) –silver-proteinate method (Courtoy and Simar, 1974). Specific controls included staining with silver-proteinate alone, periodic acid oxidation and silver proteinate, TCH and silver-proteinate without periodic acid oxidation and oxidation with periodic acid and staining with TCH and silver-proteinate. Periodic acid (3%) incubation was performed for 30 minutes and sections washed three times with ddH_2O. Reactions with TCH (0.2% in 20% acetic acid) were allowed to proceed for 4 hours before washing in 10% acetic acid, 5% acetic acid and 1% acetic acid (x3). Sections were washed in ddH_2O and incubated with silver-proteinate (1%) for 60 minutes and rinsed three times in ddH_2O. Analysis was performed with TEM.

5.2.11 Scanning electron microscopy of the salivary gland membrane system

The presence of the Golgi-apparatus was investigated by SEM using the O-D-O (osmium, DMSO, osmium) method (Tanaka and Fukudome, 1991). This method is useful to observe membrane systems within the cell. Salivary glands were fixed in 0.5% gluteraldehyde, 0.5% formaldehyde (0.075 M phosphate buffer (PB), pH 7.4) before post-fixation with 1% osmium tetroxide for 2 hours. After rinsing with buffer the glands were immersed for 30 minutes in 25% and 50% dimethyl sulfoxide (DMSO), respectively. The glands were frozen on a metal plate prechilled with liquid nitrogen and were split open with a scalpel. The split pieces were immediately thawed in 50% DMSO at room temperature, before rinsing 5 X with PB until all DMSO was removed. A further fixation of 1 hour with 1% osmium tetroxide was followed by an osmium maceration step (0.1% osmium tetroxide in PB, pH 7.4 for 72 hours at 20°C). Glands were washed with buffer (3 X PB) before incubation with 1% osmium tetroxide for 1hour followed by 2% tannic acid solution overnight and again 1% osmium tetroxide for 1 hour. Glands were

dehydrated in a graded series of ethanol (0-100%) before critical point drying. Glands were coated with a ion beam gun using platinum. Samples were observed in the JEOL 840 SEM.

5.2.12 Sub-cellular fractionation of salivary gland granules using collagenase

During the characterization of the salivary gland granules (chapter 5), granules were initially prepared using collagenase treatment of glands. Twenty glands were incubated with 0.5 mg collagenase type VII (Sigma, St. Louis, MO, USA) in digestion buffer (20 mM Tris-HCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, pH 7.4). Glands were gently resuspended every 20 minutes with a 1 ml thin tip pipette. After 1 hour, fine spherical particles could be observed that sedimented under the influence of gravitation. After 3 hours of incubation the preparation was loaded onto a 0-50% preformed Percoll (Sigma Co., USA) gradient (5% per 2 ml~20 ml in a column of ~1 cm diameter). Granules were allowed to sediment using gravity and fractions were collected after the first visible sediment reached the bottom (~ 30 minutes). All fractions were tested for apyrase activity and labeled as (1) the supernatant after collagenase treatment, (2) the first fraction that sedimented, (3) first ml from the bottom, (4) second ml from the bottom, (5) to (15). Fractions were also prepared for SEM as described for platelets (Chapter 2).

5.3 Results

5.3.1 Purification of apyrase and generation of specific polyclonal anti-sera

The generation of polyclonal anti-sera specific for savignygrin commenced at a stage when the characterization of apyrase was still in progress (Mans, 1997). Localization of another anti-hemostatic component in tick salivary glands for comparative purposes were considered to be a fruitful endeavor. To this end, apyrase was purified by use of non-specific interaction with a silica-based size exclusion matrix (Mans, 1997; Mans *et al.* 1998b), followed by AEHPLC (Fig. 5.3a and Fig. 5.3b). Tricine SDS-PAGE electrophoresis of apyrase and savignygrin was used as an extra purification step for the preparation of anti-sera (Fig. 5.3c). The specificity of the prepared anti-sera was determined using Western blot analysis of crude salivary gland extract. No cross reactivity with salivary gland antigens were observed with naïve sera. Apyrase and savignygrin specific anti-sera showed labeling of a 67 kDa and 7 kDa component, respectively (Fig. 5.3d). No other cross reactivity was observed at this dilution (x1000). This was taken as an indication that the sera were mono specific for their antigens at the dilutions used.

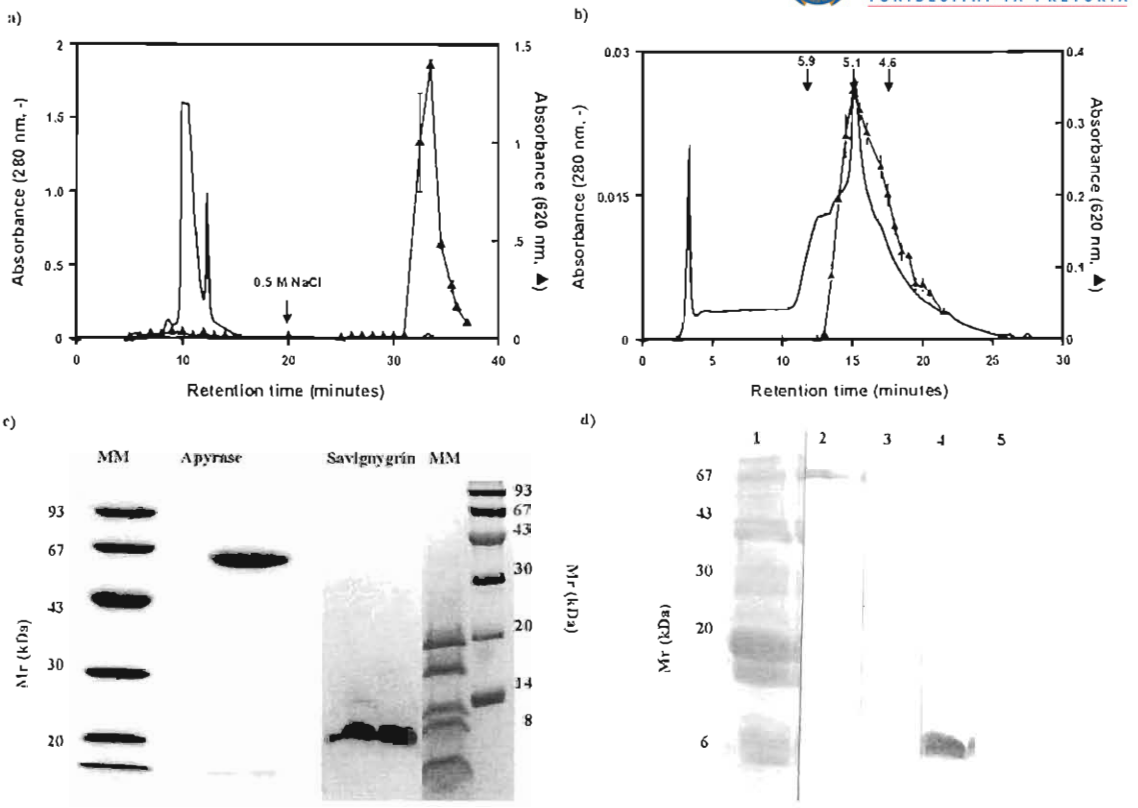

Fig. 5.3: Purification of apyrase and specificity of anti-sera. (a) SEHPLC of SGE. Apyrase was eluted from the SEHPLC matrix using a high ionic strength buffer. (b) AEHPLC of apyrase fractions from SEHPLC. Activity is indicated with triangles. (c) SDS-PAGE purification of apyrase and savignygrin for production of anti-sera. (d) Western blot analysis of α-apyrase and α-savignygrin anti-sera. Lane 1 indicates Coomassie Blue stain of salivary gland extract, lane 2 α-apyrase, lane 3 naïve sera, lane 4 α-savignygrin, lane 5 naïve sera.

5.3.2 Morphological studies on the salivary glands of *O. savignyi*

No descriptive study on the salivary gland morphology of *O. savignyi* could be located in the literature. For comparative purposes as well as for the sake of interest, morphological characterization of the salivary glands was conducted. Intact salivary glands are enclosed by a myo-epithelial sheath (L. Coons, personal communication) (Fig. 5.4a; Fig. 5.4b). Individual acini can be observed, which upon exposure to higher electron voltages show the granules inside the cells (Fig. 5.4c). At least 4 different granular cells forming pyramidal shaped cells can be distinguished. Granules ranged in size from 4-7μm in diameter (Fig. 5.4d).

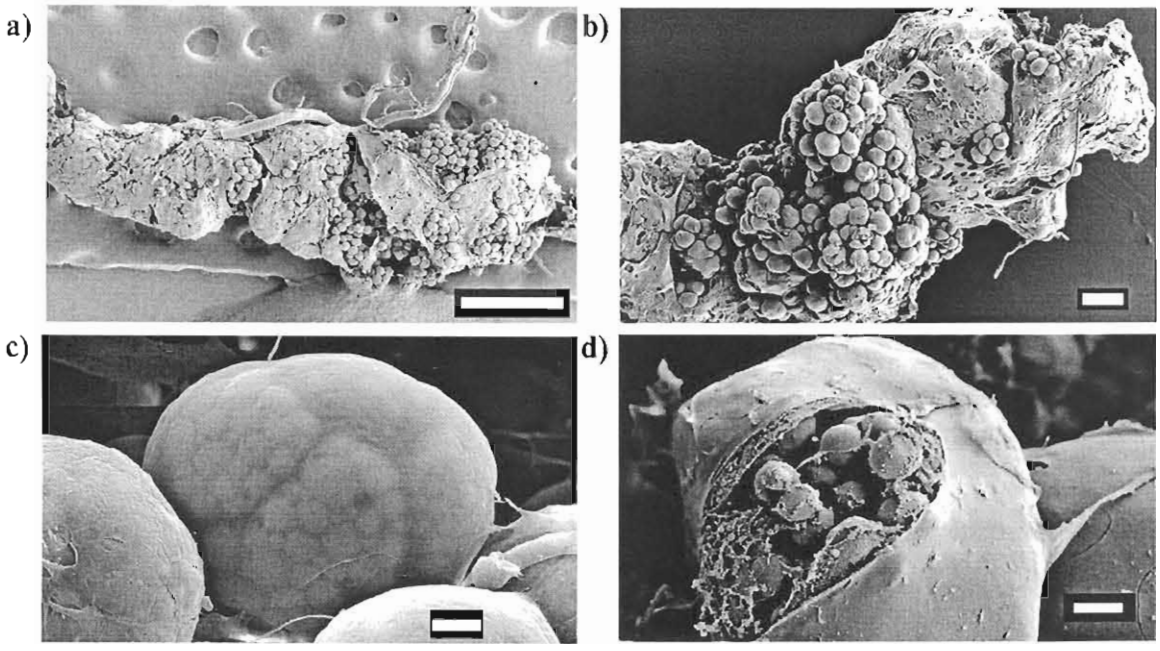

Fig. 5.4: SEM analysis of salivary glands from *O. savignyi*. (a) An intact salivary gland with the myo-epithelial sheath visible, as well as single acini (~40-70 μm diameter). Scale bar = 500 μm. (b) An intact salivary gland showing the myo-epithelial sheath as well as prominent acini, clustered together. Scale bar = 100 μm. (c) An acini at high electron voltage (30 keV) show the packing of the salivary gland granules through the cell membrane. Scale bar = 10 μm. (d) A ripped acini shows granules packaged inside a cell. Scale bar = 10 μm.

5.3.3 Ultrastructural studies of the salivary glands

Cross-sections through the salivary glands show their arrangement in acini and the pyramidal shaped granular cells (Fig. 5.5). At least three different cell types can be distinguished based on light microscopy observations. These include cell type a with dense core granules (diameter of 3-5 μm), cell type b with homogenous granules (diameter of 4-10 μm) that seem to occur more than once in an acini and cell type c with granules (diameter 1-2 μm) with electron lucent cores. No other distinctive organelles inside the granular cells could be distinguished.

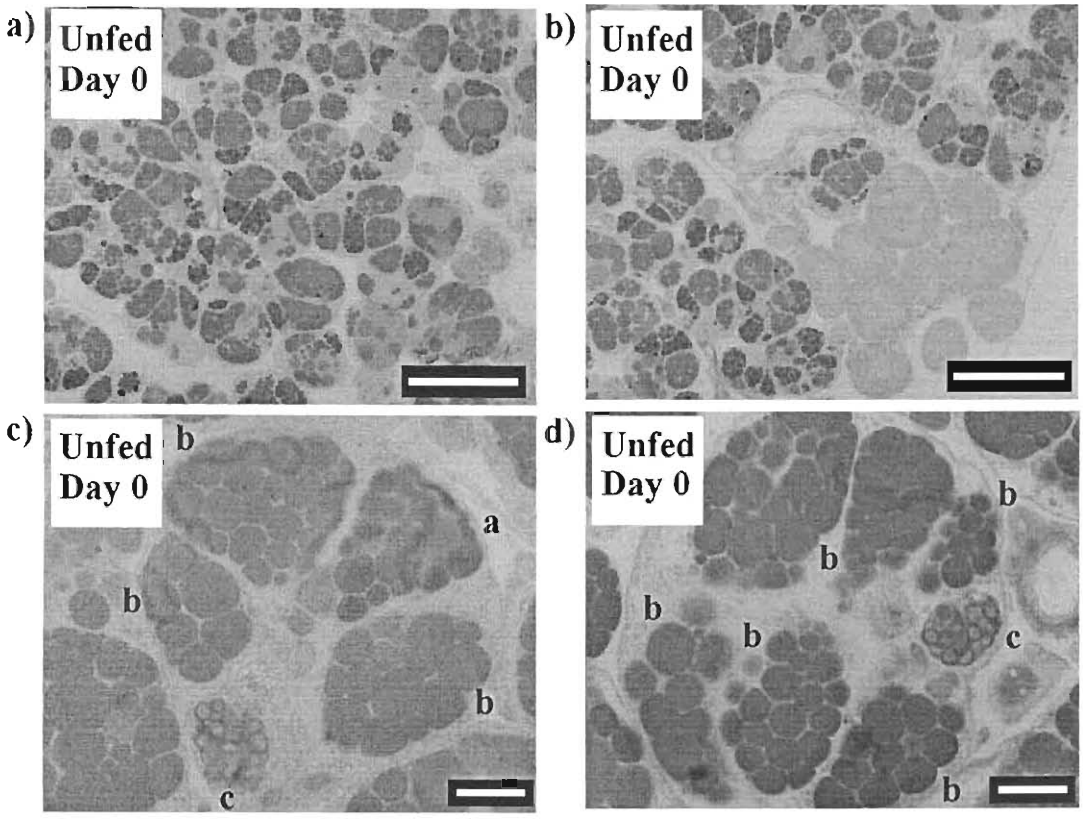

Fig. 5.5: Sections though a salivary gland cell of an unfed tick. (a) The grouping of various granular cells in acini is clear. Cells with dense core granules, as well as cells with homogenous granules can be observed. Scale bar = 100 μm. (b) Agranular acini can be observed in the lower right hand corner. Scale bar = 100 μm. (c) A single acini with at least five different granular cells. A cell with dense core granules (a), three homogenous granular types (b) and a cell with granules with an electron lucent core (c) can be observed. Scale bar = 10 μm. (d) Another acini containing at least seven cells. Scale bar = 10 μm.

5.3.4 Morphology of salivary glands from fed ticks

The hypothesis that bio-active components are stored inside the salivary gland granules, has been advanced. Salivary glands from fed ticks were analyzed to investigate secretion of granules. Salivary glands showed secretion of granules immediately after feeding, although not to the extent expected (total release of salivary gland granule contents across the whole gland). Most cells that showed granule release also retained several granules. Organelles (mitochondria) not previously observed were prominent in those cells that showed secretion (Fig. 5.6).

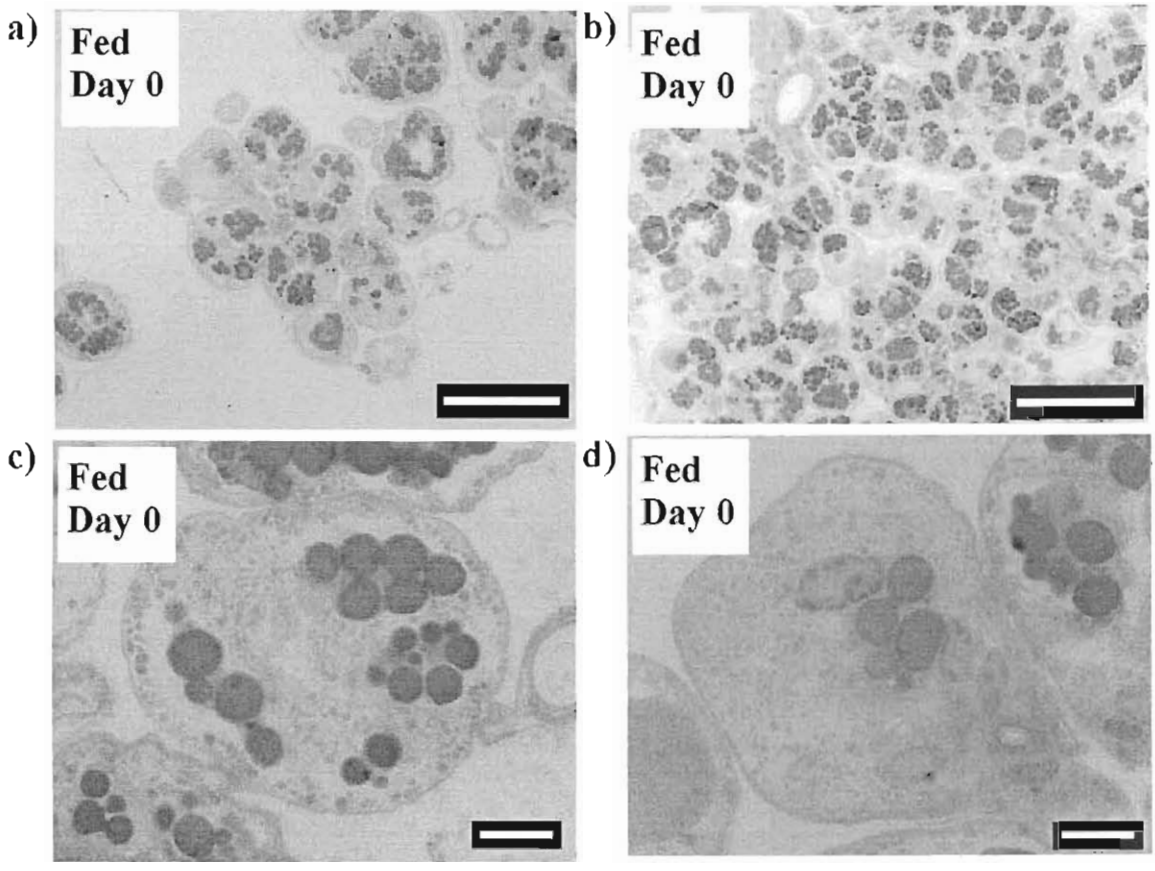

Fig. 5.6: Salivary glands from ticks dissected 10 minutes after feeding to engorgement on a rabbit. (a) Granular cells show signs of granule secretion and a dilated lumen. Scale bar = 100 μm. (b) Many acini do not show any signs of secretion or granule mobilization. Scale bar = 100 μm. (c) An acini with a reduced number of granules. Prominent are the other cellular organelles (mitochondria) visible after secretion, that's not normally observed in the salivary glands from unfed ticks. Scale bar = 10μm. (d) An acini that shows even more signs of granule secretion. Scale bar = 10 μm.

5.3.5 Morphology of salivary glands several days after feeding

Salivary glands quickly recovered and one day after feeding resumed the general morphology observed for salivary glands of unfed ticks. The only differences observed were some acini that still showed a dilated lumen, although their granular cells were still packed with granules (Fig. 5.7a; Fig. 5.7b). After 4-7 days no differences could be observed between unfed and fed tick salivary glands (Fig. 5.7c; Fig. 5.7d).

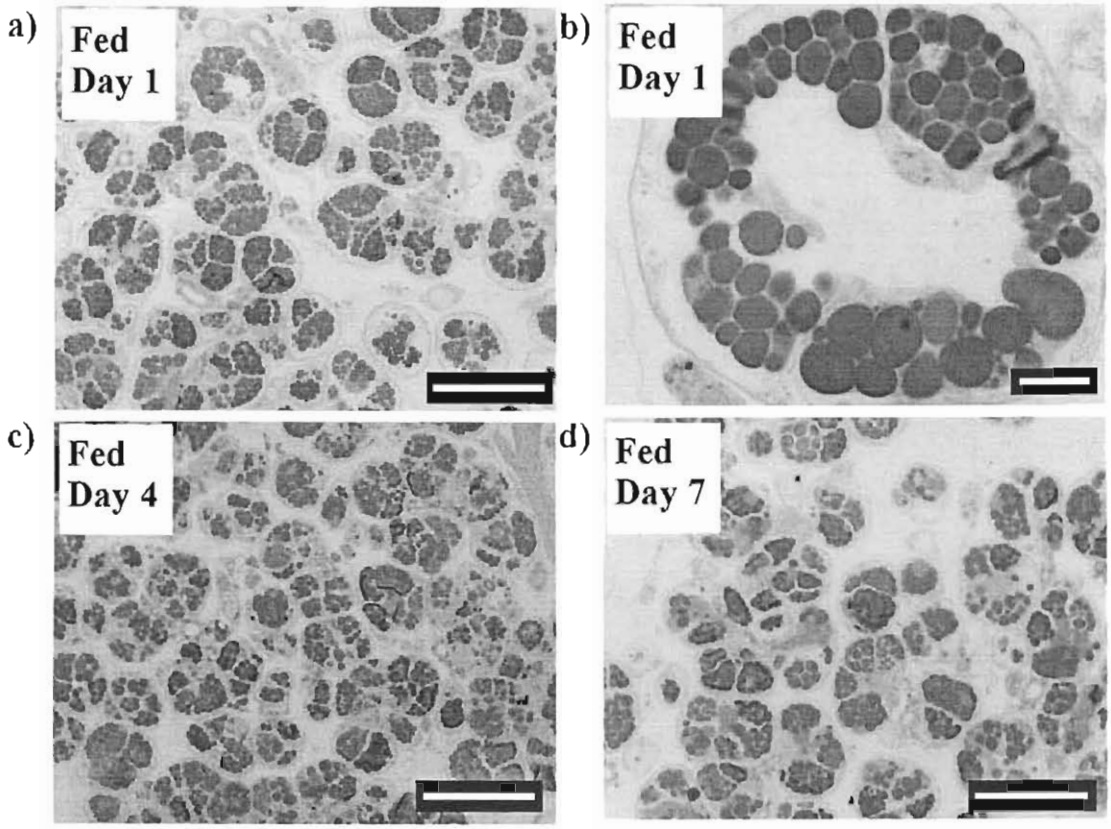

Fig. 5.7: Salivary glands from ticks at different time periods after feeding. (a) Salivary glands one day after feeding appear as those of unfed ticks, although acini with dilated lumen are still visible. Scale bar = 100 μm. (b) An acini with a dilated lumen. Scale bar = 10 μm. (c) Salivary glands four days after feeding appear as those of unfed ticks. Scale bar = 100 μm. (d) Salivary glands seven days after feeding appear as those of unfed ticks. Scale bar = 100 μm.

5.3.6 Secretion of salivary gland components induced by various stimulants

Secretion of salivary gland components was also investigated for the neurostimulants pilocarpine and dopamine. Results show that pilocarpine injected into the tick haemocoel induces salivation (Howell, 1966b). Using the same technique, dopamine failed to elicit a response. EM analysis showed that very little secretion of granules could be observed for pilocarpine-stimulated salivation. This is concurrent with results that showed that apyrase specific activity was much higher in salivary gland extracts than salivary secretion (Mans, 1997). Addition of pilocarpine to a backless tick explant induced salivation while dopamine did not. Neither pilocarpine nor dopamine induced secretion of apyrase in intact dissected salivary glands. These results did not correlate with those obtained for

hard ticks, where dopamine injected into the hemocoel or applied to isolated salivary glands induced fluid secretion (Kaufman, 1976; McSwain, Essenberg and Sauer, 1992). It was considered that the myo-epithelial sheath might hinder access to the acini in the intact salivary glands. For this purpose acini were mechanically sheared (Qian *et al.* 1998) before addition of pilocarpine or dopamine. Surprisingly dopamine induced higher secretion of apyrase than did pilocarpine, which was only slightly more than the background (Fig.5.8). EM analysis of these acini revealed no detectable secretion of granules.

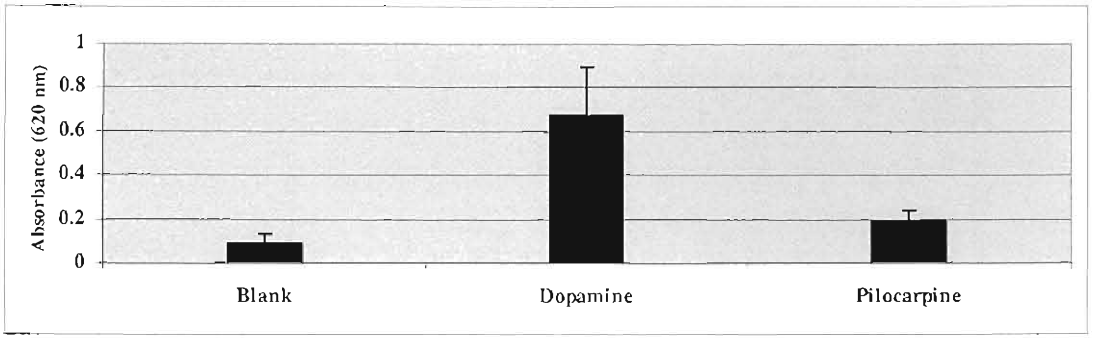

Fig. 5.8: Secretion of apyrase from exposed acini induced by dopamine. Mechanically sheared salivary glands were incubated with either dopamine or pilocarpine (1 μM) before assaying for apyrase activity.

5.3.7 Secretion efficiency of salivary glands during feeding

The low levels of secretion observed using EM analysis and chemical stimulation was further investigated using feeding ticks. Ten unfed ticks with similar masses (~0.169g) were allowed to feed to engorgement (or until they dropped off) before dissection of salivary glands and compared to salivary glands from 10 unfed ticks (Fig. 5.9). Engorged body masses from fed ticks showed a large standard deviation, while standard deviation values for both apyrase and protein concentration of the salivary glands were more comparable with those from unfed ticks. On average only 17% of the total apyrase activity and 37% of the total salivary gland protein were secreted.

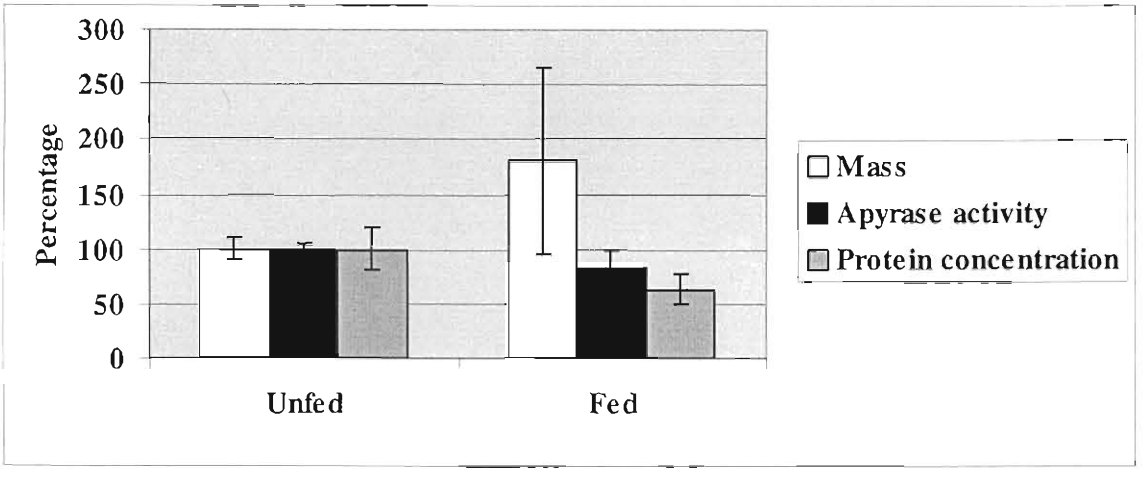

Fig. 5.9: A comparison between salivary glands of unfed and fed ticks. Indicated are body masses for ticks before and after feeding expressed as a percentage of the average unfed mass. Apyrase activity is expressed as percentage activity from the average unfed tick salivary gland extract. Protein concentration is expressed as a percentage of the average unfed tick salivary gland extract concentration.

5.3.8 Immunolocalization of apyrase and savignygrin on light microscope level

Immunolocalization of apyrase and savignygrin on light microscope level showed localization of both to granules of two different granular cell types (Fig. 5.10). Localization of savignygrin was more distinct than that of apyrase, although specific marking was evident if compared to the naïve controls. One of the granule types could be identified as cell type "a" dense core granules and the other as cell type "b" with a homogenous appearance. An additional granular cell type, "d" also had a homogenous appearance but showed no localization (Fig. 5.11). This brings the number of granular cell types observed for argasid ticks to a total of four. Note that this fits well with the results from Fig. 5.5 that indicate that the homogenous granules occur more than once per acini.

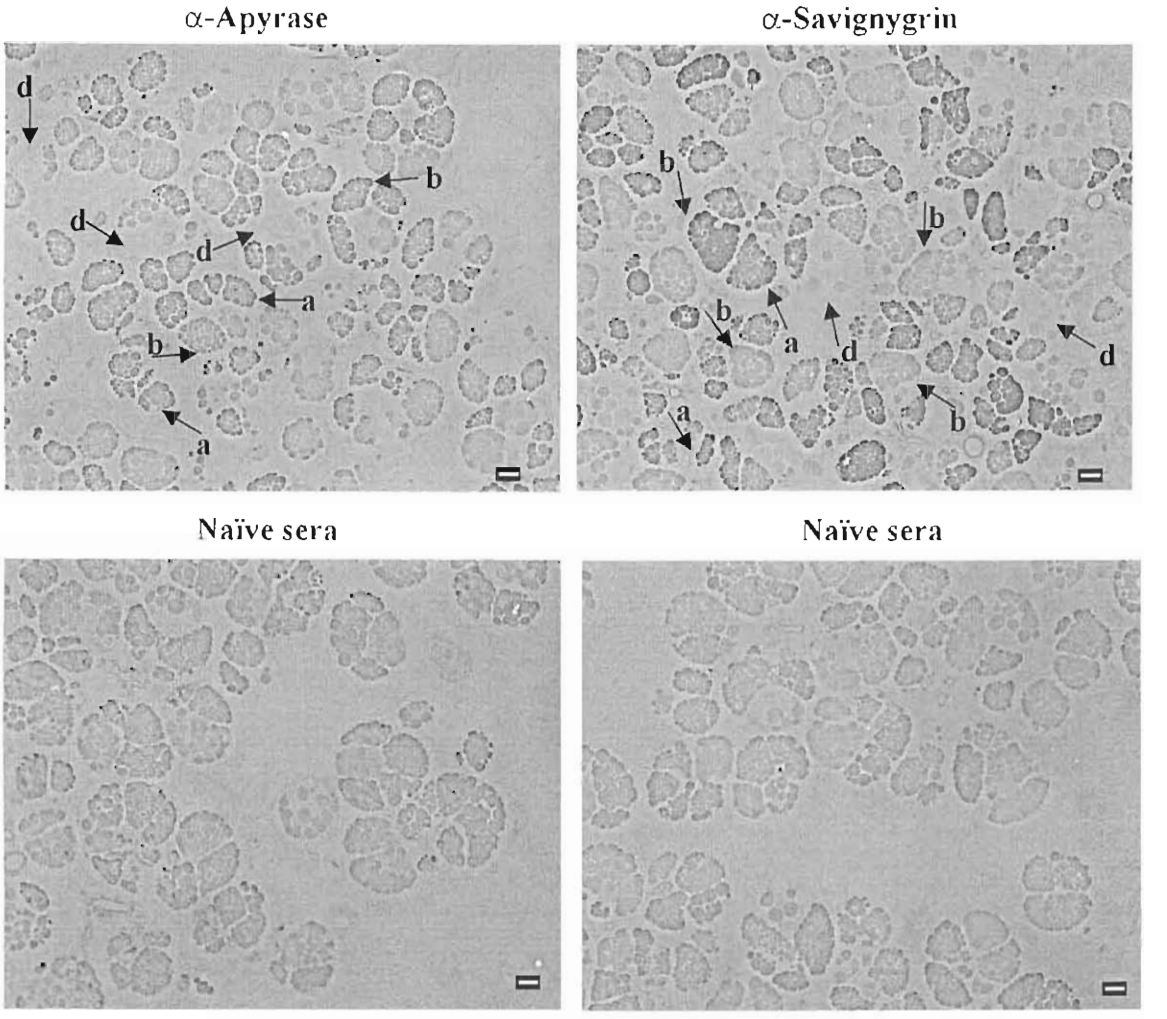

Fig. 5.10: Immunolocalization of apyrase and savignygrin. Light micrographs for sections labeled with anti-apyrase, anti-savignygrin or their respective naïve sera are indicated. Localization to dense core granules (a) and homogenous granules (b), as well as granules considered to be unlabeled (d) are indicated. Scale bar = 10 μm. All micrographs were taken using the same brightness and contrast.

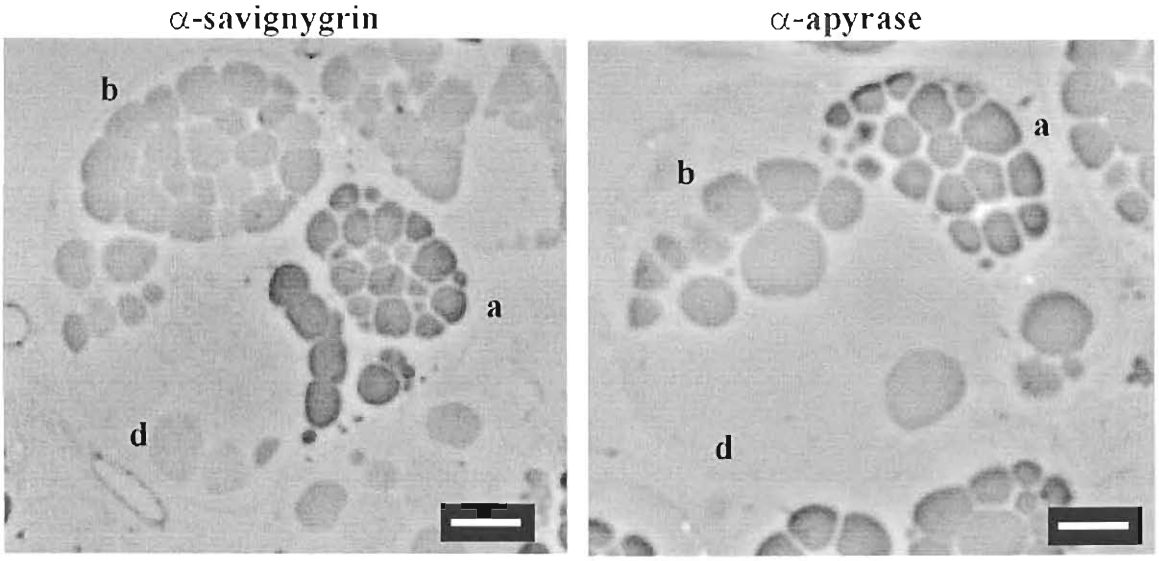

Fig. 5.11: Immunolocalization at a higher magnification. Localization to dense core granules (a) and homogenous granules (b) are indicated. Note the absence of marking in granule type (d). Scale bar = 10 μm.

5.3.9 Co-localization of apyrase and savignygrin at the ultrastructural level

It was observed that both apyrase and savignygrin were localized to the same granule types. The dense core type "a" granules and type "b" granules which have a homogenous appearance showed specific labeling (Fig. 5.12a; Fig. 5.12b). Granule types "c" and "d" that could be distinguished morphologically gave no significant labeling (Fig. 5.13). Negative controls using naïve sera at the same dilutions did not show any specific labeling (results not shown).

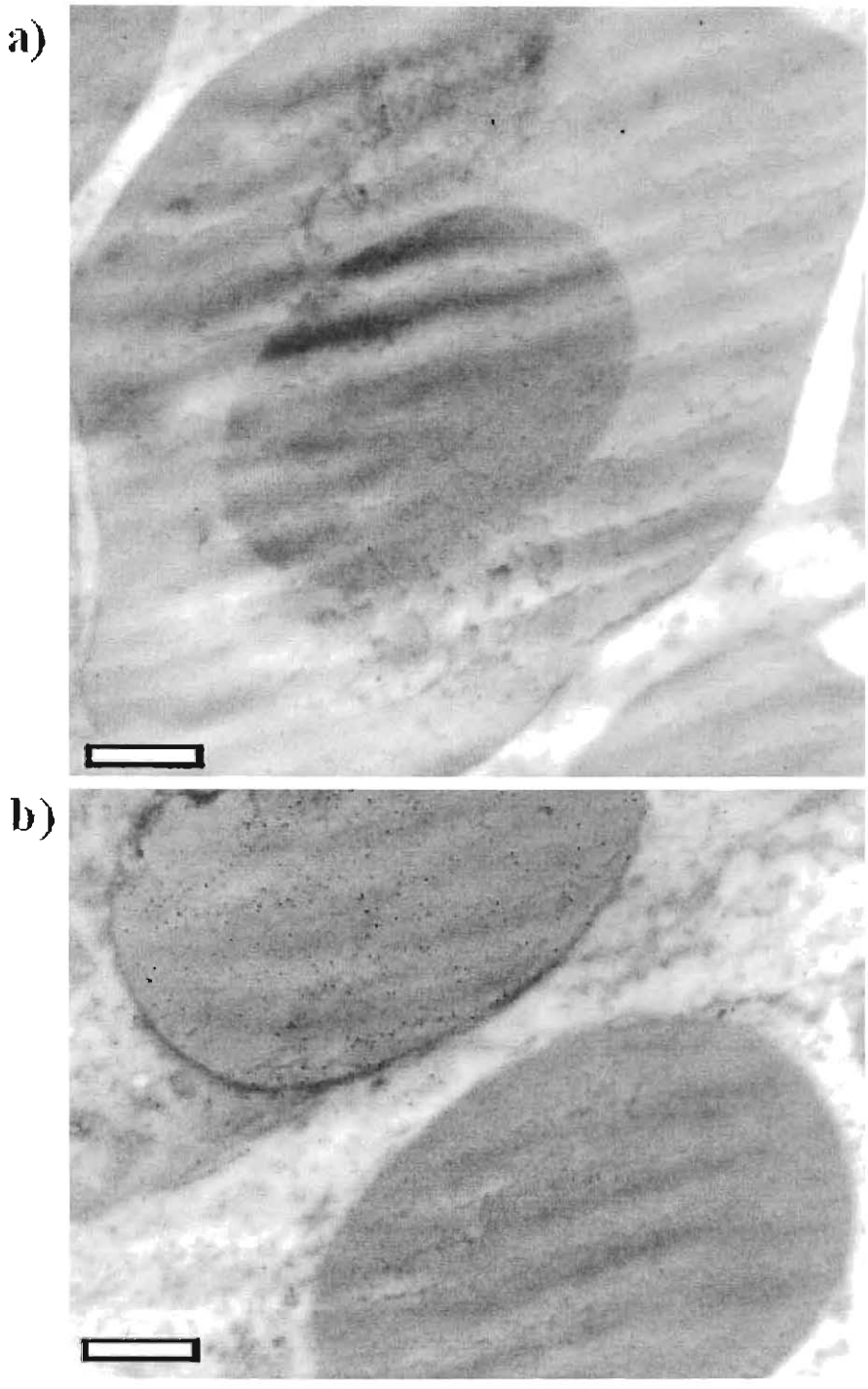

Fig. 5.12: Co-localization of apyrase and savignygrin using immuno-gold labeling. (a) Localization of both apyrase (10 nm gold) and savignygrin (20 nm gold) to the dense core granules (cell type "a"). (b) Localization of apyrase (10 nm gold) and savignygrin (20 nm gold) to homogenous granules (cell type "b"). Also indicated is a type "d" granule from another cell that shows no localization. Scale bar = 500 nm.

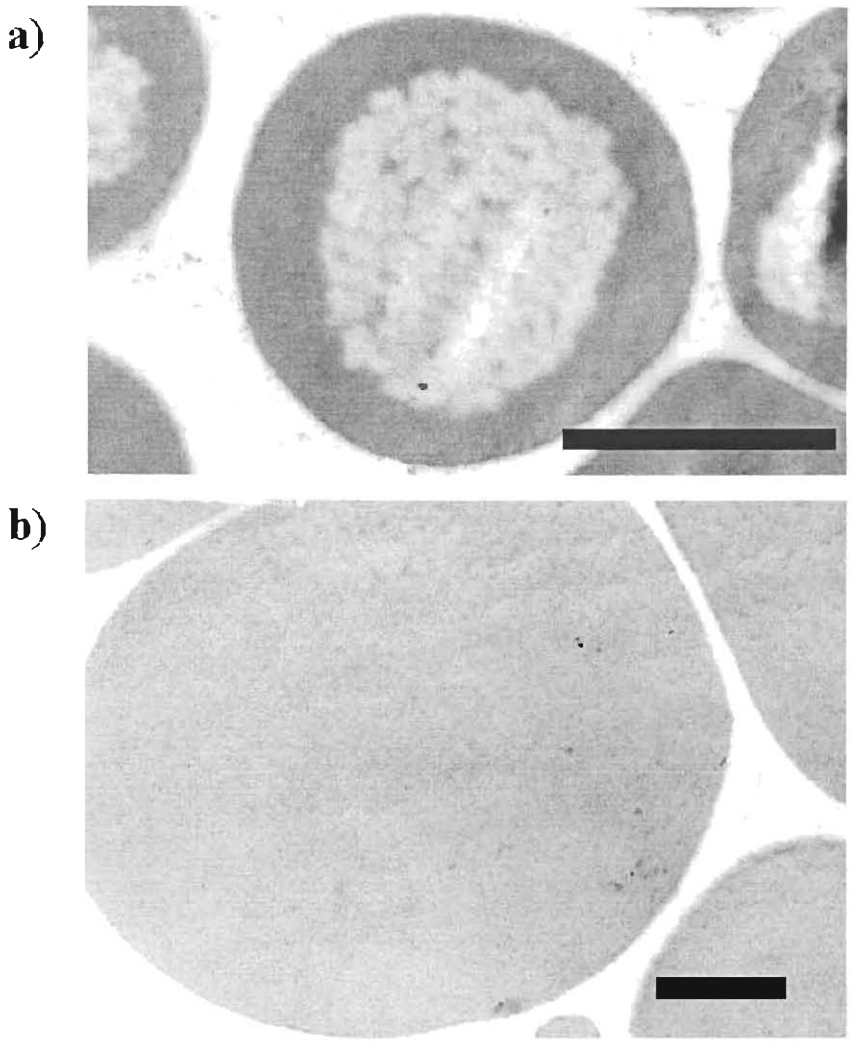

Fig. 5.13: Granular cells that did not show significant labeling. (a) Cell type "c" cells and (b) cell type "d" cells did not show any labeling. Scale bar = 1 μm.

5.3.10 Immuno-fluorescent localization of savignygrin to salivary gland granules

During a visit to the laboratory of Dr. Lewis Coons (May-June 2000, University of Memphis, Tennessee), I had the opportunity to localize savignygrin in the salivary glands using immuno-fluorescent confocal microscopy. In contrast to the light microscopy and TEM results, which showed labeling of two different granule types, consistent labeling of only one granule type per acini was obtained (Fig. 5.14a). Granules also showed higher labeling on their outer edges compared to their cores (Fig. 5.14b). An absence of labeling in some acini also suggests that there may be as yet an unidentified acini and granular cells present in soft tick salivary glands.

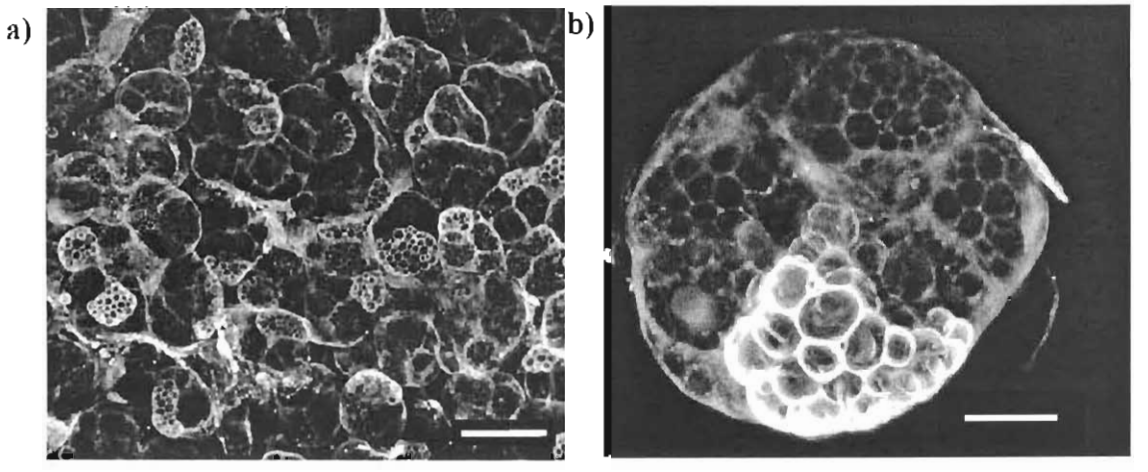

Fig. 5.14: Localization of savignygrin to a specific granule cell type with immuno-fluorescence confocal microscopy. (a) Localization of savignygrin to only one cell type per acini is clear. Note that for some acini no labeling is obtained. Scale bar = 100 μm. (b) Labeling at a higher magnification shows that granules are only labeled at their outer perimeters. Scale bar = 20 μm.

5.3.11 Absence of membrane vesicles involved in granule formation in mature cells

The absence of any membrane systems such as endoplasmic reticulum or Golgi apparatus were noticed in the thin sections prepared. To investigate this, a Thiery stain that gives good contrast for membranes and which also stains carbohydrates were employed. The only specific carbohydrate staining observed was the electron lucent cores of granule type "c" (Fig. 5.15a). In granular cells with mature granules, no ER or Golgi apparatus were observed although extensive membrane systems where found in a cell where immature granules were evident (Fig. 5.15b).

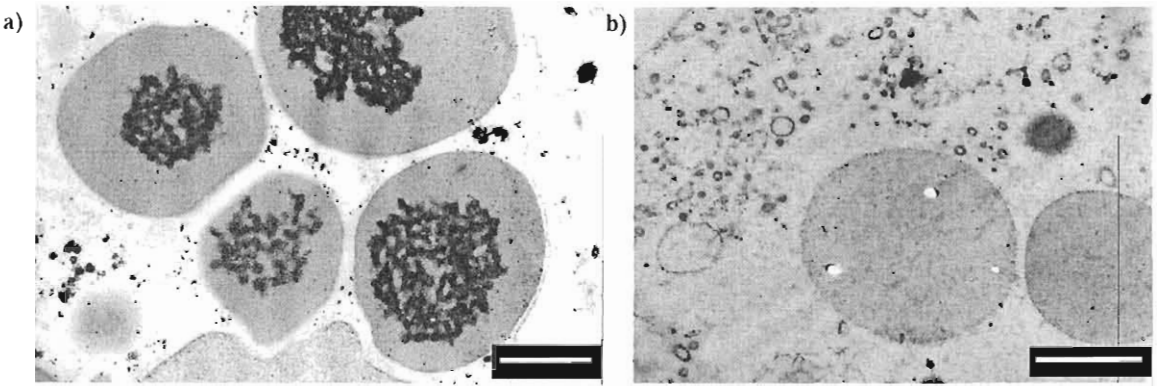

Fig. 5.15: Thiery stain of salivary glands. (a) Only cell type "c" showed positive staining for carbohydrates in its electron lucent core. (b) Membrane systems (ER, Golgi?) were only observed once in the presence of small immature granules. Scale bar = 1 μm.

5.3.12 SEM investigation into membrane systems of salivary glands

A technique that allows the selective removal of cytoplasmic components while membrane systems are retained was employed to investigate the presence of Golgi apparatus (Tanaka and Fukudome, 1991). This technique has been successfully used to investigate the three dimensional structure of various cell organelles such as ER, Golgi complex and mitochondria. In the salivary glands the only membrane systems left after osmium maceration, seem to be associated with the cytoskeleton (Fig. 5.16). All the granules seemed to have been removed completely. No structures that resemble the classic Golgi complex could be recognized.

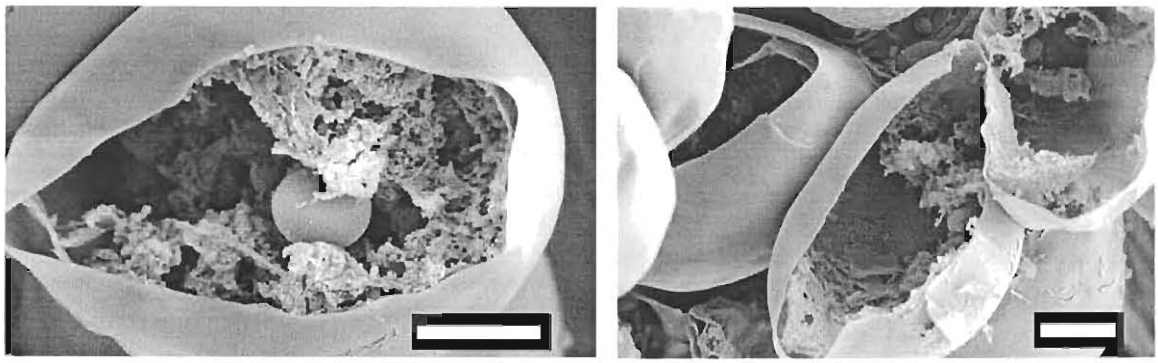

Fig. 5.16: SEM analysis of membrane systems of the salivary glands after osmium maceration. Most of the salivary gland granules were completely removed by this process and the only membrane systems present seem to be associated with the cytoskeletal network.

5.3.13 Sub-cellular fractionation of salivary gland granules after collagenase treatment

Results obtained from initial fractionation attempts of the salivary gland granules (see Chapter 6 for reason) could suggest reasons for the results obtained for the osmium maceration steps. Digestion with collagenase resulted in intact granule preparation as observed by the absence of apyrase in the supernatant fraction (fraction 15) with which the preparation was washed before loading onto the Percoll gradient. Fractionation showed that apyrase could be detected in a range of fractions, notably fraction 1, that represents the sedimented fraction, that consist of partially digested intact gland preparation and fractions (4-10) that represent granules in various degrees of association (Fig. 5.17).

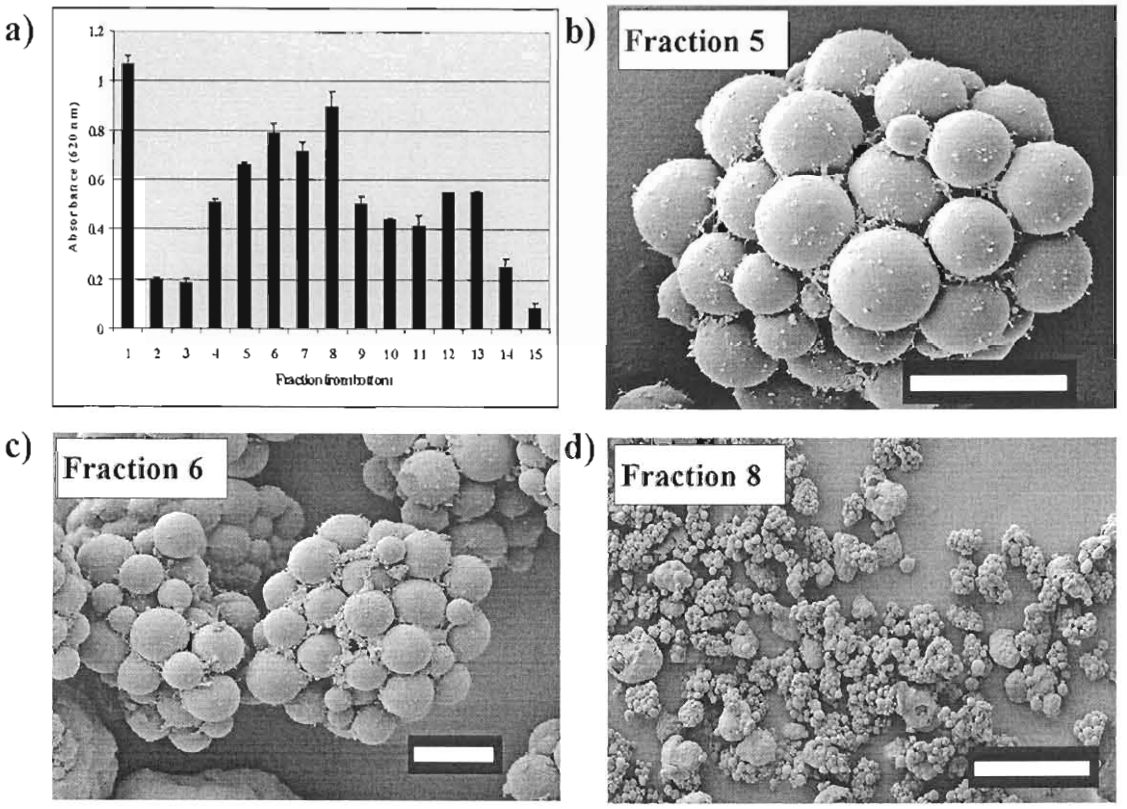

Fig. 5.17: Sub-cellular fractionation of salivary gland granules using density gradient centrifugation collagenase treatment. (a) Apyrase activity tested for different fractions collected. Fraction 1 represents sedimented material, fraction 2-14 represent 1 ml fractions (5% increments of Percoll, 0-50% gradient) collected from the bottom of the gradient and fraction 15 the supernatant of the last wash buffer before granule preparations were loaded onto the gradient. Granules preparations of fractions 5 (b), 6 (c) and 8 (d) are indicated. Scale bar = 10 μm (b, c) or 100 μm (d).

5.3.14 Immuno-localization of α-tubulin to tick salivary glands

Microtubules serve to organize the ER and Golgi complex as well as transporting secretory vesicles. Localization of the microtubules could thus indicate the possible presence of a Golgi complex. Microtubules (as detected by the presence of α-tubulin) could be detected in agranular acini of both hard and soft ticks, cells from the myo-epithelial sheath, but no extensive pattern network could be observed in granular cells. Some localization between granules is evident, although it is clear that these microtubules do not serve an organizing role (Fig. 5.18).

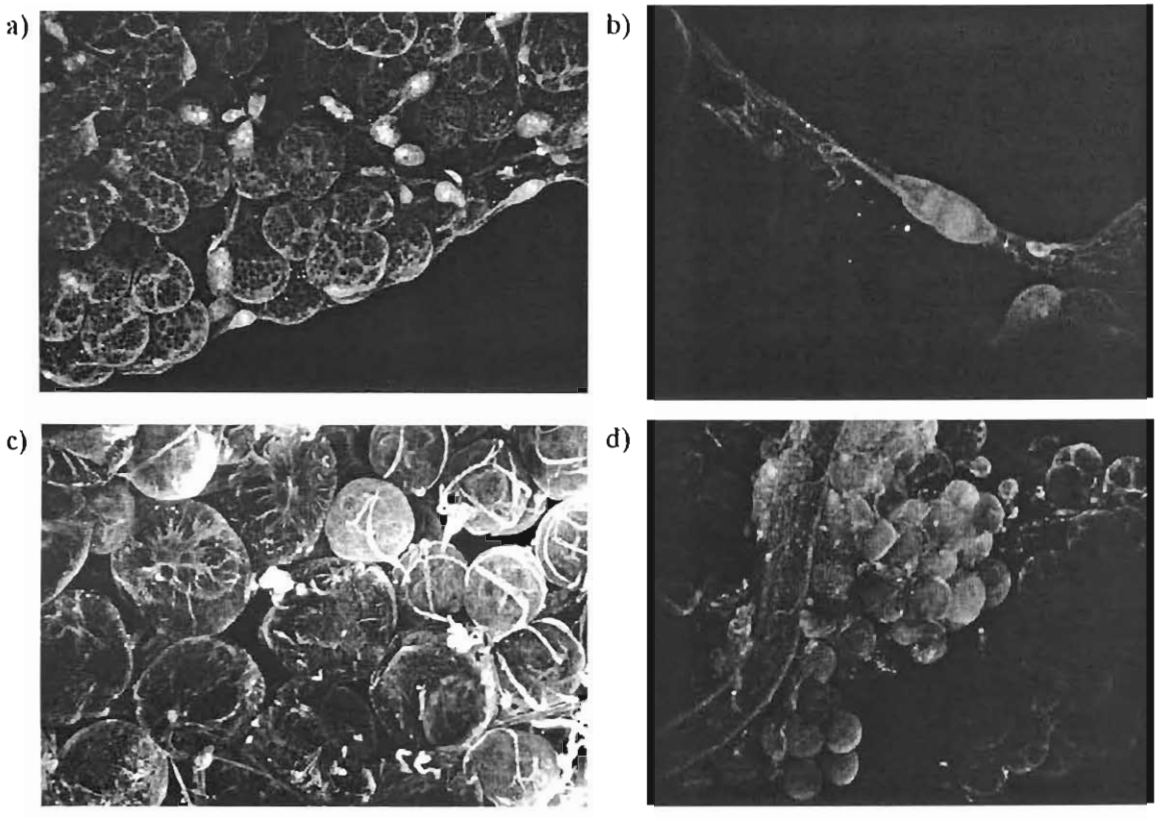

Fig. 5.18: Localization of α-tubulin to salivary gland granules. (a) Granular as well as cells from the myo-epithelial sheath are indicated. (b) A cell from the myo-epithelial sheath. (c) Agranular acini from the hard tick *Dermacentor variabilis*. (d) Agranular acini from the soft tick *O. savignyi*.

5.4 Discussion

5.4.1 Importance of the salivary glands in secretion

Tick salivary glands differentiated into highly sophisticated organs during the adaptation of ticks to a blood-feeding environment. Control of tick homeostatic volume (Sauer *et al.* 1986), secretion of cement by hard ticks (Kemp, Stone and Binnington, 1982) and proteins involved in mating (Feldman-Muhsam, 1986) can be considered as important functions performed by salivary glands. The biosynthesis, storage and secretion of bio-active compounds by feeding ticks can however, be considered as the main salivary gland function (Sauer *et al.* 1995), especially in soft ticks. For ticks to feed successfully an ample supply of feeding facilitating compounds must be available at the time of feeding (Bowman *et al.* 1997). This is especially important in the case of argasid ticks for which feeding times are relatively short (30 –60 min) and not sufficient for the induction of protein synthesis as observed for hard ticks (Fawcett, Doxley and Büscher, 1986). However, secretion of granules by the salivary glands of soft ticks seem to be very inefficient if considered that less than ~10% of a salivary gland acinus showed signs of secretion after feeding on rabbits or stimulation with chemical agonists. Only ~17% of apyrase and ~37% of the total salivary gland protein was secreted. It would seem implausible that the remaining granules are stored for future use during feeding, as the glands had recovered sufficiently a few days after feeding. The large size of the salivary glands is also totally unrelated to the amount of secretory product. In energy conservation terms this is a waste of biological resources. It can only be assumed that the tick pays this high price in order to ensure an adequate supply of anti-hemostatic factors. This again underlines the importance of the tick salivary glands in feeding and tick survival.

5.4.2 Secretory mechanisms of argasid tick salivary glands

This study did not specifically investigate the secretory mechanisms of the salivary glands. Preliminary results obtained do however suggest several possibilities. The data obtained correlate with results from studies that showed that salivary glands are not stimulated directly by cholinergic mimetics such as pilocarpine, but that this stimulation probably occurs via activation of the central nervous system (Kaufman, 1976; Kaufman and Harris, 1983). Secretion of apyrase induced by dopamine also suggests that this

adrenergic stimulant might directly stimulate salivary glands, probably through release of dopamine at the neuroeffector junction of nerves and salivary glands (Sauer, Essenberg and Bowman, 2000). In hard ticks it has been shown that dopamine can activate adenylate cyclase and PLA_2 thereby mediating the synthesis of cAMP and PGE_2 (Sauer, Essenberg and Bowman, 2000). Furtermore, PGE_2 can function as a autocrine or paracrine hormone in salivary glands that can activate the PLC pathway leading to formation of IP_3, mobilization of intra-cellular calcium and exocytosis of anti-clotting factors (Qian *et al.* 1997; Qian *et al.* 1998). It would be interesting to know whether the secretory mechanisms of hard and soft ticks differ at all. From an evolutionary viewpoint it can be predicted that these mechanisms would not differ too much, as the ancestral mite probably also secreted proteins during feeding.

5.4.3 Localization of apyrase and savignygrin

The co-localization of anti-platelet factors into specific granules suggests that other anti-hemostatic factors might be localized in the same granules. Western blot results and the high degree of localization suggest that immunolocalization was specific even though no adsorption controls were performed. Significant is the localization to dense core granules (cell type "a"). Dense core granules in other organisms are specifically involved in regulated secretion (Chidgey, 1993). It was also shown that these granules were released five minutes after the tick *O. moubata* commenced with feeding (El Shoura, 1985) implicating it in the feeding process. It has been previously shown that apyrase is secreted in tick salivary secretion after stimulation by pilocarpine (Mans *et al.* 1998a) and taken together with the results that indicate secretion of dense core granules it can be assumed that savignygrin is also secreted. The presence of a signal peptide in the sequence of savignygrin correlates with these results (Chapter 2).

The anti-hemostatic components are also localized to another granule type (cell type "b") apart from the dense core granules. The possibility that these granules may be immature forms of the dense core granules cannot be excluded. This would however, suggest a high degree of synchronization in the maturation of these granules and given the large size of these granules would seem to be unlikely. Differences in the labeling of the different

granules might be due to a higher degree of condensation inside the dense core granules that could mask the antigenic epitopes. Apyrase was labeled to a lesser extent than savignygrin that could be due to either, a lower antibody titer or a lower concentration of apyrase sequestered in the granules. Apyrase is also present at a lower concentration in the salivary gland extract (~0.7 μg per salivary gland; Mans *et al.* 1998b) compared to savignygrin (~5.0 μg per gland) and has a 9 times lower molar ratio (67 kDa: 7 kDa) compared to savignygrin. Comparisons of Western blots also show that the anti-sera directed against apyrase gave a much lower signal than that observed for savignygrin.

5.4.4 Paradoxes between light, TEM and confocal microscopy

Discrepancies were observed for the localization of savignygrin when normal immunocytochemical techniques that employ thin sections is compared to confocal microscopy that uses whole tissue. It is known that antibodies only penetrate tissue up to ~10μm during confocal microscopy. Most secretory granules are also very dense due to "condensation" of secretory proteins. Inadequate penetration of granules could thus account for labeling of only the outer surfaces of granules. Granules with dense cores can be assumed to have higher densities than homogenous granules. This could also account for labeling of only one type of granule, because penetration of the dense core granule by antibody is hindered due to high density or masked binding epitopes of the antigen. This correlates with the fact that less labeling is observed for dense core granules compared to homogenous granules as observed during TEM.

5.4.5 Thiery staining of salivary glands

Carbohydrate staining using the Thiery test showed a positive reaction with the electron lucent cores of type "c" granules. No other granule type showed any specific carbohydrate staining, but rather showed staining with the silver control. This could indicate that proteins sequestered inside these granules are not glycosylated. In contrast, the type "b" and "c" granules and the periphery of the dense core granules (type "a") in the salivary glands of *O. moubata* stained positive with the PA-TCH stain (El Shoura, 1985). Alternatively it is also possible that all the controls necessary to ensure specific carbohydrate staining were not completed in the latter study.

5.4.6 ER and Golgi apparatus in the salivary glands

The Golgi apparatus was observed in the mature granular cells of *O. moubata* (El Shoura, 1985), which indicates active biosynthesis of granule proteins. This study showed that cells with mature granules had less vesicular membrane system in contrast to cells where biogenesis of granules is still taking place. Of interest are the salivary glands of the hard tick *R. appendiculatus* where no Golgi apparatus could be observed in the "d" and "e" cells of acinus III. It was speculated that granule formation occurs directly from the endoplasmic reticulum and it was taken as confirmation that no Golgi specific glycosylation events takes place and hence the absence of positive carbohydrate staining in these granule types (Fawcett, Binnington and Voight, 1986). This observation is quite astonishing, as these are the only cells known to display this property.

5.4.7 Relationship between ER, Golgi apparatus and secretory granules

An inverse correlation between the extent of ER and the number of secretory granules was observed in secretory cells from the parotid, salivary glands and pancreas (Ghadially, 1988). It was speculated that this might either be due to the plane of sectioning or that as the granules accumulate the ER regresses. Studies on the trans-Golgi network (TGN) have shown that extensive multilayered TGN occur in cells that do not form large secretory granules, such as Sertoli cells, nonciliated cells of ductuli efferentes and spinal ganglion cells. In cells (mucous cells of Brunner's gland, pancreatic acinar cells, plasma cells, enterocytes, cardiocytes) that produce small to medium-sized secretory granules, the TGN is small. The TGN is absent in cells (secretory cells of seminal vesicles, lactating mammary glands) that form large secretory granules (Clermont, Rambourg and Hermo, 1995). Recent evidence into the nature of the Golgi complex has indeed indicated that this is a dynamic organelle not restricted to a specific morphological structure (Glick, 2000). The cisternal maturation hypothesis propose that the Golgi is a dynamic outgrowth of the ER, with ER-derived membranes coalescing into new cis-cisterna that progresses (maturates) through the different Golgi compartments until it disintegrates into transport vesicles. Experiments that inhibited ER-to-Golgi traffic caused the Golgi to collapse into the ER. Other experiments where ER exit was blocked showed that Golgi elements

disappeared in the order cis, medial, trans and when the block was released the Golgi elements reappeared in the same order (Glick, 2000). SEM, TEM and confocal microscopy in this study all showed that granular cells are filled with granules in what can be almost described as "bursting point". From this it should be clear that these cells are basically filled to capacity and as such would need some mechanism to stop granule biogenesis. It would seem logical that a removal of ER and Golgi would halt granule biogenesis. The mechanism of this process can only be speculated on. It is envisaged that the sequestration of all Golgi-network membranes into formation of granules might be part of a regulatory event in the synthesis of secretory proteins. Specific feedback mechanisms must exist that would down regulate protein synthesis and granule biogenesis when granule formation has been saturated.

5.5 Conclusions derived from this study

Apyrase and savignygrin were localized to two types of granules (type "a" and type "b"). Type "b" cannot be distinguished on a morphological or histochemical basis from another cell type (type "d") that did not show any labeling. Localization of savignygrin using immunofluoresent confocal microscopy also suggests that there might be another granular acini not previously recognized. This data imply that the morphological and histochemical classification of salivary gland cell types should be approached with caution, as granules with the same appearance do not necessarily have the same protein expression patterns. New schemes of classification should rather consider the specific composition of the granules in terms of the proteins expressed in specific cell types. This urges for a more thorough investigation of granule composition, a task that might be accomplished by the new field of proteomic analysis (Wilkins *et al.* 1997).

5.6 Salivary glands, toxins and granule formation

The large size of the granules and their dense packing in the salivary glands stimulated our interest into the processes by which these granules might form. The next chapter deals with this phenomenon and identifies possible candidates that might be involved in the process of granule biogenesis. It also looks into the nature of toxicity described for this tick species. *O. savignyi* has been implicated in pathogenesis of domestic animals,

initially thought to be due to exsanguination of the host. However, it was shown that death could be due to toxic components present in tick salivary gland secretions and a toxic protein and a non-toxic homologue has been purified (Howell, 1966b; Neitz, Howell and Potgieter, Howell, Neitz and Potgieter, 1975). Conflicting reports indicated molecular masses of 15400 Da or 6346 Da, determined by sedimentation equilibrium centrifugation or based on amino acid composition, respectively (Neitz, Howell and Potgieter, 1969; Neitz *et al.* 1983). The toxin also eluted at a position similar to that of the savignygrins during AEHPLC (Neitz, 1976). The low molecular mass reported as well as the behaviour during ion exchange suggested that the savignygrins might be the toxin previously described. It made sense that the savignygrins might have been toxic as some members of the BPTI-family are neurotoxic (Dufton, 1985). The possible toxicity of savignygrin was thus investigated. While it was eventually shown that the savignygrins are not the toxins, a firm belief was established that although specific toxic activities are present in the salivary glands, toxicity was of secondary function and that the toxins might have other more benign functions, such as a role in granule biogenesis.