Programmed cell death in the larval salivary glands of *Apis mellifera* (Hymenoptera, Apidae)

E C M Silva-Zacarin[1], G A Tomaino[2], M R Brocheto-Braga[3], S R Taboga[4] and R L M Silva de Moraes[5]

[1]*Universidade Federal de São Carlos – UFSCar, Avenida Darci Carvalho Daffener, 200 Alto da Boa Vista, Sorocaba/SP, Brasil, e-mail: elaine@power.ufscar.br*

[2,3,5]*Departamento de Biologia, Instituto de Biociências, Universidade Estadual Paulista – UNESP, Av. 24A 1515 Bela Vista, 13506-900 Rio Claro/SP, Brasil*

[4]*Departamento de Biologia, Instituto de Biociências, Letras e Ciências Exatas, Universidade Estadual Paulista – UNESP, 15054-000, São José do Rio Preto, SP, Brazil*

[1]*Corresponding author (Email, elaine@power.ufscar.br)*

The morphological and histochemical features of degeneration in honeybee (*Apis mellifera*) salivary glands were investigated in 5th instar larvae and in the pre-pupal period. The distribution and activity patterns of acid phosphatase enzyme were also analysed. As a routine, the larval salivary glands were fixed and processed for light microscopy and transmission electron microscopy. Tissue sections were subsequently stained with haematoxylin–eosin, bromophenol blue, silver, or a variant of the critical electrolyte concentration (CEC) method. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Glands were processed for the histochemical and cytochemical localization of acid phosphatase, as well as biochemical assay to detect its activity pattern. Acid phosphatase activity was histochemically detected in all the salivary glands analysed. The cytochemical results showed acid phosphatase in vesicles, Golgi apparatus and lysosomes during the secretory phase and, additionally, in autophagic structures and luminal secretion during the degenerative phase. These findings were in agreement with the biochemical assay. At the end of the 5th instar, the glandular cells had a vacuolated cytoplasm and pyknotic nuclei, and epithelial cells were shed into the glandular lumen. The transition phase from the 5th instar to the pre-pupal period was characterized by intense vacuolation of the basal cytoplasm and release of parts of the cytoplasm into the lumen by apical blebbing; these blebs contained cytoplasmic RNA, rough endoplasmic reticule and, occasionally, nuclear material. In the pre-pupal phase, the glandular epithelium showed progressive degeneration so that at the end of this phase only nuclei and remnants of the cytoplasm were observed. The nuclei were pyknotic, with peripheral chromatin and blebs. The gland remained in the haemolymph and was recycled during metamorphosis. The programmed cell death in this gland represented a morphological form intermediate between apoptosis and autophagy.

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1. Introduction

Metamorphosing insects provide very useful models for studying cell death (Lockshin 1981). Holometabolous insects undergo a dramatic form of post-embryonic development that consists of three distinct stages (larval, pupal and adult) separated by two metamorphic molts. The larval stage is a wingless form that is specialized for feeding and growth, while the pupa is a non-feeding, quiescent stage, during which the adult structures are formed. During

Keywords. Degeneration; honeybee; larva; prepupa; silk gland

Abbreviations used: CEC, Critical electrolyte concentration; HE, haematoxylin-eosin; IRS, insect Ringer solution; p-NPP, p-nitrophenyl phosphate; PP, pre-pupal phase; TEM, transmission electron microscope.

metamorphosis, most larval tissues are degraded and the adult organs arise from undifferentiated cells that proliferate slowly during larval life and very rapidly in the pupal stage.

The larval salivary glands of holometabolous insects are promising tissues for studying physiological or programmed cell death (Lockshin 1981). Differences have been observed between metamorphically induced programmed cell death in blow-fly/fruit-fly salivary glands and the changes ascribed to classic apoptosis (Wyllie 1981) or necrosis (Trump and Mergner 1974). The programmed cell death that occurs in many larval tissues, including the salivary glands, is accompanied by increases in the histochemical and biochemical activity of lysosomal enzymes (Lockshin 1981). Indeed, lysosomal and free acid phosphatases are frequently used as markers of the lytic activity associated with programmed cell death in insect tissues (Jones and Bowen 1993). Acid phosphatase activity has been mainly investigated in the larval salivary glands of Diptera (Aidells *et al* 1971; Bowen 1984; Levy and Bautz 1985; Armbruster *et al* 1986; Jones and Bowen 1993).

Several aspects of programmed cell death in larval insect salivary glands have been studied, mainly in the Lepidoptera and Diptera (Aidells *et al* 1971; Schin and Laufer 1973; Jurand and Pavan 1975; Beaulaton and Lockshin 1982; Levy and Bautz 1985; Armbruster *et al* 1986; Sehnal and Akai 1990; Jones and Bowen 1993; Bowen *et al* 1993, 1996; Jochová *et al* 1997a, b; Dorstyn *et al* 1999; Lee and Baehrecke 2001; Myohara 2004). In the blow-fly *Calliphora*, programmed cell death of the salivary glands does not emulate classical apoptosis because the cells are seen to vacuolate and swell rather than condense and shrink (Bowen *et al* 1996); additionally, there is basal fragmentation (Levy and Bautz 1985) and the nucleus does not show a distinct chromatin margination and blebbing (Bowen *et al* 1996). Histolysis of the silk gland during metamorphosis of *Bombyx mori* is carried out by autophagy, with lysosomal proteinases playing the key role in degrading cytoplasmic organelles in the autophagosomes (Lockshin *et al* 1998; Shiba *et al* 2001). However, real-time observation of autophagic programmed cell death of *Drosophila* salivary glands showed that the gland shrank due to cytoplasmic loss caused by blebbing (Myohara 2004).

Some morphological aspects of salivary gland degeneration have also been described in bees (Hymenoptera) (Cruz-Landim and Mello 1967; Silva de Moraes and Cruz-Landim 1979; Cruz-Landim and Melo 1981; Cruz-Landim and Silva de Moraes 2000), but various aspects of salivary gland autolysis in *Apis*, including morphological and histochemical features, acid phosphatase activity as well as nucleolar and nuclear alterations, have not yet been described.

The larval salivary glands of bees are composed of a secretory portion and a duct (Cruz-Landim and Mello 1967). These glands, also known as silk glands, produce the silk used to form the cocoon. In the salivary glands of *Apis mellifera*, silk is produced in the 5th larval instar. According to Silva and Silva de Moraes (2002) and Silva-Zacarin *et al* (2003), at the beginning of the 5th instar, the salivary gland lumen of *A. mellifera* is filled with a homogeneous secretion that is synthesized before silk production. In the mid-5th instar, these glands produce silk proteins that are released from the secretory cells as a homogeneous substance (monomeric silk), which subsequently polymerizes in the lumen to form silk fibrils. By the end of the 5th instar, the gland lumen is filled with a compact fibrillar secretion. When the silk is released to spin the cocoon at the end of the 5th instar, the secretion that remains in the lumen loses its compaction and its macromolecular organization.

Ultrastructural analysis of the larval salivary glands of *A. mellifera* has revealed that after the peak of protein synthesis, the secretory portion of the gland undergoes histolysis following construction of the cocoon within which the larva undergoes metamorphosis (Silva de Moraes and Cruz-Landim 1979). However, the morphological and histochemical aspects of programmed cell death in *A. mellifera* silk glands in the initial (5th instar) and late (pre-pupal period) phases remain to be determined. The aims of this study were to investigate the sequence of morphological and histochemical features, as well as the activity and distribution patterns of acid phosphatase, which is associated with programmed cell death in *A. mellifera* salivary glands for 5th instar larvae and the pre-pupal period.

2. Materials and methods

2.1 *Insects*

Larval salivary or silk glands from the honeybee *A. mellifera* were used. Fifth instar larvae and prepupae were collected from colonies in the apiary of the Biology Department, Institute of Biosciences, UNESP, Rio Claro, SP, Brazil. Fifth instar larvae were identified based on the size of the larval cephalic capsule and were classified according to their location within the hivecombs so that worker larvae collected from open cell combs corresponded to the beginning of the 5th instar, whereas larvae collected from closed cell combs corresponded to the end of the 5th instar. The prepupae (corresponding to the beginning, middle and end of the pre-pupal period) were identified based on their characteristic anatomy and position within the closed cell combs (Silva-Zacarin *et al* 2003).

2.2 *Isolation of larval salivary glands*

The salivary glands were isolated after removal of the ventral integument from 5th instar larvae and prepupae (at

the beginning of the pre-pupal period). The glands were dissected in insect Ringer solution (IRS) (128.5 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$; pH 7.4) and immediately fixed in the fixative appropriate for each method of microscopy.

2.3 *Morphological analysis by light microscopy*

The salivary glands isolated from prepupae (at the beginning of the pre-pupal period) and 5th instar larvae (at the end of this instar) were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). After fixation, the glands were rinsed in the same buffer, dehydrated in a standard ethanol series and embedded in historesin JB4 (Polyscience). Since it was impossible to isolate the silk glands from prepupae in the middle and end of this phase, whole insects were fixed after removing their cephalic and caudal extremities and then processed as usual for historesin embedding. Histological sections around 5 μm thick were stained with hematoxylin-eosin (HE) and bromophenol blue (Pearse 1960), which stained the silk proteins in the glandular lumen. The sections were subsequently examined by light microscopy.

2.4 *Morphological analysis by transmission electron microscopy*

Salivary glands isolated from prepupae (at the beginning of this period) and 5th instar larvae (at the end of this instar) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). Samples were then rinsed twice in the sodium cacodylate buffer and post-fixed in 1% osmium tetroxide in the same buffer. Following another rinse with the buffer, the samples were dehydrated in a series of increasing concentrations of acetone and embedded in Epon–Araldite resin, following the usual procedures. Ultrathin sections were obtained with diamond knives and stained with uranyl acetate and lead citrate, and then examined and photographed by a Philips CM 100 transmission electron microscope (TEM).

2.5 *Histochemical analysis*

Salivary glands were isolated from 5th instar larvae (at the end of this instar) and from prepupae (at the beginning of the pre-pupal period), and immediately fixed in ethanol–acetic acid (3:1, v/v) for 10 min, immersed in 70% alcohol and processed for embedding in historesin. Histological sections around 5 μm thick were stained with toluidine blue and a variant of the critical electrolyte concentration (CEC) method, according to Mello *et al* (1993), in order to differentiate between nucleolar and cytoplasmic RNA. This method was also found useful for detecting the presence of RNA in apoptotic bodies (Vidal *et al* 1996), by metachromatic staining, i.e., violet/blue colours. Histological sections were also stained with silver, according to Howell and Black (1980), to detect nucleolar proteins. This staining can reveal alterations in the nucleolar organizing regions (NORs) related to programmed cell death. The nucleolus was identified by its dark staining.

2.6 *Detection of acid phosphatase in whole mount preparations*

Salivary glands were isolated as described above and whole glands were prepared as described by Lewis and Bowen (1985). The glands were dissected and immediately fixed in 10% neutral buffered formalin–acetone mixture (9:1, v/v). After fixation for 1 h, the glands were briefly rinsed in acetate buffer (0.1 M, pH 4.8) and incubated in medium containing 0.46 mM naphthol AS-TR phosphate dissolved in a drop of dimethyl sulphoxide (DMSO), acetate buffer (0.1 M, pH 4.8), 10% $MnCl_2.4H_2O$ and 1.6 mM fast red violet LB salt, with each constituent being added in the order mentioned. The solution was mixed vigorously and used immediately. After incubation for 45 min at 37°C, the glands were rinsed for 1 min in acetate buffer, washed in distilled water and mounted in 50% glycerol. Acid phosphatase activity was detected by the formation of a red/pink reaction product. Glands that were incubated in medium without substrate acted as controls.

2.7 *Ultrastructural cytochemistry for acid phosphatase detection*

Salivary glands were isolated after excision of the ventral integuments from 5th instar larvae at the beginning and end of this instar. Glands were prepared according to a modified method described by Ryder and Bowen (1975). Whole glands were pre-fixed during dissection in a solution of 3% glutaraldehyde dissolved in sodium cacodylate buffer (0.1 M, pH 7.4) and fixed in the same fixative solution for 1 h at 4°C, followed by overnight washing in the sodium cacodylate buffer (0.1 M, pH 7.4) at 4°C. The glands were incubated at 37°C for 2 h in a medium containing sodium acetate buffer (0.1 M, pH 4.8) in which p-nitrophenyl phosphate (NPP; 3.8 mM) and lead acetate (2.6 mM) had been separately dissolved. Control glands were incubated in a medium without the substrate. The glands were placed in cacodylate buffer for 30 min at room temperature and post-fixed in Milling 1% osmium tetroxide for 1 h at 4°C. Following dehydration through an ascending ethanol series, the glands were embedded in Epon–Araldite. Ultrathin sections were cut, counterstained with uranyl acetate and lead citrate and examined under a EM9S2 Philips TEM.

2.8 *Biochemical assays*

Fifty salivary glands isolated from 5th instar larvae (at the beginning and end of this instar) and of prepupae (at the beginning of this period), dissected out at 4ºC in IRS, were pooled for each sample. Three samples for each developmental stage were investigated in duplicate. The glands were homogenized in IRS (0.22 ml) at 4ºC, and centrifuged at 16 000 *g* for 2 min. The enzyme activity of these supernatants, named *S-I*, was defined as soluble acid phosphatase. An aliquot (40 μl) of each *S-I* was also removed to determine protein levels according to the Bradford modified method (Sedmak and Grossberg 1977).

The pellet of each sample was resuspended in IRS (0.16 ml), at 4ºC, containing 0.5% Triton X100. They were then kept overnight at –20ºC, centrifuged at 16 000 *g* for 2 min and aliquots of the supernatant from each homogenized pellet were retained for enzyme assay. These procedures were performed to release the compartmentalized acid phosphatase and these supernatants were named *S-II*. An aliquot (40 μl) of each *S-II* was also removed to determine protein levels according to the Bradford modified method (Sedmak and Grossberg 1977).

Acid phosphatase activity was assayed according to Jones and Bowen (1993) with p-NPP (Sigma 104, 32 mM in 0.1 M acetate buffer, pH 4.8) as substrate. The amount of acid phosphatase activity was expressed as nM of p-nitrophenol liberated/microgram protein per 1 h. The acid phosphatase activity was also expressed as nanomol p-nitrophenol liberated/gland. Blanks were included in all assays.

Values obtained from the three samples per group of individuals were subjected to the nested ANOVA statistical test (Zar 1999), comparing *S-I* and *S-II* supernatants in different larval development stages. The values were also subjected to the Tukey HSD test (Zar 1999).

3. Results

3.1 *Morphological and histochemical analysis of larval salivary glands*

Glands from 5th instar larvae showed different degenerative features, depending on their physiological stage within this instar, e.g., glands with a large amount of silk in the lumen versus those with little silk in the lumen. Staining with bromophenol blue provided the best results in glands with a large amount of silk in the lumen and was therefore used to describe the morphological aspects of these glands. Similarly, HE staining was used to describe the morphological aspects of glands with little silk in the lumen and of pre-pupal glands.

In glands with a large amount of silk in the lumen, the secretory cells had a vacuolated basal cytoplasm (figure 1) and varying degrees of nuclear chromatin compaction (figure 1B). In pyknotic nuclei, the compacted chromatin was located at the centre of the nucleus, with the space between this chromatin and the nuclear envelope forming a perichromatin halo (figure 1). Figure 1A shows a cell with a high degree of cytoplasmic vacuolation and chromatin compaction. Some secretory cells did not show chromatin compaction (figure 2A).

Ultrastructural analysis showed the presence of vesicles and dilatation of the intercellular space at the apical region of the cell (figure 2B), where the microvilli appeared well structured (figure 2B). Large, degenerated areas were observed in the basal cytoplasm (figure 2C), corresponding to the vacuoles that were visible through the light microscope (figures 1, 2A). Invaginations of the basal plasma membrane became deep and sinuous at some places (figure 2C), thus characterizing partition of the basal cytoplasm. Large amounts of mitochondria and rough endoplasmic reticule, with dilated cisterns and partial loss of ribosomes (figure 2C), as well as concentric lamellar structures (figure 2D) were present in the basal cytoplasm. The median cytoplasm remained intact, and contained large amounts of rough endoplasmic reticule with dilated cisterns, free ribosomes, and mitochondria at this site (figure 2E).

The glands with little silk in the lumen contained no pyknotic nuclei but showed intense vacuolation of the basal cytoplasm (figure 4A). However, the most peculiar feature of this phase was the formation of blebs, with only one bleb being present in the apical cytoplasm of each cell (figures 3A and 4A). These blebs contained cytoplasmic RNA, as shown by the modified CEC method (figure 3B) and, occasionally, nuclear material, as shown by Ag staining (figure 3D). Portions of the cytoplasm had been released into the lumen by apical blebbing (figure 3D) and the cells that were shed into the gland lumen (figure 3C, E) had pyknotic nuclei and metachromatic cytoplasm (figure 3C) or irregular morphology (figure 3E). The cells that remained in the epithelium frequently showed an agglomeration of metachromatic granules in the cytoplasm (figure 3C).

Ultrastructurally, the cytoplasm of the protuberances primarily exhibited free ribosomes and rough endoplasmic reticule (figure 4B), with its cisterns arranged in spirals (figure 4C). Microvilli were found only at the base of the cytoplasmic protuberances and showed widened extremities (figure 4D). Vacuolation was increased in the basal cytoplasm (figure 4E), although the basal lamina remained intact (figure 4E). In some regions of the basal cytoplasm we observed rough endoplasmic reticule with a completely irregular shape and partial loss of ribosomes (figure 4E). The mitochondria and basal infoldings remained practically unaltered in comparison with the previous stage (figure 4E). The median cellular portion remained intact

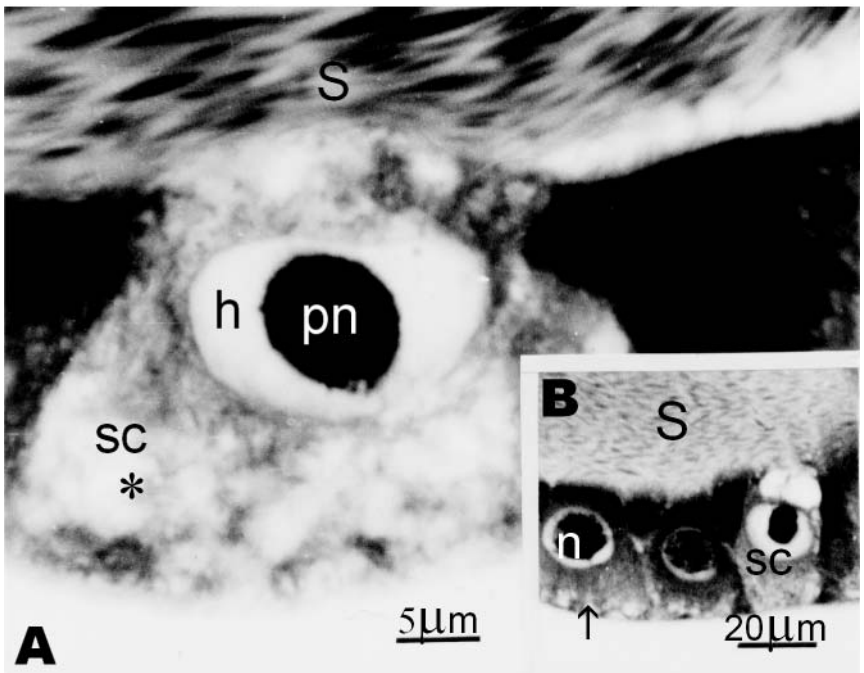

Figure 1. Histological sections of *A. mellifera* salivary glands with a large amount of silk (S) in the lumen, at the end of the 5th instar. Bromophenol blue staining. (**A)** Detail of a secretory cell (sc) with intense cytoplasmic vacuolation (*) and extensive nuclear pyknosis (pn) that produced a perichromatinic halo (h). (**B)** Note the different levels of chromatin compaction in the nuclei (n) and the vacuolated basal cytoplasm (arrow). A cell with a high level of nuclear pyknosis (sc) is observed in the epithelium.

and ultrastructural analysis showed the presence of whole mitochondria, rough endoplasmic reticule, and Golgi complexes (figure 4F).

At the beginning of the pre-pupal period the salivary gland epithelium was thinner than during the 5th instar (figures 5A and 6A). Cells with different degrees of disintegration and those with nuclear fragmentation were seen side by side in the epithelium (figure 5A). No apical blebbing was seen in this phase, but cells with an irregular morphology were still seen in the gland lumen (figure 5B). Non-pyknotic nuclei (seen with HE staining; figure 5A) stained intensely with Ag (figure 5B).

In the mid pre-pupal period, the cytoplasm of the secretory cells showed a high degree of degeneration with intense vacuolation (figure 5C). However, most of the cell nuclei remained in the gland epithelium (figure 5C), which progressively degenerated until the end of the pre-pupal period, when only nuclei and the remainder of the cytoplasm were observed (figure 5D, E). In this phase, the nuclei had an irregular morphology or were pyknotic, contained peripherally located chromatin (figure 3E) or were in the process of fragmentation with bleb formation (figure 5D).

Ultrastructural analysis of the secretory cells at the beginning of the pre-pupal period revealed that the cytoplasm of the median and apical regions appeared more preserved and dense than at the basal region, and contained vesicles filled with a material resembling the secretion found in the lumen (figure 6B). In these cells we observed structures surrounded by double membranes and with diverse contents (figure 6C, D). In the basal cytoplasm, ultrastructural analysis showed that the depth and number of infoldings of the basal plasma membrane had decreased and there was intense increase in vacuolation, while the basal lamina remained intact (figure 6E). In the median cytoplasm we noticed the presence of a few mitochondria with altered morphology, rough endoplasmic reticule with dilated cisternae, and a large amount of free ribosomes (figure 6F).

3.2 *Distribution of acid phosphatase in larval salivary glands*

A red/pink azo dye reaction product, indicative of acid phosphatase activity, was detected in the secretory portion of the gland in all the phases examined (figure 7A, D, G). Control glands showed no red/pink azo dye reaction product (figure 7C, E, I), thus confirming the specificity of this histochemical method.

At the beginning of the 5th instar, the acid phosphatase was located primarily in the intercellular spaces, although the enzyme also appeared as discrete loci within the cytoplasm

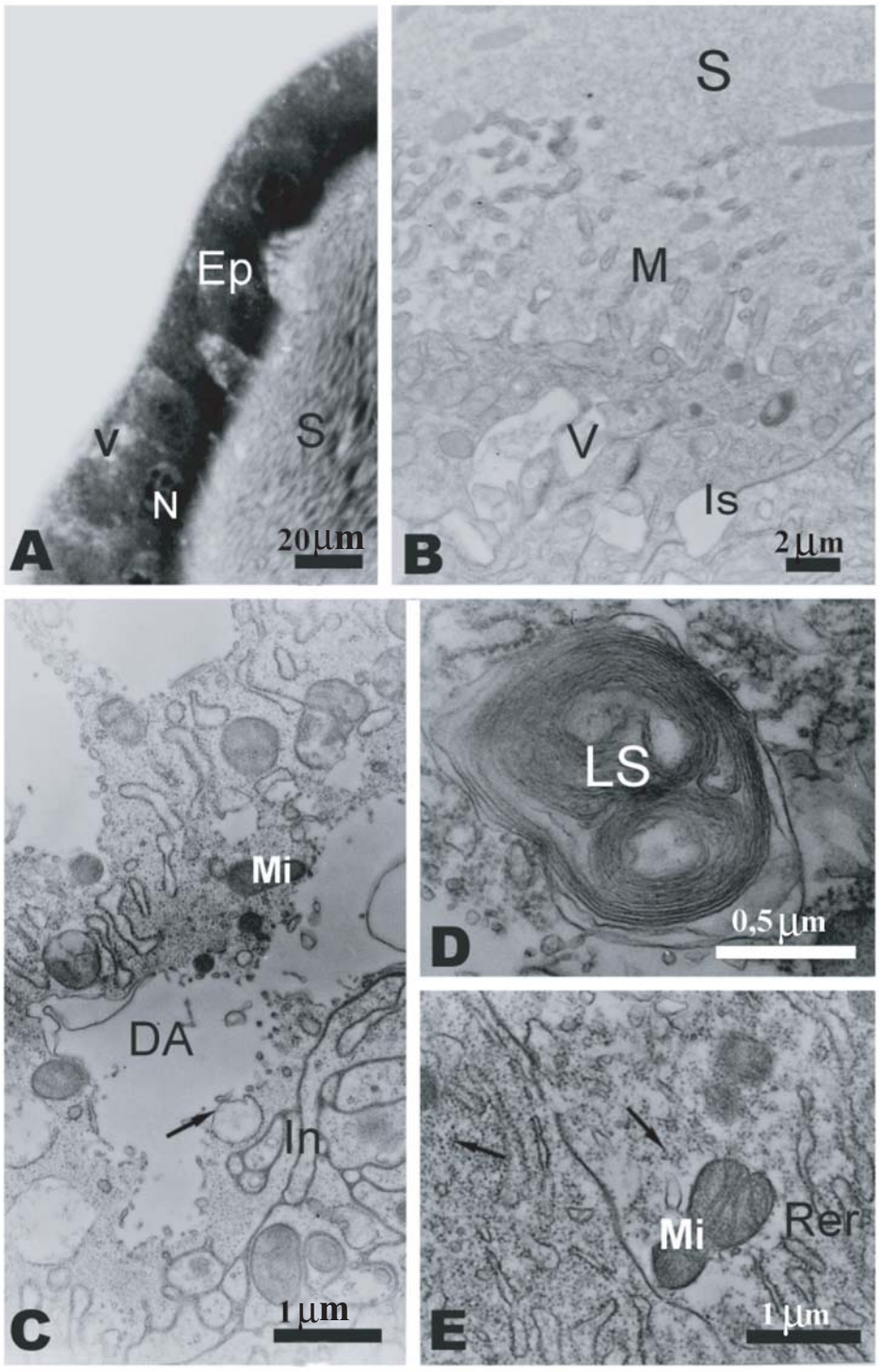

Figure 2. Secretory cells of *Apis* silk glands, with a large amount of fibrillar secretion (S) in the lumen, at the end of the 5th instar. **(A)** Histological section stained with xylidine Ponceau. The epithelium (Ep) shows cubic and condensed cells with apical nuclei (N). Most of the cells exhibit extensive areas of intense basal vacuolization (V). **B** to **E** Transmission electron microscopy. **(B)** Apical region with vacuoles (V), dilatation of intercellular space (Is) and large amount of microvilli (M); S, secretion. **(C)** Detail of basal region with degenerated areas (DA), deep invaginations of the basal plasma membrane (In), mitochondria (Mi) and rough endoplasmic reticule (Rer) with lost ribosomes (arrow). **(D)** Concentric lamellar structures (LS) in the basal region of the cells. **(E)** Middle region showing a large amount of mitochondria (Mi), rough endoplasmic reticule (Rer) with dilated cisternae and free ribosomes (arrow).

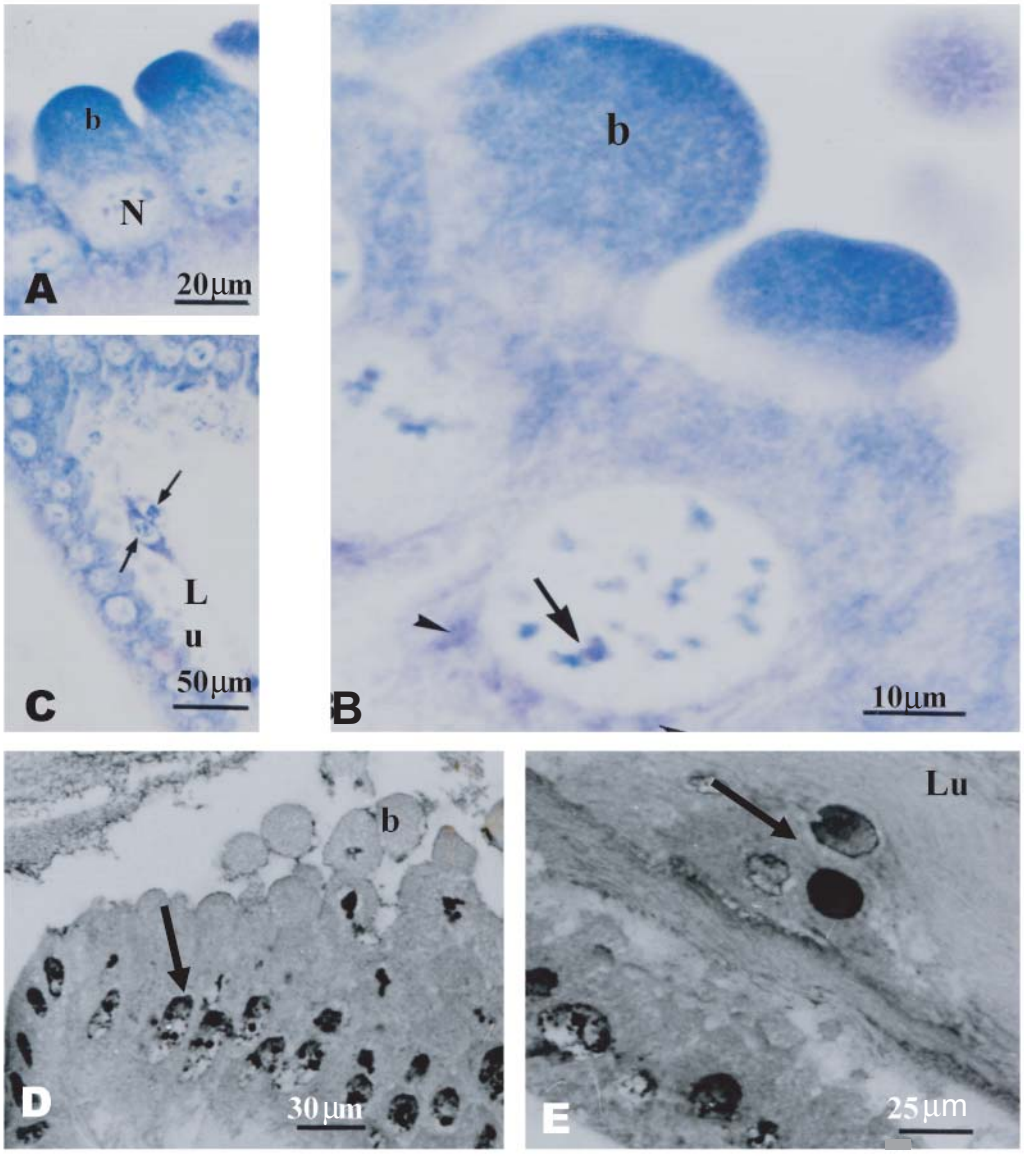

Figure 3. Histological sections of *A. mellifera* salivary glands with little silk (S) in the lumen (Lu), at the end of the 5th instar. **(A)** Toluidine blue staining. Note the metachromasy (i.e., blue colour) in the cytoplasm of the secretory cells, mainly in the apical blebs (b). Only some metachromatic granules were seen in the nuclei (N). **(B)** Variant of the critical electrolyte concentration (CEC) method. Nucleolar regions were identified in the nuclei (arrow) and some cytoplasmic areas retained their metachromasy, mainly at the nuclear periphery (arrowhead) and in the apical blebs (b), where the metachromasy was more intense. **(C)** Variant of the CEC method. Cells with intense metachromasy in their pyknotic nuclei (arrow) and cytoplasm can be seen in the gland lumen (Lu). The metachromasy was not intense in blebs that had not yet been released into the lumen. **(D)** Ag staining. Secretory cell nuclei contained large amounts of Ag-positive granules (arrow). These Ag-positive granules were also present within some apical blebs (b) that were being released from the cells into the lumen. **(E)** Ag staining. Note the cells with an irregular morphology and an Ag-positive nucleus (arrow) within the lumen (Lu).

(figure 7B). At the end of the 5th instar, the presence of a diffuse form of the enzyme within the cytoplasm (figure 7E) was accompanied by greater acid phosphatase activity (figure 7D) than at the beginning of this instar (figure 7A). This high acid phosphatase activity persisted in the cell cytoplasm through to the beginning of the pre-pupal period (figure 7G), and formed a granule-like reaction product (figure 7H).

At the beginning of the 5th instar, ultrastructural analysis showed that the cytochemical reactions for p-NPP were detected both in the middle and basal regions of the secretory cells of the gland. Reactions with high acid phosphatase

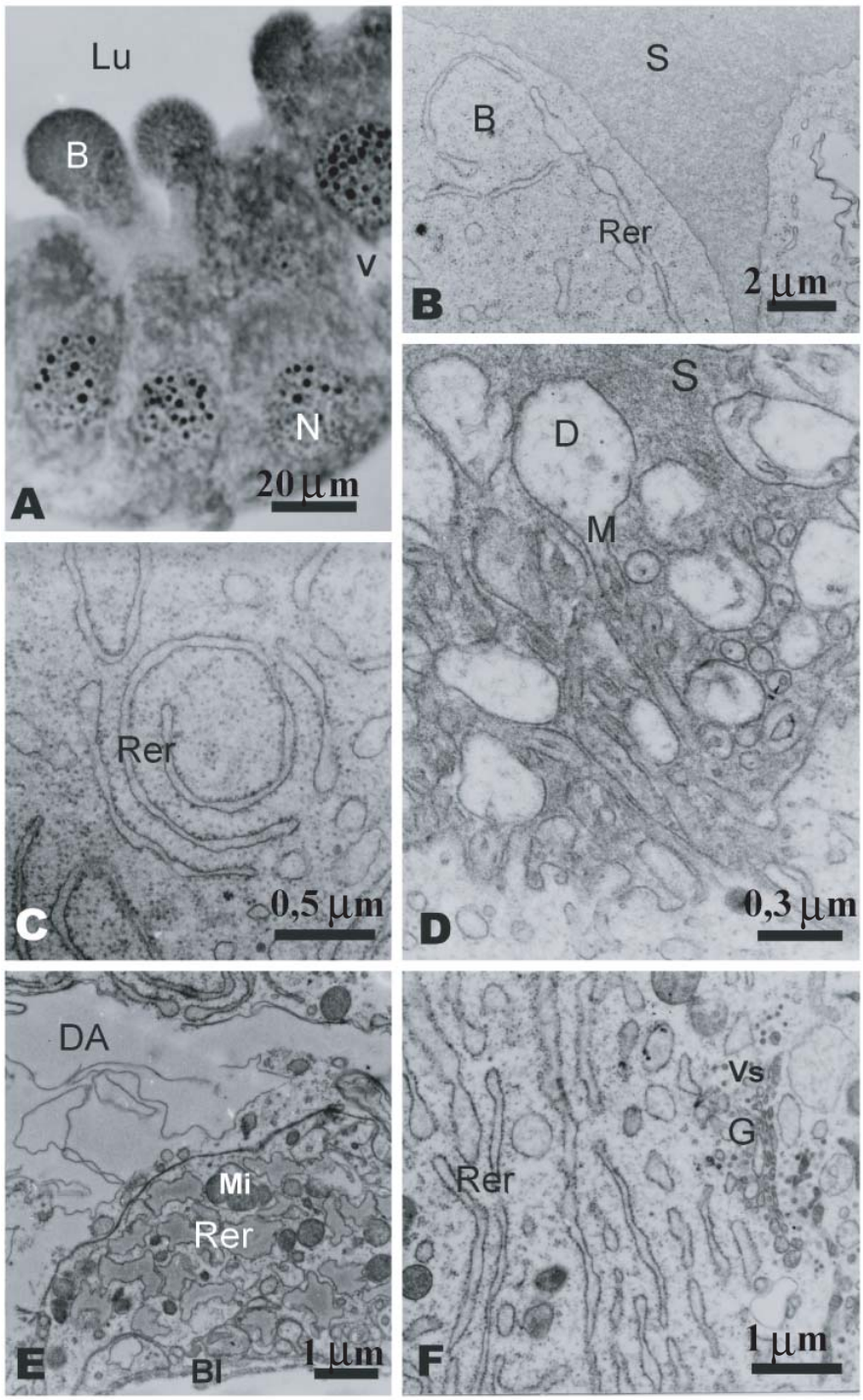

Figure 4. Secretory cells of *Apis* silk glands, with little or no fibrillar secretion (S) in the lumen, at the end of the 5th instar. **(A)** Histological section stained with haematoxylin–eosin, without secretion in the lumen (Lu). The cells have extensive areas of basal vacuolization (V). Note the apical bleb in each cell (B). N, nucleus. B to F – Transmission electron microscopy. **(B)** Detail of the apex of the apical bleb (B) with absence of microvilli. S, secretion; Rer, rough endoplasmic reticule. **(C)** Detail of the cytoplasm of the apical bleb showing rough endoplasmic reticule (Rer) with spiral cisternae. **(D)** Detail of microvilli (M) located at the bases of the blebs. Note the dilatations at the apex of the microvilli. S, secretion. **(E)** Rough endoplasmic reticule (Rer) with dilated cisternae and electron-dense material in the lumen. Extensive degenerated areas (DA) and mitochondria (Mi) are found in the basal cytoplasm. The basal lamina (Bl) remains intact. **(F)** Detail of the middle portion showing developed Golgi (G) with many vesicles (Vs) around it.

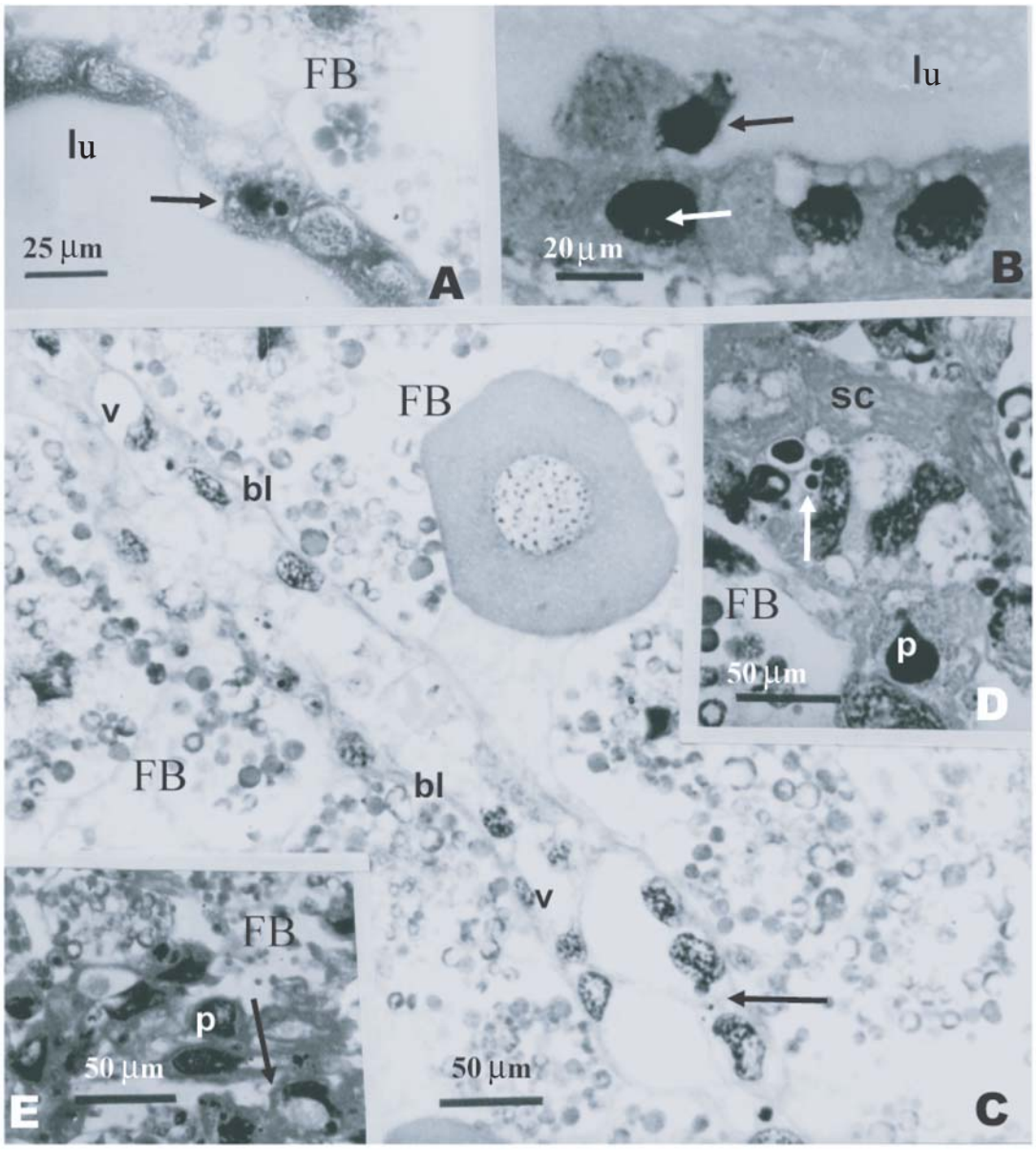

Figure 5. Histological sections of *A. mellifera* salivary glands in the pre-pupal period. **A** and **B** – Beginning of the pre-pupal period. **(A)** Haematoxylin–eosin staining. The arrow indicates a cell in an advanced stage of degeneration with a nucleus that is starting to undergo fragmentation. FB, fat body; lu, lumen. **(B)** Ag staining. Note the cells with an irregular morphology and Ag-positive nucleus (arrow) within the lumen (lu). In the epithelium, the secretory cells also have an Ag-positive nucleus (white arrow). **C**–**E**: Haematoxylin–eosin staining. FB, fat body. **(C)** In the mid pre-pupal period, the secretory cells showed practically no apical cytoplasm. The epithelial vacuolation (v) was so extensive that only a thread of cells (arrow), delimited by the intact basal lamina (bl), was seen in this phase. **(D)** and **(E)** At the end of the pre-pupal period, only nuclei and remnants of cytoplasm of the secretory cells (sc) were seen immersed in the fat body (FB). The nuclei were pyknotic (p), with peripheral chromatin (arrow) or blebs (white arrow).

activity were observed in the lysosomes, vesicular structures and Golgi (figure 8A, B, C).

A slight increase in enzyme activity was observed at the end of the 5th instar, reflected by the presence of acid phosphatase activity in areas where the reaction product had not been observed at the beginning of this instar, such as lumen secretion (figure 9A). In this larval phase, a specific lead reaction product was lying free among the microvilli and was also associated with protein structures, named tactoids, which constitute the silk present in the gland lumen

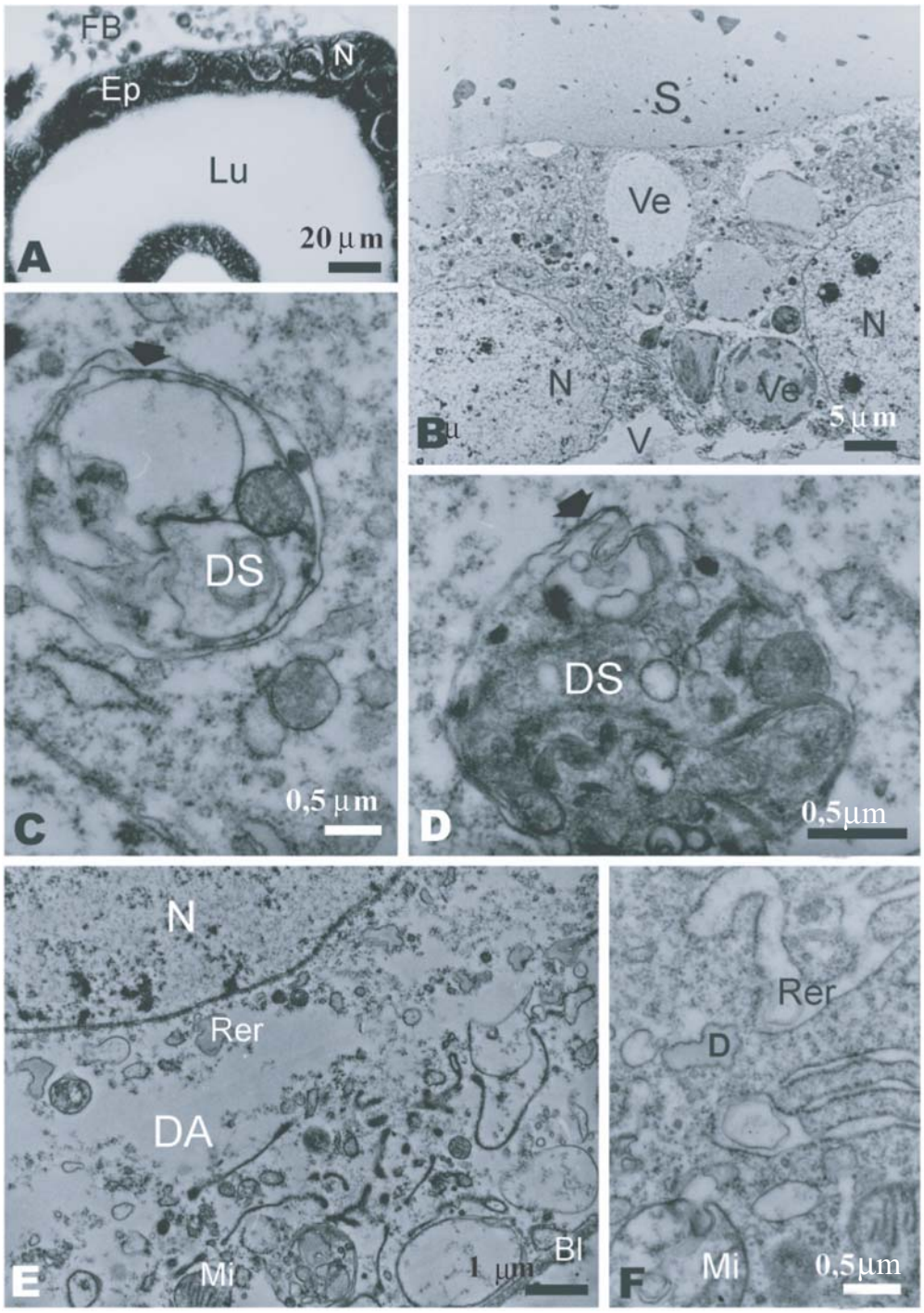

Figure 6. Secretory cells of *Apis* silk glands at the beginning of pre-pupal phase. **(A)** Histological section stained with haematoxylin–eosin. The epithelium (Ep) is thin and the cells are compacted and flattened in shape. N, nucleus; Lu, lumen; FB, fat body. Scale bar, 20 µm. (**B**–**D)** Transmission electron microscopy. **(B)** General view of cells. Vesicles (Ve) are found in the apical cytoplasm and a large zone of intense vacuolation (V) is seen in the basal cytoplasm. S, secretion; N, nucleus. **(C, D)** Degenerative structures (DS) composed of a cytoplasm mass containing organelles or parts of them, with a double membrane (arrow). **(E)** Detail of the basal portion showing the intense degenerated area (DA) and destabilization of organelles. The basal lamina (Bl) remains intact. Mi, mitochondria; Rer, rough endoplasmic reticule; N, nucleus. **(F)** Detail of the cell cytoplasm. Note the rough endoplasmic reticule (Rer) with irregular shape and with dilated cisternae (D) and electron-dense material inside it. The mitochondria (Mi) are altered.

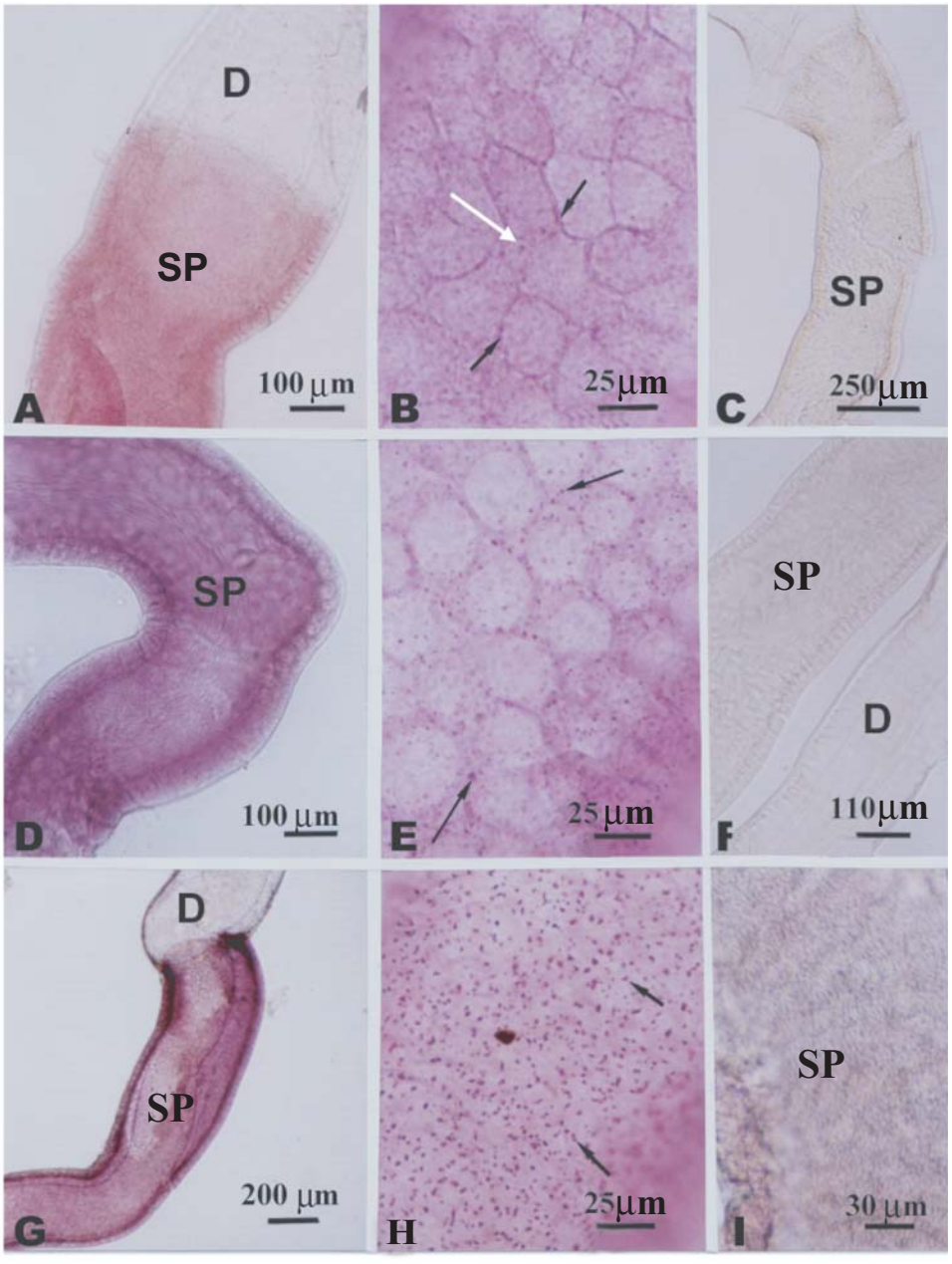

Figure 7. Whole mounts of *A. mellifera* salivary glands processed for the histochemical localization of acid phosphatase activity. **(A–C)**: Beginning of the 5th instar. **(A)** The gland duct (D) did not show the red/pink reaction product seen in the secretory portion (SP). **(B)** Note the large amount of red/pink granules (arrow) in the intercellular spaces and the presence of some granules within the cytoplasm (white arrow). **(C)** Control. Note the lack of reaction product in the secretory portion (SP). **(D–F)**: End of the 5th instar. **(D)** Acid phosphatase activity was enhanced in the secretory portion (SP) of the gland in this phase, as shown by the red/pink color. **(E)** The red/pink granules (arrow) are seen only within the cytoplasm. **(F)** Control. No reaction product was seen in the secretory portion (SP) or in the duct (D). **(G–I)**: Beginning of the pre-pupal period. **(G)** The gland duct (D) did not contain the red/pink reaction product seen in the secretory portion (SP). **(H)** Note the large amount of red/pink granules (arrow) within the cytoplasm (arrows). **(I)** Control. There was no reaction product in the secretory portion (SP).

(figure 9A). In addition, the reaction product was found in the lysosomes and vesicular structures (figure 9B, C). Autophagic structures, represented by complex vesicles containing dense material, also contained reaction products (figure 9B). Deposits of free acid phosphatase were found near the degenerated areas of the cytoplasm (figure 9B, C).

Control preparations, in which the enzyme substrates were omitted from the incubation media, showed no reactive deposits, thus confirming the specificity of the technique (figure 9D).

3.3 *Biochemical assays*

The amount of p-NPP liberated from the substrate per microgram of protein per hour was taken as a measure

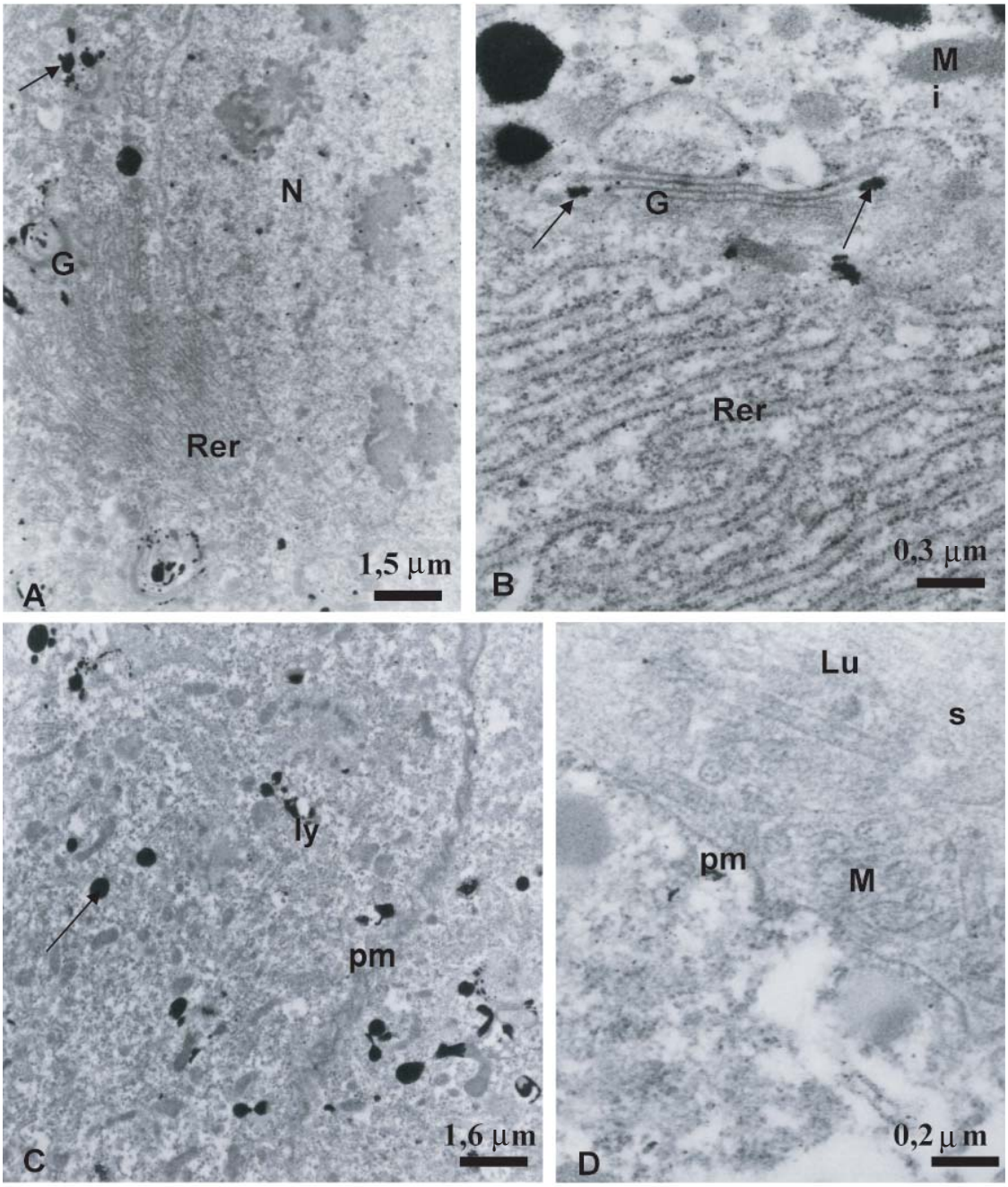

Figure 8. Transmission electron microscopy of *Apis mellifera* salivary gland at the beginning of the 5th instar. Acid phosphatase localization using p-nitrophenyl phosphate as substrate. **(A)** Middle region of secretory cell. Positive reaction in Golgi (G) and vesicles (arrow). **(B)** Detail of Golgi apparatus (G) which displays a positive reaction in its cisternae (arrow) and vesicles near it. **(C)** Basal region of a secretory cell. Positive reaction in the vesicles (arrow) and lysosomes (ly). **(D)** Apical region of a secretory cell with absence of positive reaction to acid phosphatase in the secretion (s) present in the glandular lumen (Lu).
M, microvilli; Rer, rough endoplasmic reticulum; Mi, mitochondria; pm, plasma membrane; N, nucleus.

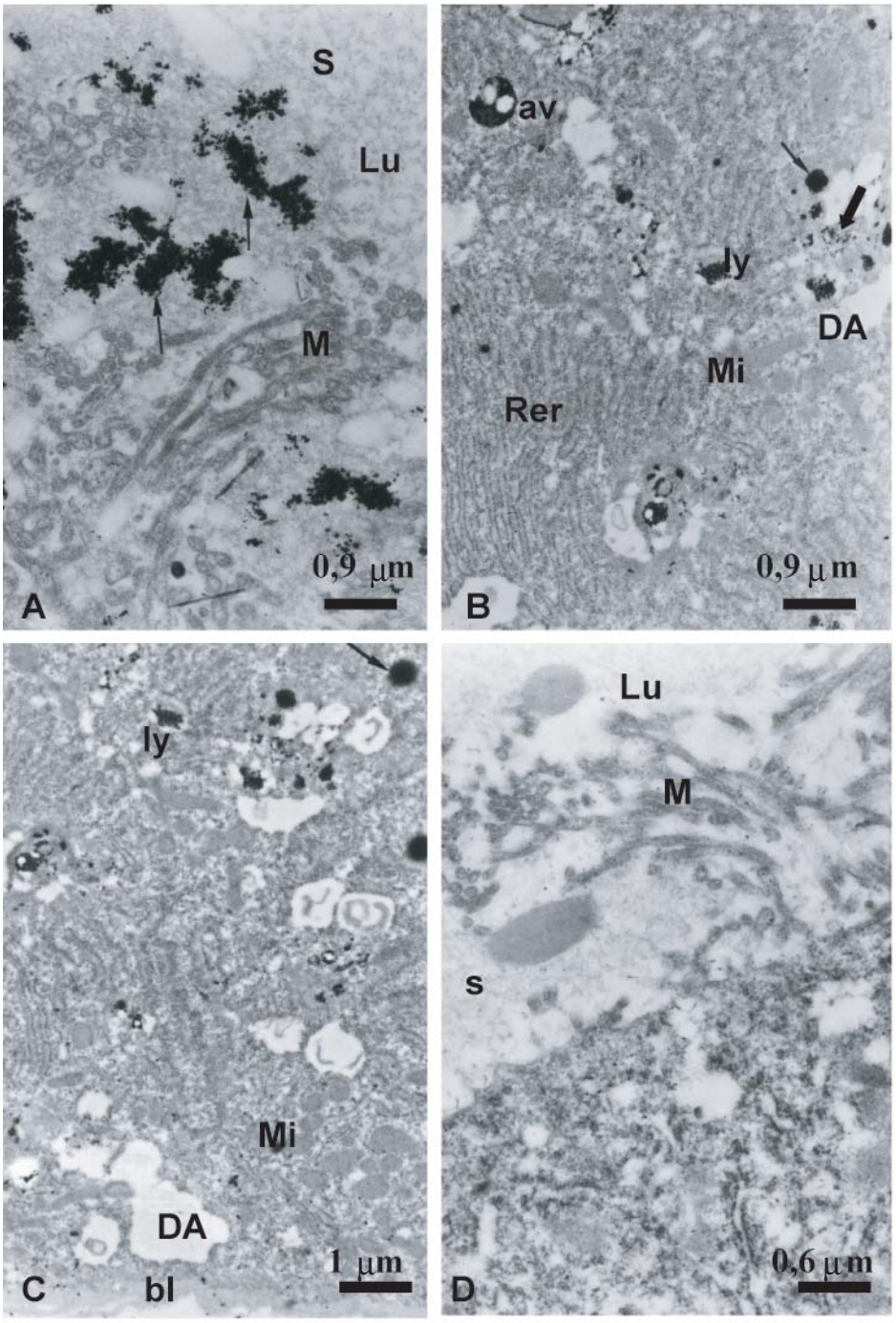

Figure 9. Transmission electron microscopy of *A. mellifera* salivary gland at the end of the 5th instar. **A**, **B**, **C** – Acid phosphatase localization using pNPP as substrate. **(A)** Apical region of secretory cell. Positive reaction (arrow) around the tactoids (*) of the secretion (S) present in the gland lumen (Lu). **(B)** Middle region of secretory cell. Vesicles (arrow), lysosome (ly) and autophagic structures (av) with positive reaction for p-nitrophenyl phosphatase (wide arrow) are located near the degenerated areas (DA) of the cytoplasm. **(C)** Basal region of secretory cell with positive reaction in the vesicles (arrow) and lysosomes (ly). **(D)** Control glands incubated in a medium without the substrate. Observe the absence of positive reaction to acid phosphatase in the cell and secretion (S). mi, mitochondria; bl, basal lamina.

Table 1. Means and SD of values of specific enzymatic activity (U/μg protein) and total enzymatic activity (U/gland) obtained to the *S-I* supernatants in salivary glands of larvae at the beginning of the 5th instar (L-5a) and end of the 5th instar (L-5b), and in prepupae at the beginning of this period (PP).

Group	Specific enzymatic activity (U)*	Total enzymatic activity (U/gland)
L-5a	$\overline{X}$ = 149 ± 0.41	$\overline{X}$ = 1868 ± 1.59
L-5b	$\overline{X}$ = 207 ± 0.21	$\overline{X}$ = 2579 ± 1.24
PP	$\overline{X}$ = 118 ± 0.27	$\overline{X}$ = 1655 ± 1.03

*U= Δ Abs/μg protein x h x 100

Table 2. Means and SD of values of specific enzymatic activity (U/μg protein) and total enzymatic activity (U/gland) obtained to the *S-II* supernatants in salivary glands of larvae at the beginning of the 5th instar (L-5a) and end of the 5th instar (L-5b), and in prepupae at the beginning of this period (PP).

Group	Specific enzymatic activity (U)*	Total enzymatic activity (U/gland)
L-5a	$\overline{X}$ = 660 ± 1.29	$\overline{X}$ = 3410 ± 1.42
L-5b	$\overline{X}$ = 721 ± 1.44	$\overline{X}$ = 3908 ± 2.37
PP	$\overline{X}$ = 499 ± 1.87	$\overline{X}$ = 2557 ± 1.68

*U= Δ Abs/μg protein x h x 100

of enzyme activity in each of the developmental stages investigated.

The biochemical assays obtained to the *S-I* and *S-II* supernatants showed that acid phosphatase-specific activity changed at the end of the larval development period. *S-I* and *S-II* supernatants demonstrated a similar pattern of acid phosphatase activity (tables 1 and 2). According to this pattern, the enzyme activity in the glands increased slowly from the beginning (L-5a) to the end (L-5b) of the 5th instar. Peak enzyme activity occurred in glands at the end of the 5th instar (L-5b), which was followed by a faster decline in activity during the pre-pupal phase (PP).

The statistical results of nested ANOVA (P<0,05), nesting *S-I* and *S-II* supernatants in different larval development stages, showed no significant difference among the specific enzymatic activities in the development stages analysed: L-5a, L-5b, PP (F = 3.636; df = 2; P = 0.058275).

There was a significant difference between the specific activity of the *S-I* and *S-II* supernatants (F = 32.820; df = 3; P = 0.000005), i.e., free acid phosphatase and compartmentalized acid phosphatase, respectively. The acid phosphatase activity of the *S-II* supernatant increased around 4-fold compared with that observed in the *S-I* supernatant. The Tukey HSD test confirmed the results obtained in the nested ANOVA test.

4. Discussion

The results of this study showed that physiologically or developmentally mediated cell death in the salivary glands of *A. mellifera* did not always emulate classic apoptosis. The metamorphic salivary gland cells investigated here did not shrink as classically seen in apoptotic cells; on the contrary, they became vacuolated and swollen towards the end of the 5th instar. However, cell death did not occur simultaneously in all of the secretory cells, which is in agreement with the findings of Bursch *et al* (1990) for apoptotic tissues. This asynchrony in degeneration has also been detected in other insect salivary glands (Aidells *et al* 1971; Lockshin and Zakeri 1994; Jurand and Pavan 1975; Sehnal and Akai 1990).

Another important feature of the programmed cell death described in this article was the nuclear pyknosis that formed a perichromatin halo within the nuclei, particularly in glands with a large amount of silk in the lumen at the end of the 5th instar. Pyknotic nuclei have frequently been described during programmed cell death in larval salivary glands (Silva de Moraes 1977; Sehnal and Akai 1990) and prothoracic glands (Dai and Gilbert 1999). However, this nuclear morphology has not been described in classic apoptosis (Wyllie 1981).

Pyknotic nuclei were not observed in *A. mellifera* salivary glands with little silk in the lumen at the end of the 5th instar, but the intense agglomeration of Ag-positive granules in these nuclei may be indicative of the presence of compacted chromatin and nucleolar regions with an affinity for Ag, as pointed out by Clavaguera *et al* (1983). The compaction of chromatin is related to a decrease in nuclear activity of tissues undergoing degeneration.

The most evident feature of programmed cell death of the *Apis* salivary gland is the vacuolation of the cytoplasm which begins at the end of the 5th instar and finishes at the beginning of the PP.

During the 5th instar, in glands with a large amount of silk in the lumen, the disintegration of the basal cytoplasm that results from the increased vacuolation observed in this cellular region is accompanied by a deepening of the plasma membrane infoldings, thus forming small intracellular compartments in the cell cytoplasm. These basal compartments contain organelles and have already been described in processes of glandular histolysis (Sehnal and Akai 1990). In this phase of the 5th instar, the concentric lamellar structures that were observed in the basal cytoplasm of the secretory cells in *Apis* have also been mentioned in the silk glands of *B. mori* (Tashiro *et al* 1968) and these

structures are considered degenerating material (Cruz-Landim and Silva de Moraes 1977).

Since the glandular cells of *A. mellifera* exhibit a dilatation of the rough endoplasmic reticule, thus acquiring a completely atypical shape and also show loss of ribosomes at various sites, the lamellar structures might represent the disintegration of the endoplasmic reticule, which would dilate and lose its ribosomes followed by a reorganization of its membranes into concentric lamellae, as described in the silk glands of *Galleria mellonella* at the beginning of the degenerative phase (Sehnal and Akai 1982).

In spite of the morphological alterations of the rough endoplasmic reticule arising at the basal region, the glandular cells of *Apis* showed typical Golgi complexes and mitochondria. Similar characteristics of programmed cell death of the apoptotic type have been described by other authors (Pollard *et al* 1987; Ferguson and Anderson 1981; Hacker 2000). The vesicular form of the rough endoplasmic reticule, which results from its dilatation and subsequent fragmentation, seems to be common during the degeneration of insect tissues (Jones 1990; Silva de Moraes and Bowen 2000).

The salivary glands of *A. mellifera* with little silk in the lumen represent a transition phase between the 5th instar and the PP since this is a post-secretory period when silk is released for cocoon spinning (Silva-Zacarin *et al* 2003). In this phase, there is an apical condensation that forms only one bleb in each cell. Because this bleb contains rough endoplasmic reticule and free ribosomes, as evidenced by metachromatic staining, and sometimes has Ag-positive nuclear material, it looks like an apoptotic body described by Kerr *et al* (1972). The metachromatic staining of the apical protuberances, which indicates the presence of cytoplasmic RNA, suggests that RNA is extruded from the secretory cells by blebbing, which has also been described in vertebrate apoptotic cells (Biggiogera *et al* 1997 and 1998). The latter authors suggested that this process of RNA extrusion was most probably linked to a decrease in the total amount of RNA in the cells rather than its degradation by RNase.

Cytoplasmic blebbing has been also described in the salivary glands of *Drosophila lebanonensis* larvae treated with the hormone ecdysone (Eeken 1977), and degenerative tissues of lepidopterans (Matsuura *et al* 1968; Zakeri *et al* 1993). The formation of apical protuberances in the glandular cells of *Apis* is probably related to the reorganization of the cytoskeleton, which has also been described in *Drosophila* salivary glands (Jochová *et al* 1997a). Such reorganization might involve the polymerization of actin and its phosphorylation by protein kinases (Mills *et al* 1998). In *Apis*, the loss of microvilli observed at the apex of the protuberances might have occurred due to modification of the actin cytoskeleton that supports the microvilli (Kondo *et al* 1997), but additional studies are needed to prove this hypothesis.

The loss of most of the microvilli during the degenerative process was also observed in the silk glands of *Melipona quadrifasciata anthidioides* (Silva de Moraes and Cruz-Landim 1979) and the hardening of their apices, together with elimination of parts of the cytoplasm, also occur in the secretory cells of the larval salivary glands of *Pseudohypocera kerteszi* during the degenerative process (Silva de Moraes and Cruz-Landim 1980). Nevertheless, the microvilli remained at the base of the cell protuberances in the silk gland of *Apis* and, since they showed extremely dilated apices, might be considered as characteristic of programmed cell death (Hacker 2000) instead of a way of elimination of secretions into the lumen (Zara and Caetano 2002). Accordingly, the fact that there was little or no silk in the lumen during this period at the end of the 5th instar indicates that most of the silk had already been eliminated to the spin the cocoon and that the larva is in the post-secretory phase.

In *Apis* salivary glands with little silk in the lumen, there was collapse of the basal cytoplasm while cytoplasmic blebbing occurred in the transition phase between the 5th instar and the pre-pupal period. Nevertheless, in the median cytoplasm of the cells, the Golgi and the rough endoplasmic reticule remained morphologically unaltered and, thus, apparently functional, as occurs in apoptotic cells (Pratt and Greene 1976; Hacker 2000) and in cells undergoing autophagic cell death (Myohara 2004), which require the synthesis of macromolecules and remain metabolically active. The agglomeration of metachromatic granules seen in the cytoplasm of the secretory cells of *A. mellifera* salivary glands by a variant of the CEC method corresponds to the large amount of free ribosomes as well as ribosomes bound to the rough endoplasmic reticule, both observed by TEM.

During the transition period between the 5th instar and the pre-pupal stage, the release of portions of the cytoplasm into the lumen by apical blebbing may be an effective mechanism of reducing the thickness of the gland epithelium seen at the beginning of the pre-pupal period in *A. mellifera* salivary glands, when the glands present a high degree of cellular disintegration.

At the beginning of the PP, the apical surface of the epithelium appeared irregular, and showed a total absence of microvilli. This fact, associated with the presence of apical vesicles containing materials that appear identical to the ones found in the glandular lumen during the previous stages, might indicate the end of the process of elimination of secretions due to cellular failure. Consequently, there is an accumulation of secretory vesicles containing polymerized silk, thus completing a set of events that accentuate cellular degeneration. Large secretion vesicles accumulated in the

apical cytoplasm of glandular cells were also described in the silk glands of *B. mori* at the time of larval moulting and, in greater amounts, in the PP (Morimoto *et al* 1968). These authors believe that the content of these vesicles, which was not eliminated into the lumen, represents an important source of amino acids required during the processes of moulting and glandular degeneration.

In the silk glands of *Apis* prepupae, the fact that the rough endoplasmic reticule remains dilated and thus able to form vesicles, together with the altered appearance of mitochondria, would indicate that the cell is in the process of losing its metabolic activity. The degenerative structures are morphologically distinct from the concentric lamellar structures detected in the glands at the end of the 5th instar; they appear enveloped by a double membrane and exhibit a diversified morphology. Probably these degenerative structures are autophagic vacuoles as reported by Beaulaton and Lockshin (1982) in insect tissues undergoing metamorphosis and during the degenerative process of the larval salivary glands of other insects (Matsuura *et al* 1968; Kloetzel and Laufer 1969; Jurand and Pavan 1975; Silva de Moraes and Cruz-Landim 1979; Lockshin and Zakeri 1994). The presence of acid phosphatase activity in the silk glands of initial prepupae, detected by histochemical and biochemical procedures, would support this hypothesis.

In the present study, biochemical assay as well as histochemical and/or cytochemical methods revealed acid phosphatase activity in all the glands analysed during the 5th instar and at the beginning of the prepupal phase.

Acid phosphatase activity increased in the salivary glands of 5th instars of *A. mellifera,* with a shift in the localization of the enzyme during development in this instar. The appearance of free acid phosphatase in the intercellular space at the beginning of the 5th instar, which was also detected by the biochemical assay, was not observed by ultrastructural cytochemistry, probably due to the diffusible reaction product in this extracellular space. The presence of free acid phosphatase near the basal degenerated areas of the secretory cells at the end of the 5th instar suggested that this enzyme had an exogenous origin and might have a role in the fragmentation of the basal cytoplasm seen in these cells. Similar data were described in the larval salivary glands of *Calliphora erythrocephala* (Armbruster *et al* 1986) and *Drosophila melanogaster* (Jones and Bowen 1993). The free cytosolic acid phosphatase activity may be derived from the movement of the enzyme from haemocytes attached to the external basal gland surface into the salivary gland tissue or from the absorption of enzyme present in the haemolymph (Levy and Bautz 1985; Armbruster *et al* 1986).

Vacuolation of the basal cytoplasm seen in the secretory cells during the 5th instar has been extensively described during the degradation of insect tissues (Aidells *et al* 1971; Schin and Laufer 1973; Armbruster *et al* 1986; Jones and Bowen 1993; Silva de Moraes and Bowen 2000). Although this intense cytoplasmic vacuolation is related to programmed cell death of the autophagic type (Beaulaton and Lockshin 1982), the presence of cytoplasmic vacuoles has been described in classic apoptosis and is probably related to the action of caspases that alter the normal turnover of cellular membranes (Hacker 2000).

Additionally, the presence of acid phosphatase activity in the intercellular space at the beginning of the 5th instar may be directly or indirectly related to the release of cells from the epithelium into the salivary gland lumen at the end of this instar. According to Kerr *et al* (1972), cells undergoing apoptosis lose contact with their neighbours. Beaulaton and Lockshin (1982) noted that the release of cells from the epithelium of insect tissues was preceded by dilatation of the intercellular space. In the present study, dilatation of the intercellular space was ultrastructurally visualized in the secretory cells during the end of the 5th instar, when cells can be observed in the gland lumen.

At the end of the 5th instar, the intracellular granules positive for acid phosphatase activity observed by histochemical procedures represent activity in the Golgi, vesicular structures and lysosomes, as seen by ultrastructural cytochemistry. The enzymatic activity in these structures represents membrane-bound acid phosphatase, as shown by the higher specific enzymatic activity in the *S-II* supernatant through the biochemical assay than in the *S-I* supernatant. The reaction products detected in the Golgi and vesicular structures of the glandular secretory cells indicated that the *Apis* salivary gland would be synthesizing acid phosphatase enzyme for exocytosis at the end of the 5th instar; hence, the Golgi is not merely a source of lysosomal acid phosphatase. The large amount of specific lead reaction product around the tactoids suggests that the gland cells produce secretory vesicles containing acid phosphatase; these are released among the microvilli. Similarly, Laicine *et al* (1991) observed that the larval salivary glands of *Bradysia hygida* produce acid phosphatase-containing secretory granules when the larvae start to build up an external collective meshwork to undergo metamorphosis. These authors speculated that the acid phosphatase present in secretory vesicles plays a role in the process by which the secreted material becomes insoluble. In this way, the large variety of acid phosphatases widely distributed in animal tissues probably has a role not only in the intracellular digestion of endogenous or exogenous components, but also in cell differentiation and production of secretion (Costa and Cruz-Landim 2001).

It is known that silk produced by insects, including the *A. mellifera* honeybee, is composed of fibrous proteins forming water-insoluble fibres that exhibit high strength and toughness (Flower and Kenchington 1967). Probably

the acid phosphatase present in the secretory vesicles of the *A. mellifera* salivary gland acts in the macromolecular array of the silk inside the lumen, which was described by Silva-Zacarin *et al* (2003), and in the process of making it insoluble, as suggested by Laicine *et al* (1991). Flower and Kenchington (1967) speculated on the possibility that the secretory cells of larval salivary glands secrete enzymes that bring about inter- or intramolecular changes in the silk secreted prior to spinning.

The increase of specific enzymatic activity detected by the biochemical assay in the *S-I* supernatant from the beginning to the end of the 5th instar would be due to exocytosis of secretory vesicles rich in acid phosphatase in the gland lumen, observed in the cytochemical analysis. The massive exocytosis could have been responsible for the increase in specific activity of the free acid phosphatase during this phase. The concentration of the compartmentalized acid phosphatase, detected in the biochemical assay, was slightly higher at the end of the 5th instar. The cytochemical analysis confirmed this fact because the presence of enzymatic activity in the vesicular structures and lysosomes was similar to that at the beginning of the 5th instar. However, autophagic vacuoles containing lead deposits were observed in the basal cytoplasm of the gland cells only at the end of the 5th instar, when the cell death process is initiated.

The presence of autophagic vacuoles positive for acid phosphatase at the end of the 5th instar and their absence at the beginning of the same instar coincided with signs of gland histolysis, such as basal cytoplasmic vacuolation, and also with peak specific enzymatic activity. Additionally, the silk secretion process coincides with cell death. These results were similar to those described by Jones and Bowen (1993) in *D. melanogaster* larval salivary glands. Indeed, increases in acid phosphatase activity during developmental histolysis have also been biochemically observed during the breakdown of *Galleria* silk glands (Aidells *et al* 1971), and larval salivary gland degeneration in *Calliphora* (Armbruster *et al* 1986) and *Chironomus* (Schin and Laufer 1973). In the hypopharyngeal glands of *Scaptotrigona postica* forager workers, the large amount of autophagic structures and lysosomes demonstrates that cellular components undergo substantial autophagy during this post-secretory phase (Costa and Cruz-Landim 2001).

The decrease in specific enzymatic activity observed in the *S-II* supernatant during the PP was probably due to the liberation of silk, whose tactoids are surrounded by free acid phosphatase, from the gland lumen in order to build the cocoon at the last larval instar. In this way, the free acid phosphatase activity in the lumen is too low in the PP. These results are in concordance with the ultrastructural findings of this study during the beginning of the PP, which showed little secretion in the lumen and a loss of cytoplasm parts from the secretory cells forming a narrow epithelium that degenerates progressively. These data also explain the decrease in concentration of the membrane-bound acid phosphatase detected by biochemical assay in our study.

The positive acid phosphatase activity within the lysosomal system is presumably related more to digestion phenomena occurring due to organelle turnover at the beginning of the 5th instar than a prelude to the cell death that begins at the end of this instar. Probably the free acid phosphatase found near the degenerated areas might have contributed to the basal vacuolation in the secretory cells, as described in the literature (Jones and Bowen 1993). Nevertheless, other mechanisms probably come into play during histolysis of the *Apis* salivary gland. In many insects, during programmed cell death of the larval salivary glands, the cells die by the autophagic destruction of organelles and activation of other proteases including lysosomal cathepsins and proteosomal proteases (Lockshin and Zakeri 2004).

The pre-pupal period was marked by intense, rapid degeneration of the gland and high acid phosphatase activity. This high acid phosphatase activity was probably related to the large amount of autophagic vacuoles, observed by TEM, and progressive vacuolation that culminates in the total degeneration of the gland at the end of this period. This has also been described in several other insect tissues (Bowen and Ryder 1976; Bowen *et al* 1982; Jones and Bowen 1980).

At the beginning of the pre-pupal period, most of the nuclei continue to show intense agglomeration of Ag-positive granules (indicative of compacted chromatin) and a high affinity for Ag ions in the nucleolar regions (Clavaguera *et al* 1983), although these nuclei apparently contained non-compacted chromatin (as shown by HE staining). The most important feature of this phase of the pre-pupal period was nuclear blebbing. This occurred only in some nuclei and was also observed at the end of the pre-pupal period when peripherally located chromatin was seen in only a few nuclei. Both these nuclear morphologies have been ascribed to classic apoptosis (Wyllie 1981), but this is the first time they have been described in the programmed cell death of larval salivary glands of Hymenoptera.

In the mid pre-pupal period, the nuclei of most of the salivary gland secretory cells remained intact, despite intense cytoplasmic vacuolation. Similar findings have also been also reported during the degeneration of the salivary glands of *Manduca sexta* (Lockshin and Zakeri 1994), *Calliphora vomitoria* (Bowen *et al* 1993), *Calliphora erythrocephala* (Levy and Bautz 1985), *B. mori* (Akai 1984) and *G. mellonella* (Sehnal *et al* 1983).

The end of the PP, when only nuclei and remnants of the cytoplasm were observed, corresponded to the final stage of cell degeneration and was characterized by an apparent lack of phagocytic intervention. This was similar to the degeneration seen in *C. erythrocephala* salivary glands

(Levy and Bautz 1985); the remnants of the salivary gland probably remained in the haemolymph to be recycled during metamorphosis.

In conclusion, the present study indicates that the programmed cell death seen in the salivary gland of larval *A. mellifera* lies between classic apoptosis and autophagy, since it exhibits some characteristics of both phenomena.

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